Anaesthetic Preconditioning;
The Role of ATP-Sensitive K^+ Channels

By Carsten Bantel, MD

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of Imperial College London

October 2009

Department of Anaesthetics, Pain Medicine and Intensive Care
Division of Surgery, Oncology, Reproductive Biology and Anaesthetics (SORA)
Faculty of Medicine
Imperial College London

I certify that unless otherwise stated, the work presented in this thesis is my own.

Carsten Bantel

1. ABSTRACT

Stroke in general but especially in the postoperative period is a serious clinical problem that warrants new therapeutic approaches. Here neuroprotective strategies and especially preconditioning have recently emerged as promising. Preconditioning was originally demonstrated in the heart but was subsequently also found in other organs. Classically it describes a phenomenon where short periods of ischaemia render tissues less vulnerable to major infarcts. In addition to ischaemia neuronal preconditioning can be achieved pharmacologically as well as through inhalational anaesthetics or drugs that open ATP-sensitive K^+(K_{ATP}) channels. However, the mechanisms through which anaesthetics produce protection remain elusive and the use of K^+ channel openers is hampered by their inability to cross the blood-brain-barrier.

This study was conducted to investigate the effects of inhalational anaesthetics on K_{ATP} channels and to explore whether their neuronal preconditioning properties were dependent on K_{ATP} channel opening. First, in whole-cell and excised patch-clamp experiments the effects of inhalational anaesthetics on recombinant wild type neuronal ($K_{ir}6.2$/SUR1) K_{ATP} and related as well as modified channels were evaluated. Secondly, the K_{ATP} channel dependence of anaesthetic preconditioning was tested in neuronal-glial co-cultures.

Recombinant K_{ATP} channels were activated by xenon, but inhibited by halogenated volatiles. Moreover, it was shown that xenon acted directly on the $K_{ir}6.2$ pore-forming subunit, reduced the ability of ATP to inhibit the channel and had no effect on the ATP-regulated $K_{ir}1.1$ channel. Functionally both sevoflurane and xenon preconditioned neurons at clinically used concentrations but only the effect of xenon was dependent on K_{ATP} channel activation.

Thus this study established xenon as a novel K_{ATP} channel opener. It interacts with the pore-forming $K_{ir}6.2$ rather than the regulatory sulphonylurea receptor subunit and disinhibits the channel from the blocking actions of ATP. As a consequence xenon but not sevoflurane is able to precondition neurons in a K_{ATP} channel-dependent manner.

Word count: 297

2. ACKNOWLEDGEMENTS

I would like to thank especially Dr Stefan Trapp and Professor Mervyn Maze for guiding me over the last years towards this PhD. Dr Trapp has given me exceptional support, advice and encouragement without which I would not have been able to finish this project successfully. Professor Maze convinced me to come to London and to join his group in such a fascinating place and creative environment.

I am also not forgetting Dr Daqing Ma who earned my respect and gratitude for his comments and suggestions and his willingness to listen even at unlikely moments.

Dr Mahmuda Hossain, Dr Cleoper Paul, Dr Christopher Edge and Mrs Raquel Yustos earned my gratitude for their invaluable support, advice and critical comments both technical and general. Without their vivid minds I would not have enjoyed my time in the lab as much as I did.

I am further thankful for the support Imperial College, Chelsea & Westminster Hospital and the Westminster Medical School Research Trust have given me.

The Westminster Medical School Research Trust supported me twice with small grants and once with a travel scholarship. Chelsea & Westminster Hospital in conjunction with Imperial College helped me organise and fit the PhD into a structured anaesthetic training programme. Representative for many more involved in this regard I would like to thank Dr Brian Porter and Dr Michelle Hayes.

Also I would like to thank Dr Stephen Tucker from Oxford University for his kind gift of DNA encoding $K_{ir}6.2\Delta C26$ and $K_{ir}1.1$ channels.

Finally I would like to thank the most important people in my live and dedicate this work to them: My wife Martina and our children Leah and Felix. The number of times they were missing a husband and dad are legend. Their love and patience stunned me and still does. I hope some day I will be able to give something back.

3. TABLE OF CONTENTS

1. ABSTRACT **3**

2. ACKNOWLEDGEMENTS **4**

3. TABLE OF CONTENTS **5**

4. LIST OF FIGURES AND TABLES **8**

5. INTRODUCTION **10**

5.1 Stroke: Defining the Problem **11**

5.2 Stroke: The General Risk **12**

5.3 Stroke: The Perioperative Risk **13**

5.4 Perioperative Stroke: Risk Determining Factors **15**

5.5 Perioperative Ischaemic Stroke: Pathophysiology **16**

5.5.1 The Cause ***16***

5.5.2 The Penumbra Concept ***17***

5.5.3 The Pathways ***20***

5.5.3.1 Peri-infarct depolarisation *23*

5.5.3.2 Excitotoxicity *23*

5.5.3.3 Reactive Oxygen Species (ROS) *25*

5.5.3.4 Tissue acidosis *26*

5.5.3.5 Inflammation *27*

5.5.3.6 Apoptosis *28*

5.6 Therapy of Ischaemic Stroke **31**

5.6.1 Risk Management ***31***

5.6.2 Treatment of Acute Stroke ***31***

5.6.3 Neuroprotection ***33***

5.6.3.1 Preconditioning *35*

5.6.3.2 Neuroresuscitation *35*

5.6.3.3 Postconditioning *37*

5.7 Preconditioning **39**

5.7.1 Pharmacological (Anaesthetic) Preconditioning ***42***

5.7.2 Preconditioning Pathways ***53***

5.7.2.1 Overview *53*

5.7.2.2 ATP-sensitive K+ (K_{ATP}) channels *57*

5.7.2.3 Mitochondrial KATP (mKATP) channels *57*

5.7.2.4 Plasmalemmal K_{ATP} (pK_{ATP}) channels *61*

5.7.2.5 Effects of anaesthetics on K_{ATP} channels *70*

5.8 Hypothesis **73**

6. METHODS **74**

6.1 Electrophysiological Experiments **74**

6.1.1 Tissue Culture *74*

6.1.2 Experimental Solutions *82*

6.1.3 Patch Pipettes *83*

6.1.4 Whole-Cell Recordings *83*

6.1.5 Inside-Out Excised Patch Recordings *87*

6.2 Cell Injury Experiments **90**

6.2.1 Neuronal Glial Cell Co-Culture Preparation *92*

6.2.2 Preconditioning *94*

6.2.3 Oxygen-Glucose-Deprivation (OGD) *95*

6.2.4 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Reduction Test *96*

6.3 Statistics **97**

7. RESULTS **98**

7.1 Whole-Cell Patch-Clamp Experiments **98**

7.1.1 Potassium Current in K_{ir}6.2/SUR1 Transfected HEK293 Cells *98*

7.1.2 Lack of Modulation of pK_{ATP} Channels via Mitochondrial ATP-sensitive K^+ Channels *103*

7.1.3 Evaluation of the Effects of Inhalational Anesthetics on pK_{ATP} Currents *109*

7.1.4 Xenon-Induced K_{ATP} Channel Opening: Insights into Possible Mechanisms *117*

7.1.4.1 Xenon as K_{ATP} channel opener: A SUR1 independent process *120*

7.1.4.2 K_{ir}6.2 activation by xenon: ATP sensitivity critically involved *123*

7.1.4.3 Specificity of involvement of ATP: Xenon does not effect K_{ir}1.1 ..... *125*

7.2 Inside-Out Macropatch Experiments **126**

7.2.1 Xenon Interaction with K_{ir}6.2: The Critical Role of ATP *126*

7.2.2 Xenon and ATP: Evidence for a Competitive Antagonism *131*

7.3 Anaesthetic Preconditioning: Functional Relevance of pK_{ATP} Channel Opening **133**

8. DISCUSSION **138**

8.1 Anaesthetic Effects on Recombinant Neuronal Plasmalemmal K_{ATP} Channels **138**

8.1.1 Contribution of Mitochondrial KATP (mKATP) Channels *138*

8.1.2 Direct Anaesthetic Effects on Plasmalemmal K_{ATP} Channels *140*

8.1.3 Mechanism of Xenon-Induced Plasmalemmal K_{ATP} Channel Opening *143*

8.1.3.1 K_{ir}6.2: Xenon's principal target *143*

8.1.3.2 Xenon induced channel opening: Independence from cellular signalling cascades *145*

8.1.3.3 Xenon modulated channel gating: An ATP-dependent process? …… *147*

8.2 Neuronal Preconditioning …… **150**

8.3 Physiological Significance of K_{ATP} Channel Inhibition …… **161**

8.4 Physiological Consequence of K_{ATP} Channel Activation …… **162**

8.5 Possible Clinical Implications for the Use of Xenon as Preconditioner ….. **164**

9. CONCLUSIONS …… **166**

10. PUBLICATIONS …… **167**

11. REFERENCES …… **181**

4. LIST OF FIGURES AND TABLES

Tables

Table 5.1: Incidence of Stroke after Surgical Procedures …… 14
Table 5.2: Morphological Characteristics of Apoptotic and Necrotic Cell Death …… 29
Table 5.3: Anaesthetic Preconditioning - *in vivo* ……... 45
Table 5.4: Anaesthetic Preconditioning - *in vitro* ……... 48
Table 5.5: Triggers and Effectors of Preconditioning …… 56
Table 5.6: K_{ATP} Channel-Dependent Preconditioning - *in vivo* ……. 59
Table 5.7: K_{ATP} Channel-Dependent Preconditioning - *in vitro* ……. 60
Table 5.8: Subunit Composition of K_{ATP} Channel Subtypes …….. 68
Table 5.9: Pharmacology of K_{ATP} Channel Subtypes …… 72

Table 7.1: Reversal Potentials …… 100

Figures

Figure 5.1: Cerebral blood flow reduction and physiological consequences …… 18
Figure 5.2: The brain after ischaemic stroke – defining the core and penumbra …… 20
Figure 5.3: Time course of events contributing to the ischaemic cascade ……... 21
Figure 5.4: Time course of damaging mechanisms and involved mediators …… 22
Figure 5.5: Neuroprotective strategies ……. 34
Figure 5.6: Main principles of endogenous neuronal protective and repair mechanisms ….. 37
Figure 5.7: Inward rectifier K^+ channels …….. 64
Figure 5.8: The $K_{ir}6.2$ pore forming unit …….. 66
Figure 5.9: Principle structure of $K_{ir}6.2$/SUR1 K^+ channel ……. 67

Figure 6.1: Schematic drawing of ATP interaction with $K_{ir}6.2$ ATP-binding site ……. 78
Figure 6.2: Human K_{ir}: Sequence identity and protein alignment …….. 80
Figure 6.3: Schematic diagram of patch clamp configuration ……. 87
Figure 6.4: Determination of slope conductance ……... 89
Figure 6.5: Time course of cell injury experiments …… 93

Figure 7.1: K^+-current of untransfected and transfected HEK cells …….. 99
Figure 7.2: Washout current …… 102

Figure 7.3: K_{ATP} currents in whole-cell recordings are not affected by specific blockers of mitochondrial ATP-sensitive K^+ channels …… 104
Figure 7.4: Whole-cell K_{ATP} currents are not affected by specific openers of mKATP channels …….. 106
Figure 7.5: Pinacidil has no effect on whole-cell K_{ATP} currents …….. 108
Figure 7.6: K_{ATP} channels expressed in HEK293 cells are mildly inhibited by sevoflurane … 110
Figure 7.7: Halogenated volatile anaesthetics inhibit pK_{ATP} channels ……... 112
Figure 7.8: Concentration-response relationships of volatile anaesthetics on $K_{ir}6.2$/SUR1 channels expressed in HEK293 cells ……... 113
Figure 7.9: Xenon activates K_{ATP} channels …….. 115
Figure 7.10: Tolbutamide reversibly inhibits pK_{ATP} currents in presence of xenon ……. 116
Figure 7.11: Xenon induced outward K^+-current is achieved by pK_{ATP} channel opening ….. 118
Figure 7.12: Effect of xenon, diazoxide and tolbutamide on pK_{ATP} currents …… 119
Figure 7.13: Xenon does not require the SUR1 subunit to activate K_{ATP} channels ……. 121
Figure 7.14: Mean effect of xenon on $K_{ir}1.1$ and $K_{ir}6.2$ channel variants ……... 122
Figure 7.15: ATP inhibition of wild type but not $K_{ir}6.2$-K185Q/SUR1 currents …… 127
Figure 7.16: Xenon enhances wild type but not $K_{ir}6.2$-K185Q/SUR1 currents …… 129
Figure 7.17: Xenon increases $K_{ir}6.2$/SUR1 currents at various ATP concentrations ……. 130
Figure 7.18: Concentration-response effect of ATP in the absence and presence of xenon … 132
Figure 7.19: No damaging effect of K_{ATP} channel openers and inhibitors on neuronal-glial cell co-cultures ……. 134
Figure 7.20: Neuronal-glial cell culturing and preconditioning experiment …… 135
Figure 7.21: Preconditioning by xenon but not sevoflurane involves K_{ATP} channels ……. 137

Figure 8.1: Suggested mechanisms of inhalational anaesthetics induced preconditioning ….. 160

5. INTRODUCTION

Pharmacological preconditioning has recently attracted attention as a therapeutic alternative for the treatment of stroke in general and perioperative stroke in particular. This is based on the recognition that current therapies of cerebrovascular accidents are either insufficient or carry their own significant risk profile.

In the following based on the existing literature the significance of stroke for the general population as well as for surgical patients is explained. This account is followed by a review of the current knowledge of causes and pathophysiology of stroke as well as currently available treatment options. In this context then the concepts of neuroprotection and preconditioning are introduced and their major pathways described.

Because the present study is mostly concerned with ATP-sensitive K^+ (K_{ATP}) channels in the final part of the introduction their potential critical involvement in pharmacological neuronal preconditioning is summarised and the reasoning explained that led to the conduct of this study.

5.1 Stroke: Defining the Problem

Stroke is defined as a rapidly developing focal neurological deficit, which lasts for more than 24 hours (Riley, 2003). It is the functional result of an acute injury to the brain secondary to either reduction of cerebral blood flow in the territory of a major artery (ischaemic stroke) or intracranial bleeding (haemorrhagic stroke) (Dirnagl et al., 1999; Rosamond et al., 2008).

Because of its frequent adverse outcome with death or disability, its associated high costs and its risk profile that is not restricted to any age, gender, race or social group, stroke is one of the most important medical conditions of our times.

With a mortality of 10-20% for ischaemic and 35-45% for haemorrhagic stroke, cerebrovascular accidents are the third commonest cause of death in the developed world (Kolominsky-Rabas et al., 1998; Thrift et al., 2001; Rosamond et al., 2008). Alone in the USA stroke contributes 6.3% of people to the annual death register whilst heart diseases and cancer do this with 27.2% and 23.1% respectively (SEER, 2008)

It is not only the high mortality rate that is staggering, it is also the impact stroke frequently has on its survivors. Recent studies found that stroke is commonly associated with neurological complications (seizures, recurrent strokes), infections (urinary, chest), immobility, thrombembolism, pain and psychological disorders (cognitive impairment, depression, anxiety) (Langhorne et al., 2000; Rabadi et al., 2008). Even minor strokes have been found to reduce quality of life of patients significantly despite a high degree of daily independence (Edwards et al., 2006).

The financial burden of stroke and therefore the burden for society as a whole is mostly dependent on the length of hospitalisation, degree of rehabilitation and ability of patients to return to work. The average length of hospital stay after stroke is between 10 and 44 days with 14 to 73% of patients being able to fully reintegrate into their previous working environments (Claesson et al., 2000; Yoneda et al., 2003; Luengo-Fernandez et al., 2006; Treger et al., 2007). This variability in stroke victim recovery is also reflected in the wide range of costs which studies were able to uncover. These costs that are normally calculated as "costs per case in the first year after stroke" are worldwide between $6887 and $49,403 (Taylor et al., 1996; Claesson et al., 2000; Dewey et al., 2001; Yoneda et

al., 2003; Luengo-Fernandez et al., 2006). However, strokes that occur in patients already in hospital for other reasons increase costs by $21,096 - $40,192 per case in the first year and are generally more expensive (Tung et al., 1999; Luengo-Fernandez et al., 2006). Therefore, the overall lifetime costs per stroke case have been estimated as $11,787 to $3,035,671 (Taylor et al., 1996; Palmer et al., 2005). Related to the spending of a single country these sums mean that the USA for example spent nearly $50 billion on stroke in 2008. In comparison, the US spending for cancer and HIV was estimated to be $70 billion and $20 billion, respectively (Kaiser-Family, 2007; National-Cancer-Institute, 2007).

5.2 Stroke: The General Risk

The incidence of a disease describes the number of new cases within a specific period of time. The incidence rate however expresses the number of new cases per unit of population per year. Both define the risk with which any given person of a population could contract that condition.

The overall annual incidence of stroke worldwide has been found to be between 149 and 174 cases per 100,000 people with a proportion of 13% haemorrhagic and 87% ischaemic strokes (Kolominsky-Rabas et al., 1998; Thrift et al., 2001; Rosamond et al., 2008). For the USA this means about 780,000 stroke cases each year and for the UK 110,000 of which approximately two thirds are first time and one-third recurrent attacks (Rosamond et al., 2008; NHS-Direct, 2009).

For comparison, cancer has an incidence of 470 per 100,000 in the USA and in Europe each year about 8000 new HIV cases are registered (Semaille et al., 2002; SEER, 2008).

The incidence of stroke doubles with every decade increase in age. Therefore the incidence of stroke beyond the age of 75 is 992 per 100,000 people, which leaves more than 50% of all stroke victims at an age older than 75 years (Kolominsky-Rabas et al., 1998; Thrift et al., 2001).

5.3 Stroke: The Perioperative Risk

Wong and colleagues (Wong et al., 2000) found an increased incidence for stroke within the first month after routine general operations and they determined the combination of surgery with anaesthesia as an independent risk factor for the development of cerebrovascular accidents. Despite this finding, the overall incidence for stroke during and after non-high risk surgery is low. It has been estimated that only 0.08-0.7% of patients develop a neurological sequel. The risk to contract a stroke after non-cardiovascular surgery is therefore approximately the same as the risk for the public in general (Kikura et al., 2004; Selim, 2007). Nevertheless, the risk is dependent on advanced age, co-morbidities and the surgical procedure (see section 5.4). Especially cardiac, aortic and carotid operations with an incidence of 1.4 to 9.7% carry an about 20fold increased chance for stroke (see Table 5.1) (Selim, 2007). Stroke is therefore still the most feared complication of cardiovascular operations despite changes in surgical techniques and patient pre-optimisation (McKhann et al., 2006).

The specific risk for cerebrovascular accidents associated with heart surgery is also emphasized by the fact that the time frame of stroke development is different in cardiac compared to non-cardiac surgery. Limburg and colleagues as well as Kikura and colleagues found the median time interval of surgery to stroke after non-cardiac operations to be between 2 and 11 days (Limburg et al., 1998; Kikura et al., 2004). In contrast, after cardiac procedures a significant number of strokes occur within 24 hours of surgery (“early strokes”). Moreover, patients contracting early strokes are considerably more likely to suffer from severe neurological complications than patients with delayed strokes (Lisle et al., 2008). This increased complication rate is also reflected by a significantly increased short- and long -term mortality rate (Toumpoulis et al., 2008).

Therefore, cardiovascular surgical patients not only face a large risk of developing stroke in the early postoperative period they are also more likely to suffer the gravest consequences. Considering the fact that in the UK alone each year more than 30,000 patient have heart surgery, early postoperative cerebrovascular accidents are a real clinical problem warranting new treatment and preventative strategies (The-Care-Quality-Commission, 2006).

Table 5.1: Incidence of Stroke after Surgical Procedures

Procedure	Risk of Stroke (%)
General Surgery	0.08-0.7
Peripheral Vascular Surgery	0.8-3.0
Head and Neck Tumor Resection	4.8
Carotid Endarterectomy	5.5-6.1
Isolated Coronary Artery Bypass Graft (CABG)	1.4-3.8
Combined CABG and Valve Surgery	7.4
Isolated Valve Surgery	4.8-8.8
Multiple Valve Surgery	9.7
Aortic Repair	8.7

Modified after Selim (2007)

5.4 Perioperative Stroke: Risk Determining Factors

Selim (Selim, 2007) classified the factors that determine the risk for the development of perioperative stroke into three groups: 1) preoperative patient-related factors; 2) intraoperative procedure-related factors and 3) postoperative factors.

In general, patient-related risk factors are advanced age (>70 years) and significant co-morbidities. Among diseases, which have been found to be stroke predictors are hypertension, diabetes mellitus, renal failure, chronic obstructive pulmonary disease, heart failure, arrhythmias, prior stroke and atherosclerotic vascular diseases (peripheral vascular, coronary, ascending aorta, carotid, cerebrovascular) (Kikura et al., 2004; Selim, 2007).

In addition, Toumpoulis and colleagues determined specific predictors for the development of early and delayed stroke after cardiac surgery: Predictors of early strokes were age, recent myocardial infarction, smoking, femoral vascular disease and obesity whereas predictors for delayed stroke were female gender, white race and preoperative renal failure (Toumpoulis et al., 2008).

Intraoperative predictors for stroke overall are type, nature and duration of anaesthesia and surgery as well as the development of arrhythmias, hyperglycaemia as well as hypo- and hypertension. Specific cardio-surgery related risks were the duration of cardiopulmonary bypass, aortic cross-clamp time and manipulations of an atherosclerotic aorta (Kikura et al., 2004; Selim, 2007).

Postoperative predictors for early stroke according to Toumpoulis and colleagues are reoperation for bleeding, sepsis and respiratory failure. Risk factors for delayed stroke are respiratory and renal failure (Toumpoulis et al., 2008). But it is principally intrinsic cardiogenic factors that place the patients at risk in the postoperative period. Heart failure, infarction and atrial fibrillation in particular, which occurs in up to 50% of patients after cardiac surgery, have been strongly associated with postoperative cerebrovascular accidents (Selim, 2007).

5.5 Perioperative Ischaemic Stroke: Pathophysiology

5.5.1 The Cause

As discussed in the previous sections early postoperative stroke after cardiac surgery has a high incidence and is associated with an elevated disability and mortality rate.

Perioperative stroke is mostly caused by thrombembolic events (Selim, 2007). The risk to contract such a cerebrovascular accident has therefore been linked to conditions that carry an increased chance for the development of thrombi. Atrial fibrillation for instance, that predisposes the heart for thrombus formation, occurs in more than 30% of heart surgical patients in the early postoperative period. Conversely, 36% to 75% of patients with stroke after cardiac procedures had at least one episode of atrial fibrillation, which has therefore been identified as a predictor for the development of postoperative stroke (McKhann et al., 2006).

Also manipulation of an atherosclerotic aorta during surgery has been strongly correlated to the development of stroke (McKhann et al., 2006). Emboli for that reason have long been suspected to be the main cause of brain infarction (McKhann et al., 2006). Their composition can be of air or lipid but is most likely of platelet-fibrin aggregates or atheromatous debris that can emerge principally from the heart, the ascending aorta or any cervicocranial artery (Caplan and Hennerici, 1998). Indeed their formation has been confirmed in Doppler ultrasound studies during and after cardiac surgery in both aorta and cerebral circulation, but especially in the middle cerebral artery (Caplan and Hennerici, 1998).

In addition to embolisation cerebral hypoperfusion during heart surgery is now suspected to contribute to stroke development in the perioperative period as well. This is based on the fact that reduced tissue perfusion per se deprives organs from provision with nutrients and oxygen. Such deprived organs might either become directly injured or more vulnerable to the impact of an acute, even partial, arterial occlusion. Also, diminished perfusion is suspected to impair the washout and clearance of emboli deposited in the cerebral circulation or even predispose to thrombus formation at sites of stenotic lesions (Caplan and Hennerici, 1998). However, the probably most important consequence of cerebral hypoperfusion is

the unmasking of a normally subcritical arterial stenosis, which under such circumstances might become clinical relevant. McKhann and colleagues argue that subcritical vascular lesions can be assumed in a considerable proportion of patients especially in those with co-existing atherosclerotic disease in peripheral vascular systems (McKhann et al., 2006). Indirect evidence for the presence and unmasking of a subcritical cerebro-vascular stenosis as a cause for stroke comes from the POISE trial (Devereaux et al., 2008). Here the perioperative administration of the beta-blocker metoprolol significantly increased the risk of stroke compared to placebo. This increased risk of stroke in the treatment group was attributed to the frequent occurrence of bradycardia and hypotension in this group and hence cerebral hypoperfusion (Sear et al., 2008). Consequently perioperative stroke can be regarded as a multifactorial process, which does not only include vascular occlusion but also cerebral perfusion abnormalities. Surgery and anaesthesia as well as concomitant therapies can therefore be expected to each contribute to the mechanism of stroke.

5.5.2 The Penumbra Concept

Thrombembolic occlusion of a blood vessel in the brain leads to a reduction in cerebral blood flow (CBF), a condition called cerebrovascular ischaemia. Should this ischaemia persist oxygen supply to the affected brain area will become significantly impaired, and the partial pressure of oxygen in the affected cells will subsequently fall to or below a critical level (tissue hypoxia). The consequence of such a focal hypoxic- process will ultimately be brain injury or stroke.

The occlusion is hardly ever total and some residual perfusion almost always persists, for instance through facilitation of collateral blood vessels (Moustafa and Baron, 2008). Consequently, the degree of blood flow reduction and associated hypoxia determines the severity of injury. Mild ischaemia leads only to functional impairment, moderate ischaemia additionally suppresses metabolic activity but severe and ongoing malperfusion will finally destroy the structural integrity of the affected brain area (Hossmann, 2006). Therefore hypoxia-ischaemia will unmask the hierarchy of energy dependent cellular functions with each function being

reliant on a certain minimal blood flow (Back, 1998). In other words the impact of stroke can be expressed in terms of CBF thresholds (Figure 5.1). Two thresholds are of particular interest in this regard since they not only describe the degree of damage that has been inflicted upon the brain but also determine the fate of the affected neurons. The upper threshold describes the degree of CBF reduction (usually mild) that is sufficient to induce electrical impairment in neurons. The lower threshold in contrast is only reached when flow is severely affected and is defined as the threshold for cellular energy failure (Astrup et al., 1981).

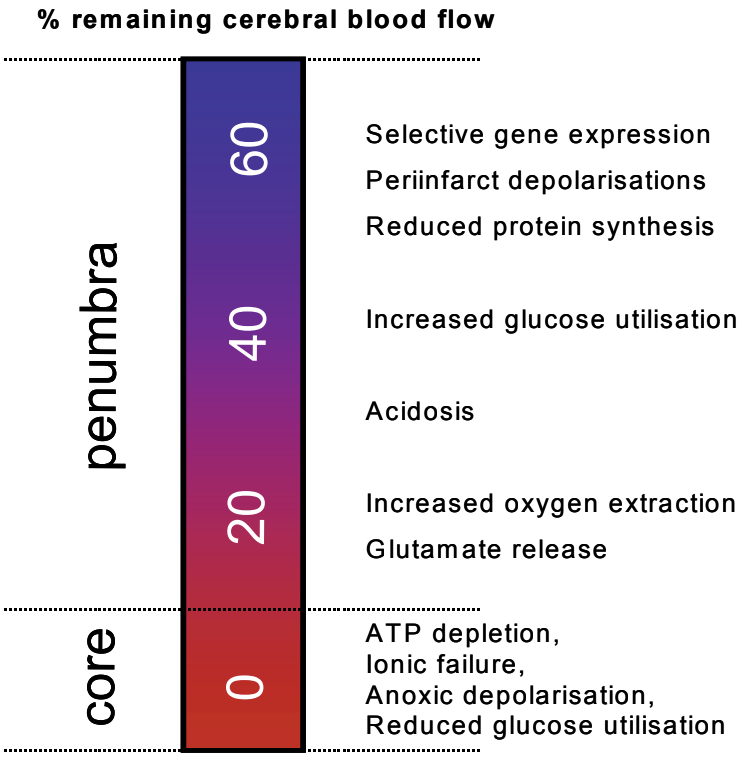


Figure 5.1: Cerebral blood flow reduction and physiological consequences
Remaining cerebral blood flow (CBF) in % from normal and biochemical and physiological consequences of CBF reduction. The figure associates also the remaining blood flow with the core and penumbral region of an ischaemic brain infarct. Figure modified after Symon (1977) and Hossmann (1994).

Tissue perfused at or below the lower threshold is termed the (infarct) “core” (Figure 5.1). Cells in the core are irreversibly damaged and frequently display the features of necrotic cell death (Moustafa and Baron, 2008). Because of the depletion of energy metabolites and the subsequent failure of ion pumps that lead to a breakdown of physiological gradients across the cell membranes (membrane failure) the core has further been characterised through the generation of anoxic depolarisations (Hossmann, 2006).

In contrast to the core, tissue with maintained energy metabolism despite exposure to hypoperfusion has been termed “penumbra” (Figure 5.2). In stroke the penumbra surrounds the infarct core and despite the selective loss of neurons its texture and morphological integrity are preserved as well as its fate remains largely undetermined for a prolonged period of time (Astrup et al., 1981; Back, 1998). The penumbra is the tissue between the upper threshold of electrical and the lower threshold of energy failure. In absolute terms, that refers to tissue exposed to CBF between 0.12 and 0.22ml/g/min as a guide (Fiehler et al., 2002). Since CBF is age-dependent (higher in the young) penumbral blood flow thresholds are better defined in relation to the normal flow of the population of interest (Obrist et al., 1975). Therefore, a penumbral area can be assumed if cerebral blood flow is between to 20% to 70% of normal.
Furthermore, it has been found that neurons in the penumbra experience graded functional loss as blood flow is more impaired and the more severe hypoxia becomes (Figure 5.1). As a result, thresholds for metabolic and electrical cellular functions have been determined: Protein synthesis is abolished prior to glucose utilisation followed by lactic acidosis development. Electrophysiologically, 30% reduction in perfusion leads to the generation of peri-infarct depolarisations and 50-70% flow impairment to reduced EEG signals and evoked potential responses. Finally, in the core a flow less than 20% of normal will lead to anoxic depolarisations (Back, 1998; Hossmann, 2006).

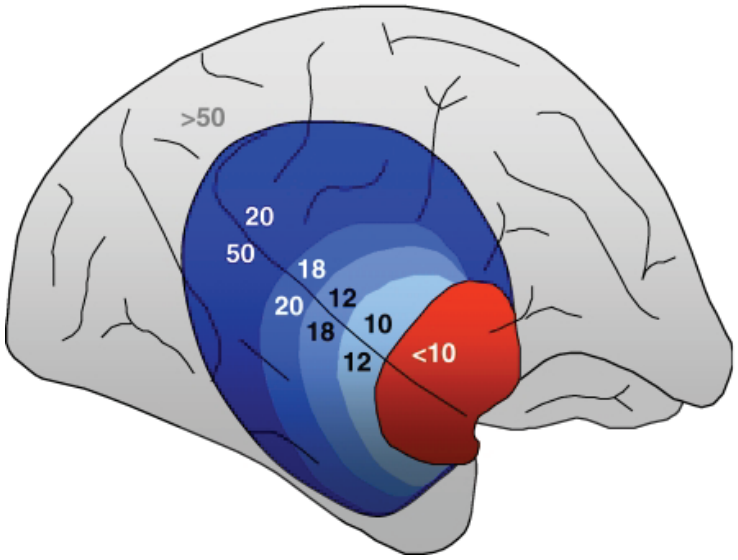


Figure 5.2: The brain after ischaemic stroke – defining the core and penumbra

Spatial pattern of cerebral blood flow (CBF) reduction following middle cerebral artery (MCA) occlusion in a baboon model of stroke. Red denotes the core; blue the penumbra and grey normally perfused brain tissue. Numbers represent CBF in ml/100g tissue/minute. CBF values greater 50ml/100g/min are assumed normal. Figure modified after Moustafa and Baron (2008).

5.5.3 The Pathways

As described above, brain tissue affected by ischaemia and subsequent hypoxia can be divided into a core and the penumbra. In the core the fate of the affected neurons is clear from the beginning of the blood vessel occlusion: Fulminant intra- and extra-cellular pathway activation will lead to rapid cell death. In the penumbra, in contrast, cells are injured but the signalling cascades that are activated are not instantaneously lethal. If neuronal death develops this process will take up days to build up rather than hours in the core. The penumbra has therefore been regarded as potentially salvageable since it is currently understood that the damaging pathways remain reversible (Weinberger, 2006).

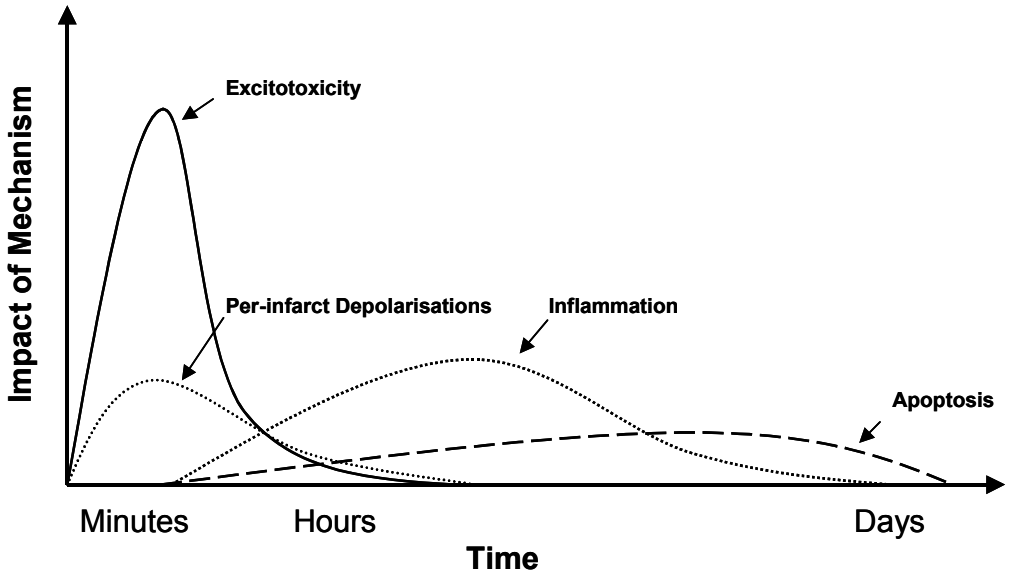


Figure 5.3: Time course of events contributing to the ischaemic cascade

This figure depicts the impact and time course of the four main pathophysiological principles causing neuronal damage and death after stroke ('ischaemic cascade').

Figure modified after Dirnagl (1999).

Over recent years some of those pathways have emerged to be particularly important for the development of neuronal injury and subsequent death. They are distinct in their temporal profile after the insult as well as in their complex signal transduction. Because these cascades are initiated by ischaemia they have been summarised under the term 'ischaemic cascade' (Figure 5.3) (Dirnagl et al., 1999; Weinberger, 2006).

Dirnagl and colleagues (Dirnagl et al., 1999) reviewed events leading to early and delayed cell death during stroke and identified four main mechanisms (Figure 5.4): 1) Excitotoxicity; 2) peri-infarct depolarisation; 3) inflammation and 4) apoptosis. Mergenthaler and colleagues added free oxygen radicals (ROS) and tissue acidosis (Mergenthaler et al., 2004).

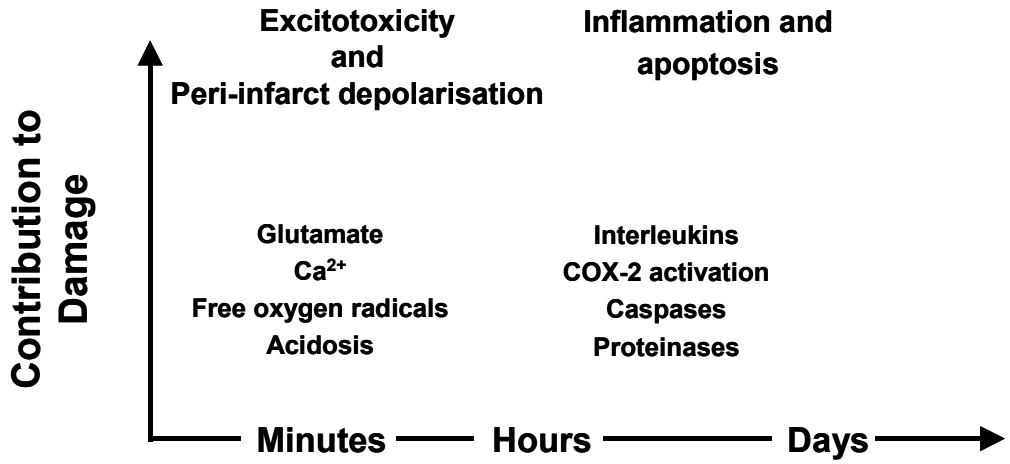


Figure 5.4: Time course of damaging mechanisms and involved mediators

Cerebral ischaemia induces a cascade of events that damage brain cells. The figure shows the involved major mechanisms and their mediators in relation to the time course of injury.

Figure modified after Dirnagl (2003).

5.5.3.1 Peri-infarct depolarisation

Peri-infarct depolarisation develops within minutes after a severe insult to the brain (Dirnagl et al., 1999). The neurons of the core depolarise because of a shortage of energy. The associated failure of ion pumps prevents re-uptake of potassium into the cells, which then accumulates in the extracellular space. Moreover, as another consequence of the depolarisation large amounts of glutamate are released into the synaptic cleft (Mergenthaler et al., 2004). Glutamate then accumulates outside the cells particularly since the shortage of ATP impairs glutamate re-uptake transporters. The surplus of glutamate and K^+ subsequently spreads from the core into the penumbra. There it induces more excitation and therefore propagation and spreading of the depolarisation (Mergenthaler et al., 2004). The repetitive depolarisations in the infarcted region as cause and consequence of neuronal damaging events are called "peri-infarct depolarisations" (Dirnagl et al., 1999; Mergenthaler et al., 2004).
In contrast to the neurons in the core, which cannot repolarise after anoxic depolarisation, the penumbra maintains its capability of repolarisation (Dirnagl et al., 1999; Bramlett and Dietrich, 2004). This presents an additional threat to the penumbral cells because of the energy consuming nature of the processes involved that might further aggravate the injury.

5.5.3.2 Excitotoxicity

Excitotoxicity is strongly linked to the action of extracellular glutamate and resulting cellular Ca^{2+} overload.
Hypoxia-triggered membrane depolarisation activates voltage gated Ca^{2+} channels. This process in turn increases the concentration of intracellular Ca^{2+}, which subsequently induces glutamate release (Small et al., 1999). The release together with the impaired re-uptake leads to an accumulation of glutamate in the extracellular space within minutes after hypoxic-ischaemic brain injury (Dirnagl et al., 1999). The extracellular glutamate then stimulates glutamate receptors in the neuronal cell membrane and consequently intracellular damaging pathways.

Glutamate receptors can principally be divided into metabotropic (G-protein coupled) and ionotropic receptors (Small et al., 1999). Especially the role of ionotropic receptors has been suggested to play a key role in glutamate mediated excitotoxicity. Ionotropic glutamate receptors are ion channels and three main classes have been identified so far: 1) N-methyl-D-aspartate (NMDA) receptors, 2) α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors and 3) kainate (KA) receptors (Small et al., 1999; Weinberger, 2006).

NMDA receptors are highly permeable to Ca^{2+}, Na^{+} and K^{+}. Na^{+} influx through NMDA receptors primarily depolarises the cell but has also been associated with altered neuronal osmotic homeostasis leading to cellular swelling (Small et al., 1999).

An increase of intracellular free Ca^{2+} through glutamate induced NMDA receptor opening is suggested to significantly contribute to neuronal Ca^{2+} overload, which has been identified as an important mechanism of rapid neuronal death (Won et al., 2002).

The role of AMPA and KA receptors however is less clear, but they are suggested to contribute to cascades mediating delayed cellular death (Won et al., 2002).

Neuronal free calcium overload triggers a plethora of pathways that all lead to cellular damage or even death within minutes to hours after the initiating event. Here the interaction of calcium with either protein expression or protein function has been suggested to play a key role. For instance, increasing nuclear $[Ca^{2+}]$ affects gene expression via the cAMP-response element (CRE) and CRE-binding protein (CREP) whereas increased cytoplasmic $[Ca^{2+}]$ activates protein transcription through the serum-response element (SRE) (Hardingham et al., 1998).

In addition, an increase of intracellular free calcium also affects protein homeostasis from a different angle by activating various proteases such as calpains (family of cysteine proteases), phospholipase A2, protein kinases and endonucleases (Small et al., 1999; Won et al., 2002).

Cells aim to mitigate the effects of the increased intracellular Ca^{2+} concentration. One of those mechanisms is the activation of the Ca^{2+}/Na^{+} antiporter. But instead of alleviating the Ca^{2+} effect the increase of $[Na^{+}]_i$ aggravates the injury. Na^{+}

influx - as explained above - is accompanied by water inflow. This will cause cellular swelling and subsequently structural damage to the affected neurons.
The attempt of neurons to buffer increased intracellular Ca^{2+} through uptake into mitochondria is only sufficient if the free cellular calcium concentration does not exceed a certain limit. Above that threshold further mitochondrial calcium uptake will lead to an overload of mitochondria with calcium and a subsequent increase in the permeability of the inner mitochondrial membrane. This mechanism then triggers the release of cytochrome c and the initiation of an apoptotic cascade (Small et al., 1999).
Apart from having consequences for mitochondrial membrane integrity, Ca^{2+} overload also influences the electro-chemical gradient of mitochondria. Increased Ca^{2+} will lead to mitochondrial depolarisation with subsequent inhibition of oxidative phosphorylation and ATP production. This in turn aggravates the energy deficit of the cell and reduces its chance of survival (Small et al., 1999).

5.5.3.3 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) or free radicals are generated by several physiologic reactions in the body. Because of their unpaired free electron they are highly reactive and therefore capable of rapidly altering structure proteins, enzymes, channels, membranes and DNA. This makes them potentially harmful to the cell and therefore mandates tight regulation of their intracellular concentration by means of radical scavengers like superoxide dismutase (SOD) or vitamins C and E (Dwenger et al., 1994). However, radicals apart from being potentially damaging to cells are also vital for many cellular mechanisms. For instance ROS are key effectors in defence pathways protecting cells against intruders. In addition they have also been found to have a role in intracellular signal transduction cascades (Genestra, 2007).
During the process of hypoxic-ischaemia in the brain ROS accumulate in cells and exhaust intrinsic scavenging capabilities, which subsequently leads to cellular damage (Won et al., 2002).

Radical production during stroke is reliant on three main pathways: The first pathway is calcium-dependent and therefore activated in response to calcium overload during excitotoxicity. In the second pathway ROS are generated as products of chemical reactions critically involving transition metals such as Fe^{2+} and Zn^{2+}. Finally ROS are also produced in mitochondria. Under aerobic conditions most oxygen is used for energy production via the electron transport chain in the inner mitochondrial membrane. However, even under physiological conditions a proportion of the intracellular oxygen is also metabolised in reactions that generate oxygen free radicals. This mechanism is aggravated under circumstances of hypoxic-ischaemia because of the then disrupted electron transport chain. Hence in situations of metabolic stress the remaining O_2 is increasingly used for ROS production. The radicals subsequently leak into the cytoplasm to cause structural damage (Won et al., 2002).

But it is not only ROS potentially harming cells during ischaemia, nitrogen radicals are also suggested to play a major role. Neuronal nitric oxide synthase (nNOS) is activated by increased intracellular calcium concentrations. The subsequent excess of nitric oxide (NO) is believed to contribute directly to excitotoxic neuronal damage via reaction with superoxide (O_2^-). The resulting peroxynitrate ($ONOO^-$) has been suggested to be directly cytotoxic (Radi et al., 1991).

5.5.3.4 Tissue acidosis

To maintain some basic level of ATP neurons switch their metabolism during hypoxia from aerobic to anaerobic. As a side-product of this process lactate is generated which accumulates in the compromised cell and induces acidosis once it exceeds the buffering capability of the cell (Mergenthaler et al., 2004). The precise consequences of acidosis are currently not fully understood but is has been suggested that it might lead to oxygen radical (ROS) production. There is also some evidence that acidosis interferes with protein synthesis and function (Mergenthaler et al., 2004).

5.5.3.5 Inflammation

In addition to their role in reconstruction and remodelling of the brain after injury, inflammatory pathways are currently believed to contribute significantly to cellular damage and death during stroke (Dirnagl et al., 1999). Inflammation usually develops within minutes after the hypoxic-ischaemic insult but reaches its peak only several hours afterwards (Figure 5.3) (Dirnagl et al., 1999). Triggers of the inflammatory response are, amongst others, increased intracellular Ca^{2+} concentrations, ROS and hypoxia itself. These triggers lead to an expression of pro-inflammatory genes with the subsequent production of mediators such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and platelet-activating factor (PAF). Furthermore vascular endothelial cells of the affected brain area will produce adhesion molecules, which are complementary to receptors on neutrophils. This will facilitate leukocyte adherence to the vessel walls and subsequent migration into the brain parenchyma (Dirnagl et al., 1999; Mergenthaler et al., 2004). The journey of the cells is guided by the release of chemotaxins from injured neuronal cells. The chemotaxins not only attract migrating cells but also activate resident astrocytes and microglia to release further pro-inflammatory mediators (Dirnagl et al., 1999; Mergenthaler et al., 2004). This leads to a spread of the inflammatory response throughout the injured brain area, predominantly the penumbra. There is now growing evidence that cellular activation during this process critically involves stimulation and up regulation of inducible nitric oxide synthesis (iNOS) with subsequent increased NO production as well as induction of cyclooxygenase-2 (COX-2) activity (Small et al., 1999; Mergenthaler et al., 2004).

5.5.3.6 Apoptosis

Apoptosis or programmed cell death is classically regarded as a late event in hypoxic-ischaemic stroke with gradual onset after hours and days (Figure 5.3). In contrast to necrosis, which traditionally has been viewed as the major form of accidental or pathological cell death and hence as the predominant type of death in the core, apoptosis was seen as the endpoint of death pathways in the penumbra (Small et al., 1999; Bramlett and Dietrich, 2004). However, this strict distinction of necrosis and apoptosis based on histopathological characteristics and spatial distribution has recently been challenged. Necrosis and apoptosis are now increasingly regarded as two poles of a continuous mechanism leading to cellular death after stroke (Small et al., 1999; Schaller, 2007). Whether a cell dies from necrosis or apoptosis in this process is probably dependent on the nature, and most of all intensity, of the damaging stimulus (Dirnagl et al., 1999).

Classically necrosis and apoptosis have been morphologically characterized according to criteria listed in Table 5.2.

Programmed cell death is the result of an activation of a cascade of specific enzymes, the caspases. Caspases are a family of cysteine proteases that cleave proteins next to aspartic acid residues (Boatright and Salvesen, 2003). According to their function and place in the apoptotic cascade caspases can be classified as ‘initiator’ or ‘executioner’ caspases (Boatright and Salvesen, 2003).

For the activation of initiator caspases an intrinsic pathway has been described which is distinct from an extrinsic pathway. The extrinsic pathway involves caspase-8 and is under physiological conditions responsible for the elimination of unwanted cells during development and immune system maturation. The extrinsic pathway is started by the activation of a transmembrane death receptor of the tumor necrosis factor receptor family (FAS), which is located on the outer cell membrane. FAS in turn activates caspase-8 which stimulates pro-caspases-3 and –7 to form caspases-3 and –7, respectively (Sugawara et al., 2004).

Table 5.2: Morphological Characteristics of Apoptotic and Necrotic Cell Death

Apoptosis	Necrosis
Usually individual cells	Several cells involved
Early stage:	***Early stage:***
Cell shrinkage	Cell swelling
Chromatin condensation against nuclear membrane	Modest chromatin condensation
DNA fragmentation and degradation	
DNA digestion produces small double-stranded fragments	
> fragments show distinct ladder pattern on electrophoresis gels	
Cytoplasmic organelles remain largely intact	Cytoplasmic vacuolisation
	Breakdown of organelles
	Rupture cell membrane
	Release of cellular contents
Late stage:	***Late stage:***
Disintegration of nucleus and cytoplasm into apoptotic bodies	Shrinkage and loss of nuclear chromatin
	Random single-strand DNA breaks
Phagocytosis of apoptotic bodies through immune and surrounding cells	
Mild inflammatory response	Strong inflammatory response with immune cell invasion

Modified after Oppenheim (1999).

The intrinsic pathway has been demonstrated to eliminate cells in the body in response to ionising radiation, chemotherapy or mitochondrial damage. The initiating stimuli, for example Ca^{2+} or ROS, alter the permeability of mitochondrial membranes, which subsequently leads to the release of cytochrome c into the cytoplasm (Mergenthaler et al., 2004; Sugawara et al., 2004). Cytosolic cytochrome c than forms a complex with the protein 'Apaf-1' to form an apoptosome. This apotosome is the activator for caspase-9, which as caspase-8 is capable of converting pro-aspases-3 and –7 into their active forms (Boatright and Salvesen, 2003; Sugawara et al., 2004).

Caspases-3 and -7 are currently the most extensively examined executioner caspases. Their proposed role in the apoptotic pathway is the direct interference with the transcription of genetic information for instance via inactivation of DNA repair enzymes (Mergenthaler et al., 2004).

5.6 Therapy of Ischaemic Stroke

The current management of stroke follows two main strategic paradigms: 1) prevention of stroke by modifying its risks and 2) treatment of the acute event.

5.6.1 Risk Management

The overall risks for stroke are the same regardless as to whether a patient is at home, in hospital or has been subjected to surgery (see also sections 5.2 and 5.3) (Szeder and Torbey, 2008).

Clinicians divide these risks into factors that are not modifiable (age, male sex, non-white ethnicity, family history, previous stroke) and those that are (Dahlof, 2007). The main modifiable factors are hypertension, diabetes mellitus, atrial fibrillation, carotid artery disease, hyperlipidaemia, cigarette smoking, obesity and high alcohol consumption (Alberts, 2003; Dahlof, 2007). Successful management of those risks has been found to significantly reduce the individual chance of contracting cerebrovascular accidents. In this regard management of high blood pressure (>140/90 mmHg) has become the single most important factor in risk management since studies have shown untreated hypertension to be associated with the highest incidence of stroke (Dahlof, 2007).

The use of anti-platelet agents like aspirin and clopidogrel has also been advocated to improve blood rheology. These drugs are especially recommended to prevent recurrent strokes (Alberts, 2003; Klijn and Hankey, 2003).

5.6.2 Treatment of Acute Stroke

Since acute vascular occlusion is the underlying cause for hypoxic-ischaemic stroke (see section 5.5), revascularisation of the affected brain area is considered the *conditio sine qua non* of any stroke therapy. Consequently strategies to re-open obstructed blood vessels have been explored and especially thrombolytic agents have been found to be effective. Intravenous treatment with tissue

plasminogen activator (tPA, alteplase) within 3 hours of stroke onset reduced death and disability by 44% in a placebo controlled study (Klijn and Hankey, 2003). Therefore administration of tPA is currently the only approved therapy for acute cerebrovascular accidents (Alberts, 2003). However, this treatment concept is not without problems: The main concerns about thrombolytic agents are the induction of bleeding complications like intracranial haemorrhage which has been found to occur in 6-7% of treated patients (Alberts, 2003; Moustafa and Baron, 2008). For this reason, costly diagnostic procedures like computed tomography (CT) or magnetic resonance imaging (MRI) are currently warranted in patients with suspected stroke. They are intended not only to confirm the diagnosis of stroke but also to exclude a haemorrhagic event. This is important because application of lysis therapy during an acute haemorrhage may have disastrous consequences for the patient.

The diagnostic requirements together with an often delayed time to hospital admission frequently result in a breach of the three-hour threshold for thrombolytic therapy. This precludes most stroke patients in daily clinical practice from an effective therapy. Studies have estimated that in the USA only 2-3% of patients with stroke are currently treated with tPA (Alberts, 2003). The usefulness of the three-hour cut-off for tPA application has recently been questioned on the basis of studies showing beneficial effects of alteplase up to six hours after stroke onset (Moustafa and Baron, 2008). Nevertheless, even if new guidelines would recommend a more liberal use of lysis therapy, it is unlikely that substantially more patients would receive it.

Moreover, patients suffering from stroke in the perioperative period are altogether excluded from thrombolysis due to concerns of severe bleeding complications (Szeder and Torbey, 2008). This leaves a significant patient population without the option of the currently most effective therapy. Outcome in these patients therefore relies completely on their intrinsic ability to dissolve obstructive blood clots or to open collateral blood vessels (Kidwell et al., 2003). They would most likely benefit from a therapeutic concept that could strengthen the resistance of neurons against hypoxia. The aim of such a therapy would be to allow brain tissue to withstand the effects of stroke until re-opening of affected blood vessels has

occurred. Such a concept is pursued with the introduction of the idea of neuroprotection into the search for treatments of stroke.

5.6.3 Neuroprotection

Neuroprotection is a therapeutic concept that aims to limit the deleterious effects of ischaemia on neurons in the affected brain area (see section 5.5.3) (Green and Shuaib, 2006). Since the core of the brain tissue subjected to a cerebrovascular accident is regarded as unsalvageable the target of neuroprotective treatments is the penumbra with its undecided fate (Back, 1998; Weinberger, 2006; Moustafa and Baron, 2008).

Neuroprotection in its strictest definition is a generic term for different strategies that are distinct for their time of application in relation to the onset of stroke (Figure 5.5). Treatments can be given either before (preemptive) or immediately after the start of ischaemia (neuroresuscitation) as well as in the reperfusion period (Fukuda and Warner, 2007). Preemptive therapy is now known as preconditioning, whilst treatment administered during reperfusion is termed postconditioning (Ferdinandy et al., 2007; Zhao, 2007). However, currently in most of the literature neuroprotection is being used as synonym for neuroresuscitation (Fukuda and Warner, 2007).

Neuroprotection, especially pharmacologically induced, can either be direct or indirect. Whilst neurons are the targets of direct treatments, indirect therapies alter the conditions brain cells are exposed to. Drugs that alter cerebral blood flow and therefore help to maintain cellular oxygen supply are examples for indirect protection. Since the focus of the current study is on direct neuroprotection only mechanisms concerned with this principle will be discussed here.

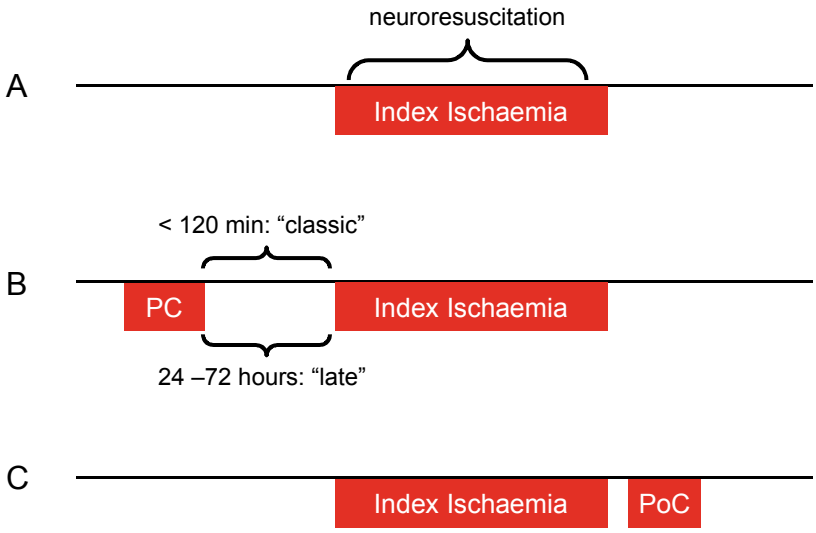


Figure 5.5: Neuroprotective strategies

This figure depicts the possible application of neuroprotective strategies in relation to the damaging ischaemic insult (index ischaemia; InI).

A) Neuroresuscitation: Application of neuroprotective treatments during the damaging period. B) Preconditioning (PC): Brief exposure to a sublethal insult (preconditioning stimulus; PCS) increases tissue resistance to a subsequent ischaemic (InI) event. Early (classical) PC: short time interval (hours) between PCS and InI. Delayed or late PC: Prolonged time interval (days) between PCS and InI. C) Postconditioning (PoC): Brief cycles of a sublethal insult in the period immediately after InI.

Figure modified after Ferdinandy (2007).

5.6.3.1 Preconditioning

Preconditioning describes the principle of applying subthreshold damaging stimuli to tissues with the aim to induce resistance against a subsequent more severe insult (Dirnagl et al., 2003; Steiger and Hanggi, 2007). Classically ischaemic preconditioning was evoked by temporarily interrupting the blood supply to the organ of interest, e.g. the heart (Murry et al., 1986). However, recent research has also found that the heart can be protected against the consequences of coronary occlusion by means of remote preconditioning. Here myocardial protection is induced by exposing a remote organ such as gut or kidney to brief episodes of ischaemia and reperfusion (Gho et al., 1996). In addition there is now evidence indicating that remote preconditioning might also be applicable to the brain (Dave et al., 2006).
A detailed account on preconditioning will be given in the next chapter of this introduction (chapter 5.7).

5.6.3.2 Neuroresuscitation

The inevitable prerequisite of all neuroresuscitation is the reinstitution of sufficient blood flow to the infarcted brain area. However, since the damaging pathways of the 'ischaemic cascade' might be active until well after reperfusion has been achieved and are not terminated by mere tissue reoxygenation, treatments have long been sought to reduce the impact of these mechanisms (Fukuda and Warner, 2007). Therefore so far all drugs used with potential neuroprotective properties were aimed against specific targets to interrupt the injury-mediating signal transduction cascade (Figure 5.4 and 5.6) (Ferriero, 2005). Amongst compounds that were successful in animal models and subsequently tested in clinical trials were NMDA receptor antagonists, calcium channel blockers, anti-inflammatory agents, γ-aminobutyric acid (GABA) agonists, growth factors and antioxidants (Green and Shuaib, 2006; Savitz and Fisher, 2007). Unfortunately, all but the ROS scavenger NXY-059 showed disappointing results in those trials (Lees et al., 2006). However, in a follow-up trial also NXY

failed (Shuaib et al., 2007). In response to this failure researchers determined some reasons why neuroprotection might fail when given after the onset of stroke in man: It was suggested that the patient selection for the trials was too broad-based and disregarded the fact that different circumstances might involve different signalling pathways (Savitz and Fisher, 2007; Hussain and Shuaib, 2008). Also, achieving the same and therefore sufficient drug concentration in the human brain as in test animals was found to be difficult. This was not only because of drug toxicity but also because of a lack of ability to monitor effective brain concentrations (Savitz and Fisher, 2007; Hussain and Shuaib, 2008). Moreover, it was appreciated that the achievement of effective concentrations of neuroprotectants in the brain was dependent on the level of reperfusion established. Finally, timing of treatment implementation was regarded as another key factor to success since too late an application might have missed the window of opportunity with the penumbra already converted to core tissue (Hussain and Shuaib, 2008). The preemptive treatment (preconditioning) in high-risk patients, for instance in patients prior to cardiovascular surgery, might offer an alternative since cerebral perfusion can then be expected to be still grossly intact. Preconditioning should preferably be done with compounds that readily cross the blood brain barrier and whose brain concentrations could be monitored (see below).

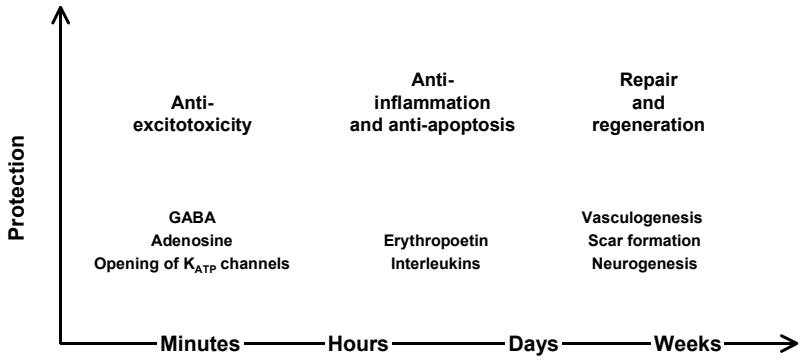


Figure 5.6: Main principles of endogenous neuronal protective and repair mechanisms

The mechanisms are depicted in relation to their suggested time of involvement in cellular defensive strategies and repair mechanisms.

Figure modified after Dirnagl (2003)

5.6.3.3 Postconditioning

Postconditioning is a novel concept that has been developed only recently (Zhao et al., 2003). It consists of short bursts of perfusion impairments in the reperfusion period after a lethal event (Zhao, 2007). It was first described by Zhao and colleagues in 2003. These authors found in dogs that short periods of coronary artery re-occlusion in the reperfusion phase after myocardial infarction significantly reduced myocardial damage (Zhao et al., 2003). In their experiments postconditioning was nearly as effective as myocardial preconditioning.

Subsequent research has demonstrated myocardial postconditioning also in other species such as rats (Obal et al., 2005), mice (Kin et al., 2005), rabbits

(Krolikowski et al., 2006), pigs (Iliodromitis et al., 2006) and even man (Staat et al., 2005).

In 2006 Zhao and colleagues (Zhao et al., 2006) demonstrated a beneficial effect of postconditioning also on rat brains subjected to focal ischaemia. However, data about effectiveness and the involved pathways are so far are only limited and the clinical usefulness of this principle elusive (Zhao, 2007).

5.7 Preconditioning

Preconditioning is the concept of achieving organ tolerance against critical ischaemia through brief subcritical or pharmacological challenges (Dirnagl et al., 2003; Steiger and Hanggi, 2007).

It started to gain widespread attention in 1986 after Murry and colleagues found a reduction of myocardial infarct size in dog hearts that had been pre-treated with short periods of ischaemia prior to prolonged coronary artery occlusion (Murry et al., 1986). Despite the fact that reports of experimentally-induced tolerance had been published before, the Murry paper marked the beginning of a new era of research (Dirnagl et al., 2003). Although originally a cardiac domain the potential of preconditioning for tissue protection was quickly realised also by scientists from other fields. However, most of the groundbreaking work was still done in cardiology laboratories.

In their experiments Murry and colleagues had used four sequences of five-minute coronary occlusion followed by five-minute reperfusion immediately before a forty-minute myocardial ischaemia to induce cardioprotection (Murry et al., 1986). From these experiments it became obvious that myocardial cells were able to adapt swiftly to acute changes in blood supply. Subsequent work not only confirmed Murry's data but also found this response being a transient phenomenon lasting only a few hours (Kuzuya et al., 1993). However, in their study Kuzuya and colleagues also demonstrated a recurrence of tolerance twenty-four hours after the preconditioning stimulus, hence the presence of a second phase of protection (Kuzuya et al., 1993).

It is now established that in the heart tolerance towards ischaemia induced by preconditioning follows a distinct pattern: There is an early phase of protection termed in reference to Murry's description 'classic' or 'early' preconditioning and a late phase that is present between twenty-four and seventy-two hours after pre-treatment. For the late phase the terms 'second window of protection', 'delayed preconditioning' or 'late phase preconditioning' are in use in the literature (Ferdinandy et al., 2007). The protection conferred to cardiomyocytes in the second window is usually less pronounced than that from the early phase and an involvement of different pathways in both phases has been suggested (Zaugg et

al., 2003). In contrast to the early phase, the second window of protection has been associated for instance with substantial *de novo* synthesis of proteins (Zaugg et al., 2003).

Soon after the discovery of preconditioning in preclinical studies scientists started to explore whether this phenomenon could be observed under clinical circumstances as well. Up to now there have been several studies reporting preconditioning in cardiac patients (Rezkalla and Kloner, 2007). For instance there is evidence that the severity of myocardial damage is attenuated and the outcome of patients improved when they had experienced pre-infarct angina (Kloner et al., 1995; Kloner et al., 1998). Moreover, data from patients after coronary balloon angioplasty as well as off-pump coronary bypass surgery suggests, that short periods of coronary occlusion during these procedures reduces the deleterious effects of prolonged ischaemia in man (Argaud et al., 2004; Karthik et al., 2004).

Because of the limited therapeutic options for stroke and encouraged by the promising results from cardiac laboratories, neuroscientists in the early 1990s started to systematically explore preconditioning as a means of brain protection.

Kitagawa and colleagues were the first who demonstrated preconditioning in hippocampal neurons (Kitagawa et al., 1990). They had subjected gerbils to cerebral ischaemia for five minutes and measured the number of dead cells in the CA1 hippocampal region. Hippocampi from animals that had received a short period of non-damaging ischaemia twenty-four hours earlier presented a reduced number of dead cells compared to control. In a subsequent study using the same model the authors demonstrated significant protection not only in other hippocampal regions but also in the cerebral cortex, the caudoputamen and the thalamus (Kitagawa et al., 1991). The phenomenon of tolerance in the brain induced by preconditioning was later confirmed in other animal models of global and focal ischaemia (Kirino, 2002). These studies largely corroborated the results of research in the field of cardioprotection and many similarities between hearts and brains in their responses to ischaemia have subsequently been reported (Kirino, 2002; Zaugg et al., 2003). However, apart from these similarities, it became also clear that some mechanisms were distinct in the brain. For instance,

Kato as well as Barone and colleagues found the time course of cerebral protection after preconditioning in whole animal experiments different from that described in heart (Kato et al., 1991; Barone et al., 1998). They were unable to find evidence for an early window of protection but rather showed that protective mechanisms started to become effective one day after the preconditioning stimulus. Furthermore compared to cardiac tissue they were able to demonstrate more sustained protection that could last for as long as seven days (Kato et al., 1991; Barone et al., 1998). This finding has important consequences for clinical practice especially for patients with an increased risk of contracting a stroke (see 5.2 to 5.4). It suggests for example, that cardiovascular surgical patients would be unprotected in the early perioperative phase, a period associated with highest risk of thrombembolic events.

Shortly after the demonstration of the phenomenon of preconditioning in animal brains neuroscientists started to search for evidence of its occurrence in man as well. Although limited there are some data now indicating that neuronal preconditioning might exist in patients (Johnston, 2004). However, all studies in man are hampered by the fact that experimental temporary cerebral blood flow interruptions similar to intermittent coronary artery occlusions in hearts are not possible due to ethical considerations. Brain tissue is exceptionally sensitive to hypoxic-ischaemia so that the risk for volunteers even under controlled conditions in a laboratory would be unforeseeable (Kirino, 2002). Therefore most information so far is gained from clinical studies comparing the outcome of stroke victims with and without subcritical transient ischaemic attacks (TIAs). TIA prior to stroke could alleviate neurological symptoms as well as improve outcome and possibly reduce infarct volume (Johnston, 2004). Moreover, some additional data suggested an even better outcome if TIA and stroke were separated by some time, usually days (Johnston, 2004), again arguing against an early protective phase.

5.7.1 Pharmacological (Anaesthetic) Preconditioning

Although ischaemic preconditioning is regarded as the archetypical form of preconditioning and suggested to be an evolutionary conserved strategy of organisms to cope with restricted substrate delivery, recent research has shown, that preconditioning is also possible by the application of other stimuli (Dirnagl et al., 2003; Gidday, 2006). Gidday stated that every mechanism with the potential of causing brain injury could also induce protection when applied at right (lower) concentrations (Gidday, 2006). Therefore physical stimuli (hyperthermia), exogenously applied substances (inflammatory mediators, glutamate, ROS), changes of endogenous conditions (hypoglycaemia, spreading depressions, epilepsy) as well as chemically available drugs have been shown to induce neuroprotection via preconditioning (Kirino, 2002; Gidday, 2006; Steiger and Hanggi, 2007).

The application of drugs for induction of cerebral tolerance has been termed ‘pharmacological preconditioning’ (De Hert, 2004). Here inhalational anaesthetics have been regarded as a particularly interesting class of compounds because of the long-term experience clinicians have in their use as well as their favourable safety profile. They are capable of rapidly inducing a variety of clinically important effects, which they normally achieve in a dose-dependent and reversible manner (Campagna et al., 2003). Moreover, since the target of preconditioning is the brain one concern associated with other classes of drugs namely the ability to cross the blood-brain barrier (BBB) in sufficient concentrations can be neglected with anaesthetics. It is even possible to assess brain concentrations of anaesthetics by relatively simple means in daily practice. For instance, the degree of sedation or depth of anaesthesia a person experiences after inhalation of an anaesthetic gas is directly correlated to the achieved cerebral concentration. This concentration is determined by the partial pressure of the anaesthetic in the brain. However the cerebral partial pressure of an anaesthetic is directly proportional to its partial pressure in the blood, which itself is a function of the partial pressure of the agent in the alveoli (P_A). It follows that the achieved brain concentration is directly proportional to P_A and hence to the alveolar concentration (Peck and Williams, 2000). Measuring the end-tidal concentration of an anaesthetic provides therefore

a good estimate for the achieved brain concentration of that anaesthetic (Sice, 2005).

The most commonly used inhalational anaesthetics worldwide today are the nitrate derivative nitrous oxide (N_2O) as well as the ethers isoflurane, desflurane and sevoflurane. The latter group had largely replaced the hydrocarbon halothane as the leading volatile by the turn of the millennium (Campagna et al., 2003). As with the recognition of potential problems with N_2O such as pollution (green house gas) and neurotoxicity in developing brains clinicians have sought for alternatives (Soriano and Anand, 2005). Consequently, the noble gas xenon, which anaesthetic and analgesic properties are known for some time, has gained much attention recently. Although burdened with high production costs mandating a special delivery system, xenon has a favourable pharmacokinetic and -dynamic profile that is in contrast to the properties of other inhalational anaesthetics (Sanders et al., 2003).

There is now a growing body of evidence that the anaesthetics desflurane, halothane, isoflurane, sevoflurane and xenon all possess the ability to precondition neuronal tissues either *in vivo* or *in vitro* or both. Tables 5.3 and 5.4 give a comprehensive overview about studies investigating the preconditioning effects of those anaesthetics.

In whole animals isoflurane so far is the most extensively studied agent and has been shown to be an efficient preconditioner. It has been found effective in different species as well as in different models of cerebral ischaemia (Table 5.3). However to this point preconditioning with isoflurane could only be shown in males and seems therefore gender specific (Clarkson, 2007; Kitano et al., 2007; Wang et al., 2008a). In addition there is growing evidence that isoflurane is neurotoxic in neonates and infants (Mellon et al., 2007; Sanders et al., 2009).

In contrast to isoflurane, data on the preconditioning effect of sevoflurane in neurons is limited but evidence suggests that it is capable of inducing tolerance against the damaging effect of global ischaemia in rats (Payne et al., 2005). Halothane on the other hand has mostly been studied under conditions of focal ischaemia and demonstrated to be protective there (Clarkson, 2007; Wang et al., 2008a).

In addition to *in vivo* studies, recent *in vitro* experiments established halothane, isoflurane, desflurane and sevoflurane as preconditioners of brain slices and neuronal cultures against the deleterious effects of several neuronal injury-inducing stimuli (Table 5.4).

Also, there is mounting evidence now that the noble gas xenon has organo-protective properties and is capable of preconditioning neuronal tissues. A study by Ma and colleagues (Ma et al., 2006) for instance explored the preconditioning effect of xenon on cell injury induced by oxygen-glucose deprivation in neuronal-glial cell co-cultures. They found that two-hours of xenon exposure twenty-four hours prior to the damaging stimulus reduced cell injury in a concentration-dependent manner. Moreover, in the same study the authors were able to show that xenon preconditioning reduced the size of a hypoxic- iachaemic infarct in seven-day old rats significantly (Ma et al., 2006).

These data indicate that pharmacological (anaesthetic) preconditioning is as effective in neuronal tissue as it is in hearts, although with a different time-course. However, the cellular mechanisms involved in anaesthetic-induced neuronal preconditioning remain highly elusive.

Table 5.3: Anaesthetic Preconditioning - *in vivo*

Agent	Species	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Isoflurane (fixed dose)	Dog	GI	yes	n/d	↓ neurological deficit preservation of CaMKII	Blanck 2000
Isoflurane (fixed dose)	Rat	FI	n/d	yes	↑ iNOS	Kapinya 2002
Isoflurane (dose-effect)	Rat	FI	n/d	yes	↓ neurological deficit ↓ infarct volume K_{ATP}-dependent	Xiong 2003
Isoflurane (fixed dose)	Rat (male)	FI	n/d	yes	MAPK dependent ↓ neurological deficit ↓ infarct volume	Zheng 2004
Isoflurane (fixed dose)	Rat (neonatal)	FI	n/d	yes	↔ mortality rate ↓ infarct volume iNOS dependent	Zhao 2004
Isoflurane (fixed dose)	Rat	FI	yes	n/d	↓ neurological deficit ↓ infarct volume adenosine A1-receptor dependent	Liu 2006
Isoflurane (dose-effect)	Rat	FI	yes	n/d	↓ cortical cell death ↑ neuromotor score doses < 1.5 MAC more beneficial	Nasu 2006

Table 5.3 (cont.): Anaesthetic Preconditioning - *in vivo*

Agent	Species	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Isoflurane (fixed dose)	Mice (neonatal)	FI	n/d	yes	↓ Mortality rate ↓ striatal function ↔ spatial memory	McAuliffe 2007
Isoflurane (fixed dose)	Rat (neonatal)	FI	n/d	yes	↔ Mortality rate ↓ neuronal death ↑ motor coordination ↔ learning and memory ↑ Bcl-2 iNOS dependent	Zhao 2007
Isoflurane (fixed dose)	Mice (male & female)	FI	n/d	yes	↓ Infarct volume in males ↑ Infarct volume in females ↑ Akt in males	Kitano 2007
Isoflurane (fixed dose)	Rat (male)	FI	n/d	yes	↓ Infarct volume ↓ Neurological deficit ↑ Bcl-2 ↓ caspase 3 ↓ cytochrome c release	Li 2008
Isoflurane (fixed dose)	Mice (female)	FI	n/d	yes	↓ Infarct volume PC effect reversed with estradiol	Wang 2008

Table 5.3 (cont.): Anaesthetic Preconditioning - *in vivo*

Agent	Species	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Isoflurane (fixed dose)	Rat (neonatal)	FI	yes	n/d	↓ neuronal death	Sasaoka 2009
Halothane (fixed dose)	Rat	FI	n/d	yes	↑ iNOS	Kapinya 2002
Sevoflurane (fixed dose)	Rat	GI	yes	yes	preserved neuronal morphology and function	Payne 2005
Sevoflurane (fixed dose)	Rat	GI	yes	n/d	↓ neuronal death	Wang 2007
Sevoflurane (fixed dose)	Rat (neonatal)	GI	yes	n/d	↑ neuromotor function ↓ infarct size	Luo 2008
Sevoflurane + Xenon coadministration (fixed dose)	Rat (neonatal)	GI	yes	n/d	↑ neuromotor function ↓ infarct size	Luo 2008
Xenon	Rat (neonatal)	GI	n/d	yes	↓ neurological deficit ↑ pCREB, ↑ Bcl-2, ↑ BDNF	Ma 2006
Xenon (fixed dose)	Rat (neonatal)	GI	yes	n/d	↑ neuromotor function ↓ infarct size	Luo 2008

Table 5.4: Anaesthetic Preconditioning - *in vitro*

Agent	Models	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Isoflurane	PNC	OGD	n/d	yes	↓ LDH release	(Kapinya 2002)
Isoflurane (concentration-effect)	Rat Cerebellar slices	OGD	yes EC_{50}: 1.17%	n/d	Glutamate transporter dependent ***Not*** K_{ATP}-dependent	(Zheng and Zuo, 2003)
Isoflurane (fixed dose)	PNC	OGD	n/d	yes	↓ LDH release MKATP-dependent	(Kaneko 2005)
Isoflurane (concentration-effect)	Rat Hippo-campal cultures	OGD	n/d	yes	↓ neuronal death ↑ intracellular Ca2+ ↑ MAP kinase ↑ Akt	(Bickler 2005)
Isoflurane (fixed dose)	Rat Brain slice	Glutamate toxicity	yes	n/d	↓ neuronal death PKC and iNOS dependent	(Zheng and Zuo, 2005)
Isoflurane	Human Neuro-blastoma SH-SY5Y cells	OGD	n/d	yes	ERK activation ↑ Bcl-2, ↑Egr-1	(Zuo 2006)
Isoflurane (concentration-effect)	Rat Cerebellar slices	OGD	yes EC_{50} 221µM	n/d	Glutamate transporter dependent	(Wang 2007a)

Table 5.4 (cont.): Anaesthetic Preconditioning - *in vitro*

Agent	Models	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Isoflurane (concentration-effect)	PNC PC12 cells	Isoflurane induced damage	yes	n/d	↓ cell death	(Wei 2007)
Isoflurane (concentration-effect)	Mouse Microglial cells	LPS + IFNγ	yes	n/d	↓ glutamate release, PKC dependent, ↓ iNOS expression	(Xu 2008)
Isoflurane	Hippo-campal neurons	OGD	n/d	yes	↑ cell viability, ↑ HO-1 expression	(Li 2008a)
Isoflurane (concentration-effect)	Hippo-campal neurons	OGD	n/d	yes	↓ cell injury, HIF-1α activation, ERK1/2 activation. ↑ iNOS expression	(Li 2008b)
Isoflurane (fixed dose)	NGCC	OGD	n/d	yes	PC MT-I/II dependent	(Edmands and Hall, 2009)
Halothane (fixed dose)	PNC PC12 cells	Isoflurane induced damage	yes	n/d	↓ cell death	(Wei 2007)
Halothane (concentration-effect)	Rat Cerebellar slices	OGD	yes EC_{50} 173μM	n/d	Glutamate transporter dependent	(Wang 2007a)

Table 5.4 (cont.): Anaesthetic Preconditioning - *in vitro*

Agent	Models	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Sevoflurane (concentration-effect)	Rat Hippo-campal slices, cellular recording	hypoxia	yes	n/d	↑ neuronal function, MKATP-dependent	(Kehl 2004)
Sevoflurane (fixed dose)	Rat Hippo-campal slices, cellular recording	OGD	yes	n/d	MKATP-dependent, ↓ morphologic changes	(Wang 2006)
Sevoflurane (high fixed dose)	PNC PC12 cells	Isoflurane induced damage	yes	n/d	no effect	(Wei 2007)
Sevoflurane (concentration-effect)	Rat Cerebellar slices	OGD	yes EC_{50} 184µM	n/d	Glutamate transporter dependent	(Wang 2007a)
Sevoflurane (concentration-effect)	Rat Hippo-campal slices	Hypoxia, Intra-cellular recordings	yes	n/d	↑ repolarisations ↓ depolarisations PKC dependence	(Wang 2007b)
Sevoflurane (concentration-effect)	PNC, NGCC	OGD	n/d	yes	↑ cellular viability	(Luo 2008)

Table 5.4 (cont.): Anaesthetic Preconditioning - *in vitro*

Agent	Models	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Sevoflurane & Xenon coadmini-stration (fixed dose)	PNC, NGCC	OGD	n/d	yes	↑ cellular viability, P3K dependent	(Luo 2008)
Sevoflurane (concentration-effect)	NGCC	OGD	yes	n/d	↓ LDH release, K_{ATP} and ROS dependent	(Velly 2009)
Sevoflurane (fixed dose)	Rat Hippo-campal slices,	OGD	yes	n/d	↑ FAK ↓caspase 3	(Sigaut 2009)
Desflurane (concentration-effect)	Rat Cerebellar slices	OGD	yes EC_{50} 929µM	n/d	Glutamate transporter dependent	(Wang 2007a)
Xenon (concentration-effect)	NGCC, Hippo-campal slice culture	OGD	n/d	yes	↓ LDH release, ↓ cell death	(Ma 2006)
Xenon (concentration-effect)	PNC, NGCC	OGD	n/d	yes	↑ cellular viability, P3K dependent	(Luo 2008)

Akt	Protein kinase	**LPS**	lipopolysccharide
Bcl-2	B-cell lymphoma-2 protein	**MAC**	minimum alveolar concentration
BDNF	brain-derived neurotrophic factor	**MAPK p38**	mitogen-activated protein kinase
CaMKII	Ca2+/calmodulin-dependent protein kinase	**MT-I/II**	Metal-regulatory genes, metallothioneins-I/II
DP	delayed preconditioning	**NGCC**	Neuronal-glial cell coculture
Erg-1	early growth response gene 1	**n/d**	not determined
EP	early preconditioning	**OGD**	oxygen-glucose deprivation
ERK	extracellular signal-regulated kinase	**PC**	preconditioning
FAK	focal adhesion kinase	**PC12 cells**	phaeochromocytoma neurosecretory cells
FI	focal ischaemia	**pCREB**	phosphorylated cAMP response element
GI	global iscaemia	**PKC**	protein kinase C
HIF-1α	hypoxia inducible factor-1alpha	**PNC**	primary neuronal cultures
HO-1	heme oxygenase isoenzyme 1	**P3K**	phosphoinositol-3-kinase
IFNγ	interferon gamma	**ROS**	reactive oxygen species
INOS	inducible notric oxide synthase		

5.7.2 Preconditioning Pathways

5.7.2.1 Overview

In his comprehensive review Sapolsky (Sapolsky, 2001) identified six main intrinsic principles of how neurons defend themselves against excitotoxic insults. Since excitotoxicity is a main contributor for the development of cerebral injury (see section 5.5.3.2) after hypoxic-ischaemia these mechanisms can be expected to be crucial for endogenous and drug-induced neuroprotective and preconditioning pathways as well.

The first defence mechanism Sapolsky (Sapolsky, 2001) describes is a decrease in neuronal excitability via opening of potassium channels. Indeed over the past 10 years various classes of K^+ channels have been shown to be involved in neuronal protective mechanisms. Tanabe and colleagues (Tanabe et al., 1999b) for instance showed hypoxia-induced activation of Ca^{2+}-dependent K^+ conductance in hippocampal neurons, which was viewed as a positive adaptive process. Heurteaux and colleagues (Heurteaux et al., 2004) demonstrated an increased sensitivity of two-pore K^+ channel (TREK-1) knock-out mice to ischaemia and epilepsy. Misonou and colleagues (Misonou et al., 2005) found delayed rectifier voltage-dependent K^+ channels to contribute to a reduction in neuronal excitability during periods of hypoxia and suggested a role of these channels in intrinsic neuronal protection. Also, an increasing body of evidence suggests an involvement of ATP-sensitive K^+ (K_{ATP}) channels in neuroprotective and preconditioning pathways. Trapp and Ballanyi demonstrated in 1995 that K_{ATP} channels in rat dorsal vagal neurons open in response to anoxia (Trapp and Ballanyi, 1995). These data were in agreement with studies by Heurteaux and colleagues indicating a critical participation of K_{ATP} channels in basic neuronal protective as well as preconditioning pathways (Heurteaux et al., 1993; Heurteaux et al., 1995).

The second principle Sapolsky (Sapolsky, 2001) lists is a mechanism through which neurons limit the accumulation of glutamate in the synaptic cleft. This can be achieved by either up regulation of glutamate reuptake or decreased release of glutamate from cells. Increased glutamate transporter expression has in fact been

demonstrated in experimental preconditioning paradigms (Zhang et al., 2007). Also glutamate release can be reduced for example through opening of K_{ATP} channels (Lee et al., 1996) or activation of adenosine A1 receptors (Li and Eisenach, 2005). In addition, scientists have also tried to diminish the effects of the released glutamate on NMDA receptors, which are suggested to mediate glutamate's deleterious action on neurons. Although successful *in vitro* (Kaku et al., 1991) and *in vivo* (Park et al., 1988) models, NMDA receptor antagonists have failed to show any benfit in stroke outcome in clinical trials (Clark et al., 2000; Albers et al., 2001; Warach et al., 2006).

The third and fourth principle Sapolsky (Sapolsky, 2001) describes are concerned with neuronal calcium homeostasis (see section 5.5.3). To prevent possible harmful effects of increased intracellular calcium concentrations, neurons have adopted mechanisms to limit its rise and to abolish its consequences. For example an increase of $[Ca^{2+}]_i$ via opening of voltage gated Ca^{2+} channels is partially offset by a negative feedback on Ca^{2+} influx through NMDA receptors (Vyklicky, 1993; Lieberman and Mody, 1994). This negative feedback of Ca^{2+} on NMDA receptors might be mediated via the activation of the calcium-dependent phosphatase calcineurin (Vyklicky, 1993; Lieberman and Mody, 1994). Also to reduce the damaging effects increased $[Ca^{2+}]_i$ has on cellular proteins, neurons facilitate heat-shock proteins for protection (Sharp et al., 1994).

In an attempt to reduce calcium influx under situations of hypoxia, L-type Ca^{2+} channels inhibitors have been trailed in pre-clinical and clinical settings. However, similar to the results with NMDA receptor antagonists this concept failed to show a benefit in clinical trials (Mandir et al., 2000; Fogelholm et al., 2004).

Sapolsky's fifth defence principle is concerned with mechanisms by which neurons preserve energy or increase the availability of substrates for energy metabolism. This principle, compared to all others, is the only one directly dealing with energy depletion as the cause of cellular distress under conditions of hypoxic-ischaemia.

Up regulation of glucose transporters and hence cellular glucose uptake represents one example of such a mechanism (Lee and Bondy, 1993). An additional one is glycogenolysis in glial cells with subsequent lactate production and release

(Wiesinger et al., 1997). This lactate then becomes the preferred source of energy for neurons under conditions of glucose depletion (Bliss and Sapolsky, 2001). To preserve energy *in vivo* it is now also the possible to expose the brain to mild hypothermia (33°C). This has been shown in a rat model of focal ischaemia to reduce the development of stroke and neurological deficit (Yanamoto et al., 2001).

The sixth and final defences mechanism of cells is the up regulation of anti-apoptotic proteins like bcl-2 and the subsequent cleavage of pro-apoptotic caspases (Clark et al., 1999). In addition, the proteolytic degradation of glutamate (AMPA) receptors, which reduce the neuronal vulnerability to the excitotoxic stimulus has been described (Glazner et al., 2000).

However, one of the most important intrinsic cellular defence mechanisms that contribute to energy preservation is the down regulation of neuronal excitability as mentioned above. Here K_{ATP} channels are suggested to play a crucial role since their activity has been directly linked to cellular metabolism (Ashcroft and Trapp, 2001). These channels will be discussed in more detail in the next section.

As discussed above there is evidence that all six principles might play a role in neuroprotection and possibly also in preconditioning. However, apart from determining the cascades involved in tolerance, scientists are also interested to uncover the hierarchy of the events. Therefore, Dirnagl and colleagues as well as Gidday distinguished between triggers, sensors, transducers and effectors (Table 5.5), with the triggers being the process-initiating stimuli. The sensors pick up the initiating signal and transfer it to the transducers. These integrate and transmit it to the end-effectors, which finally confer protection to the cells (Dirnagl et al., 2003; Gidday, 2006). Triggers for instance are hypoxia, ischaemia or drugs. Sensors are adenosine and adenosine A1 receptors. Interestingly, K_{ATP} channels have been categorized amongst transducers as well as effectors (Schulz et al., 2001; Dirnagl et al., 2003; Zaugg et al., 2003; Gidday, 2006). For these channels there is an increasing body of evidence indicating a pivotal role in neuronal preconditioning (for overview see Tables 5.6 and 5.7).

Table 5.5: Triggers and Effectors of Preconditioning

Triggers	Sensors	Transducers	Effectors
Hypoxia; Ischaemia; Inflammation; Chemicals	Adenosine A1 receptors; Hypoxia inducible factor (HIF); Reactive oxygen species (ROS); Excitatory amino acid transporter	ATP-sensitive K^+ (K_{ATP}) channels; Nitric oxide (NO); Erythropoetin; Protein kinase C; Tumor necrosis factor; Immediate early genes	ATP-sensitive K^+ (K_{ATP}) channels; Heat shock protein; Radical scavengers; Brain-derived neurotrophic factor

The table gives an overview over suggested main triggers, sensors, transducers and effectors of preconditioning. As can be seen K_{ATP} channels might play a key role in preconditioning. Table modified after Dirnagl (2003).

5.7.2.2 ATP-sensitive K^+ (K_{ATP}) channels

Potassium channels can be classified according to the International Union of Pharmacology XLI Compendium into four main classes depending on architectural similarities as well as phylogenetic relationships. These defined classes are voltage-gated (K_V); calcium-activated (K_{Ca}); two-pore (K_{2P}) and inward rectifier (K_{ir}) potassium channels (Gutman et al., 2003).
Adenosine 5'-triphosphate sensitive potassium (K_{ATP}) channels have been defined as a subfamily of potassium channels because of their hallmark feature of inhibition through intracellular ATP. They have been found to be an integral part of several cellular membranes but functional the most important subgroups are plasmalemmal and mitochondrial K_{ATP} channels (Ashcroft and Trapp, 2001).

5.7.2.3 Mitochondrial KATP (mKATP) channels

First evidence for the presence of mitochondrial KATP channels (mKATP) was provided in 1991 by Inoue and colleagues who identified a small conductance channel highly selective for K^+ in the inner membrane of fused giant mitoplasts prepared from rat liver mitochondria (Inoue et al., 1991). They further observed that ATP, 4-aminopyridine and glybenclamide applied to the matrix side in inside-out patch-clamp experiments could reversibly inactivate this channel. A year later Paucek and colleagues reported the reconstruction of a purified inner mitochondrial membrane potassium channel protein from rat liver and beef heart that was inhibited by ATP and glybenclamide (Paucek et al., 1992). Evidence for the existence of neuronal mKATP channels came from a study published in 2001: Here Bajgar and colleagues stated that they had partially purified and reconstituted mKATP channels from brain mitochondria (Bajgar et al., 2001).
Although some data suggest mKATP channels to be identical with $K_{ir}6.1$ this notion has been subsequently challenged leaving the molecular identity of these channels still elusive (Suzuki et al., 1997; Hanley and Daut, 2005).
Since these initial reports numerous studies have been published investigating the role of mKATP channels in organ protection and preconditioning. The attention

mKATP channels have attracted over the recent years is likely due to the fact that they are suggested to a) be involved in the regulation of mitochondrial volume (Szewczyk and Marban, 1999), b) be a limiting factor for the increase of the mitochondrial membrane potential during respiratory H^+ transport (Ashcroft and Trapp, 2001) and most importantly c) reduce ATP synthesis via mitochondrial membrane depolarisation (Holmuhamedov et al., 1998). This reduction of ATP synthesis, which resembles situations of hypoxia and ischaemia, might in turn also induce adaptive protective processes (O'Rourke, 2000b).

Channel opening is believed to be the initiating step of mKATP channel-dependent protective pathways. These pathways might involve reactive oxygen species (ROS) and protein kinase C (PKC) as messengers, which have been shown to be important for organ protection (Carroll et al., 2001; Schulz et al., 2001; Costa and Garlid, 2008).

However, the evidence for the existence of mKATP channels is met with some reservations among the scientific community concerned with K_{ATP} channels as nicely laid out in a recent comprehensive review by Henley and Daut (Hanley and Daut, 2005). In this very detailed article the authors critically appraise the currently available results, methods and techniques that build the basis for the mKATP channel hypothesis. They come to the conclusion that at this time data supporting the presence of mKATP channels are incomplete and that therefore conclusions drawn from studies based on their existence should be handled with caution.

Table 5.6: K_{ATP} Channel-Dependent Preconditioning - *in vivo*

Preconditioning	Species	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
K_{ATP} opening (diazoxide)	Rabbit	Spinal cord ischaemia	yes	yes	Improvement of neurological symptoms	(Caparrelli 2002)
Ischaemia	Gerbil	Cerebral ischaemia	yes	n/d	K_{ATP} channels and adenosine A1 receptors ***not*** involved in early phase preconditioning	(Sorimachi and Nowak, 2004)
Ischaemia	Rat	Focal cerebral ischaemia	n/d	yes	K_{ATP} channels and adenosine A1 receptors involved in preconditioning	(Yoshida 2004)
K_{ATP} opening (diazoxide)	Rat	Focal cerebral ischaemia	yes	n/d	Reduction of proteolysis and immediate early gene expression	(Jiang 2005)
Remote Ischaemia	Mouse	Cerebral ischaemia	yes	n/d	K_{ATP} channels and adenosine A1 receptors involved in preconditioning	(Rehni 2007)
K_{ATP} opening (diazoxide)	Rat	Focal cerebral ischaemia	n/d	yes	K_{ATP} channels involved in delayed preconditioning	(Mayanagi 2007)

Table 5.7: K_{ATP} Channel-Dependent Preconditioning - *in vitro*

Agent	Models	Injury	Preconditioning		Outcome / Mechanism	Reference
			early	delayed		
Chemical (3-NP)	Hippo-campal slices	Chemical	yes	n/d	K_{ATP}-dependent	(Riepe 1996)
Chemical (3-NP)	Hippo-campal slices	Hypoxia	yes	n/d	K_{ATP} and ROS dependent	(Riepe 1997)
K_{ATP} opening (diazoxide)	Primary neuronal cultures	OGD	n/d	yes	ROS and PKC dependent	(Kis 2003)
Hypothermia	Cerebellar slices	OGD	yes	n/d	K_{ATP}-dependent	(Yuan 2004)
Ischaemia, PKC, K_{ATP} opening (diazoxide, pinacidil)	Hippo-campal slices	OGD	n/d	yes	and PKC preconditioning are K_{ATP}-dependent	(Raval 2007)

n/d not determined
3-NP 3 -nitropropionic acid
OGD oxygen-glucose deprivation
PKC protein kinase C
ROS reactive oxygen species

5.7.2.4 Plasmalemmal K_{ATP} (pK_{ATP}) channels

Plasmalemmal K_{ATP} channels are located in the outer membrane of cells. They were first described by Noma in 1983 in cardiomyocytes but soon afterwards evidence for their presence was found in other tissues as well (Noma, 1983; Jenkinson, 2006). In 1984 Ashcroft and colleagues were the first to link K_{ATP} channels to a specific function; they showed their involvement in insulin secretion from pancreatic β-cells (Ashcroft et al., 1984).
Both Noma's and Ashcroft's work set the stage for the future research into the properties and functions of pK_{ATP} channels. Since their initial work has been with cardiac and pancreatic tissues it is not surprising that pK_{ATP} channels have been subsequently best characterised in those two tissues. However, evidence for the existence of pK_{ATP} channels in brains is early as well and comes from radioligand binding experiments performed in 1985 (Geisen et al., 1985).

In the cell membrane the assembly of two subunits is necessary to form a plasmalemmal K_{ATP} channel: An inwardly rectifying pore forming unit ($K_{ir}6$) is combined with a regulator unit, the sulphonylurea receptor (SUR) in a stoichiometry of 4:4. Since it is known that four $K_{ir}6$ subunits are needed to form the functional channel pore, four sulphonylurea receptors are also required, which makes the wild type pK_{ATP} channel an octameric complex (Figure 5.8 and 5.9) (Clement et al., 1997).
1995 marked a decisive year for the research of plasmalemmal K_{ATP} channels. For the first time cDNA of both a ubiquitously expressed K_{ir} ($K_{ir}6.1$) subunit and a pancreas-specific unit ($K_{ir}6.2$) was cloned and reconstituted successfully into cells (Inagaki et al., 1995a; Inagaki et al., 1995c; Sakura et al., 1995). In the same year, the sulphonylurea receptor (SUR) of pancreatic β-cells was also cloned (Aguilar-Bryan et al., 1995). This gave scientists not only new insights into the composition of pK_{ATP} channels but provided also new opportunities for functional studies.

As stated above the $K_{ir}6$ subunit of pK_{ATP} channels belongs to the class of inward rectifier potassium channels of which to date seven different subclasses are known. These are the strong rectifiers $K_{ir}2$ and $K_{ir}3$, the weak inward rectifiers $K_{ir}1$, $K_{ir}4$, $K_{ir}5$ and $K_{ir}6$ as well as $K_{ir}7$ (Isomoto et al., 1997; Nichols and Lopatin, 1997; Partiseti et al., 1998).

$K_{ir}2$ is the classical inwardly rectifier K^+ channel, whereas $K_{ir}3$ comprises G protein-activated channels. All ATP-sensitive potassium channels are subsumed under $K_{ir}6$. They are structurally related to $K_{ir}1$, which also contain a binding domain for ATP (Isomoto et al., 1997; Nichols and Lopatin, 1997). However, ATP-binding to $K_{ir}6$ occurs with an about thousand-times greater affinity than to $K_{ir}1$ (Wu et al., 2004). $K_{ir}6$ have therefore been termed ATP-sensitive whereas $K_{ir}1$ are regarded as ATP-dependent.

Two isoforms of the pore forming subunits of pK_{ATP} channels are known to date, $K_{ir}6.1$ and $K_{ir}6.2$ (Ashcroft and Trapp, 2001). $K_{ir}6.2$ is the predominant pore-forming unit in most tissues although $K_{ir}6.1$ might have a physiological role in some types of smooth muscles. Despite 71% molecular identity, the two forms display distinct characteristics (Yamada et al., 1997). Single channel conductances of $K_{ir}6.1$/SUR2B for instance are with 30pS considerably smaller than for $K_{ir}6.2$/SUR2B (70pS) (Repunte et al., 1999). Also, there is some evidence that ATP inhibits $K_{ir}6.1$ with lower affinity than $K_{ir}6.2$ (Yamada et al., 1997).

The general structure of $K_{ir}6.2$ demonstrates the prototypic topology of K_{ir} channels. It has two hydrophobic membrane-spanning domains, M1 and M2, one pore-forming loop and cytoplasmic hydrophilic N- as well as C-terminal domains (Figure 5.9) (Isomoto et al., 1997; Baukrowitz and Fakler, 2000).

Inward rectification means that a current of positive charge can pass more easily into than out of a cell. This flux is dependent on the extracellular K^+ concentration in a way that potassium conductance increases with increased external K^+ and decreases with falling extracellular K^+ levels (Lopatin and Nichols, 1996). Although potassium influx into the cell is always possible, potassium outflow becomes increasingly reduced the more positive the membrane potential becomes in relation to the K^+ equilibrium potential. This has physiologic consequences for the generation of action potentials: A potassium current that is equally possible in

both directions through the channel would immediately offset any sodium influx during depolarisation and hence significantly reduce cellular excitability (Nichols and Lopatin, 1997; Lopatin and Nichols, 2001; Lu, 2004). Therefore inward rectifiers stabilise the resting membrane potential near the K^+ equilibrium potential without impairing the cells' general excitable actions.

The property of inward rectification is caused by the effects of intracellular Mg^{2+} and polyamines on the channel. These substances act from the inside of the pore to obstruct potassium flow in an unidirectional manner (Figure 5.7) (Horie et al., 1987; Ciani and Ribalet, 1988; Fakler et al., 1994; Fakler et al., 1995). An additional mechanism contributing to rectification has been described as intrinsic gating. This means that the current flow through a channel is dependent on the state of that channel and therefore on its regulation. Opening or closing of $K_{ir}6.2$ is facilitated via cytoplasmic variations of the [ADP]/[ATP] ratio (see below) (Isomoto et al., 1997).

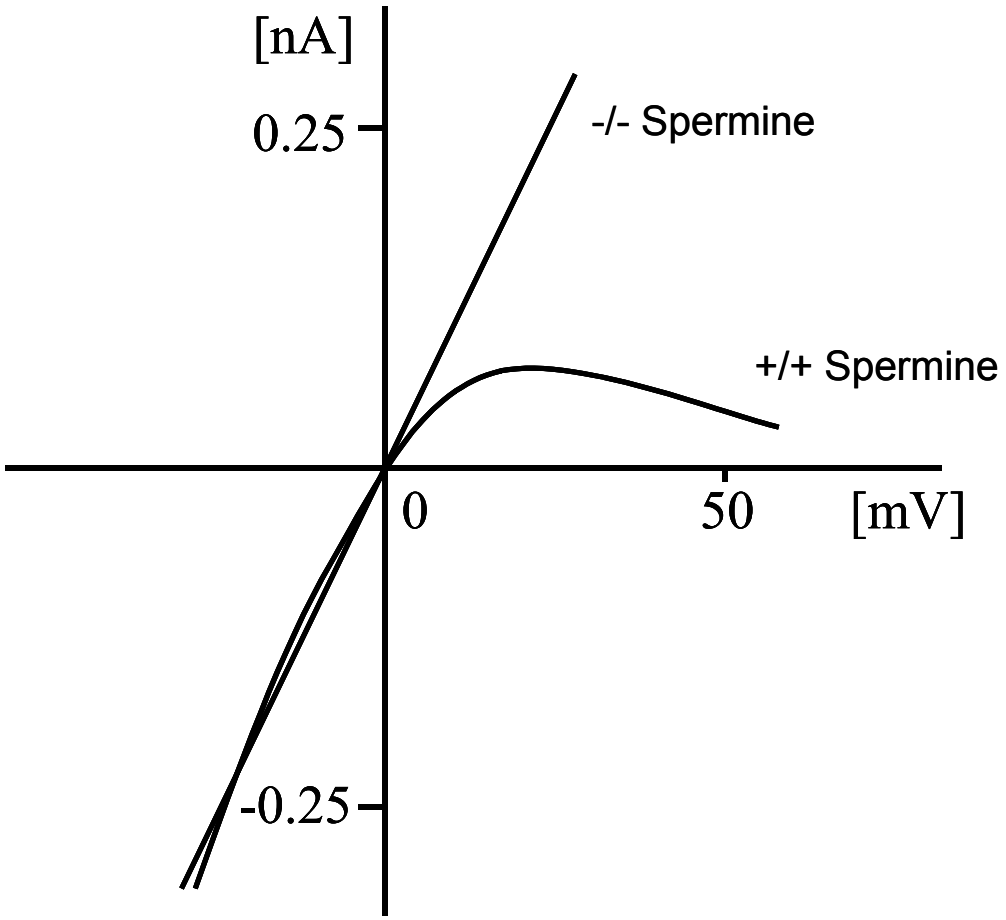


Figure 5.7: Inward rectifier K^+ channels

Figure exemplifies inward rectification. Rectification is elicited from $K_{ir}1.1$ through polyamine block. The major polyamines in cells are spermine and spermidine. A similar type of block is usually achieved under physiological conditions by the action of Mg^{2+}. Hence polyamines and Mg^{2+} represent the two main rectifying principles. Figure modified from Glowatzki (1995).

$K_{ir}6.2$ needs to combine with SUR in a 1:1 ratio to form the functional octameric pK_{ATP} channel. As demonstrated in figures 5.8 and 5.9 the four pore-forming $K_{ir}6.2$ subunits are located in the centre of the channel complex whereas SUR subunits surround them peripherally (Antcliff et al., 2005). Binding of SUR to $K_{ir}6$ has been suggested to fulfil two purposes. The first is that the assembly of the two subunits allows the translocation of the channel from the endoplasmatic reticulum (ER) to the cell membrane. Conversely, a lack of building these complexes would lead to retention of channel proteins in the ER (Burke et al., 2008). Most importantly, the combination $K_{ir}6$/SUR is also a suggested part of channel regulatory mechanisms (Burke et al., 2008).

The name sulphonylurea receptor for the regulator subunit of pK_{ATP} channels was derived after binding studies showed a high affinity of the anti-hyperglycaemic sulphonylurea glibenclamide to that subunit (Babenko et al., 1998).

The sulphonylurea receptor SUR1 had been cloned in 1995 and its molecular structure subsequently resolved (Aguilar-Bryan et al., 1995). It is a large transmembrane protein that belongs to the ATP-binding cassette (ABC) family, which function as transporters, ion channels and channel regulators. Together with the cystic fibrosis regulator protein (CFTR) and the multidrug-resistance-related protein 1 (MRP1) SUR is part of the ABC-C subfamily (Campbell et al., 2003; Gribble and Reimann, 2003a).

ABC proteins in general are composed of four structural parts, two transmembrane domains (TMD1, TMD2) and two cytosolic nucleotide-binding domains (NBD1, NBD2). SUR1 grossly follows this blueprint with TMD1 and TMD2, which contain 6 helices each. However, SUR is also somewhat atypical since it has an additional 5-helix transmembrane domain, TMD0 that most likely links the pore forming $K_{ir}6.2$ to the rest of SUR (Campbell et al., 2003; Neagoe and Schwappach, 2005; Burke et al., 2008). Therefore sulphonylurea receptors are made of a total of 17 transmembrane helices (Figure 5.9) (Babenko et al., 1998; Campbell et al., 2003; Burke et al., 2008).

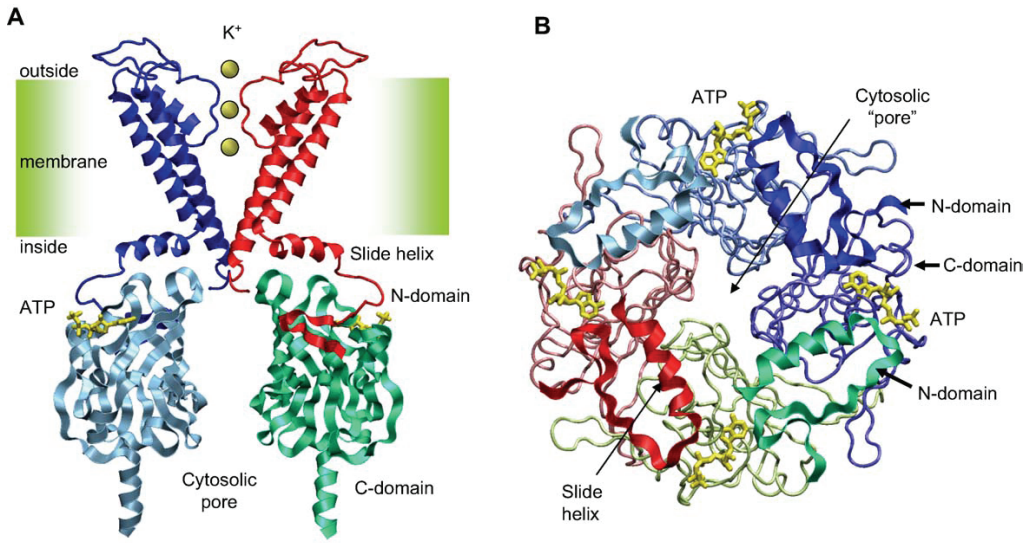


Figure 5.8: The $K_{ir}6.2$ pore forming unit

*(**A**) Side view of $K_{ir}6.2$ with the ATP binding site. ATP (yellow) is shown attached to the binding pocket. (**B**) $K_{ir}6.2$ tetramer viewed from above with the transmembrane domains removed for clarity. ATP (yellow) is attached to its binding sites. The N domain is shown in ribbon whereas the C domain is shown in backbone format.*

Figure modified after Antcliff (2005).

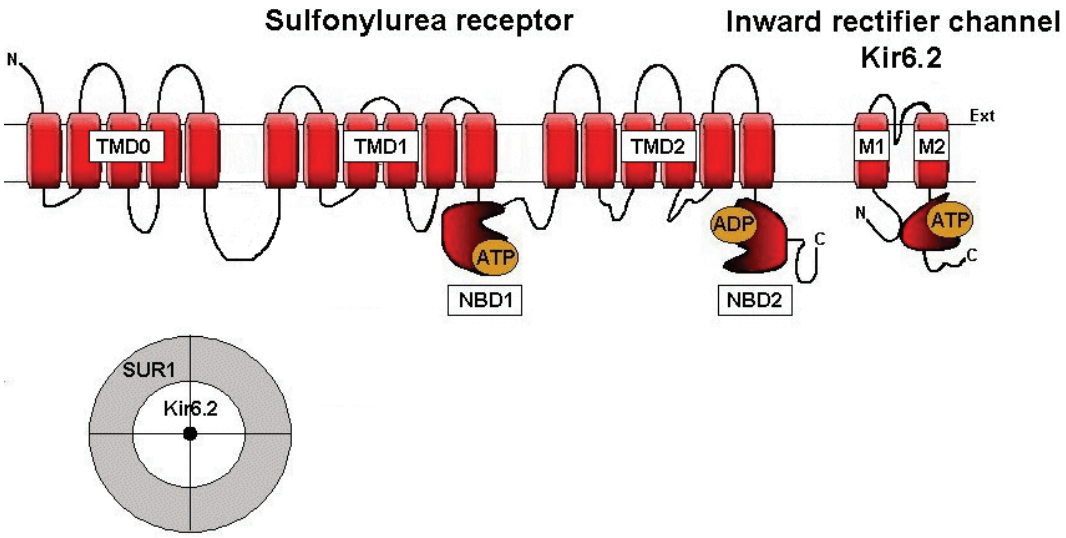


Figure 5.9: Principle structure of $K_{ir}6.2$/SUR1 K^+ channel

The upper part of the figure depicts the hypothetical organisation of a plasmalemmal K_{ATP} channel. Four inwardly rectifying $K_{ir}6.2$ and four sulphonylurea subunits (SUR1) form a functional channel octamer. $K_{ir}6.2$ has two transmembrane helices (M1, M2) and a large cytoplasmic domain containg an inhibitory ATP binding site. SUR is made of three transmembrane domains (TMD) and two cytoplasmic nucleotide binding domains (NBD). The bottom part of the figure shows a schematic view of the channel complex from above.
'Ext': external side of the membrane; figure modified after Moreau (2005).

The nucleotide-binding domains of SUR are highly conserved structures. Each NBD contains a Walker A (W_A) and a Walker B (W_B) motif, a linker motif and a histidine as well as glutamine residue (Ashcroft and Trapp, 2001; Campbell et al., 2003). All of these structures are likely involved in nucleotide binding and hydrolysis. ATP for instance when bound to SUR lies in a sandwich-like pocket formed by the W_A and W_B motifs (Campbell et al., 2003).

Two sulphonylurea receptor genes have been cloned so far, SUR1 and SUR2, from which additional splice variants are also known. Of those splice variants SUR2A and SUR2B are the most important (Burke et al., 2008). Therefore six different combinations of subunits are theoretically possible to form the main types of plasmalemmal ATP-sensitive potassium channels in tissues. This generates the possibility that pK_{ATP} channels are tissue-specific. Indeed a preponderance of some combinations could be demonstrated in various organs (Table 5.8). For instance $K_{ir}6.2$/SUR1 is the predominant K_{ATP} channel in neurons and pancreatic β-cells whereas $K_{ir}6.2$/SUR2A has been demonstrated to be the main channel in the heart. $K_{ir}6.2$/SUR2B and $K_{ir}6.1$/SUR2B can be found mainly in smooth muscle cells. $K_{ir}6.1$/SUR2A is most likely physiologically not relevant (Babenko et al., 1998; Matsuo et al., 2005; Burke et al., 2008).

Table 5.8: Subunit Composition of K_{ATP} channel Subtypes

K_{ATP} channel subtype	**Composition**
Pancreatic and neuronal type	SUR1 + $K_{ir}6.2$
Cardiac type	SUR2A + $K_{ir}6.2$
Vascular smooth muscle type	SUR2B + $K_{ir}6.1$
Smooth muscle type	SUR2B + $K_{ir}6.2$

Modified after Matsuo (2005)

There is now consensus that the $K_{ir}6.2$ subunit regulates the transport of potassium and contains the principal ATP binding site whereas SUR1 modulates channel activity (Burke et al., 2008). This is also supported by the fact that the clinically available pK_{ATP} channel openers as well as inhibitors exert their effect via interaction with SUR (Moreau et al., 2005). However, normally the channel activity is regulated by the ratio of intracellular ATP to ADP concentrations (Ashcroft and Trapp, 2001). This interaction most likely represents a complex interplay of nucleotide effects at the K_{ir} and SUR subunits. This notion is supported by experiments from Tucker and colleagues who examined the inhibitory potency of adenine nucleotides on truncated channels, i.e. channels which do not need SUR to form a functional molecule. The results showed that both ATP and ADP potently inhibited $K_{ir}6.2$ with nearly equal K_i's of 115μM and 260μM, respectively. AMP in contrast only displayed a low affinity effect (K_i 9.2mM) (Tucker et al., 1998). The inhibitory effect of ATP and ADP is Mg^{2+} independent and does not require channel phosphorylation (Ashcroft and Trapp, 2001).

In addition to the inhibitory effect of magnesium-free ATP and ADP on $K_{ir}6.2$, Gribble and colleagues found that Mg-ATP and Mg-ADP opened pK_{ATP} channels. However, the potency of this stimulatory effect was less compared to the potency of inhibition displayed by these compounds (Gribble et al., 1998a). The channel opening is suggested to be mediated via nucleotide interaction with SUR1 rather than $K_{ir}6.2$ (Gribble et al., 1997b). This is supported by evidence that shows high affinity binding of ATP and ADP to NBD1 of SUR1 as well as low affinity binding to NBD2 (Matsuo et al., 2000). It is has been proposed that ATP binds only to NBD1 and NBD2 when pK_{ATP} channels are closed. However, this nucleotide binding requires the presence of Mg^{2+}. Mg-ATP is subsequently hydrolysed to Mg-ADP, which is believed to induce such conformational changes at SUR1 that ATP inhibition of K_{ir} is overridden and the channel is opened (Matsuo et al., 1999; Ashcroft and Trapp, 2001).

In cardiomyocytes as well as in neurons pK_{ATP} channels are likely to be closed under physiological conditions. However, they are suggested to open during phases of ischaemia and hypoxia when the cell is exposed to metabolic stress and

intracellular ATP concentrations are diminished. The opening of pK_{ATP} channels facilitates an outward K^+ current that shifts the membrane potential towards the potassium equilibrium potential of –80 mV (Babenko et al., 1998). This in turn limits cellular excitability and influences action potential duration and frequency. Therefore, K_{ATP} channels are suggested to couple cell metabolism to electrical activity and K^+ flux. Moreover, by stabilising the resting membrane potential they help to reduce the influx of Ca^{2+} and its possible deleterious consequences (Babenko et al., 1998; Ashcroft and Trapp, 2001).

5.7.2.5 Effects of anaesthetics on K_{ATP} channels

First evidence suggesting a possible contribution of K_{ATP} channels to the effects of the volatile anaesthetics halothane, isoflurane and sevoflurane were published in the early 1990s and started with the observation that the airway relaxing effect of halothane was dependent on opening of K_{ATP} channels (Mehr and Lindeman, 1993). Subsequent studies confirmed this initial result and it was established that halothane and isoflurane dilated blood vessels most likely via pK_{ATP} channel ($K_{ir}6.2$/SUR2B) dependent smooth muscle relaxation (Cason et al., 1994; Crystal et al., 1997; Crystal et al., 2000).

Since the knowledge about the effects of volatile anaesthetics on vascular smooth muscles was largely gained through investigations of the coronary system it is not surprising that scientists tried to characterise also the nature of the interaction of volatile anaesthetics with cardiomyocytes directly. In functional essays they not only found that the cardio-protective effect of isoflurane and sevoflurane is partially dependent on K_{ATP} channel opening they also showed in patch-clamp experiments with cardiac muscle cells that isoflurane could directly participate in pK_{ATP} channel opening (Kersten et al., 1996; Nakayama et al., 1997; Kwok et al., 2002; Mullenheim et al., 2003). In contrast, the effects of halothane on the cardiac type pK_{ATP} channel ($K_{ir}6.2$/SUR2A) in electrophysiological experiments were less clear but suggested a possible channel inhibition by halothane (Kwok et al., 2002).

So far data of volatile anaesthetic actions on K_{ATP} channels in the brain exists only for sevoflurane and only for its effect on mKATP channels. Kehl and colleagues as well as Wang and colleagues found that sevoflurane concentration-dependently preconditioned rat hippocampal slices against global hypoxia and that this effect was dependent upon activation of mitochondrial KATP channels (Kehl et al., 2004; Wang et al., 2006). Therefore, it is still elusive if and how the volatile anaesthetics halothane, isoflurane and sevoflurane interact with the predominant neuronal type pK_{ATP} channel $K_{ir}6.2/SUR1$.

One problem so far with most studies investigating the effects of anaesthetics on pK_{ATP} channels is that they lack a clear pharmacological distinction between the different pK_{ATP} channel subtypes. Cardiac type pK_{ATP} channels have a different composition compared to neuronal type channels (Table 5.8) and hence display a different pharmacology. Table 5.9 gives an overview about commonly used K_{ATP} channel agonists and antagonists and their differential actions on K_{ATP} channel subtypes.

Evidence that xenon might exert an effect on potassium channels is also scarce. So far only one study has addressed this question: In 2004 Gruss, Bushell and colleagues found that xenon has differential actions on two-pore potassium channels. Whilst they could demonstrate an activation of a member of the TREK subfamily of two-pore potassium channels (TREK-1) in response to 80% xenon, they found no effect of xenon on a channel of a different subfamily (TASK-3) (Gruss et al., 2004). However, the effect of xenon on K_{ATP} channels had not yet been investigated.

Table 5.9: Pharmacology of K_{ATP} Channel Subtypes

Agonist	**Neuronal (K_{ir}6.2/SUR1)**	**Cardiac (Kir6.2/SUR2A)**	**Mitochondrial KATP channels**
Diazoxide	Yes	No	Yes
Pinacidil	No	Yes	Yes
Antagonist			
Tolbutamide	Yes	No	No
Glibenclamide	Yes	Yes	Yes
5-HD	(Yes)	No	Yes

Yes or No describe whether the drug has an effect on the channel subtype.

5.8 Hypothesis

There is a growing body of evidence suggesting an involvement of K_{ATP} channels in pharmacological preconditioning. However, it still remains undetermined whether pK_{ATP} channels are involved in anaesthetic induced tolerance to ischaemia in neurons. The present study was conducted to address this question.

If $K_{ir}6.2$/SUR1 were to mediate preconditioning by modern inhalational anaesthetics it would be expected that those anaesthetics open K_{ATP} channels. Therefore the first part of this study examined electrophysiologically whether the anaesthetics halothane, isoflurane, sevoflurane and xenon open K_{ATP} channels in a heterologous expression system. For those compounds that were found to be pK_{ATP} channel openers it was further determined which channel subunit was targeted and by what mechanism.

The second part of this study addressed the physiological consequences of the results obtained in part one. Neuronal glial cell co-cultures were taken as a model system to verify that K_{ATP} channel opening is mandatory for preconditioning by those anaesthetics that had been established as K_{ATP} channel openers.

6. METHODS

6.1 Electrophysiological Experiments

6.1.1 Tissue Culture

Human embryonic kidney (HEK) cells are an invaluable and widely used tool for the evaluation of the pharmacological properties, signal transduction mechanisms or structure-activity relationships of many ion channels. Because of their ease of handling, reliable protein expression and low background currents they have become a well-accepted recombinant expression system for electrophysiological studies. They are especially popular in neurobiological research since they have been found to display similar characteristics to those of early differentiating neurons (Thomas and Smart, 2005). With an average diameter of 20-40μm HEK cells are considerably smaller than the other popular tool for electrophysiological studies, the *Xenopus oocyte*, which usually has a diameter of around 1mm (Sigel and Minier, 2005; Thomas and Smart, 2005). Because of the size *Xenopus ooctytes* usually allow easy generation of excised macropatches. In addition, oocytes contain intrinsically only few ion channels on their outer cell membrane, which provides them with the advantages of generating minimal noise during single channel recordings (Sigel and Minier, 2005). However despite the relative ease of use of oocytes, some experts regard the fact that they comprise non-human tissue, with consequently different enzymatic systems and signalling cascades, as a major disadvantage (Sigel and Minier, 2005). HEK cells because of their human origin have hence become the preferred tool in neuroscientific channel research.

HEK cells were sourced from American Type Culture Collection (ATCC; Manassas, USA) under the designation ‘293’, accession code CRL-1573 (Thomas and Smart, 2005).

They were stored in aliquots in liquid nitrogen until needed for experiments. When required one aliquot was removed from the store and thawed in a waterbath at 37°C for 5-10 minutes. The cell suspension was then transferred into a 50ml falcon tube. 10ml of growth medium were added slowly to reduce the likelihood

of cell damage caused by a rapid change of media. Growth medium was made of minimum essential medium (MEM; Gibco, Paisley, UK) that was enriched per 500ml stock solution with 50,000 units penicillin (Sigma, Poole, Dorset, UK), 50mg streptomycin (Sigma, Poole, Dorset, UK), 5ml non-essential amino acids (Gibco, Paisley, UK), 50ml foetal bovine serum (Gibco, Paisley, UK) and 2mM L-Glutamine (Gibco, Paisley, UK).

After centrifuging at 1100rpm for 5 minutes at 25°C the supernatant was discarded and the pellet re-suspended in 15ml growth medium. The cells were finally transferred to a T75 flask (Fisher Scientific, Loughborough, Leicestershire, UK) and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C overnight. Twenty-four hours later the attachment of the cells to the flask surface was checked under the microscope and the old growth medium was exchanged for 15ml of fresh medium. Cells were subsequently grown in an incubator at 5% CO_2 and 95% air at 37°C and passaged when about 60% confluent as evidenced by light microscopy. This was usually the case after a period of 5-7 days. Cells were used for experiments from the second to the 15^{th} passage after thawing.

Cell passaging normally included plating of one aliquot of cells destined for experiments on glass coverslips, re-growing of another aliquot in a T75 flask and storing the remaining cells in liquid nitrogen. The process of passaging was initiated by removing the growth medium from the T75 flask and replacing it with 5ml of 0.05% Trypsin-EDTA in growth medium (Gibco, Paisley, UK) to detach the cells from the flask surface. To neutralise the trypsin 5ml of growth medium was added and the suspension transferred to a 50ml falcon tube. Cells were centrifuged for 5 minutes at 1100rpm at 25°C. The supernatant was discarded and HEK293 cells re-suspended in 10ml of growth medium. The number of cells in the suspension was determined on a haemocytometer.

$2.5x10^6$ of cells were re-suspended in 15ml of growth medium and transferred to a new T75 flask. They were subsequently grown in 5% CO_2/95% air in a humidified incubator at 37°C for about a week until passaged again.

Cells for experiments were plated onto glass coverslips coated with 1mg/ml poly-D-lysine in 4-well plates (Nunc, Roskilde, DK) at a density of $2.5x10^3$ cells in 3ml growth medium per well. The remaining cells of the passage were transferred into cryovials at $4\text{-}10x10^6$ cells per vial and stored in liquid nitrogen.

After resting for 24 hours HEK293 cells dedicated for experiments were transiently transfected using the calcium phosphate method (Chen and Okayama, 1987). To each coverslip plasmids containing the coding sequences of either the wild type ($K_{ir}6.2$/SUR1), a truncated ($K_{ir}6.2\Delta C26$), a K_{ATP} channel mutant with reduced ATP-sensitivity ($K_{ir}6.2$-K185Q) or that of a related inwardly rectifying potassium channel ($K_{ir}1.1$) were added respectively (see details below). Also added was pcDNA3 containing green fluorescent protein (GFP) cDNA to be able to verify successful transfection. Cells were then incubated at 3% CO_2/ 97% air for 6-8h. Subsequently cells were washed twice with phosphate-buffered saline (PBS) and incubated in growth medium in 5% CO_2 and 95% air in a humidified incubator at 37°C for 24-96h until use for electrophysiological recordings.
Successful channel expression was verified microscopically. Since abnormal cell morphology indicates ill-health of cultured HEK cells with consequently possible erratic channel protein expression only cells with normal shape and appearance were used for experiments (Thomas and Smart, 2005). In addition, cells needed to show also green fluorescence that directly signifies protein transfection to be further used. However, green fluorescence is a rather unspecific parameter that does not allow identification of either the gene product of interest or its expression levels. Therefore electrophysiological parameters were also employed to further verify the presence of pK_{ATP} and $K_{ir}1.1$ channels.

For experiments with the wild type pK_{ATP} channel HEK293 cells were transfected with 0.25µg/well pcDNA3 vectors containing the coding sequence of mouse $K_{ir}6.2$ (Genbank D50581 (Inagaki et al., 1995b; Sakura et al., 1995)) and 0.35µg/well pcDNA3 vectors of rat SUR1 (Genbank L40624 (Aguilar-Bryan et al., 1995)).
The wild type pK_{ATP} channel requires the assembly of both $K_{ir}6.2$ and SUR1 subunits to become a functional channel that is able to migrate to the cell surface (Schwappach et al., 2000). However, truncation of the last 26 C-terminal amino acids from $K_{ir}6.2$ allows the pore-forming subunit to integrate into the plasmamembrane without the presence of the sulphonylurea subunit. This $K_{ir}6.2\Delta C26$ complex therefore forms a functional channel in the absence of SUR1 (Tucker et al., 1997).

To produce a C-terminal truncated $K_{ir}6.2$ a stop codon was introduced at the appropriate residue by site directed mutation. The resulting construct makes it then possible to attribute drug effects to actions on the different subunits (Tucker et al., 1997). The $K_{ir}6.2\Delta C26$ in pcDNA3 was used at a concentration of 5ug/ml.

To explore whether the xenon effect on the wild type pK_{ATP} channel was dependent on the binding of ATP to $K_{ir}6.2$ a pore unit was constructed that displayed reduced sensitivity to ATP. This was achieved through site-directed mutagenesis where the positive lysine residue at position 185 on the C-terminus was neutralised by introduction of glutamate (K185Q) (Tucker et al., 1997). This mutation was chosen because it had been shown that lysine in position 185 (K185) of the C-terminus is part of the ATP binding pocket of $K_{ir}6.2$ (Tanabe et al., 1999a). There is now convincing evidence for the phosphate tail of ATP to make electrostatic interactions with three positively charged residues at the binding site (Figure 6.1): The ATP α-phosphate interacts with arginine in position 201 (R201), the β-phosphate with the discussed lysine (K185) and the γ-phosphate with arginine in position 50 (R50) (Trapp et al., 2003). In previous studies it was indeed demonstrated that a $K_{ir}6.2$-K185Q mutation not only had a reduced sensitivity to ATP but also left the intrinsic open probability of the pK_{ATP} channel grossly unchanged (Tucker et al., 1998). This is important since increasing the channel's intrinsic open probability would effectively also decrease its sensitivity to ATP as shown for mutations in position 166 (cysteine to threonine, alanine or methionine) (Trapp et al., 1998). However, altering also the channel's open probability would have impaired the ability to test drug effects on that mutant and to compare it to the wild type channel.

$K_{ir}6.2$-K185Q was generated by site-directed mutagenesis using standard techniques (Stratagene, Agilent Technologies UK Ltd, Stockport, Cheshire, UK) and verified by direct sequencing. For experiments HEK293 cells were then transiently transfected with 2.5μg/ml $K_{ir}6.2$-K185Q and 0.35μg/ml SUR1 pcDNA3 plasmids.

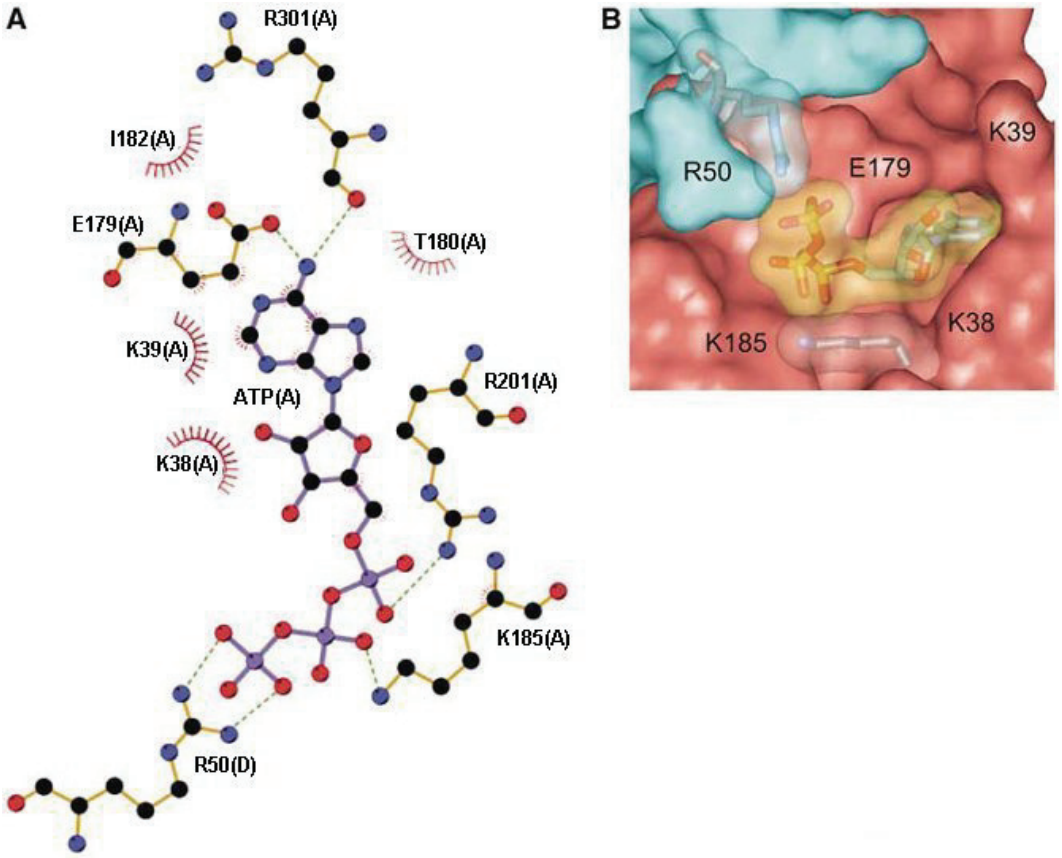


Figure 6.1: Schematic drawing of ATP interaction with K_{ir}6.2 ATP-binding site

*(**A**) Interaction between ATP and amino acid residues in the K_{ir}6.2 ATP-binding pocket; hydrogen bonds are indicated by dashed lines and hydrophobic interactions by sunbursts. Panel (**B**) shows a space-filling model of the ATP-binding pocket. The electron shells of R50, K185 and ATP are shown in transparency.*

The figure is modified after Antcliff (2005).

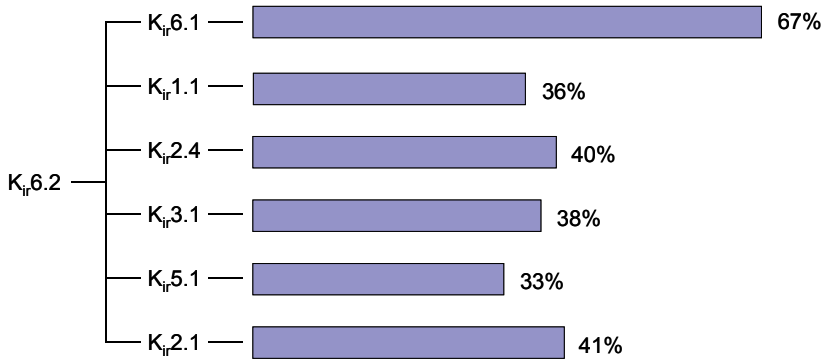
To exclude that the action of xenon on $K_{ir}6.2$/SUR1 represented a selective rather than a specific effect on channels containing ATP binding sites the influence of xenon on a different type of inwardly rectifying K^+ channel was tested.

For this purpose the renal outer-medullary K^+ channel 1 (ROMK1, $K_{ir}1.1$) was chosen. This channel is primarily located in the thick ascending limb of the loop of Henle in the kidney. Its physiological role is to participate in the regulation of the body's K^+ homeostasis through secretion of K^+ into the tubule lumen (Schulte and Fakler, 2000). The channel is mainly regulated via intracellular [H^+] and hence is pH dependent. Acidosis has been found to inhibit $K_{ir}1.1$ whereas alkalosis stimulates K^+ conductance (McNicholas et al., 1998). Although the channel is inhibited by ATP, compared to $K_{ir}6.2$ it is regarded only as ATP dependent (Wu et al., 2004). This is shown by the fact that the K_i for ATP of $K_{ir}1.1$ is in the millimolar and that of $K_{ir}6.2$ in the micromolar range (Gribble et al., 1997a; McNicholas et al., 1998).

Phylogenetically $K_{ir}1.1$ and pK_{ATP} ($K_{ir}6.2$/SUR1) channels are closely related showing considerable sequence identity (Figure 6.2) (Nichols and Lopatin, 1997). Any effect of xenon on $K_{ir}1.1$ would therefore indicate a selective interaction with proteins that are capable of binding ATP rather than a specific action on $K_{ir}6.2$.

For experiments HEK293 cells were transiently transfected with 0.5µg/well pcDNA3 vectors coding for rat $K_{ir}1.1$ (Genbank X72341).

A)



B)

```
Kir6.2_KCN      ----MLSRKGIIPEEYVLTR-----LAEDPA---------KPRYRARQRRARFVSKKGNC
KIr6.1_KCN      ----MLARKSIIPEEYVLAR-----IAAENLR--------KPRIRDRLPKARFIAKSGAC
Kir1.1_KCN      ---MNASSRNVFDTLIR--------VLTESMFKHLRKWVVTRFFGHSRQRARLVSKDGRC
Kir2.4_KCN      MGLARALRRLSGALDSGDSRAGDEEEAGPGLCRNGWAPAPVQSPVGRR-RGRFVKKDGHC
Kir3.1_KCN      --MSALRRKFGDDYQVVTTS-----SSGSGLQPQGPGQDPQQQLVPKKKRQRFVDKNGRC
Kir5.1_KCN      -MSYYGSSYHIINADAKYPG-----YPPEHIIAE----------KRRARRRLLHKDGSC
Kir2.1_KCN      MGSVRTNRYSIVSSEE------DGMKLATMAVANGFGNGKSKVHTRQQCRSRFVKKDGHC

                          50
Kir6.2_KCN      N--VKNIR--EQGRFLQDVFTTLVDLKWPHTLLIFTMSFLCSWLLFAMAWWLIAFAHGDL
Kir6.1_KCN      NLAHKNIR--EQGRFLQDIFTTLVDLKWRHTLVIFTMSFLCSWLLFAIMWWLVAFAHGDI
Kir1.1_KCN      NIEFGNVEAQSRFIFFVDIWTTVLDLKWRYKMTIFITAFLGSWFFFGLLWYAVAYIHKDL
Kir2.4_KCN      NVRFVNLGG-QGARYLSDLFTTCVDVRWRWMCLLFSCSFLASWLLFGLAFWLIASLHGDL
Kir3.1_KCN      NVQHGNLGS-ETSRYLSDLFTTLVDLKWRWNLFIFILTYTVAWLFMASMWWVIAYTRGDL
Kir5.1_KCN      NVYFKHIFG-EWGSYVVDIFTTLVDTKWRHMFVIFSLSYILSWLIFGSVFWLIAFHHGDL
Kir2.1_KCN      NVQFINVGE-KGQRYLADIFTTCVDIRWRWMLVIFCLAFVLSWLFFGCVFWLIALLHGDL

                                                185             201
Kir6.2_KCN      QNIVGLMINAIMLGCIFMKTAQAHRRAETLIFSKHAVIALRHGRLCFMLRVGDLRKSMII
Kir6.1_KCN      QNIVGLIINAVMLGCIFMKTAQAHRRAETLIFSRHAVIAVRNGKLCFMFRVGDLRKSMII
Kir1.1_KCN      QSILGVIINSFMCGAILAKISRPKKRAKTITFSKNAVISKRGGKLCLLIRVANLRKSLLI
Kir2.4_KCN      QCIAGCVLDAFVVGAVMAKMAKPKKRNETLVFSENAVVALRDHRLCLMWRVGNLRRSHLV
Kir3.1_KCN      QSILGSIVDAFLIGCMFIKMSQPKKRAETLMFSEHAVISMRDGKLTLMFRVGNLRNSHMV
Kir5.1_KCN      QSILSCIINTFIIGAALAKMATARKRAQTIRFSYFALIGMRDGKLCLMWRIGDFRPNHVV
Kir2.1_KCN      QSIVGCIIDAFIIGAVMAKMAKPKKRNETLVFSHNAVIAMRDGKLCLMWRVGNLRKSHLV

                                        301
Kir6.2_KCN      PSDLHHHQDLEIIVILEGVVETTGITTQARTSYLADEILWGQRFVPIVAEE-DGRYSVDY
Kir6.1_KCN      ATDLAN-QDLEVIVILEGVVETTGITTQARTSYIAEEIQWGHRFVSIVTEE-EGVYSVDY
Kir1.1_KCN      AETLLQ-QDFELVVFLDGTVESTSATCQVRTSYVPEEVLWGYRFAPIVSKTKEGKYRVDF
Kir2.4_KCN      RAELAR-ADFELVVILEGMVEATAMTTQCRSSYLPGELLWGHRFEPVLFQR-GSQYEVDY
Kir3.1_KCN      QRSMQT-EQFEIVVILEGIVETTGMTCQARTSYTEDEVLWGHRFFPVISLE-EGFFKVDY
Kir5.1_KCN      RKAVAK-DNFEILVTFIYTGDSTGTSHQSRSSYVPREILWGHRFNDVLEVK-RKYYKVNC
Kir2.1_KCN      KQDIDN-ADFEIVVILEGMVEATAMTTQCRSSYLANEILWGHRYEPVLFEE-KHYYKVDY
```

Figure 6.2: Human K_{ir}: Sequence identity and protein alignment

*(**A**) depicts the amino acid sequence identity of the most important K_{ir}'s in relation to K_{ir}6.2 in percent. As expected, K_{ir}6.1 shows the greatest identity with K_{ir}6.2. Figure modified after Ashcroft and Gribble (1998)*

(B) *Although K_{ir}1.1 shares only 36% identity with K_{ir}6.2, the amino acid sequences of the ATP binding site are highly similar. The numbers above the letters represent the position of the respective amino acid in K_{ir}6.2. The letters aligned in columns show amino acids of other channels in equivalent positions compared to K_{ir}6.2.*

The human sequences for K_{ir} were taken from the protein library of the National Centre for Biotechnology Information (NCBI - PubMed; Bethesda, Maryland, USA). The NCBI reference sequences were encoded in the library with NP_000516.3. To obtain images of alignments sequences were copied into ClustalW (www.clustal.org).

6.1.2 Experimental Solutions

Stock solutions of tolbutamide (50mM) diazoxide (20mM) and pinacidil (10mM) were prepared in 100mM NaOH. Each day fractions of these stock solutions were added directly to fresh experimental solutions to achieve their final concentrations. Before use experimental solutions containing test drugs were pH checked and adjusted to pH 7.3 if needed. Saturated aqueous solutions of volatile anaesthetics were also freshly made each day and stored in airtight glass containers at room temperature until use. In saturation the concentrations for isoflurane, halothane and sevoflurane were taken as 15.3mM, 17.5mM and 11.8mM, respectively (Jenkins et al., 1999). Volume fractions of such saturated anaesthetic solutions were then mixed with experimental solution to obtain a test solution with the final concentration of the respective anaesthetic.

Solutions for the xenon experiments were prepared by bubbling extracellular solution with pure gases either of xenon, nitrogen or oxygen at a rate of 30ml/min for 20min in a Drechsel bottle. This produced saturated solutions for each of the individual gases (Gruss et al., 2004). For experiments solutions with fractional gas concentrations were then obtained by mixing adequate volumes of the individual saturated solutions. The final test solutions always contained 20% oxygen. The remaining 80% were made up by varying amounts of nitrogen- and xenon-saturated solutions. Immediately after preparation the test solutions were transferred to glass syringe barrels. The fluid in the barrels was separated from air by a polypropylene float, thus preventing gases from escaping (Gruss et al., 2004).

All chemicals used were purchased from Sigma (Poole, Dorset, UK). Xenon was obtained from Air Products (Basingstoke, UK) and nitrogen as well as oxygen from BOC (Guildford, UK).

6.1.3 Patch Pipettes

Patch pipettes were pulled from thin-walled borosilicate glass (GC150TF; Harvard Apparatus, Edenbridge, Kent, UK) and tips polished with a Zeitz-DMZ puller (Zeitz Instruments, Martinsried, Germany). The pipettes when filled with solution had resistances of 3-5MΩ. The pipette solution contained (in mM): 120 KCl, 1 NaOH, 1 $MgCl_2$, 1 $CaCl_2$, 5 EGTA, 5 HEPES, 0.3 K_2ATP (pH adjusted to 7.3 with KOH; final $[K^+]$ ~140mM).

6.1.4 Whole-Cell Recordings

For electrophysiological recordings a coverslip with transfected HEK cells was transferred into a recording chamber mounted under an upright microscope (Zeiss Axioskop 2FS) with epifluorescence. Only cells with healthy morphology and successfully transfected with GFP as evident from green fluorescence (excitation: 395-440nm, emission: 470-600nm) were selected for electrophysiological recordings (Thomas and Smart, 2005).

During the course of the experiment cells were constantly superfused at 3-5ml/min with extracellular solution of the following composition (in mM): 118 NaCl, 3 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 25 HEPES (pH adjusted to 7.4 with NaOH). Test drugs were added to this solution either directly or from stock solutions as described above.

Whole-cell currents were recorded at a holding potential of -20mV and at 20-24°C. Currents were evoked by 700ms hyperpolarizing voltage ramps from -20mV to -120mV every 15s and recorded using an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Recordings were digitised at 0.5kHz and analysed using Pulse/Pulsefit software (HEKA Elektronik, Lambrecht, Germany) running on a personal computer. This protocol allows characterisation of the properties of currents through specific ion channels. It is routinely used when examining recombinant channels as done here, but has also been used in neurons (Millar et al., 2000; Hopwood and Trapp, 2005).

To confirm that all effects on the holding current at -20mV were indeed caused by modulation of K^+ currents, reversal potentials were determined for each experimental condition and compared (see results Table 7.1).

The net ionic flow over a membrane can be expressed with equation 6.1 (Offner, 1991):

$$I_{net} = I_{in} - I_{out}$$

Equation 6.1

I_{net} represents the net flow whereas I_{in} and I_{out} express the current into and out of the cell respectively.

At the reversal potential equation 6.2 can be re-written so that

$$I_{net} = 0 \ .$$

Equation 6.2

The reversal potential is therefore the membrane potential at which there is no net flow of ions over the membrane (Offner, 1991). Furthermore it is the potential at which the direction of the ion flow across the membrane changes from either inward to outward or vice versa.

In a single-ion system the reversal potential is the same as the equilibrium potential of that particular ion. This happens under physiological conditions when all channels amongst a population of different ion channels are closed but only one channel type with selectivity for a specific ion remains open. The resting membrane potential of a cell is the prototypic example for such a condition because then the flow across the membrane is dominated and the ensuing membrane potential characterised by K^+ ions. The resting potential becomes therefore close to the equilibrium potential of K^+.

The equilibrium potential is a function of all forces across a membrane – electrical and osmotic - which influence the movement and distribution of ions on both sides of that membrane. It is termed equilibrium potential because when

established it represents the membrane potential at which all those forces are in equilibrium. As a consequence there will be no net current flow into or out of the cell.

When a cell has significant permeabilities for more than one ion species the equilibrium potential can be calculated accurately with the Goldman-Hodgkin-Katz equation (Pancrazio, 1997). However, in systems where the ion current is determined mostly through a single ion species the Nernst equation describes the equilibrium potential best (Pancrazio, 1997). Since the heterologous HEK293 cell expression system of the present study can be assumed to come close to a single-ion system, the calculation of the equilibrium potential with the Nernst equation will also determine the reversal potential.

$$E = \frac{RT}{zF} \ln \frac{[ion]_o}{[ion]_i}$$

Equation 6.3

R is the gas constant, *T* is the temperature in Kelvin, *F* is Faraday's constant and *z* is the valance of the ion. *RT/zF* are usually assumed constant. For a monovalent cation like K^+ its value is about 26mV at 25°C and 27mV at 37°C.

$[ion]_o$ denotes the concentration of the ion on the outside of the cell and $[ion]_i$ is the concentration of that ion in the cytoplasm.

Translated into the conditions of the experiments in the present study at 25°C with $[K]_o$ of 3mM and $[K]_i$ of 140mM the equation can be rearranged as

$$E = 25 \ln(\frac{3}{140})$$

Equation 6.4

This then equates to

$$E \approx -96mV$$

Equation 6.5

In the present study a reversal potential between -105 and -65mV has been accepted as representative for K^+ currents. The reversal potentials for all treatment groups of the present study were not corrected for the liquid junction potential, which was calculated as 4.0mV at 24ºC with Clampex (Molecular Devices, Sunnyvale, California, USA). The values for the reversal potentials as determined experimentally ranged from -76mV to -84mV (Table 7.1) with no statistical significant difference between the groups.

Concentration-response relationships for the volatile anaesthetics were obtained by alternating the control solution with a test solution containing a defined concentration of anaesthetic. The extent of inhibition by the anaesthetic was then expressed as a fraction of the mean of the value obtained in the control solution before and after anaesthetic application. Concentration-response curves were fitted to a modified Hill equation:

$$I / I_c = \frac{1}{(1+\left(\frac{[VA]}{K_i}\right)^h)}$$

Equation 6.6

I is the holding current at -20mV in the presence of the drug, I_c is the current in the absence of the drug, $[VA]$ is the volatile anaesthetic concentration, K_i is the anaesthetic concentration at which inhibition is half maximal and h is the slope factor (Hill coefficient).

6.1.5 Inside-Out Excised Patch Recordings

Inside-out excised membrane patches of HEK293 cells transfected with either wild type (K_{ir}6.2/SUR1) or K_{ir}6.2-K185Q/SUR1 channels were obtained as described by Hamill and colleagues (Hamill et al., 1981). A patch pipette was moved toward the cell surface and attached to it. After formation of a gigaohm seal the pipette was rapidly withdrawn from the cell. This left a crescent shaped patch of cell membrane in the pipette lumen close to the tip with the former inside of the membrane facing now out towards the bath solution (Figure 6.3). The pipette was then carefully placed in the inflow stream of the test solutions to optimise the exposure of the membrane patch to drugs and to allow rapid exchange of solutions. To achieve this the pipette was positioned close to the mouth of a perfusion pipe that supplied the bath constantly with external solution.

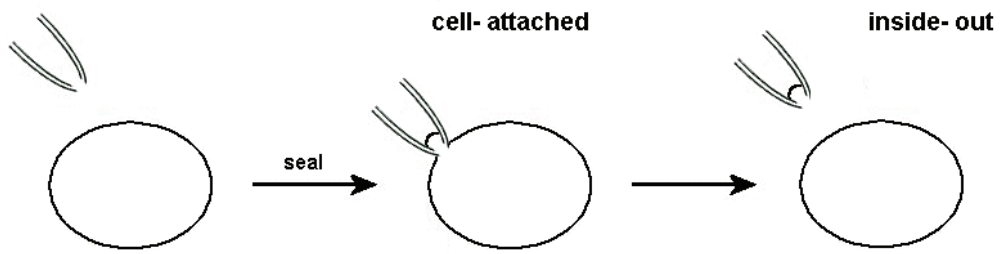


Figure 6.3: Schematic diagram of patch clamp configuration

For details see text. A pipette is moved toward the cell (left) to form a gigaseal (middle). To achieve the inside-out configuration the pipette is quickly withdrawn from the cell (right).

Figure modified after Cahalan (1992)

Macroscopic inside-out currents were recorded from excised patches at a holding potential of 0mV and at 20-24°C in response to 700ms voltage ramps from -110mV to +100mV. The pipette (external) solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH). The standard intracellular (bath) solution contained (mM): 107 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES (pH 7.2 with KOH; final $[K^+]$ ~140mM).

All inside-out currents were filtered at 1kHz, sampled at 3kHz and analysed using Pulse/Pulsefit software (HEKA Elektronik, Lambrecht, Germany). The slope conductance was measured by fitting a straight line to the current-voltage relation between -20mV and -100mV (Figure 6.4): the average of 5 consecutive ramps was calculated in each solution. To control for rundown, that is the spontaneous decay of current flow through the channel (Trube and Hescheler, 1984; Hussain and Wareham, 1994), the control conductance was usually taken as the mean of that obtained in control solution before and after the application of the test compound.

ATP concentration-inhibition curves for $K_{ir}6.2$/SUR1 currents were fit to a modified Hill equation (Equation 6.7):

$$G/G_c = \frac{1}{(1+\left(\frac{[ATP]}{IC_{50}}\right)^h)}$$

Equation 6.7

G is the conductance in the presence of ATP, G_c is the conductance in the absence of ATP, [ATP] is the ATP concentration, IC_{50} is the ATP concentration at which inhibition is half maximal and h is the Hill coefficient (slope factor). Fits were obtained using Origin Pro software (Origin Lab Corporation, Northampton, MA, USA).

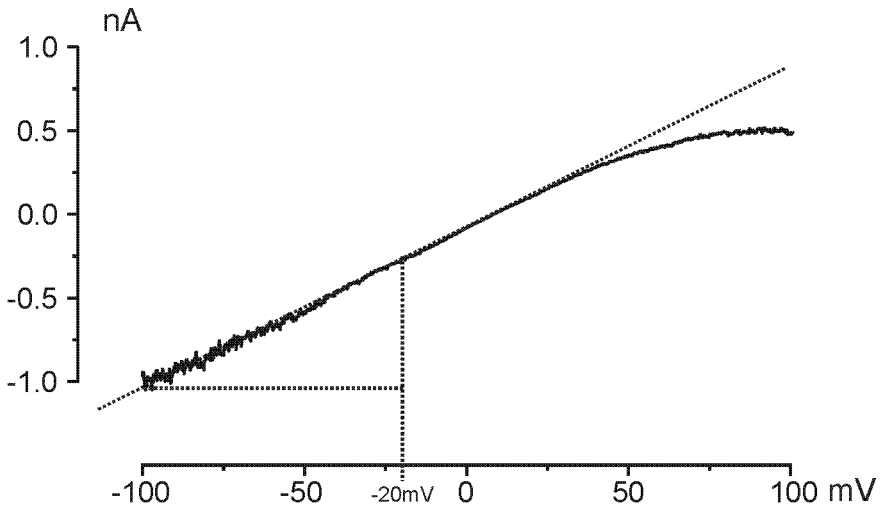


Figure 6.4: Determination of slope conductance

The slope conductance was determined by fitting a straight line through the linear part of each original trace. To calculate the slope of this straight line a linear regression curve fit was performed using Pulse/Pulsefit software (HEKA Elektronik, Lambrecht, Germany).

The straight line fit was based on the formula $y = mx + b$, where m is the slope of the line and b the intercept value.

6.2 Cell Injury Experiments

To gain information about pathways involved in the development of disease processes or to increase our knowledge of mechanisms of action of therapeutic concepts several basic experimental strategies can be applied. Principally all these strategies involve either *in vivo* or *in vitro* models with each of them displaying specific advantages and disadvantage in addition to different degrees of complexity (Abdulla and Campbell, 1993; Stacey and Viviani, 2001). It remains the researcher's responsibility to choose the right experimental approach to test an explicit hypothesis. In the following a short description will be laid out about the reasoning that led to the employment of neuronal glial cell co-cultures over other models in the present study before in the second part a detailed account of the used experimental protocol will be given.

Whilst *in vivo* or whole animal models in neurobiology research have been found to be especially valuable to test complex integrated responses, *in vitro* (tissue or cell culture) models usually allow a more detailed analysis of pathways down to the molecular level. In neuroscience in particular three different cell culturing principles have emerged over the years to explore the specific involvements of mechanisms on an intercellular, cellular or subcellular level: These cultures include single cell, mixed cell or brain slice preparations (Abdulla and Campbell, 1993; Gahwiler et al., 1997).

Primary neuronal cultures although theoretically having the advantage of experimental results to be completely independent of secondary influences by other cell lines are nevertheless hampered by potentially significant shortcomings: Neurons in single cell culture are notoriously difficult to mature and their life span is reduced under such artificial conditions. Moreover, it has been found surprisingly challenging to obtain pure neuronal cultures to the extent that nearly all cultures remain contaminated to some degree with glial cells (Cestelli et al., 1992; Abdulla and Campbell, 1993). Alternatively, and in addition to acute brain slice preparations, which are only short-lived, recently organotypic brain slice cultures have been developed (Gahwiler et al., 1997). These preparations have the advantage of containing more integrated networks, which come close to the *in situ*

situation. Organotypic brain slices can be generated from various brain regions and therefore display region-specific characteristics. They usually survive for several weeks. However, they have been found to be relatively susceptible to influences from their culturing environment. Additionally, it has been suggested that they might not be ideal for the investigation of death pathways since there is evidence for them to be more sensitive to excitotoxic stimuli than what was expected from acute slice experiments. Moreover, microglial cells, which are also present in these organotypic slices, have been found activated for some days to potentially influence cell injury mechanisms. Finally, in these preparations lactate dehydrogenase (LDH) isoenzymes have been demonstrated to show various degrees of maturation indicating an incomplete shift from anaerobic to aerobic glycolysis (Gahwiler et al., 1997).

In the present study neuronal glial cell co-cultures were used because the presence of differentiated glial cells encourages neuronal maturation and makes culturing of neurons easier, most likely due to the release of growth factors. Additionally, this model has been shown to come closer to the physiological conditions in the brain than pure neuronal cultures but without displaying the drawbacks attributed to the complexity of the slice cultures (Abdulla and Campbell, 1993; Gahwiler et al., 1997). Neuronal glial co-cultures therefore represent an established and validated model used to investigate the mechanisms involved in hypoxic-ischaemic brain injury (Goldberg and Choi, 1993).

6.2.1 Neuronal Glial Cell Co-Culture Preparation

Neuronal glial cell co-cultures were prepared as described previously (Wilhelm et al., 2002). First glial cells were cultured. To obtain them 1-2 day old BALB/c mouse pups were anaesthetised with isoflurane and decapitated. Heads were immediately placed in ice-cold high sucrose-glucose solution (HSG) that was made from Hank's balanced salt solution (HBSS, Gibco, Paisley, UK) enhanced with 4mM $NaHCO_3$, 20mM sucrose, 30mM D-glucose and commercially available antibiotic-antimycotic solution (AAS, Gibco, Paisley, UK).

Brains were removed from the skull and micro-dissected to get whole cerebral neocortices devoid of the hippocampus, basal ganglia and meninges. Neocortices were then immersed in 10ml of culture medium containing 0.05% trypsin and shaken at 37°C in a chamber containing an atmosphere of 5% CO_2 and 95% air. After 50 minutes DNAse (20-30µl, Sigma, Poole, UK) was added. The solution was mixed for another 15 minutes before it was centrifuged at 1600rpm for 10 minutes at 4°C. The supernatant was discarded. Glia cells were resuspended in culture medium that contained Eagle's minimum essential medium supplemented with 20mM glucose, 26mM $NaHCO_3$, 10% foetal bovine serum, 10% heat-inactivated horse serum, 2mM glutamine (Sigma, Poole, UK), 10ng/ml murine epidermal growth factor (Gibco, Paisley, UK) and Antibiotic-Antimycotic Solution (AAS; Gibco, Paisley, UK) containing 100U/ml penicillin, 0.1mg/ml streptomycin and 0.25µg/ml amphotericin B. Finally the cells were plated at a density of $6.25x10^4$ cells/cm^2 on 4-well plates (Nunc, Roskilde, DK) and incubated at 37°C in humidified air enriched with 5% CO_2.

About 5 days after plating the cells reached confluence and were therefore ready to be used as culture ground for newly harvested neuronal cells. These neuronal cells were prepared in a similar procedure as the glial cells. They were obtained from the neocortices of foetal BALB/c mice at 14-16 days of gestation and plated onto the glial cell monolayer at a density of 1.25 X 10^5 cells/cm^2. After 5 days of neuronal growth co-cultures were exposed for 48 hours to 10µM cytosine arabinoside (Sigma, Poole, UK) to stop glial cell proliferation. After 3 additional days of culturing and a total of 15+/-1 days neuronal glial cell co-cultures were ready for preconditioning experiments.

Cultures were assigned to one of the following protocols: Preconditioning, oxygen-glucose deprivation only (OGD) or naïve control. Cultures in the preconditioning group were exposed to either 1 MAC xenon, 2 MAC sevoflurane, or 0.01mM diazoxide for 2 hours in the presence or absence of the pK_{ATP} channel inhibitor tolbutamide (0.1mM) or the mKATP inhibitor 5-HD (0.5mM). The time course of the experiment is depicted in Figure 6.5.

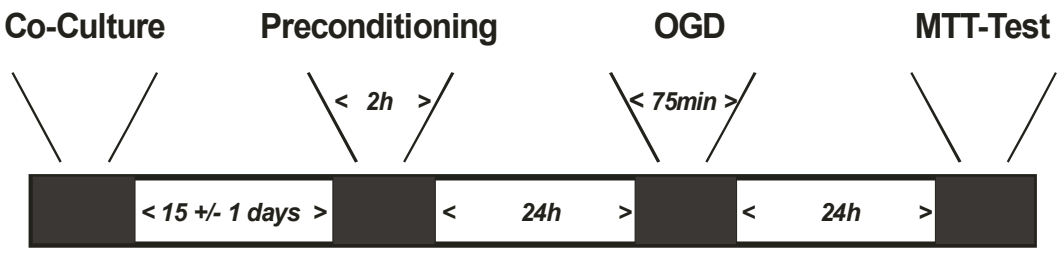


Figure 6.5: Time course of cell injury experiments

Glial and neuronal cells were co-cultured for a total of two weeks before they underwent cell injury experiments. Some cultures (treatment groups) were exposed for two hours to a preconditioning stimulus (anaesthetics or diazoxide) 24 hours before a damaging insult (oxygen-glucose deprivation; OGD). Cultures assigned to oxygen-glucose deprivation only (OGD group) underwent a media change 24 hours prior to OGD without preconditioning whereas naïve controls were just washed at the appropriate time points.

Twenty-four hours after OGD cell viability was tested with 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction test (for details see text).

6.2.2 Preconditioning

For the preconditioning experiments a model was used, that was previously established by Ma as well as Luo and colleagues (Ma et al., 2004; Luo et al., 2008).

Anaesthetic-enriched medium was obtained by bubbling xenon or sevoflurane for 20 minutes through experimental medium containing Eagle's minimal essential medium, 21.1mM glucose and 38mM $NaHCO_3$ in a Drechsel bottle. This period was found to be sufficient to allow equilibrium between the gas phase and the liquid phase in the bottle as assessed by gas chromatography (Ma et al., 2003).

Neuronal glial cell co-cultures were washed twice with HEPES-buffered solution (120mM NaCl, 5.4mM KCL, 0.8mM $MgCl_2$, 15mM glucose and 2mM HEPES, titrated to pH 7.4 using 1M NaOH) and then incubated with anaesthetic-enriched medium, or with experimental medium containing 0.01mM diazoxide. The pK_{ATP} channel inhibitor tolbutamide (0.1mM) or the mKATP channel inhibitor 5-hydroxydecanoate (5-HD, 0.5mM) were added to some dishes as appropriate. Co-cultures were then transferred to an airtight, temperature-controlled gas exposure chamber.

Preliminary experiments had established that xenon and sevoflurane needed a part of 75% and 3.3% respectively, of the surrounding gas atmosphere to achieve maximum preconditioning (Ma et al., 2006; Luo et al., 2008). If converted to the commonly used endpoint for inhalational anaesthetics - the minimum alveolar concentration (MAC), which is the concentration in the lungs that is able to prevent a purposeful response to painful stimuli (Campagna et al., 2003) - 75% of xenon and 3.3% sevoflurane in humans are equal to about 1 MAC and 2 MAC, respectively (Cullen et al., 1969; Behne et al., 1999).

Furthermore the model validation found also a two-hour exposure period of the gases to the cultures to be sufficient to achieve maximal preconditioning (Ma et al., 2006). This was hence employed in the present study.

To maintain a constant concentration of gases in the experimental solutions of the culture dishes during preconditioning the gases in the culture solutions had to be in equilibrium with the gas atmosphere in the airtight chamber. The gas flows necessary to achieve the respective atmospheric gas composition were determined

by Ma and colleagues (Ma et al., 2003). The gas composition in the chamber for the xenon experiments consisted of 20% O_2, 5% CO_2 and 75% xenon (1 MAC). The gas composition in the sevoflurane experiments was 20% O_2, 5% CO_2, 71.7% N_2 and 3.3% sevoflurane (2 MAC). The atmosphere in the chamber for diazoxide preconditioning consisted of 20% O_2, 5% CO_2 and 75% N_2. After preconditioning the co-cultures were washed twice with HEPES-buffered solution and stored in culture medium in an incubator at 37°C in a humidified mixture of 5% CO2 and 95% air.

Co-cultures in the OGD group underwent the same protocol as the cultures in the preconditioning group except that instead of being exposed to HEPES-buffered solution containing the preconditioning drugs they were washed with HEPES–buffered solution only. Cultures in the naïve control group received the same pattern of solution changes, but were not exposed to the preconditioning drugs or OGD.

6.2.3 Oxygen-Glucose-Deprivation (OGD)

Oxygen-glucose deprivation in neuronal glial cell co-cultures is an established model to investigate pathways and mechanisms of ischaemic brain injury (Goldberg and Choi, 1993). In preliminary studies by Ma and colleagues this model was further modified to meet the needs of our laboratory and was subsequently validated: For instance the time needed to induce maximal damage to neurons was determined and it was found that 60 minutes of OGD were needed to develop most significant injury. However extending OGD to up to 100 minutes did not achieve any further effect and hence 75 minutes of substrate deprivation were applied in the experiments of the present study (Ma et al., 2003; Ma et al., 2004).

Additionally, through the use of phase-contrast microscopy, Ma and colleagues also excluded a significant influence of OGD on the glial cells of the co-cultures (Ma et al., 2005) and therefore confirmed Goldberg's data indicating that up to 6 hours of OGD selectively harmed only neuronal cells (Goldberg and Choi, 1993).

In the present experiments twenty-four hours after preconditioning co-cultures assigned to the OGD group were washed twice with HEPES-buffered solution and once with pre-warmed OGD medium. OGD medium contained 116mM NaCl, 5.4mM KCl, 0.8mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $CaCl_2$ and 26mM $NaHCO_3$ and was bubbled with 5% CO_2 and 95% N_2 in a Drechsel bottle at 37°C. Cultures were then placed into an airtight gas exposure chamber with an anaerobic environment consisting of 5% CO_2 and 95% N_2 at 37°C for 75 minutes. Oxygen-glucose deprivation was terminated by washing cultures twice with HEPES-buffered solution before being incubated in culturing medium and stored at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.
Finally, cell viability testing was performed 24 hours after OGD. This time point was chosen since Rajakumaraswamy and colleagues in accordance with Goldberg and colleagues had found the extent of the cell damage to be unchanged between 6 and 24 hours after injury (Goldberg and Choi, 1993; Rajakumaraswamy et al., 2006).

6.2.4 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Reduction Test

The MTT reduction test is a quantitative measurement of cellular metabolic activity and therefore widely used for the assessment of cellular viability and cell proliferation (Berridge and Tan, 1993; Takahashi et al., 2002). In the presence of active mitochondrial and cytosolic redox systems the tetrazolium salts of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) are reduced to intensely blue coloured formazans, which can be quantified in a spectrophotometer. The measured optical density corresponds directly to the viability of the cells in culture.

Twenty-four hours after OGD neuronal-glial co-cultures were incubated for 2 hours in 0.6ml phosphate-buffered saline with 0.5mg/ml MTT (Merck, Darmstadt, Germany) to allow uptake of MTT into the cells. After removing MTT from cultures, cells were dissolved and homogenised in 1ml dimethyl sulfoxide

(DMSO). 0.3ml of each sample was then transferred to one well of a 96-well plate. The optical density (OD) was measured in triplicates at 570nm in a MRX Microplate Reader (Dynex Technologies, Chantilly, VA, USA).
The OD readings were then converted into normalised cell viability readings using the following equation:

$$\%\, C_{via} = \frac{(OD_{drug} - OD_{OGD})}{(OD_{control} - OD_{OGD})} \times 100 \qquad \text{Equation 6.8,}$$

where % C_{via} is cell viability expressed in percent, OD_{drug} is the optical density reading of preconditioned cells, $OD_{control}$ is the optical density reading of untreated naïve cells and OD_{OGD} is the optical density reading of non-preconditioned cells, which were exposed to OGD only. Uninjured neurons in co-cultures would therefore express a C_{via} of 100%, whereas a C_{via} of 0% represents maximal neuronal damage. In preliminary experiments the possible damaging effect of test drugs on neuronal-glial cell co-cultures was assessed (Figure 7.19). Neuronal injury in those experiments was quantified by the amount of lactate dehydrogenase (LDH) released into the culture medium. LDH activity in each sample was determined spectrophotometrically (MRX Microplate Reader; Dynex Technologies, Chantilly, VA, USA) at room temperature using a standard colorimetric enzyme kit (Sigma 500 C; Sigma-Aldrich, Poole, UK). The degree of neuronal damage was shown to be proportional to the LDH activity in the sample (Wilhelm et al., 2002).

6.3 Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc. San Diego, California, USA). All data were expressed as mean +/- SEM. To test for differences between groups two-tailed unpaired t-test or one-way ANOVA followed by Tukey's multiple comparison test were used as appropriate. A value of $p < 0.05$ was considered significant.

7. RESULTS

7.1 Whole-Cell Patch-Clamp Experiments

7.1.1 Potassium Current in K_{ir}6.2/SUR1 Transfected HEK293 Cells

K_{ir}6.2 and SUR1 subunits as the predominant constituents of neuronal plasmalemmal K_{ATP} channels (Karschin et al., 1997; Liss and Roeper, 2001) were expressed in a HEK293 cell recombinant system. Successful expression of K_{ir}6.2/SUR1 was confirmed morphologically as described in the methods (see chapter 6.1). In addition, electrophysiological determination of current characteristics also verified the presence of plasmalemmal K_{ATP} channels in transfected cells.

Under voltage clamp conditions naïve HEK293 cells normally display a very small intrinsic membrane conductance (Gruss et al., 2004). This was also found in the current study where untransfected HEK293 cells showed whole-cell current amplitudes of 22+/-10pA at -20mV (n=12). This current could not be enhanced by the application of the specific K_{ATP} channel opener diazoxide (0.2mM; n=6). Only upon transfection with K_{ir}6.2 and SUR1 500 times larger outward currents were recorded at -20mV, which also responded to diazoxide application (Figure 7.1).

Also analysis of the zero-current potentials provided further evidence for the presence of neuronal type pK_{ATP} channels in transfected HEK293 cells. The zero-current potential for K_{ir}6.2 and SUR1 transfected HEK293 cells was –77+/-2mV (n=30), consistent with the fact that most of the whole-cell conductance was made up by K_{ATP} channels. In contrast, untransfected HEK293 exhibited a zero-current potential of –40+/-5mV (n=13).

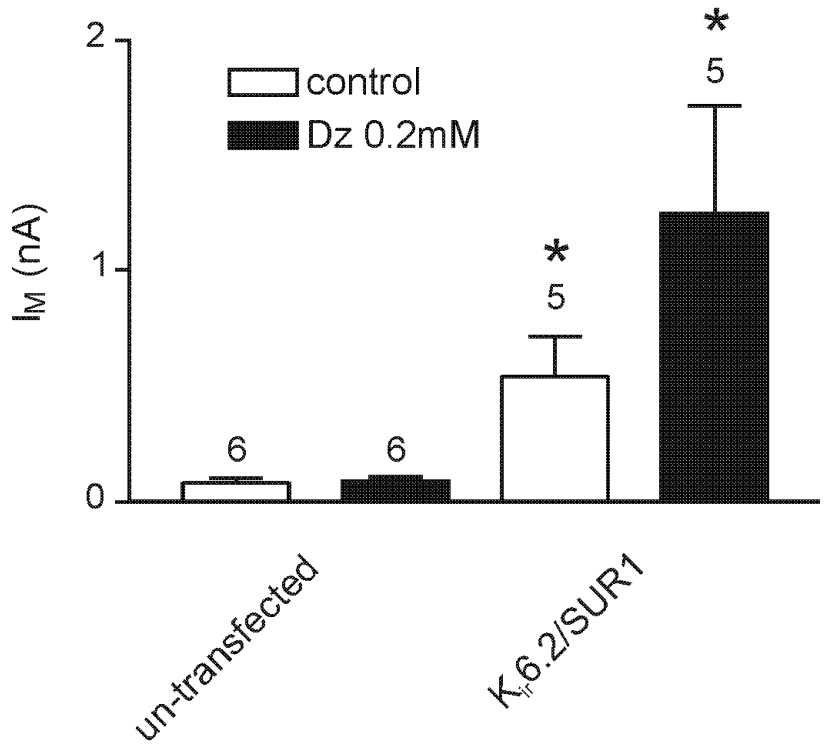


Figure 7.1: K^+-current of untransfected and transfected HEK cells

Mean K^+-current at a holding potential of -20mV of untransfected and K_{ir}6.2/SUR1 transfected HEK293 cells. Diazoxide (Dz; 0.2mM) was bath applied to some cells. N-numbers are given above the bars.

** indicates $p < 0.05$ compared to respective untransfected group.*

Thirdly, the potential at which the drug-modified current changes its direction, the reversal potential, was determined for every experimental group each time an experiment was conducted. Reversal potentials served as a quality control to exclude currents other than potassium for analysis. They should be close to the K^+ equilibrium potential (E_K) as determined by the Nernst equation (see methods Equation 6.3).

Experiments showing reversal potentials more negative than -105mV or more positive than -65mV were discarded. The average reversal potentials for each experimental group are shown in table 7.1. Their values were not significantly different.

Table 7.1	**Reversal Potential (mV)**	**n**
Xenon	-80 +/- 3	10
Sevoflurane	-83 +/- 3	22
Halothane	-76 +/- 2	22
Isoflurane	-77 +/- 2	14
Diazoxide	-77 +/- 3	10
Tolbutamide	-84 +/- 3	10
Washout	-80 +/- 2	12

Table 7.1 shows the reversal potential of the current elicited or inhibited by the drugs indicated. No significant difference was observed between any of the treatment groups. The reversal potentials are not corrected for the liquid junction potential, which was determined as 4.0mV (see methods).

Finally, in HEK293 cells transfected with $K_{ir}6.2$/SUR1 there was an increase of outward current during the first 5 minutes of whole-cell voltage-clamp recordings (Figure 7.2). It was then followed by a stable holding current of 1.05+/-0.12nA (n=30) (Figure 7.2A, B). The initial increase in current is a common phenomenon in whole-cell patch-clamp experiments involving pK_{ATP} channels and has been attributed to the washout of ATP from the cytosol and subsequent progressive opening of plasmalemmal ATP-sensitive K^+ channels (Trapp et al., 1994). In support of this hypothesis, the stable holding current was reversibly inhibited by the specific pK_{ATP} channel blocker tolbutamide (0.1mM; n=17; Figure 7.2A,C).

In summary, the expression of plasmalemmal K_{ATP} channels in the HEK293 cell system of the current study was suffienctly validated through the presence of increased current amplitudes, characteristic zero-currents and reversal potentials as well as observed washout currents.

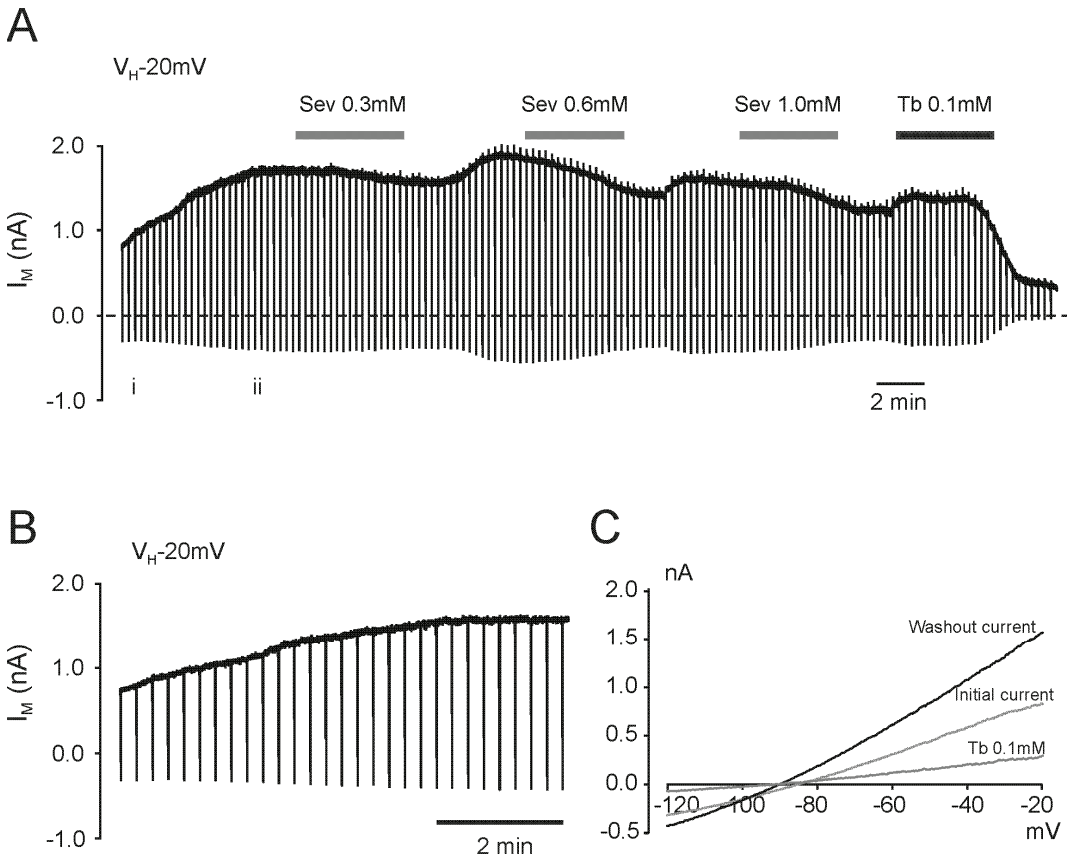


Figure 7.2: Washout current

*(**A**) Whole-cell recording at a holding potential of -20mV from a HEK293 cell transfected with $K_{ir}6.2$ and SUR1. The trace is taken from a sevoflurane experiment that will be explained in further detail below (Figure 7.6). It exemplifies the washout current between points i and ii. The dashed line indicates the zero-current level. Vertical deflections indicate the current response to a 700ms voltage ramp from -20mV to -120mV. These current responses between points i and ii are shown in greater detail in (**B**). In (**C**) they were plotted against the command voltage to obtain current voltage relations. These demonstrate that the washout current has a reversal potential near the K^+ equilibrium potential (E_K). The plot further shows the sensitivity of the current to the selective pK_{ATP} channel inhibitor tolbutamide (Tb;0.1mM).*

7.1.2 Lack of Modulation of pK_{ATP} Channels via Mitochondrial ATP-sensitive K^+ Channels

Studies, mostly on cardiomyocytes, have identified both pK_{ATP} and mKATP channels as key mediators of preconditioning. Independent of type and tissue distribution the initiating event of K_{ATP} channel-dependent protection is channel opening (O'Rourke, 2000a; Hanley and Daut, 2005). To what extend both channels contribute to organ protection and whether there are tissue differences remains still unclear. However, as reviewed by Zaugg and colleagues there is the possibility that both channels interact on a sub-cellular level. In this model mKATP channels are seen as central mediators that could activate pK_{ATP} channels as downstream end-effectors (Zaugg et al., 2003).

To exclude such modulatory effects of mKATP channels on whole-cell channel activity in HEK293 cells a series of experiments with specific K_{ATP} channel openers and inhibitors were performed (see Table 5.9).

Tolbutamide has become the standard $K_{ir}6.2$/SUR1 pK_{ATP} channel antagonist because it closes the channel with high affinity but leaves $K_{ir}6.2$/SUR2A unaffected (Table 5.9) (Ashcroft and Gribble, 2000; Gribble and Ashcroft, 2000; Gribble and Reimann, 2003a). Furthermore, Sato and colleagues demonstrated that 0.1mM of tolbutamide, which efficiently closes pK_{ATP} channels, had no effect on mKATP channels (Sato et al., 2006). As with tolbutamide for $K_{ir}6.2$/SUR1, 5-hydroxydecanoate (5-HD) at a concentration of 0.5mM is regarded as the prototypical inhibitor of mKATP channels (Table 5.9). In contrast, it has no effect on cardiac type pK_{ATP} channels (Jaburek et al., 1998; Sato et al., 1998; O'Rourke, 2000a; Lebuffe et al., 2003). Therefore, in a first set of experiments we determined whether tolbutamide and 5-HD could suppress K^+ currents in $K_{ir}6.2$/SUR1 transfected HEK293 cells. Whilst 0.1mM tolbutamide (n=10) decreased current amplitude dramatically by 84+/-4%, no significant reduction in whole-cell current was observed using 0.5mM 5-HD (n=5) (Figure 7.3).

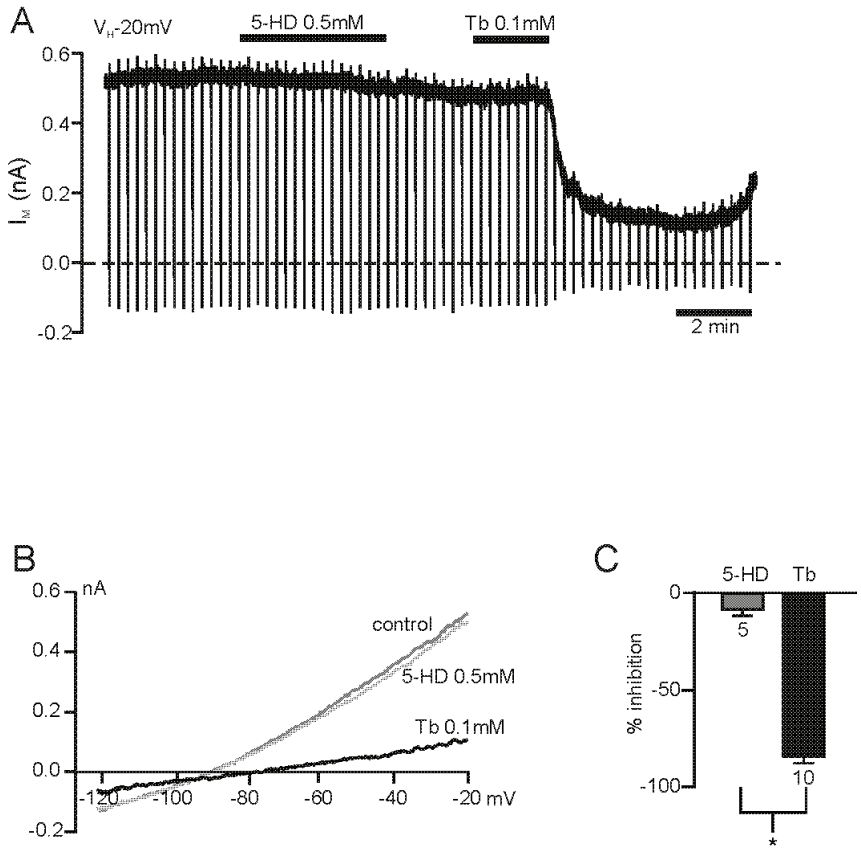


Figure 7.3: K_{ATP} currents in whole-cell recordings are not affected by specific blockers of mitochondrial ATP-sensitive K^+ channels

*(**A**) Whole cell recording demonstrating the lack of effect of the mitochondrial KATP channel blocker 5-HD (0.5mM) on K_{ATP} channels made up of K_{ir}6.2 and SUR1. In contrast, tolbutamide (Tb, 0.1mM) strongly and reversibly inhibited the whole-cell current. The drugs were applied as indicated by the bars above the recording. (**B**) Current-voltage relations in the absence (control) and presence of 0.5mM 5-HD or 0.1mM tolbutamide. (**C**) Mean data from experiments as that shown in (**A**). N-numbers are given above the bars. * indicates $p < 0.05$.*

Equivalent results were obtained when using K_{ATP} channel openers. Pinacidil has been shown to open mKATP and cardiac type $K_{ir}6.2$/SUR2A pK_{ATP} channels (Table 5.9) (Shindo et al., 1998; Sato et al., 2006). In contrast, it has no effect on $K_{ir}6.2$/SUR1 (Gribble et al., 1998b; Ashcroft and Gribble, 2000; Gribble and Reimann, 2003a). Therefore under conditions where $K_{ir}6.2$/SUR2A channel types are absent - such as in neurons or in the heterologous HEK cell expression system of the present experiments - pinacidil at a concentration of 0.1mM can be assumed to be mKATP channel specific (O'Rourke, 2000b). In addition to pinacidil diazoxide has also been reported to open mKATP channels (Table 5.9) (Garlid et al., 1996; Garlid et al., 1997; Liu et al., 1998). However, in contrast to pinacidil it also activates K_{ATP} channels containing SUR1 with an EC_{50} of 30μM but has negligible effects on $K_{ir}6.2$/SUR2A (Garlid et al., 1997; Liu et al., 1998; Lawson, 2000; Dabrowski et al., 2003). Consequently, diazoxide is expected to be mKATP channel specific in systems that do not contain SUR1 (e.g. heart muscle).

In the present study diazoxide at concentrations of 0.01mM (n=10) and 0.2mM (n=5) significantly increased whole-cell currents in HEK293 cells expressing $K_{ir}6.2$ and SUR1 by 44% and 120%, respectively (Figure 7.4A). Since 0.1mM pinacidil (n=8) failed to show any effect in these experiments it is unlikely that opening of mKATP channels modulates pK_{ATP} currents (Figures 7.4A and 7.5). These results rather indicate a direct concentration-dependent effect of diazoxide on $K_{ir}6.2$/SUR1 channels (Figures 7.4A).

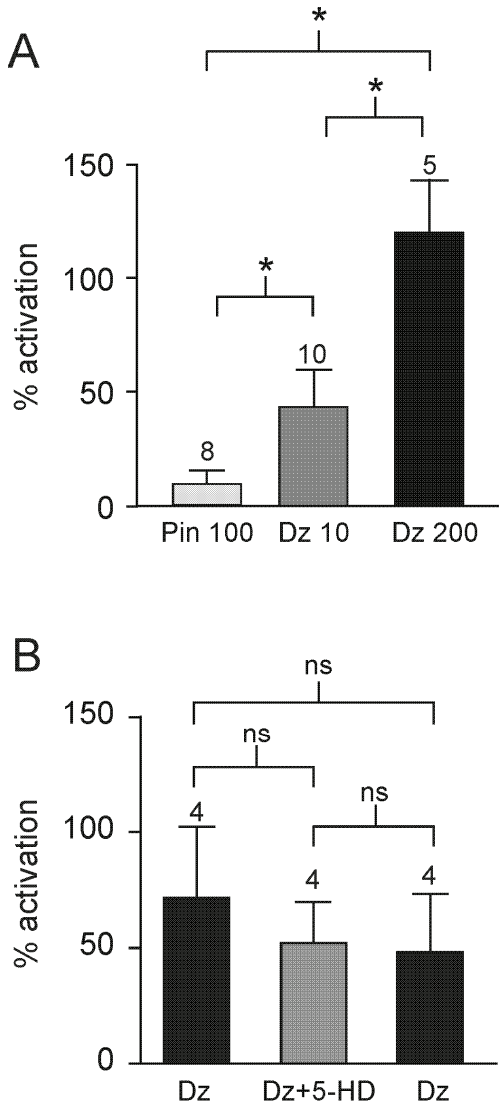


Figure 7.4: Whole-cell K_{ATP} currents are not affected by specific openers of mKATP channels

*(**A**) Mean effects of various K-channel openers on $K_{ir}6.2/SUR1$ currents. Diazoxide (Dz) at concentrations of 0.01mM (Dz10) and 0.2mM (Dz200) increases K^+-current significantly by 44+/-13% and 120+/-22%, respectively. In contrast, pinacidil (Pin; 0.1mM) increased the current only by 10+/-4%. This was not significantly different from baseline.*

*(**B**) Diazoxide at 0.01mM increases the pK_{ATP} current independently of its potential effect on mKATP channels, as demonstrated by the lack of effect of the mKATP channel inhibitor 5-HD on the diazoxide activation of the whole-cell current. N-numbers are given above the bars. * indicates $p < 0.05$; ns denotes non significant results.*

However, because diazoxide potently opened mitochondrial ATP-sensitive K^+ channels in other studies it was further evaluated whether mKATP channel activation, and subsequent alterations in mitochondrial function, might contribute significantly to the K_{ATP} current seen with 0.01mM diazoxide (Garlid et al., 1996; Garlid et al., 1997; Liu et al., 1998). In order to test this hypothesis the K_{ATP} current was enhanced with 0.01mM diazoxide (n=4) and then 0.5mM 5-HD (n=4) was added. No significant reduction of the diazoxide-elicited current was observed under these conditions and the results established that 0.01mM diazoxide activates pK_{ATP} channels independently from its effects on mKATP channels (Figure 7.4B). Taken together these results provide strong evidence that the effects of inhalational anaesthetics on K_{ATP} whole-cell currents, which will be discussed below, are not secondary to an interaction of these drugs with mitochondrial ATP-sensitive K^+ channels.

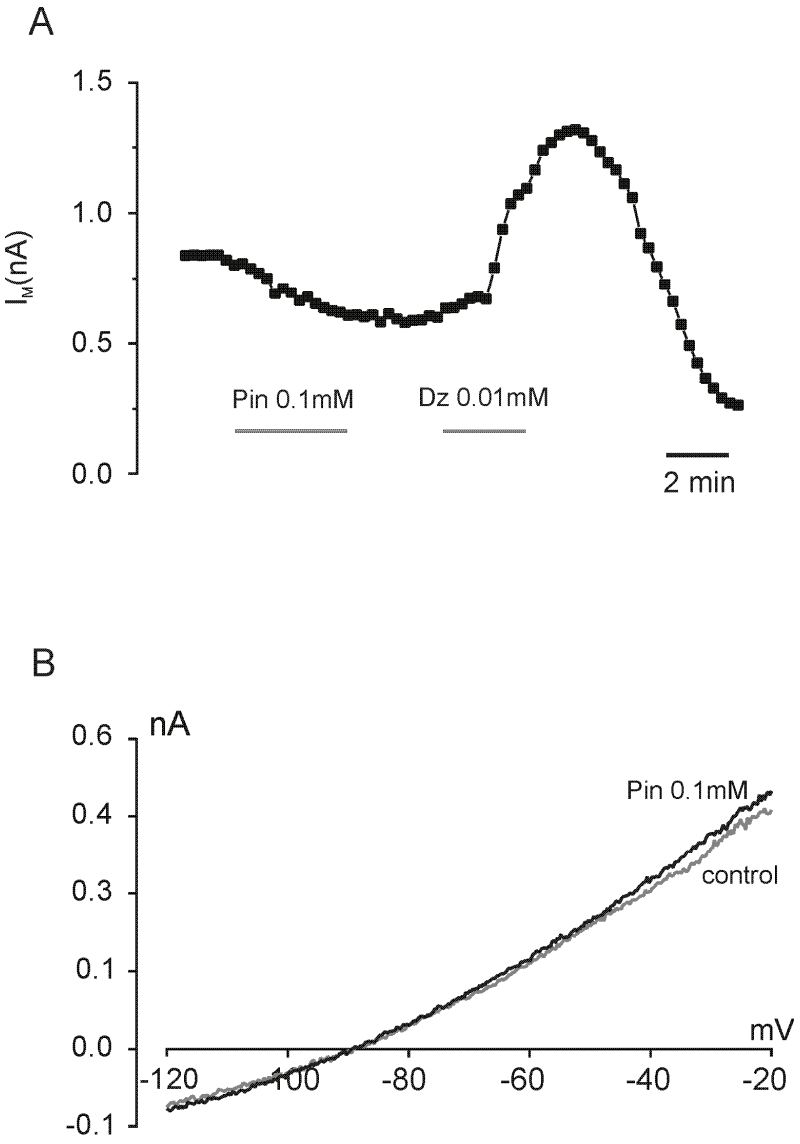


Figure 7.5: Pinacidil has no effect on whole-cell K_{ATP} currents

*(**A**) Whole-cell recording from a HEK293 cell expressing K_{ir}6.2 and SUR1. Plotted is the mean holding current at -20mV every 15s. The SUR1-specific K^+-channel opener diazoxide (Dz; 0.01mM) as well as the mKATP channel specific opener pinacidil (Pin; 0.1mM) are applied as indicated by the solid bars. Diazoxide but not pinacidil potentiated the whole-cell current. (**B**) Current-voltage relations demonstrating the lack of effect of pinacidil (0.1mM) on K_{ir}6.2/SUR1 currents in HEK293 cells.*

7.1.3 Evaluation of the Effects of Inhalational Anaesthetics on pK_{ATP} Currents

Three different classes of inhalational anaesthetics were tested for their effects on $K_{ir}6.2$/SUR1 channels. Xenon represented the noble gases with anaesthetic properties, halothane the hydrocarbons and isoflurane as well as sevoflurane the ethers (Campagna et al., 2003). Isoflurane and sevoflurane are standard drugs in daily anaesthetic practice whilst xenon is precluded from widespread use mainly because its high costs. Halothane in contrast is outdated clinically but still widely used experimentally (Sanders et al., 2003; Hecker et al., 2004).

The addition of all tested volatile anaesthetics (halothane, isoflurane, sevoflurane) to the superfusate in the recording chamber reduced the K_{ATP} current reversibly in a concentration-dependent manner. This is exemplified for sevoflurane in Figure 7.6.

Interestingly this lack of activation of pK_{ATP} current by volatiles was seen at clinically relevant concentrations (Figure 7.7 and 7.8). Halothane for instance was given in a concentration range of 0.125–2.0mM which equates to 0.9-7.1 MAC whereas the range for isoflurane was 0.25–2.0mM (0.5-8.7 MAC) and that for sevoflurane 0.3-2.5mM (0.9-7.5 MAC) (Jenkins et al., 1999). Doses higher than maximum concentrations used in these experiments were applied in preliminary experiments but was found to damage the cells.

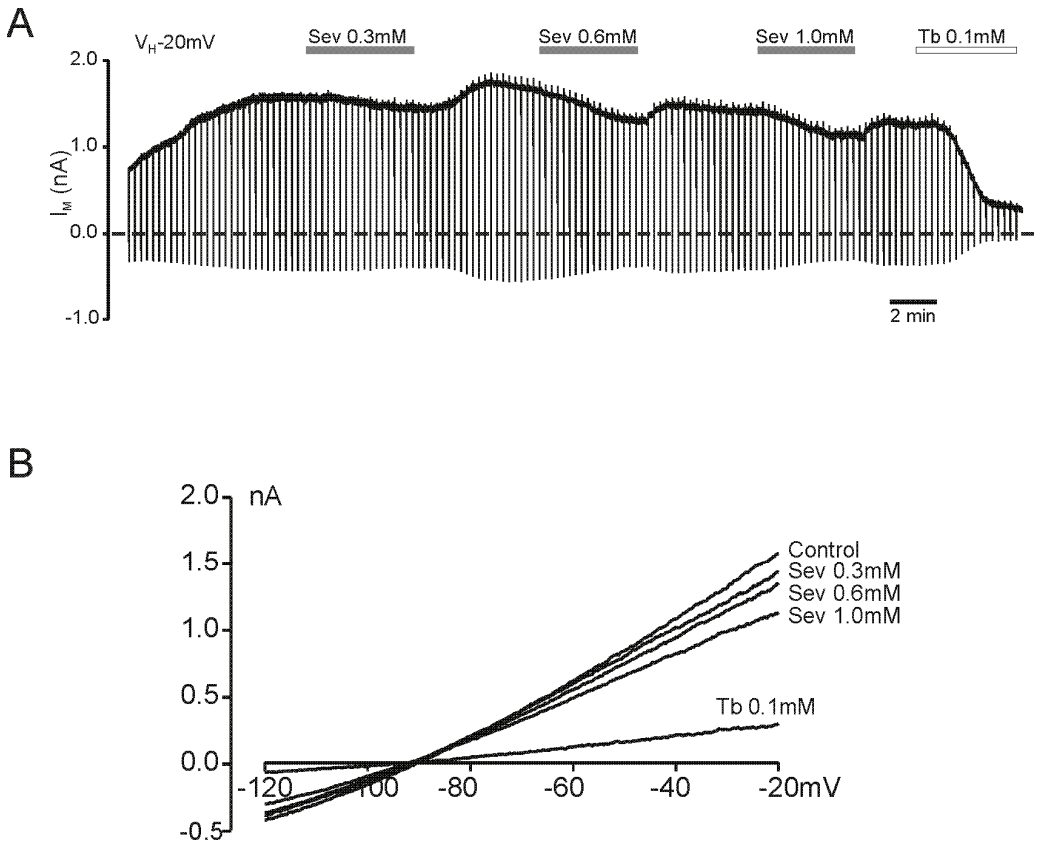


Figure 7.6: K_{ATP} channels expressed in HEK293 cells are mildly inhibited by sevoflurane

*(**A**) Whole-cell recording at a holding potential of -20mV from a HEK293 cell transfected with $K_{ir}6.2$ and SUR1. Sevoflurane (Sev) and tolbutamide (Tb) were bath-applied as indicated by the horizontal bars. The dashed line indicates the zero-current level. Vertical deflections indicate the current response to a 700ms voltage ramp from -20mV to -120mV. (**B**) Current voltage relations taken from (**A**) showing the concentration-dependent small inhibition of the K_{ATP} current by sevoflurane (Sev) at clinically relevant concentrations, compared to the block by tolbutamide (Tb).*

The tested volatile anaesthetics displayed different potencies in their inhibitory effect on pK_{ATP} channels composed of $K_{ir}6.2$/SUR1. This is represented in Figure 7.7 where equimolar concentrations of halothane, isoflurane and sevoflurane reduce whole-cell K_{ATP} currents to varying degrees.

The difference in potency is also reflected in the concentration-response curves of each volatile (Figure 7.8). Halothane inhibited pK_{ATP} currents with an IC_{50} of 1.3+/-0.4mM (n=4-8), isoflurane had an IC_{50} of 2.1+/-0.6mM (n=3-5), and the effect of sevoflurane was extrapolated to an IC_{50} of approximately 50mM (n=3-8).

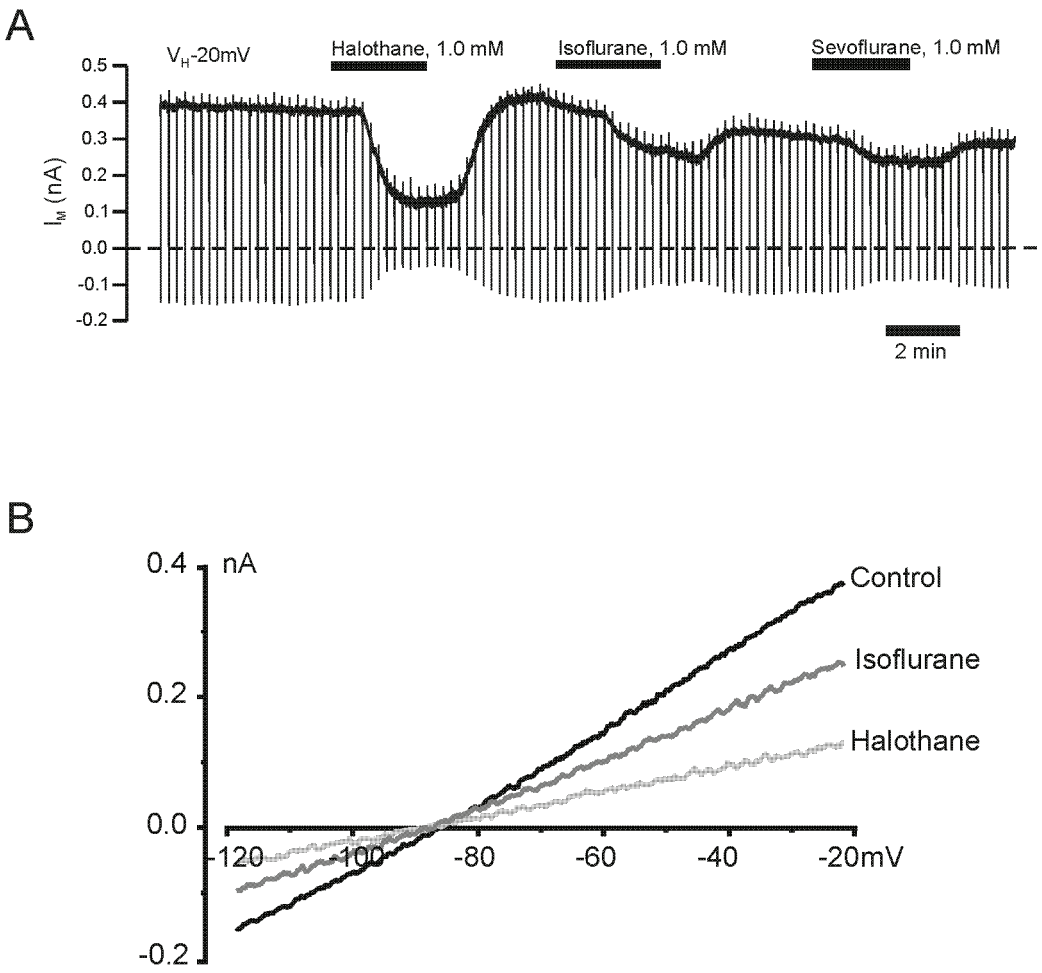


Figure 7.7: Halogenated volatile anaesthetics inhibit pK$_{ATP}$ channels

*(**A**) Whole-cell recording at a holding potential of -20mV from a HEK293 cell transfected with $K_{ir}6.2$ and SUR1. The dashed line indicates the zero-current level. Halothane, isoflurane and sevoflurane (1mM each) were bath-applied as indicated by the horizontal bars. Each drug reversibly inhibited the whole-cell K_{ATP} current. (**B**) Current voltage relations taken from a recording as that shown in (**A**) demonstrating that the current inhibited by the volatile anaesthetics has a reversal potential near E_K.*

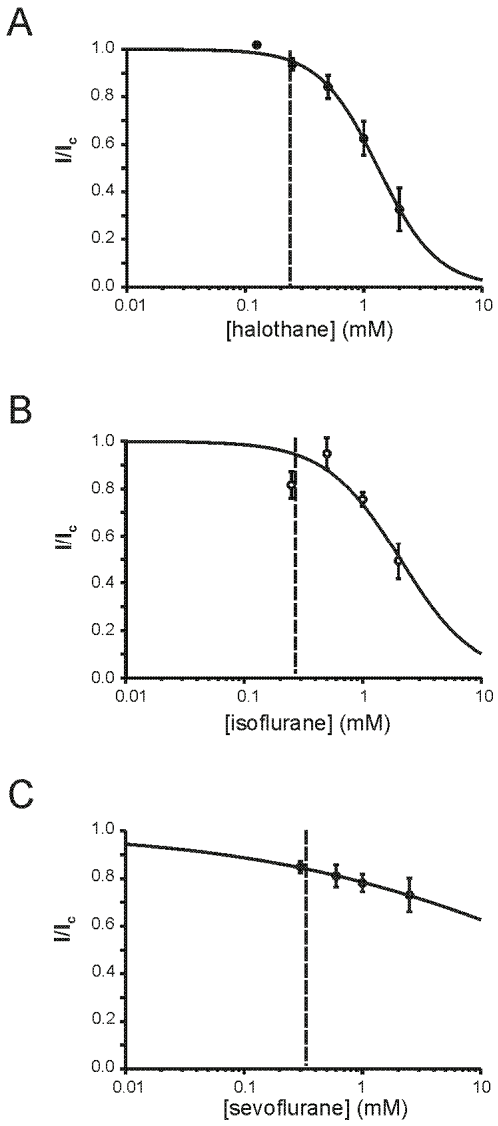


Figure 7.8: Concentration-response relationships of volatile anaesthetics on K_{ir}6.2/SUR1 channels expressed in HEK293 cells

*Concentration-response curves of the inhibitory effect of (**A**) halothane (n=4-8), (**B**) isoflurane (n=3-5) and (**C**) sevoflurane (n=3-8) on whole-cell K_{ATP} currents. The current in presence of the drug (I) is expressed as a fraction of the current in its absence (I_C). The solid lines describe the best fit with a modified Hill equation using K_i = 1.32mM and Hill coefficient (h) = 1.75 for halothane, K_i = 2.08mM and h = 1.39 for isoflurane and K_i = 47mM and h = 0.34 for sevoflurane. In each case it is assumed that complete inhibition of the pK_{ATP} current could theoretically be achieved with high enough concentrations of the anaesthetic. 1 MAC is indicated by the vertical dashed line in each graph.*

Next the effects of xenon were tested on pK_{ATP} currents. Xenon was applied at two concentrations: 50% and 80%. An 80% xenon solution is equivalent to 1 MAC in humans or 3.4mM of xenon (White et al., 2005). Consequently, 50% xenon equates to 0.6 MAC and 2.1mM. HEK293 cells transfected with $K_{ir}6.2$ and SUR1 showed an increase in outward current by 19+/-7% when superfused with a 50% xenon solution (n=5) and by 44+/-7% (n=5) when exposed to 80% xenon solution as compared to control solution (80% N_2; Figure 7.9). The reversal potential of this current (Figure 7.10B; Table 7.1) indicated it being a K^+ current. Furthermore, the current was inhibited by 0.1mM tolbutamide (Figure 7.10). These results strongly suggest that the xenon-induced K^+ current was due to the opening of $K_{ir}6.2$/SUR1 channels.

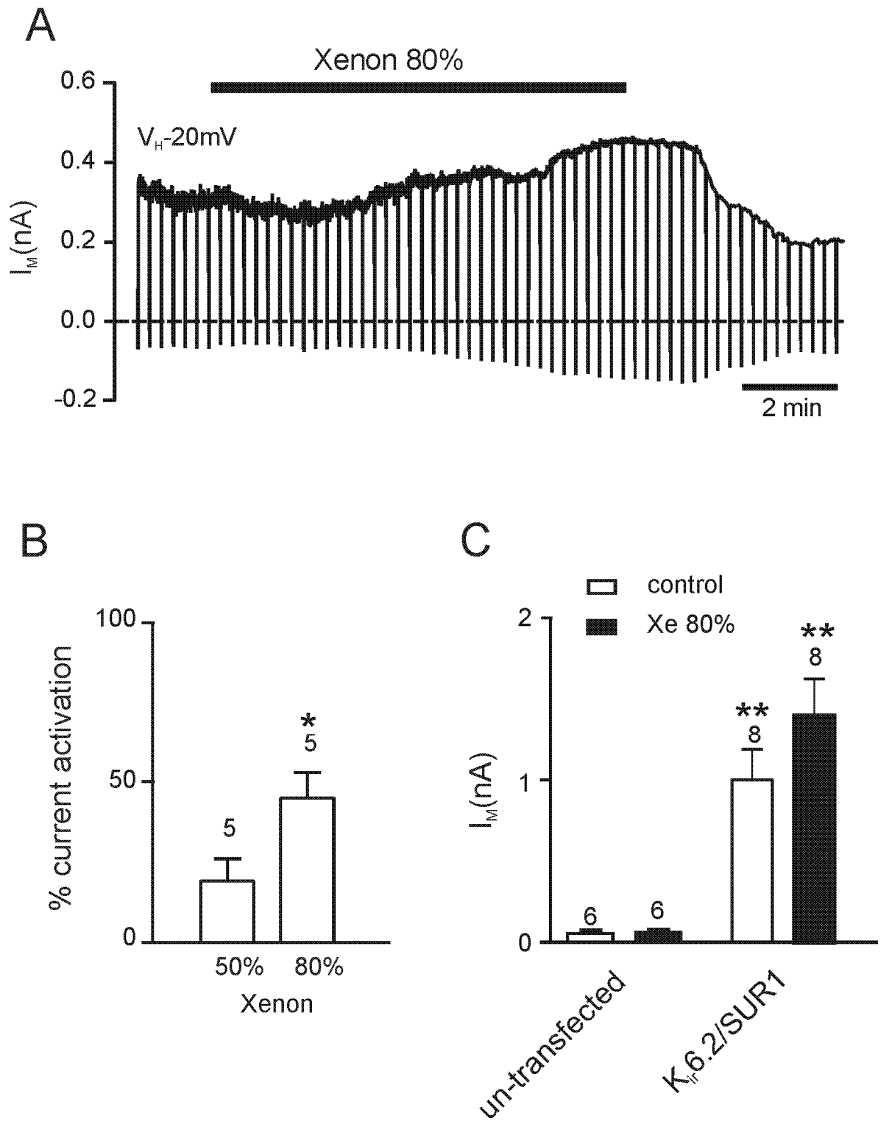


Figure 7.9: Xenon activates K_{ATP} channels

*(**A**) Whole-cell recording froma a HEK293 cell transfected with K_{ir}6.2 and SUR1 demonstrating reversible activation of the current by 80% xenon. Xenon was applied as indicated by the horizontal bar. (**B**) Mean effects of the two test-concentrations of xenon on the pK_{ATP} current at a holding potential of -20mV. (**C**) Mean affects of xenon (80%) on K^+-currents at a holding potential of –20mV of transfected and untransfected cells.*

*N-numbers are given above the bars. * indicates $p < 0.05$ compared to baseline; ** indicates $p < 0.01$ as compared to untransfected control.*

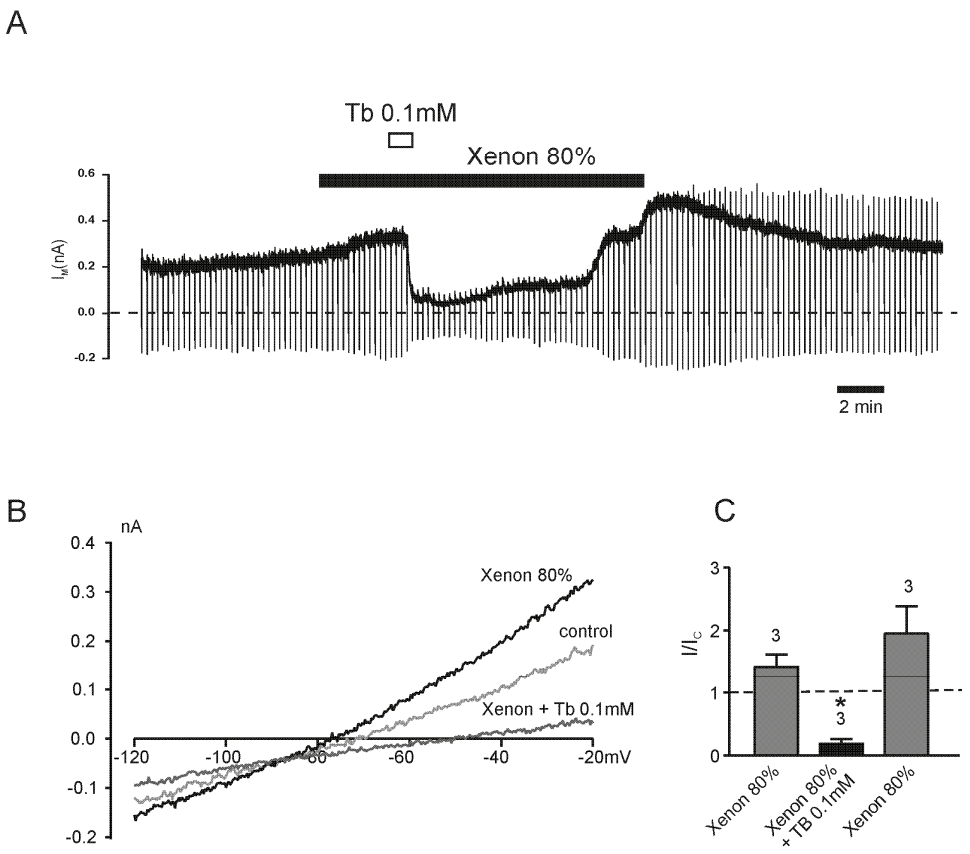


Figure 7.10: Tolbutamide reversibly inhibits pK_{ATP} currents in presence of xenon

*(**A**) Whole-cell recording from a HEK293 cell transfected with $K_{ir}6.2$ and SUR1 showing an activation of the K^+-current by 80% xenon. Tolbutamide (Tb;0.1mM), even in the presence of xenon, reversibly inhibited the pK_{ATP} current. Xenon and tobutamide were applied as indicated by horizontal bars.*

*(**B**) Current-voltage relations in the absence and presence of 80% xenon and xenon + 0.1mM tolbutamide (Tb). Xenon potentiated the pK_{ATP} current, whereas tolbutamide completely inhibited the current, even in the presence of xenon. (**C**) Mean data from experiments as depicted in (**A**). Mean holding current at -20mV in the presence of the drug expressed as a fraction of that in the absence of any drug. Xenon led to an increase in holding current and tolbutamide blocked both the xenon-potentiated current and the current in the absence of xenon (dashed line). N-numbers are given above the bars. * indicates $p < 0.05$ compared to groups exposed only to xenon.*

7.1.4 Xenon-Induced K_{ATP} Channel Opening: Insights into Possible Mechanisms

In the previous section xenon was established as an opener of plasmalemmal ATP-sensitive potassium channels. This conclusion was drawn from the following data: **a**) When applied at a concentration of 80%, which is the concentration that achieves anaesthesia in humans (Rossaint et al., 2003), xenon increased the outward current in $K_{ir}6.2$/SUR1 transfected HEK293 cells by 49+/-8% (n=7) (Figure 7.12B). **b**) The reversal potential of this current indicated it to be a K^+ current (Figures 7.10B and 7.11B). **c**) No increase in outward current in response to 80% xenon was observed when the current was pre-blocked with tolbutamide (n=7) (Figure 7.11A). **d**) 0.1mM tolbutamide were still capable of completely blocking K^+-currents, even in the presence of xenon (Figure 7.10A). **e**) Xenon increased an outward current in $K_{ir}6.2$/SUR1 transfected but not in untransfected HEK293 cells (n=6-8; Figure 7.11D).
For comparison, the traditional K_{ATP} channel opener diazoxide was also tested at a concentration of 0.01mM (typically used for preconditioning) and at 0.2mM, a concentration routinely used to activate the channel *in vitro*. As expected, diazoxide increased the whole-cell $K_{ir}6.2$/SUR1 current at both concentrations (Figure 7.12).

However, from these results it was not possible to draw any conclusions as to how xenon might facilitate pK_{ATP} channel opening. Therefore in this section experiments are presented that were designed to shed some light into the mechanism of xenon induced K_{ATP} channel activation. For this two different electrophysiological approaches were used: The whole-cell patch clamp as well as the excised inside-out macro-patch technique. In the following the findings from the whole-cell recordings are described first.

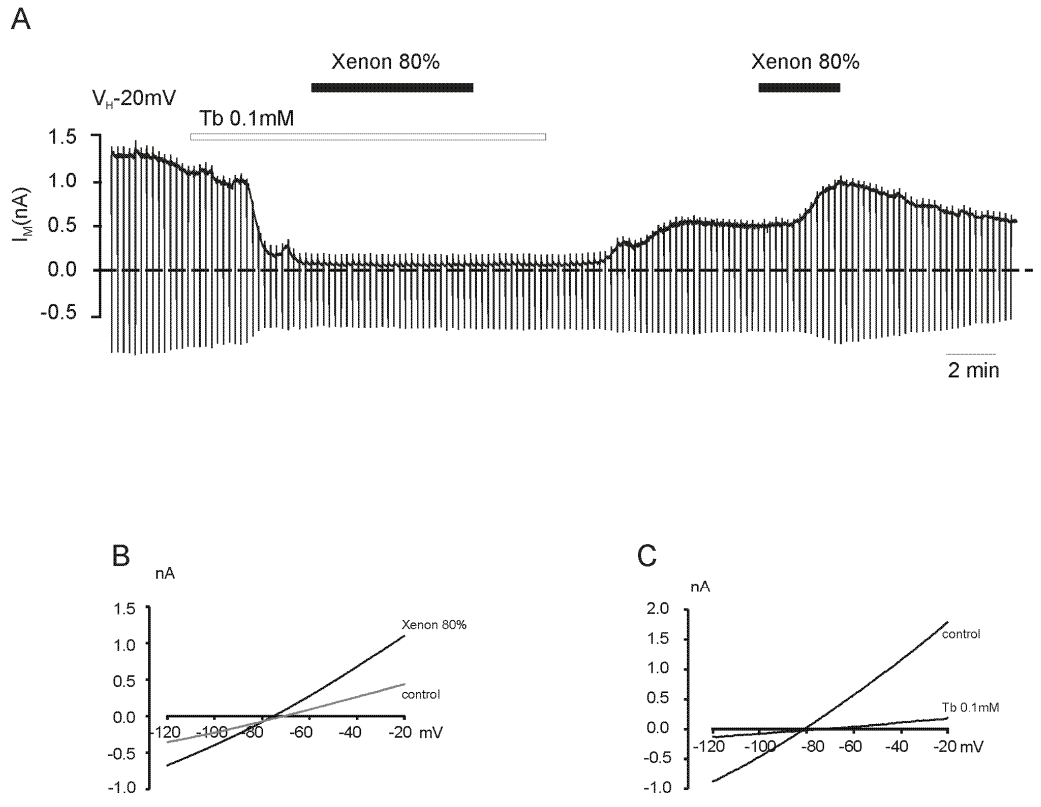


Figure 7.11: Xenon induced outward K^+-current is achieved by pK_{ATP} channel opening

*(**A**) Whole-cell recording at a holding potential (V_H) of -20 mV from a HEK293 cell transfected with $K_{ir}6.2$ and SUR1 demonstrating the reversible activation of the current by 80% xenon and it's preclusion by tolbutamide. The dashed line indicates the zero-current level. Xenon and tolbutamide (Tb;0.1mM) were bath-applied as indicated by the horizontal bars. (**B**) Current-voltage relations demonstrating the activation of the pK_{ATP} current by xenon compared to the block by tolbutamide (**C**).*

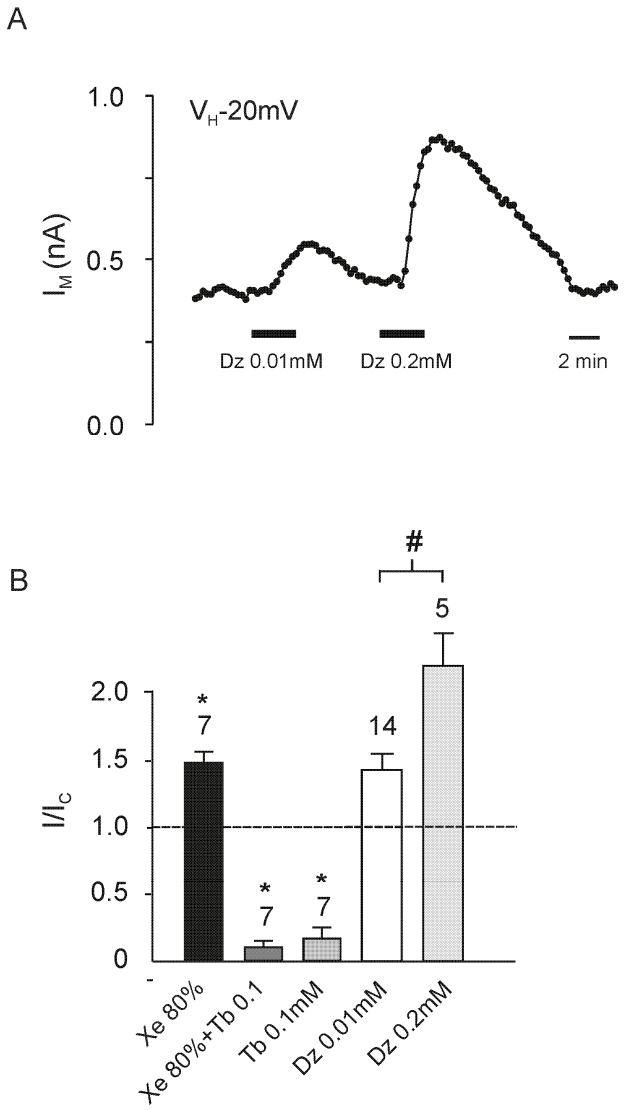


Figure 7.12: Effect of xenon, diazoxide and tolbutamide on pK$_{ATP}$ currents

*(**A**) Whole-cell recording from a HEK293 cell expressing K_{ir}6.2 and SUR1. Plotted is the mean holding current at -20mV applied every 15s. The SUR1-specific K^+ channel opener diazoxide (Dz) is applied as indicated by the solid bars. (**B**) Mean effects of the K-channel openers xenon and diazoxide on K_{ir}6.2/SUR1 currents. Expressed is the holding current at -20mV in the presence of the drug as a fraction of the current in the absence of the drug. Xe+Tb: effect of xenon (80%) on current pre-blocked with tolbutamide (0.1mM). N-numbers are given above the bars.* indicates $p < 0.05$ as compared to control. # indicates $p < 0.05$ as compared to lower drug concentration.*

7.1.4.1 Xenon as K_{ATP} channel opener: A SUR1 independent process

Since neuronal plasmalemmal K_{ATP} channels are built of $K_{ir}6.2$ and SUR1 subunits in a 4:4 stoichiometry (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997), xenon can potentially interact with either of these components to induce channel opening.

Normally both $K_{ir}6.2$ and SUR1 carry a signal that prevents travelling of each component from the endoplasmic reticulum to the cell membrane on its own. Only co-expression of the two subunits masks that signal and allows trafficking to the cell surface (Schwappach et al., 2000). This signal, which has been termed endoplasmic reticulum retention signal, is present in the C-terminal region of $K_{ir}6.2$ and in an intracellular loop between the 11^{th} transmembrane region and the 1^{st} nucleotide-binding fold of SUR1 (Zerangue et al., 1999; Seino and Miki, 2003). However, truncation of the C terminus of $K_{ir}6.2$ with deletion of the last 26 amino acids removes the retention signal. Such $K_{ir}6.2\Delta C26$ units form functional channels without the need to assemble with SUR1 (Tucker et al., 1997; Tucker and Ashcroft, 1998). This makes it possible to study the pharmacology of $K_{ir}6.2$ independently of SUR1. To address the question which subunit is essential for xenon induced pK_{ATP} channel opening, whole-cell experiments were performed in HEK293 cells expressing $K_{ir}6.2\Delta C26$ without SUR1.

The results of these experiments showed that xenon increased K^+ current through the truncated channel to the same extent as to the wild type channel (Figures 7.13A and 7.14). In contrast diazoxide and tolbutamide, which both require the SUR1 subunit in order to open and close the channel, respectively, were ineffective (Figure 7.14). These results provided evidence that the SUR1 subunit is not required for xenon induced pK_{ATP} channel opening.

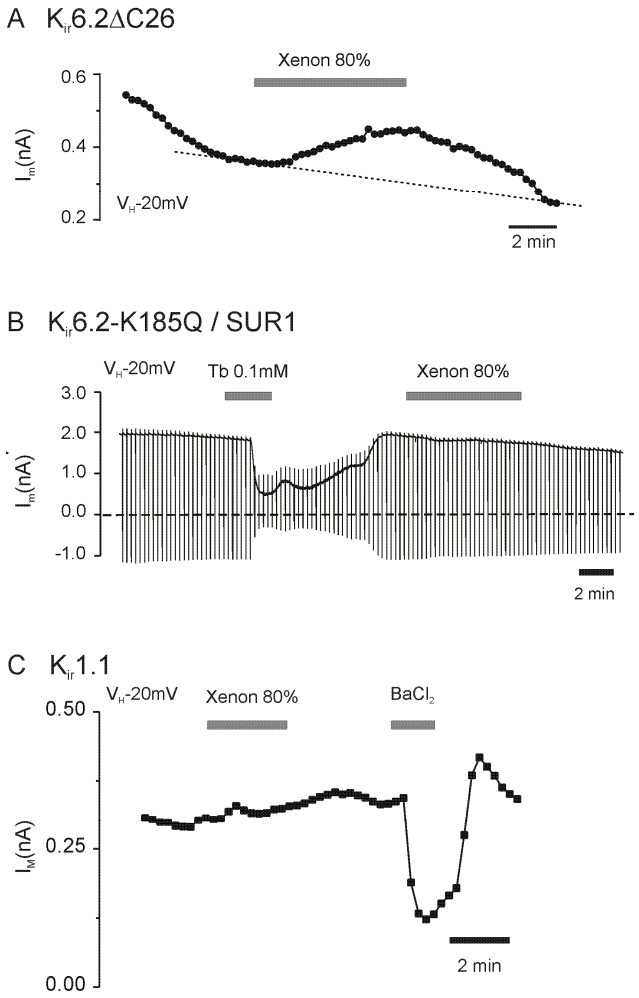


Figure 7.13: Xenon does not require the SUR1 subunit to activate K_{ATP} channels

*(**A**) Whole-cell recording from a HEK293 cell expressing K_{ir}6.2ΔC26. Plotted is the mean holding current at -20mV every 15s. Xenon activates K_{ir}6.2ΔC26 currents. Note the enhanced rundown of current compared to channels containing the SUR1 subunit (Tucker et al., 1997; Trapp et al., 1998). Dashed line: approximation of slower phase of rundown. (**B**) Whole-cell recording from a HEK293 cell expressing K_{ir}6.2-K185Q and SUR1. Tolbutamide (Tb;0.1mM) inhibited this current, but xenon failed to affect it. (**C**) Whole-cell recording from a HEK293 cell expressing K_{ir}1.1. Shown is the mean current at a holding potential of –20mV every 15s. Xenon does not affect K_{ir}1.1 activity. In contrast, barium chloride (1mM) inhibited the channel verifying the potential responsiveness of the channel in the HEK293 expression system.*

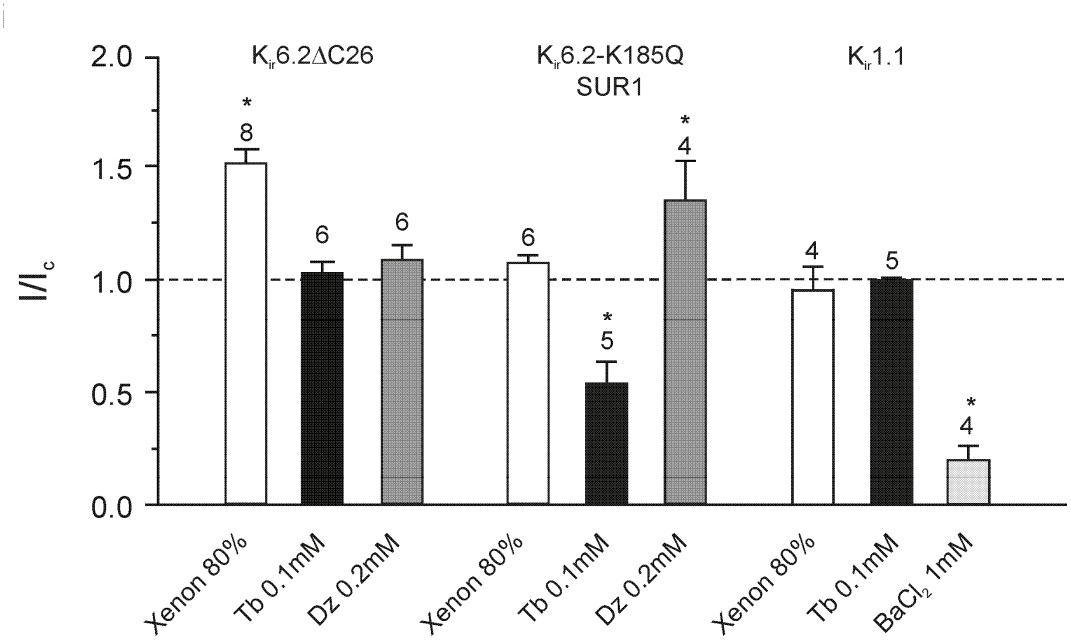


Figure 7.14: Mean effect of xenon on K_{ir}1.1 and K_{ir}6.2 channel variants

Mean data from experiments with K_{ir}6.2ΔC26, K_{ir}6.2-K185Q/SUR1 and K_{ir}1.1 as shown in Figure 7.13. Note the lack of effect of tolbutamide (Tb) and diazoxide (Dz) on K_{ir}6.2ΔC26 currents.

*N-numbers are given above the bars. * indicates $p<0.05$ as compared to control.*

7.1.4.2 K_{ir}6.2 activation by xenon: ATP sensitivity critically involved

After it was established that xenon interacts with K_{ir}6.2 to induce pK_{ATP} channel opening the question remained by what mechanism this stimulating effect was achieved. Given that K_{ir}6.2 is inhibited by ATP it seemed plausible to hypothesise an interference of xenon with the inhibitory action of ATP to aid channel activation. This essentially implies pK_{ATP} channel disinhibition by xenon as its underlying mechanism of action. However such a mechanism would be reliant on the presence and activity of ATP at K_{ir}6.2. It can consequently be expected, that any reduction of the sensitivity of the channel pore to ATP should also reduce the effect of xenon.

Recently mutations of K_{ir}6.2 have been employed to reduce its sensitivity to ATP (Tucker et al., 1997; Tucker et al., 1998). Since the nucleotide exerts its effect from the cytoplasmic site onto the channel, it became feasible to postulate an interaction with the intracellular termini of K_{ir}6.2. Therefore the mutations were directed towards the N- and C-terminus of K_{ir}6.2 (Tucker et al., 1998; Proks et al., 1999). However, although most of the channel mutations maintained channel function, channel characteristics - especially channel open probabilities - were often considerably altered (Trapp et al., 1998; Tucker et al., 1998). In this regard the exchange of the positively charged lysine to the negatively charged glutamine at position 185 at the C-terminal end of K_{ir}6.2 has been shown to be exceptional: This mutation (K_{ir}6.2-K185Q) displays a markedly reduced sensitivity to ATP because of a disrupted interaction with the β-phosphate of the nucleotide (Reimann et al., 1999; Trapp et al., 2003). However, channel function as well as channel open probability are maintained, thus excluding changes in ligand-sensitivity secondary to intrinsic gating (Trapp et al., 1998; Tucker et al., 1998).

When co-expressed with SUR1 in the present study this channel (K_{ir}6.2-K185Q/ SUR1) expressed large whole-cell currents, but addition of 80% xenon had no significant further effect (Figure 7.13B). It increased the current at -20mV by 7+/-3% (n=6; Figures 7.14). In contrast, diazoxide (n=4) enhanced and tolbutamide (n=5) inhibited these whole-cell currents (Figure 7.14).

These results implicate that a loss of ATP-sensitivity of K_{ir}6.2 leads also to a loss of xenon's ability to induce channel opening. Furthermore, these results also add

further evidence that xenon mediated activation - unlike that of diazoxide - is independent of the SUR subunit.

Since ATP is a strong inhibitor of $K_{ir}6.2$ it could be argued that xenon's effect is state dependent, relying on a closed channel conformation. However, the results of experiments where the channel was pre-blocked with tolbutamide (Figure 7.11) do not support this hypothesis. If xenon's mechanism of action were to be dependent on a closed channel state pre-blocking with tolbutamide should have magnified the xenon effect. These results support the findings with $K_{ir}6.2$-K185Q/SUR1, arguing for a xenon induced ATP disinhibition at $K_{ir}6.2$.

7.1.4.3 Specificity of involvement of ATP: Xenon does not affect $K_{ir}1.1$

In order to explore whether xenon stimulated K_{ATP} channel opening relies specifically on the presence and action of ATP at $K_{ir}6.2$, another inward rectifier K^+ channel was exposed to xenon. For those experiments $K_{ir}1.1$ was chosen because it shares significant sequence identity with $K_{ir}6.2$. In addition $K_{ir}1.1$ is the only inward rectifier outside the $K_{ir}6.x$ subfamily that is also inhibited by ATP, albeit in the millimolar range (Ho et al., 1993; Vanoye et al., 2002; Haider et al., 2007a).

When expressed in HEK293 cells, $K_{ir}1.1$ exhibited whole-cell currents of 672+/-96pA (n=8) at -20mV. The addition of 80% xenon did not enhance those currents (Figure 7.13C and 7.14) but decreased the amplitude marginally by 4+/-9% (n=4). In contrast and as expected $K_{ir}1.1$ was sensitive to $BaCl_2$, that blocked the channel by 80+/-6% (n=4) (Figure 7.13C and 7.14) (Bhandari and Hunter, 2001). $K_{ir}1.1$ was also not affected by 0.1mM tolbutamide (99+/-1% of control current remaining; n=5; Figure 7.14).

Since xenon does not activate another inwardly rectifying K^+ channel ($K_{ir}1.1$), which has an intrinsically low affinity to ATP, and since xenon loses its channel opening properties once the ATP sensitivity of $K_{ir}6.2$ is reduced, it is feasible to suggest that the xenon induced K_{ATP} channel activation is specifically linked to an ATP-dependent mechanism.

7.2 Inside-Out Macropatch Experiments

The results presented so far demonstrate that the SUR1 subunit of plasmalemmal K_{ATP} channels is not essential for xenon activation and that activation is not universal between inwardly rectifying K^+ channels. The results further suggest that the effect of xenon in contrast to that seen with the prototypical K^+ channel opener diazoxide requires the integrity of ATP-inhibition on pK_{ATP} channels in order to stimulate K^+ currents. However, these results do not exclude a possible interaction of xenon with intracellular pathways such as mitochondrial metabolism and subsequent reduction of ATP concentration within the cell which then in turn causes an increase in K_{ATP} currents. Consequently, inside-out patch-clamp recordings were used to separate the pK_{ATP} channels from potential influences of either cell metabolism or intracellular signalling cascades. This technique also allows the direct exposure of pK_{ATP} channels to defined ATP and test-drug concentrations to further explore the relation between ATP-inhibition and xenon-activation of K_{ATP} currents.

7.2.1 Xenon Interaction with $K_{ir}6.2$: The Critical Role of ATP

In the previous section it was described how mutations at the C- or N-terminal cytoplasmic endings of $K_{ir}6.2$ could reduce the channel's sensitivity to ATP. With the help of those mutations it was subsequently possible to identify and model the binding site of ATP on $K_{ir}6.2$ (Trapp et al., 2003; Antcliff et al., 2005). Part of this binding site is lysine at position 185 on the C-terminus (Tucker et al., 1997; Tanabe et al., 1999a). Mutations in this position, for instance lysine to glutamine (K185Q) as used in the present study, will therefore reduce the sensitivity of $K_{ir}6.2$ to ATP. This is because the mutation is suggested to impair the ability of the β-phosphate of ATP to interact with the channel protein (Trapp et al., 2003).
If such a mutation with reduced sensitivity to ATP would also show diminished responsiveness to xenon, even under excised patch conditions, that would further strengthen the hypothesis that the xenon effect on pK_{ATP} channels is crucially dependent on ATP.

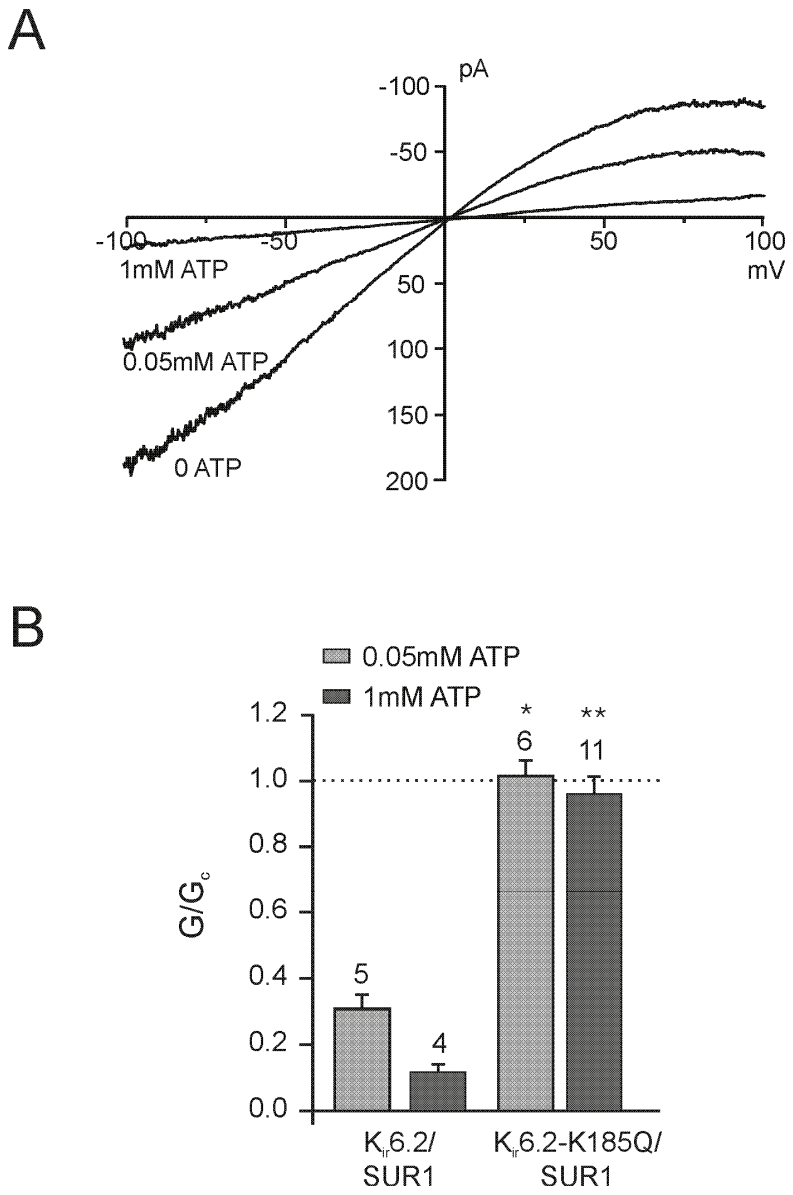


Figure 7.15: ATP inhibition of wild type but not K_{ir}6.2-K185Q/SUR1 currents

*(**A**) Inside-out patches excised from a HEK293 cell expressing K_{ir}6.2/SUR1 revealed macroscopic inwardly rectifying ATP-sensitive currents under symmetrical K^+ concentrations. (**B**) Mean slope conductance in the presence of ATP expressed as a fraction of the current in the absence of ATP. ATP-inhibition was concentration-dependent in the wild type and absent in the K_{ir}6.2-K185Q/SUR1 mutant. N-numbers are given above the bars. * indicates $p<0.05$ compared to K_{ir}6.2/SUR1. ** indicates $p<0.01$ compared to K_{ir}6.2/SUR1.*

Figure 7.15A shows typical current-voltage relations for $K_{ir}6.2$/SUR1 currents in inside-out macropatches. The wild type $K_{ir}6.2$/SUR1 current was highly sensitive to ATP, with 0.05mM ATP inhibiting the current by ~70% and 1mM ATP causing virtually complete inhibition. In contrast, $K_{ir}6.2$-K185Q/SUR1 currents were not reduced by 0.05mM or 1mM ATP (Figure 7.15AB). These results verified the effectiveness of the K185Q mutation on ATP sensitivity of $K_{ir}6.2$.

Similar to what was found in whole-cell experiments, xenon enhanced wild type $K_{ir}6.2$/SUR1 currents, but not mutant $K_{ir}6.2$-K185Q/SUR1 currents (Figure 7.16). This distinctiveness also remained at various ATP concentrations (Figure 7.16 and 7.17): In the nominal absence of ATP xenon did not activate $K_{ir}6.2$-K185Q/SUR1 currents but was still able to increase the slope conductance of the $K_{ir}6.2$/SUR1 current by about 20%, although this was significantly less than found in the presence of ATP (Figure 7.17B). In the presence of 0.05mM ATP xenon enhanced wild type currents by more than 50% (Figure 7.17B). This is comparable to the situation in whole-cell recordings where similar increases were achieved by xenon. In contrast, xenon had no effect on $K_{ir}6.2$-K185Q/SUR1 currents (Figure 7.17B).

Since the results of these inside-out patch-clamp experiments were similar to what was demonstrated in whole-cell experiments, they also support the idea that xenon-induced with K_{ATP} channel opening is critically dependent on the presence of ATP.

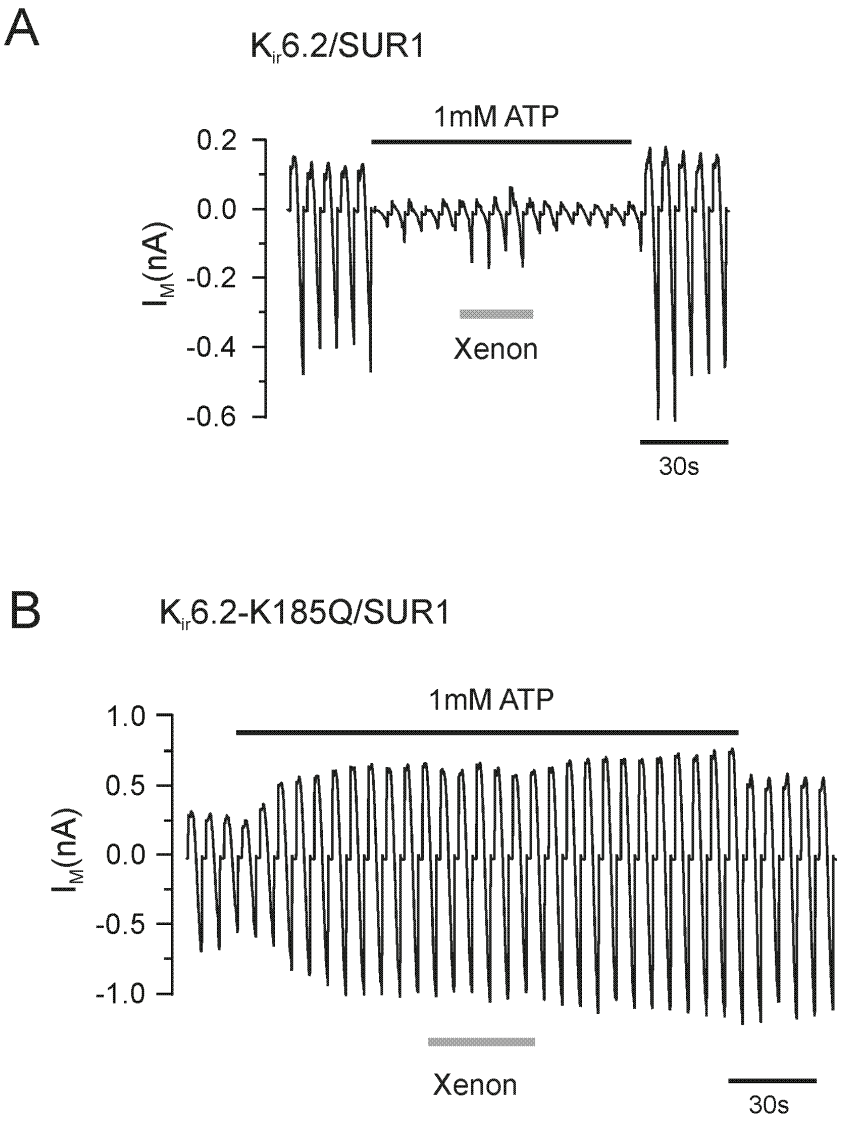


Figure 7.16: Xenon enhances wild type but not K_{ir}6.2-K185Q/SUR1 currents

*(**A**) Xenon enhanced K_{ir}6.2/SUR1, but not K_{ir}6.2-K185Q/SUR1 currents in the presence of 1mM ATP. (**B**) In contrast, the K_{ir}6.2-K185Q/SUR1 current was increased by 1mM ATP due to Mg-ATP-dependent refreshment, i.e. reconstitution of a greater and stable channel conductance that was previously diminished by rundown (Tucker et al., 1997; Xie et al., 1999b). However, xenon could exert no further effect.*

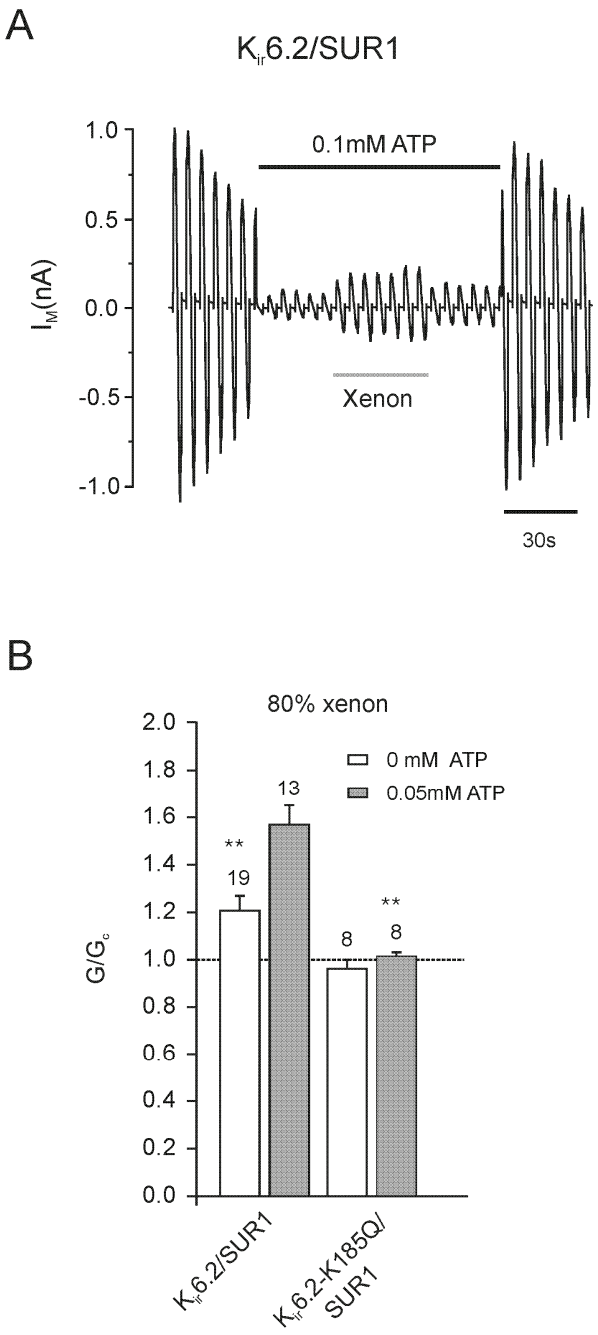


Figure 7.17: Xenon increases K_{ir}6.2/SUR1 currents at various ATP concentrations

*(**A**) Xenon enhanced K_{ir}6.2/SUR1 currents in the presence of 0.1mM ATP. (**B**) Xenon enhancement of the wild type channel was reduced in the absence of ATP and it was abolished in the K_{ir}6.2-K185Q mutant both in the absence and in the presence of 0.05mM ATP. N-numbers are given above the bars. ** indicates $p<0.01$ compared to K_{ir}6.2/SUR1 in presence of 0.05mM ATP.*

7.2.2 Xenon and ATP: Evidence for a Competitive Antagonism

The final experiments were conducted to further clarify the nature of the interference of xenon with ATP at the pK_{ATP} channel. For this purpose concentration-response relationships of ATP on $K_{ir}6.2$/SUR1 were constructed in the absence or presence of 80% xenon. Also, to minimize a theoretical influence of an ATP effect on SUR1 as a contributing factor to channel opening, recordings were performed in Mg^{2+}-free solutions (Gribble et al., 1998a). Data showed a parallel shift of the ATP concentration-inhibition curve to the right when xenon was added (Figure 7.18). This was reflected by an increase of the K_i of ATP from 26+/-4μM (n=5) in the absence to a K_i of 96+/-6μM (n=6) in the presence of 80% xenon. These results therefore showed a reduced potency of the inhibitory action of ATP at $K_{ir}6.2$/SUR1 in the presence of xenon, which is suggestive of a competitive interaction of xenon with ATP at the pore-forming subunit $K_{ir}6.2$.

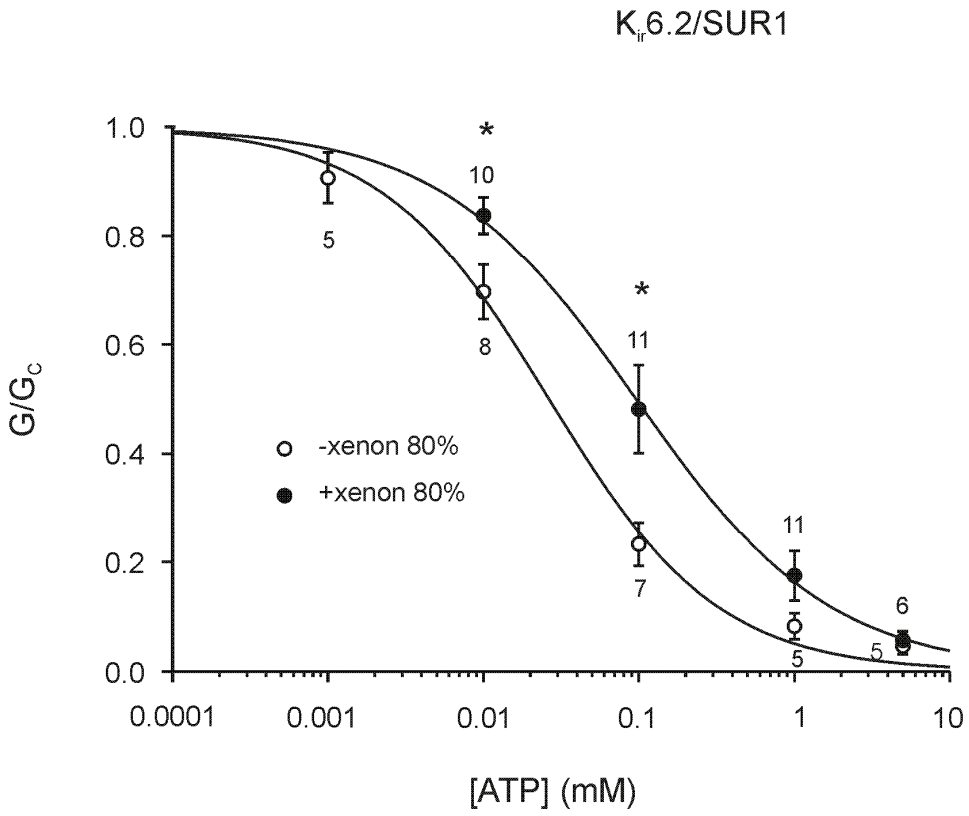


Figure 7.18: Concentration-response effect of ATP in the absence and presence of xenon

ATP concentration-inhibition curves for K_{ir}6.2/SUR1 currents in the absence of $MgCl_2$. Experiments were performed either in the absence (open circle) or presence of 80% xenon (filled circle). Solutions containing ATP were alternated with ATP-free solutions. Slope conductance (G) in the presence of the drug is expressed as a fraction of the slope conductance in its absence (G_c). The slope conductance was determined between -20 and -100mV and is the mean of five voltage ramps. The lines are the best fit to the data using a modified Hill equation (Equation 6.7).

7.3 Anaesthetic Preconditioning: Functional Relevance of pK_{ATP} Channel Opening

The establishment of halothane and isoflurane as potent pK_{ATP} channel inhibitors in the present study made them unlikely candidates for any neuronal preconditioning that is dependent on pK_{ATP} channel opening. Therefore they were not further tested in a functional preconditioning assay. Sevoflurane in contrast exhibited only minor channel inhibitory properties in the heterologous HEK cell expression system. However, to what degree the sevoflurane induced channel inhibition had consequences for sevoflurane preconditioning remained elusive. Therefore experiments investigating the pK_{ATP} channel dependence of sevoflurane induced preconditioning were conducted. In addition the functional relevance of xenon's newly established pK_{ATP} channel opening properties were investigated in these experiments as well.

The final part of the present study was therefore designed to analyse the role of K_{ATP} channels in preconditioning induced by xenon and sevoflurane using oxygen-glucose-deprivation (OGD) in neuronal-glial co-cultures as an *in vitro* model of ischaemic brain injury. In this model 70 to 80 minutes of OGD was sufficient to maximally damage neurons, with glial cells being left largely unharmed (Ma et al., 2004; Rajakumaraswamy et al., 2006). The extent of the injury was found to remain unchanged from 6 to 24h after OGD with no further evidence for either a delayed second burst of damage or repair mechanisms taking place (Ma et al., 2004). In preliminary experiments it was also determined that the pK_{ATP} channel openers and inhibitors used in the preconditioning experiments had no intrinsic damaging effect on neuronal-glial co-cultures (Figure 7.19).

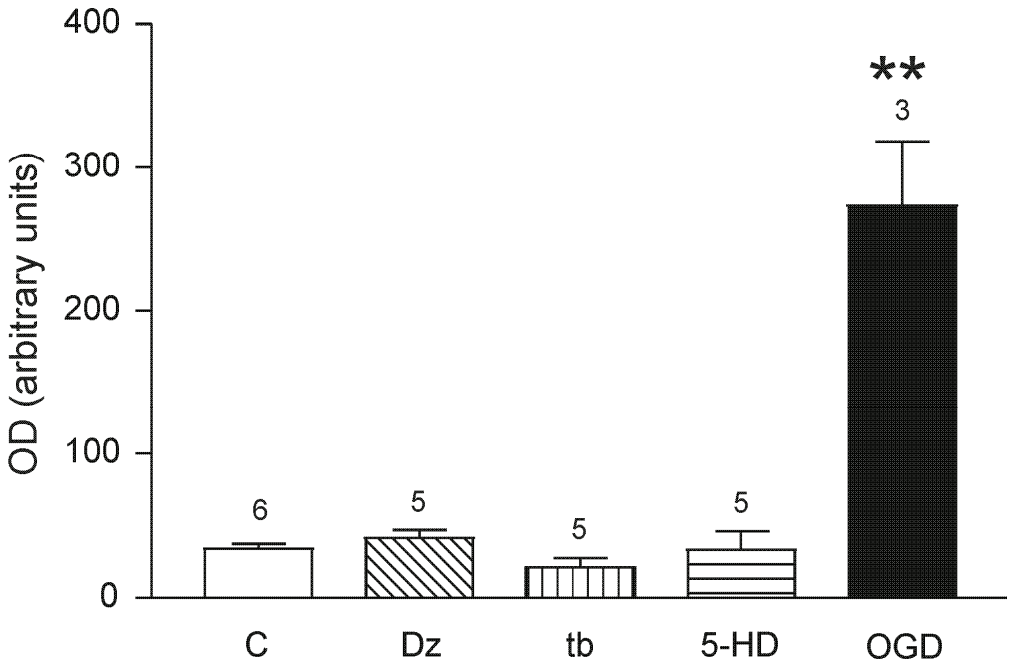


Figure 7.19: No damaging effect of K_{ATP} channel openers and inhibitors on neuronal-glial cell co-cultures

Direct damaging effects of test drugs on neuronal-glial co-cultures were excluded in preliminary experiments.

*Neuronal-glial co-cultures were exposed for 2 hours to either the K_{ATP} channel opener diazoxide (Dz; 0.01mM) or to the channel inhibitors tolbutamide (Tb; 0.1mM) and 5-HD (0.5*m*M). Cell damage was unchanged compared to naïve control (C) but significantly less compared to co-cultures exposed to oxygen-glucose deprivation (OGD).*

Cell damage was assessed through the amount of lactate dehydrogenase (LDH) released from the cells into the culture medium (see methods). LDH concentrations were determined spectrophotometrically. Optical density (OD) is given in arbitrary units. Cell damage is proportional to the OD reading.

*** indicates $p<0.01$ compared to control. N-numbers are given above the bars.*

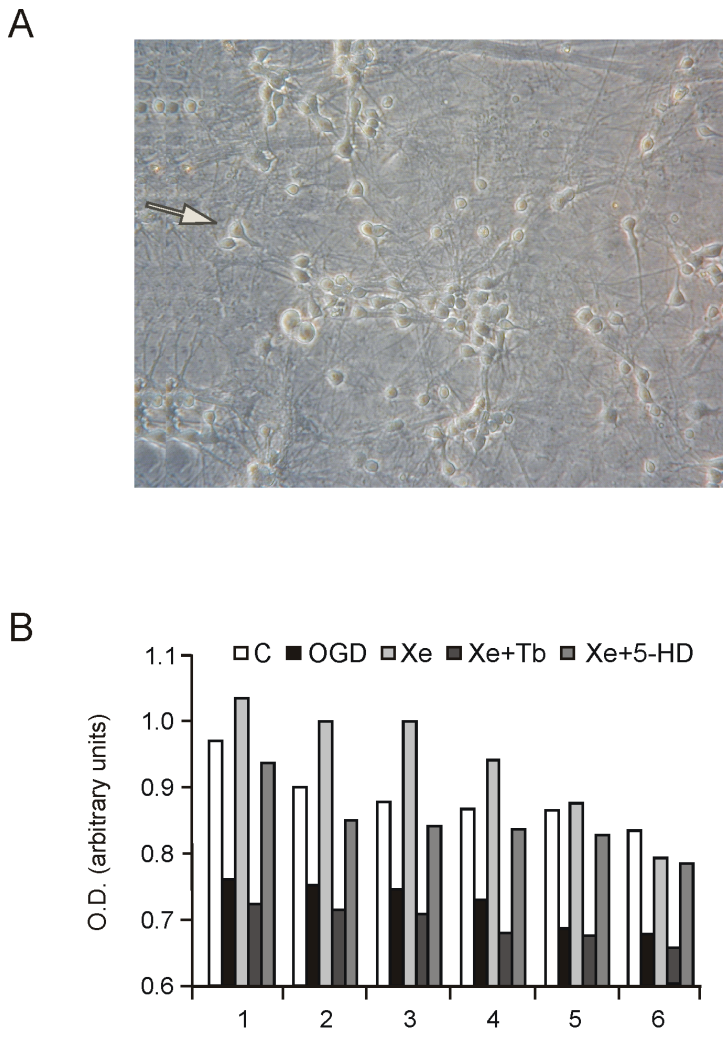


Figure 7.20: Neuronal-glial cell culturing and preconditioning experiment

*(**A**) Phase-contrast microscopical image under 120x magnification of a typical neuronal-glial co-culture ready for use in preconditioning experiments. Clearly visible are intact neurons (arrow) on a dense layer of glial cells.*

*(**B**) Representative raw data of different preconditioning experiments (1-6). Cell viability was assessed with 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction test and expressed in arbitrary units of optical density reading. Viability (survival) is proportionate to OD with a low reading being indicative for cellular damage.*

C: naïve control cultures; OGD: co-cultures exposed to oxygen-glucose deprivation; Xe: xenon treated cultures; Xe+Tb: cultures exposed to xenon and tolbutamide; Xe+5HD: cultures exposed to xenon and 5-HD

Exposure to the anaesthetics xenon or sevoflurane, or to the potassium channel opener diazoxide 24 h prior to the OGD insult, effectively prevented neuronal cell loss (survival: 80-100%; Figure 7.21). The addition of the pK_{ATP} channel inhibitor tolbutamide to the xenon group during preconditioning completely abolished the protective effect of xenon (Figure 7.21A). Xenon's protective effect was also slightly reduced by the addition of the mKATP channel inhibitor 5-hydroxydecanoate (5-HD). Although statistically significant, the reversal was not complete since cell viability remained high in cultures treated concomitantly with xenon and 5-HD (Figure 7.21A). In contrast, the preconditioning effect of sevoflurane could not be reversed with either the pK_{ATP} channel inhibitor tolbutamide or the mKATP channel inhibitor 5-HD (Figure 7.21B). Finally, diazoxide preconditioning was completely abolished by 5-HD and to a slightly lesser extent by tolbutamide (Figure 7.21C). Diazoxide, at a concentration of 0.01mM, is widely seen as specific for mKATP channels (Garlid et al., 1996; Garlid et al., 1997). However, this is only true for cardiac tissue where K_{ATP} channels have a SUR2A subunit. The SUR1 subunit of neuronal pK_{ATP} channels has also been shown to respond to diazoxide (Dabrowski et al., 2003). These findings were supported by the results of whole-cell recordings in the present study (Figures 7.4, 7.5 and 7.12). Thus it is not surprising that the preconditioning effect of diazoxide was sensitive to the pK_{ATP} channel blocker tolbutamide as well as the mKATP channel antagonist 5-HD, suggesting a role for both channels. These results indicated that opening of plasmalemmal K_{ATP} channels, but not mKATP channels, is essential for neuronal preconditioning by xenon, and demonstrated that sevoflurane preconditioning does not involve the opening of either plasmalemmal ATP-sensitive K^+ channels or mKATP channels. The clear pK_{ATP} channel-dependence of xenon preconditioning and the lack of K_{ATP} channel involvement in sevoflurane preconditioning were consistent with the results from of the electrophysiological experiments. Therefore these results suggest that xenon's pK_{ATP} channel opening properties are functionally important for its neuroprotective actions. In contrast, this study provides evidence that sevoflurane preconditions via other, not yet determined, pathways.

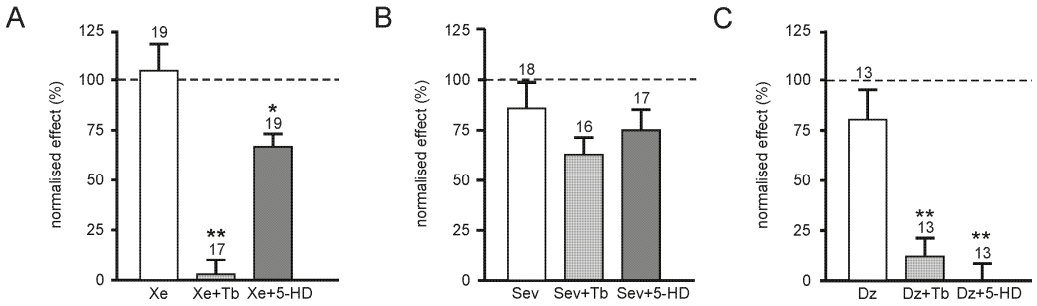


Figure 7.21: Preconditioning by xenon but not sevoflurane involves K_{ATP} channels

*(**A**), (**B**) and (**C**) show the mean +/- SEM data for preconditioning experiments. Cell viability was measured with MTT as optical density (O.D.) at 570nm. The 'normalised effect' describes the relative viability compared to maximal damage (OGD only, no preconditioning) and maximal survival (no OGD; naïve cultures). 100% cell viability therefore represented the viability observed in naïve cultures at the end of the protocol, whereas 0% cell viability indicated the viability found in cultures exposed to OGD only. (**A**) Xenon preconditioning (Xe; 1MAC) is abolished by the K_{ATP} channel inhibitor tolbutamide (Xe+Tb; 0.1mM), but not the mKATP channel blocker 5-HD (0.5mM). (**B**) Sevoflurane (Sev; 2MAC) effectively preconditions, however this effect is not significantly reduced by tolbutamide (Tb) or 5-HD. (**C**) Preconditioning by the K_{ATP} channel opener diazoxide (0.01mM) is blocked by either tolbutamide or 5-HD. N-numbers are given above the bars. * indicates $p<0.05$, ** indicates $p<0.01$ compared to groups only treated with either xenon or diazoxide, respectively.*

8. DISCUSSION

8.1 Anaesthetic Effects on Recombinant Neuronal Plasmalemmal K_{ATP} Channels

This is the first study to show that the noble gas xenon opens plasmalemmal ATP-sensitive K^+ channels whereas the volatile anaesthetics halothane, isoflurane and sevoflurane were established as pK_{ATP} channel inhibitors. It was further demonstrated that the pK_{ATP} channel opening effect of xenon is mediated via its interaction with the pore forming $K_{ir}6.2$ rather than the regulatory SUR1 channel subunit. For this interaction not only the presence of ATP was found to be essential but data also indicated a direct competition of xenon with ATP at the channel's ATP binding site. From these results it can therefore now be hypothesised that xenon induced pK_{ATP} channel opening is caused by a disinhibiton of $K_{ir}6.2$ from ATP.

8.1.1 Contribution of Mitochondrial KATP (mKATP) Channels

Recent research has suggested the possibility that mitochondrial KATP channels, could interfere with the activity of plasmalemmal K_{ATP} channels (Zaugg et al., 2003).

Two different pathways could achieve mKATP channel induced pK_{ATP} channel opening: The first is mediated via intracellular signalling cascades whereas the second involves changes of intracellular ATP concentration.

Nitric oxide (NO), reactive oxygen species (ROS) and protein kinase C (PKC) amongst others have been suggested as mediators of those pathways between mKATP and pK_{ATP} channels (Zhuo et al., 2005). For instance opening of mKATP channels has been proposed to induce NO and ROS generation as well as PKC translocation (Lebuffe et al., 2003; Xi et al., 2005; Roth et al., 2006). In this pathway ROS are located up-stream from PKC, which in turn increases K^+ conductance of cardiac and pancreatic type pK_{ATP} channels by increasing the channel open probability (Light et al., 2000; Juhaszova et al., 2004; Jung et al.,

2004). NO on the other hand can independently induce both mKATP and pK_{ATP} channel opening. The effect on vascular smooth muscle type ($K_{ir}6.2$/SUR2B) and likely also on neuronal type pK_{ATP} channels has been suggested to be mediated via a mitogen-activated protein kinase pathway whereas opening of mKATP channels in cardiomyocytes is achieved via a cGMP-protein-kinase G mechanism (Lin et al., 2004; Oldenburg et al., 2004). However, mKATP channel opening through NO might also induce the described ROS and PKC dependent cascade with the subsequent opening of pK_{ATP} channels (Zaugg et al., 2003).

In addition to the interaction of mKATP and pK_{ATP} channels via signalling cascades it has also been suggested that mKATP channel activity might influence opening of pK_{ATP} channels via regulation of mitochondrial oxidative phosphorylation and hence cellular ATP production. This hypothesis is supported for example by data showing that myocardial pK_{ATP} channels are directly regulated via cytosolic ATP that was predominantly produced through oxidative phosphorylation in the mitochondria (Knopp et al., 2001).

If in contrast ATP hydrolysis would occur in mitochondria instead of ATP production, the cytoplasmic ATP concentration could be expected to drop. Subsequently, plasmalemmal ATP-sensitive K^+ channels as the archetypal ATP sensors would likely become activated, which would be followed by an increased outward K^+ current (Ashcroft and Trapp, 2001; Sasaki et al., 2001). Mitochondrial ATP hydrolysis has been suggested to occur if the inner mitochondrial membrane is depolarised as a consequence of increased potassium influx through mKATP channels. Furthermore, inner mitochondrial membrane depolarisation has also been suggested to directly reduce ATP production via inhibition of oxidative phosphorylation (Holmuhamedov et al., 1998). Therefore activation of mKATP channels is hypothesised to lead to mitochondrial uncoupling with the consequence of reduced ATP production (Kopustinskiene et al., 2003).

Any significant interaction between mKATP and pK_{ATP} channels in the heterologous HEK293 cell system of the present study could be expected to be reflected in an increased K^+ current measured in whole-cell patch clamp recordings in response to the addition of the mKATP channel opener pinacidil. Conversely, the mKATP channel inhibitor 5-HD should be able to reduce K^+ current. However, our observation that neither pinacidil nor 5-HD had any effect

on the activity of plasmalemmal K_{ATP} channels, indicates that modulation of mKATP channels cannot influence pK_{ATP} channels in our system. Thus the observed effects of anaesthetics on these channels cannot be explained by an indirect effect via mKATP channels.

8.1.2 Direct Anaesthetic Effects on Plasmalemmal K_{ATP} Channels

One exciting finding of the present study was that the noble gas xenon was able to open K_{ATP} channels in whole-cell patch-clamp experiments. So far in the search for the mechanisms of the anaesthetic actions of xenon its effects on some ion channels had already been investigated (for reviews see Sanders (Sanders et al., 2003) and Preckel (Preckel et al., 2006)). However in most cases xenon was incapable of influencing the current through the channels in question. De Sousa and colleagues for instance found xenon to be ineffective on $GABA_A$, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate receptors (de Sousa et al., 2000). In addition Stowe as well as White and colleagues could not find evidence in their experiments for xenon being able to alter current flow through Na^+ or L- and N-type Ca^{2+} channels (Stowe et al., 2000; White et al., 2005).

These data would have easily led to the suggestion that xenon is unlikely to be capable of interacting with ion channels in keeping with its role as an inert gas. In contrast and as early as these 'negative' results there have also been reports of xenon as having inhibitory properties on some channels. Suzuki for example demonstrated the inhibition of neuronal type nicotinic acetylcholine receptors by xenon in voltage-clamp experiments (Suzuki et al., 2003). However, the so far most important finding in the search to uncover the mechanisms of xenon-induced anaesthesia was its establishment as N-methyl-d-aspartate (NMDA) receptor antagonist by de Sousa in 2000 (de Sousa et al., 2000). This antagonistic effect was later shown by Dickinson to be mediated through the interaction of xenon with the glycine-binding site at the NMDA receptor (Dickinson et al., 2007).

Xenon's effect on K^+ channels has also been investigated recently: In the above described publication by Stowe and colleagues the authors looked at the actions of

xenon on inward rectifying K^+ currents in isolated cardiomyocytes (Stowe et al., 2000). Although they found that xenon was ineffective in altering K^+ currents in their experiments, the study was hampered by the fact that it did not distinguish between the different types of inwardly rectifying potassium channels. This is especially important in the heart since at least three different K_{ir} subfamilies ($K_{ir}2$, $K_{ir}3$ and $K_{ir}6$) have been described in myocardial cells (Ehrlich, 2008). Stowe's results therefore most likely reflect a net-effect of xenon on the various cardiac type inwardly rectifying K^+ channels.

First evidence that xenon might be able to influence K^+ currents came from whole-cell patch-clamp experiments in human cardiomyocytes. In those preparations Hueneke and colleagues demonstrated a small inhibitory effect of xenon on voltage-gated K^+ channels (Huneke et al., 2001). Furthermore, Gruss and colleagues investigated xenon's action on 2-pore-domain potassium channels and found subtype specific xenon effects (Gruss et al., 2004). Whilst xenon was unable to alter TASK-3 currents, the authors were able to demonstrate xenon dependent activation of TREK-1 channels. Therefore their results provided the first evidence that xenon is able to open K^+ channels. The current results with xenon enhancing current flow through pK_{ATP} channels advance Gruss' findings to another family of potassium channels.

The physiological role of this newly discovered K^+ channel opening in the context of xenon induced preconditioning will be discussed in detail below.

In contrast to xenon the volatile anaesthetics halothane, isoflurane and sevoflurane failed to activate plasmalemmal K_{ATP} channels. In fact, dose dependent inhibition was observed. At 1 MAC halothane and isoflurane inhibited pK_{ATP} channels mildly but more substantially than sevoflurane at higher concentrations. These results were similar to what has been described previously for the effects of barbiturates: At supra-clinical concentrations they are capable of inhibiting neuronal K_{ATP} channels (Ohtsuka et al., 2006). Indirect evidence that halothane might act as an inhibitor of K_{ATP} channels came also from experiments with likely $K_{ir}6.2$/SUR2B involvement: Larach and colleagues instead of reversing halothane induced smooth muscle relaxation with the K_{ATP} channel inhibitor glybenclamide found an increased effect (Larach and Schuler, 1993). These results were later supported by Fukushima and colleagues who found no evidence that smooth

muscle relaxation through halothane is mediated by K_{ATP} channel opening (Fukushima et al., 1996). More direct evidence for halothane and isoflurane being inhibitors of K_{ATP} channels was provided by studies of Roscoe and Han, respectively. They found a reduction of K_{ATP} currents in whole-cell patch-clamp experiments in cardiomyocytes in keeping with inhibitory effects of both agents on cardiac type pK_{ATP} channels (Han et al., 1996; Roscoe et al., 2000) Furthermore, a recent study investigating the acute effects of 4% sevoflurane on the membrane potential of hippocampal CA1 pyramidal cells also failed to see an activation of neuronal type K_{ATP} channels despite the fact that hypoxia elicited such a hyperpolarisation, which was also enhanced after preexposure to sevoflurane (Wang et al., 2007b). These results probably reflect our finding that sevoflurane is able to precondition, but not in a K_{ATP} channel dependent manner.

Inhibition by halogenated anaesthetics might be a general feature of inwardly-rectifying K^+ channels as several members of this ion channel family were inhibited to varying degrees by these agents (Yamakura et al., 2001). In the case of the K_{ATP} channel the SUR subunit might have an additional modulatory influence on the anaesthetic sensitivity as Bienengraeber and colleagues reported that the SUR2A subunit of cardiac plasmalemmal K_{ATP} channels conferred isoflurane activation of the K^+ current under acidic conditions (Bienengraeber et al., 2006).

8.1.3 Mechanism of Xenon-Induced Plasmalemmal K_{ATP} Channel Opening

In the previous section the data was discussed that established xenon as an opener of plasmalemmal K_{ATP} channels. It was further argued that mitochondrial KATP channels do not contribute to xenon induced pK_{ATP} channel opening. However from this data the mechanism of interaction of xenon with $K_{ir}6.2$/SUR1 could not be deduced. This will be done in the following section using the results of further whole-cell and excised patch electrophysiology experiments.

8.1.3.1 $K_{ir}6.2$: Xenon's principal target

In addition to the more general observation, which found xenon to be capable of activating K_{ATP} channels, the present study also provides more insight into possibly involved mechanisms.

Most neuronal pK_{ATP} channels are complexes composed of $K_{ir}6.2$ and SUR1 subunits. Since xenon could theoretically interact with each of those subunits to induce channel opening, this study sought to determine the principally involved target. This was especially important since to this point endogenous modulation of SUR1 has been predominantly linked with channel opening whereas actions on $K_{ir}6.2$ are classically inhibitory (Tucker and Ashcroft, 1998). However, the data presented here indicate that xenon interacts with $K_{ir}6.2$ rather than SUR1 to facilitate channel opening. This notion is based on the results obtained from experiments using truncated pK_{ATP} channels ($K_{ir}6.2\Delta C26$). Tetrameric assembly of $K_{ir}6.2\Delta C26$ forms a functional channel in the absence of sulphonylurea receptors that has similar characteristics to the wild type channel. $K_{ir}6.2\Delta C26$ has therefore become an invaluable tool to study the channel pore independently of SUR1 (Tucker et al., 1997). Since xenon activated $K_{ir}6.2\Delta C26$ channels to a similar extent as $K_{ir}6.2$/SUR1 channels it is feasible to assume that $K_{ir}6.2$ comprises xenon's principal target. This is in contrast to all other pK_{ATP} channel openers (Lawson, 2000). Although chemically diverse they all interact mainly with the second transmembrane domain (TMD2) of the sulphonylurea receptor (Jahangir and Terzic, 2005). Therefore the data of the present study suggest xenon

to be not simply another K_{ATP} channel opener but possibly to represent an entirely different class of K_{ATP} channel modulators.

This idea is further supported by experiments using Mg^{2+} free solution. Mg^{2+} is the compulsory co-factor that enables nucleotides but especially di-phosphate-nucleotides like ADP or GDP to exert their stimulatory (opening) action on wild type pK_{ATP} channels (Nichols, 2006). Furthermore the nucleotide binding domains (NBD) on sulphonylurea receptors (SUR) have been identified as the molecular target of Mg^{2+}-nucleotides (Ueda et al., 1997; Sakura et al., 1999; Moreau et al., 2005). Therefore the ability of xenon in the present study to open $K_{ir}6.2$/SUR1 even under Mg^{2+} free experimental conditions excludes a possible indirect effect via interaction with Mg^{2+}-nucleotides on SUR1 (Craig et al., 2008). This again is in contrast to classical K-channel openers, since the action of these drugs is abolished in the absence of Mg^{2+} or when the nucleotide binding domains of SUR are functionally disabled by point mutation (Gribble et al., 1997b).

Additional evidence that supports a SUR1 independent action of xenon is provided by the results of experiments using the K185Q mutation of $K_{ir}6.2$, which reduces the ATP sensitivity of the pore forming subunit (Tucker et al., 1997). The present study not only verified that $K_{ir}6.2$-K185Q is ATP insensitive but also demonstrated that $K_{ir}6.2$-K185Q is unresponsive to xenon. Since these experiments were performed in the presence of SUR1 it is unlikely that SUR is of importance for xenon induced channel opening.

8.1.3.2 Xenon induced channel opening: Independence from cellular signalling cascades

After establishing $K_{ir}6.2$ as the principal target for xenon it remained undetermined whether the xenon effect was dependent on intracellular signalling cascades or not.

Since the hallmark feature of ATP-sensitive potassium channels is their inhibition by intracellular ATP (Ashcroft and Trapp, 2001), it is reasonable to hypothesise that xenon might activate the channel via xenon-induced changes in cellular ATP concentrations. Here mKATP channels have been suggested to play a major role since their activation has been found to reduce ATP synthesis (Holmuhamedov et al., 1998). However, this study provides no evidence for a contribution or an involvement of mKATP channels in xenon induced pK_{ATP} channel opening. Furthermore, instead of decreasing mitochondrial ATP production via mKATP channel activation, xenon might have a direct blocking effect on the electron transport chain hence serving as metabolic inhibitor. Xenon might therefore act similarly to what has been found for volatile anaesthetics, which are capable of reducing respiratory chain function (Hanley et al., 2002b; Bains et al., 2006).

This hypothesis is not supported by the present study since xenon not only enhances K_{ATP} currents in intact cells, but also in inside-out macropatches where ATP concentrations are dictated by the bath solution. These findings rule out the possibility that xenon exerts its effect indirectly, via altering cellular metabolism and thus intracellular ATP content.

In addition to its possible interaction with mitochondria xenon could potentially also interfere with cellular signalling cascades that are known to regulate pK_{ATP} channels. Protein kinase C (PKC) for instance increases the open probability of cardiac and pancreatic pK_{ATP} channels via phosphorylation of a threonine residue on $K_{ir}6.2$ (Light et al., 2000). As xenon has been shown to induce PKC in rat hearts, one could hypothesise a PKC-dependent mechanism of xenon induced neuronal pK_{ATP} channel opening as well (Weber et al., 2005). However, the results of the excised macropatch experiments where intracellular signalling cascades are eliminated refute this assumption. The data are rather highly

suggestive of a direct effect of xenon on the channel, although membrane-delimited signalling cascades cannot be excluded.

Probably the most important membrane-restricted signalling pathway for modification of K_{ATP} channel activity involves phospholipids such as phosphatidylinositol-4, 5-bisphosphate (PIP2) and phosphatidylinositol-4-phosphate (PIP). Phospholipids are modulated by phosphatidylinositol-4-phosphate 5 (PIP5)-kinase (Shyng et al., 2000), some neurotransmitters (Baukrowitz et al., 1998; Xie et al., 1999b) and phospholipase C (PLC), with most information so far available for PLC mediated K_{ATP} channel regulation. Since PLC has been demonstrated to reduce pK_{ATP} currents, a PLC blocking mechanism of xenon could be hypothesised (Xie et al., 1999a). The substrate of PLC, phosphoinositol bisphosphate (PIP2), is known to activate pK_{ATP} channels via reducing the sensitivity of the channel to ATP. However, other studies have found that PIP2 activation of the channel is not precluded by tolbutamide, but on the contrary, PIP2 decreases the efficacy of tolbutamide and other sulphonylureas (Koster et al., 1999; Krauter et al., 2001). This is in contrast to the findings described here where tolbutamide not only reversed xenon induced channel activation (Figure 7.10) but also prevented it (Figure 7.11). Furthermore phospholipid activation is a common feature for inward rectifying K^+ channels (Huang et al., 1998; Zhang et al., 1999). Since xenon opened $K_{ir}6.2$ but not $K_{ir}1.1$ this further supports the idea that xenon induced pK_{ATP} channel activation does not involve PLC or phospholipids. However, a definite answer would require the crystallisation of the channel in the presence of xenon (and ATP).

8.1.3.3 Xenon modulated channel gating: An ATP-dependent process?

A simple explanation for xenon's effect on K_{ATP} channels would be that it reverses ATP-inhibition at $K_{ir}6.2$. Support for this hypothesis was gained in the present study from concentration-effect experiments. Here it was found that xenon shifted the K_i of ATP to a fourfold higher concentration. These results are indicative of a competition of xenon with ATP at the channel pore (Tallarida, 2000). More precisely, the fact that the xenon effect could be overcome (surmounted) fulfils the classical criteria of competitive antagonism (Taylor and Insel, 1990). This notion appears even stronger since Mg^{2+} free experimental conditions were employed to exclude a possible interference of Mg-nucleotides with the xenon effect as discussed in section 8.1.3.1 (Craig et al., 2008).

Furthermore, it was found that xenon activation of K_{ATP} currents in inside-out patches was more pronounced in the presence of ATP than in its nominal absence. These observations - the xenon induced shift of K_i for ATP inhibition and the increased efficacy of xenon in the presence of ATP - would be consistent with xenon interfering with ATP inhibition of the K_{ATP} channel. Alternatively, xenon might require a pre-blocked channel in order to cause activation. However, the finding that inhibition of the channel by tolbutamide prevents xenon activation argues against the latter possibility.

Xenon therefore seems to specifically involve an ATP-dependent process in its K_{ATP} channel opening mechanism. This idea is supported by the data from some additional experiments:

In employing a site-directed mutation of $K_{ir}6.2$ ($K_{ir}6.2$-K185Q/SUR1) as described by Tucker and colleagues (Tucker et al., 1998) not only the sensitivity of the channel to ATP was greatly diminished but also the opening effect of xenon lost. This failure of xenon to enhance $K_{ir}6.2$-K185Q/SUR1 currents was observed both in intact cells and in inside-out patches. The residue K185 of $K_{ir}6.2$ interacts with the β-phosphate of ATP and thus contributes to the ATP-binding site (Trapp et al., 2003; Antcliff et al., 2005). This mutant has a greatly reduced ATP-sensitivity, but compared to the wild type channel a virtually identical intrinsic open probability (Tucker et al., 1998). This is important because ligand binding to the K_{ATP} channel tends to be state-dependent (Trapp et al., 1998; Enkvetchakul et

al., 2000; Krauter et al., 2001). A number of additional residues have been shown to contribute to the ATP binding site on $K_{ir}6.2$ by interacting with the adenine and ribose ring of ATP, but these were not tested with xenon because they have a more profound effect on the intrinsic open probability of the mutant channel (Antcliff et al., 2005). With the data available from experiments using the K185Q mutant it was not possible to distinguish whether xenon activation is lost in the mutant channel, because K185 forms part of the xenon interaction site, or whether the lack of ATP inhibition simply prevented any xenon enhancement. However, the latter possibility would be in agreement with the hypothesis that xenon disinhibits the channel from the action of ATP. In any case, it is clear, that xenon works very differently from other K-channel openers, as diazoxide activation remains unchanged by the K185Q mutation.

Further support for an ATP-dependent effect of xenon was gained from experiments with a different inward rectifier K^+ channel, $K_{ir}1.1$. Phylogenetically $K_{ir}1.1$ is a close relative of $K_{ir}6.2$ (Isomoto et al., 1997) that shares about 36% sequence homology with the K_{ATP} channel (Proks et al., 2001). It even contains a lysine in position 185 and therefore an ATP binding-site. The most obvious difference between $K_{ir}6.2$ and $K_{ir}1.1$ channels however is that the former, but not the latter, is inhibited by micromolar concentrations of ATP (Tucker et al., 1997; Hebert et al., 2005). Therefore, under physiological conditions, ATP does not seem to regulate $K_{ir}1.1$ (Schulte and Fakler, 2000). An effect of xenon on this channel would have been an indication for an ATP independent, in this regard unspecific, action of xenon. The results presented here show that xenon did not exert any significant change in current flow through $K_{ir}1.1$. Hence all data discussed in this section are in agreement with the idea of xenon specifically interfering with the action of ATP on $K_{ir}6.2$. However, the precise mechanism of this interference remains elusive. For example, xenon atoms could physically obstruct the ATP-binding pocket to prevent ATP gaining access to an empty binding site. Since ligand binding is a dynamic process with molecules constantly associating to or dissociating from their receptor, over time xenon might just displace ATP and thus disinhibit the channel. The kinetics of this process would be entirely determined by the concentration of each ligand. Furthermore, such a mechanism would be in agreement with the observed competitive antagonism

between xenon and ATP, which can be described by the law of mass action (Taylor, 1990).

Based on the assumption of 'intrinsic gating', which describes the observation of ATP-sensitive K^+ channels being spontaneously active in the absence of nucleotides (Proks and Ashcroft, 2009) another model could also explain xenon's effect. This model would be similar to what has been described to explain the channel opening properties of the already discussed phosphoinositol bisphosphate (PIP_2): PIP_2 has been demonstrated to have its binding site close to that of ATP on $K_{ir}6.2$ (Baukrowitz et al., 1998; Shyng and Nichols, 1998; Haider et al., 2007b). Functional studies have further shown that binding of PIP_2 leads to displacement of ATP from $K_{ir}6.2$ (Wang et al., 2002), which subsequently increases the intrinsic open probability of pK_{ATP} channels (Shyng and Nichols, 1998) and therefore blocks the inhibitory effect of ATP at $K_{ir}6.2$ (Fan and Makielski, 1999). Applied to a potential xenon model of action this would imply that - instead of physically obstructing the ATP-binding site on $K_{ir}6.2$ - xenon associates with the pore-forming unit most likely close to the ATP binding site. Although this association of xenon with $K_{ir}6.2$ might not lead to a significant conformational change at the pore it might nevertheless change the energy status of the ATP binding site. This in turn might make ATP binding energetically less desirable and therefore reduce the re-association rate of ATP with $K_{ir}6.2$. However, given the nature of xenon as noble gas, its effect can be expected not to be very strong so that it could be overcome by a concentration effect, i.e. by increasing the number ATP molecules.

However, at present all the above suggested models are speculative and therefore future research necessary to establish the precise mechanism of xenon induced pK_{ATP} channel opening. Here uncovering the exact site of interaction of xenon with $K_{ir}6.2$ could provide some valuable information to further characterise the xenon-ATP relationship.

8.2 Neuronal Preconditioning

Evidence for the involvement of ATP-sensitive potassium channels in organ protection and especially in preconditioning so far is mostly derived from studies in myocardial tissues. Especially the exploit of $K_{ir}6.2^{-/-}$ knockout mice has provided valuable information in this regard (Seino and Miki, 2003). For instance, Suzuki and colleagues as well as Gumina and colleagues found a lack of preconditioning in cardiac muscle of $K_{ir}6.2^{-/-}$ animals (Suzuki et al., 2002; Gumina et al., 2003). Furthermore, the observation that K_{ATP} channel openers mimic whilst K_{ATP} channel inhibitors reverse preconditioning established the role of K_{ATP} channel opening as a major pathway in myocardial preconditioning (Cavero et al., 1995). In addition to the suggested involvement of plasmalemmal K_{ATP} channels in cardiac preconditioning, recent research indicated a critical role of mitochondrial KATP channels as well. As a matter of fact at present the predominant opinion favours mKATP rather than pK_{ATP} channels as central effectors in myocardial protection. However, this notion is not undisputed since some researcher regard the support for the presence of mKATP channels in heart muscle cells as unconvincing (Seino and Miki, 2003; Zaugg et al., 2004; Hanley and Daut, 2005).

This 'K_{ATP} channel controversy' is even stronger amongst neuroscientists since evidence for the presence of mitochondrial KATP channels in the brain is fairly recent and therefore scarce. Bajgar and colleagues were the first to report partial purification and reconstitution of mKATP channels from rat mitochondria in proteoliposomes and to successfully measure K^+ flux through these channels (Bajgar et al., 2001). They even claim that the concentration in the brain of those newly characterised mKATP channels exceeded the concentration of mKATP channels in heart and liver by six to seven times. Additional functional evidence for the involvement of neuronal mKATP channel in preconditioning seems to be provided by a study from Kis and colleagues. They report that pre-treatment with a newly developed allegedly selective mKATP channel opener was able to protect primary neuronal cultures against OGD induced injury (Kis et al., 2004). Nevertheless, evidence for the involvement of plasmalemmal K_{ATP} channels in protection and preconditioning of the brain is more substantial. For instance, over-

expression of the channel pore $K_{ir}6.2$ in mice provided increased protection against hypoxic-ischaemic injury compared to wild type mice (Heron-Milhavet et al., 2004). Conversely, mice lacking functional pK_{ATP} channels were more vulnerable to focal ischaemia *in vivo* (Sun et al., 2006). Also $K_{ir}6.2$ knockout mice developed enhanced seizure activity in response to hypoxia compared to normal controls (Yamada et al., 2001).

Despite the mounting data that K_{ATP} channels might also play a significant role in neuronal preconditioning the involvement of ATP-sensitive potassium channels in pharmacological, especially anaesthetic, preconditioning is still poorly characterised. The present study aimed to shed some light onto the kind of interaction of some inhalational anaesthetics with plasmalammal K_{ATP} channels and the physiological consequences of it. After the establishment of xenon as plasmalemmal K_{ATP} channel opener the functional relevance of this newly discovered property was determined in the context of neuronal preconditioning. The effect of xenon was compared to the actions of the established K_{ATP} channel opener diazoxide and the volatile anaesthetic sevoflurane. Sevoflurane was chosen because of all volatiles tested in this study it had displayed the least inhibitory effect on pK_{ATP} channels.

The present study showed that all three different drugs were able to precondition neuronal cells against injury induced by oxygen-glucose deprivation *in vitro*. However, the results also suggest that individual inhalational anaesthetics facilitate different pathways to provoke protection in this model. This hypothesis is deduced from the observation that xenon preconditioning required opening of plasmalemmal K_{ATP} channels, whereas sevoflurane preconditioning was pK_{ATP} channel independent.

Also, neither of the anaesthetics showed a substantial involvement of mKATP channels in its preconditioning action. This finding is unlikely to be attributable to a systemic error in the experimental set-up since preconditioning with diazoxide that is known to open both pK_{ATP} and mKATP channels also induced protection. Moreover the effect of diazoxide was also sensitive to the actions of both the pK_{ATP} channel inhibitor tolbutamide and the mKATP channel blocker 5-HD.

However, whilst appreciating a potential effect of diazoxide on mKATP channels, it has to be emphasized that diazoxide – despite being regarded as the gold

standard mKATP channel opener in cardiac tissue – is not specific for mKATP channels in organs that have pK_{ATP} channels with SUR1 subunits (Garlid et al., 1996; Quast et al., 2004).

This notion is supported by Kis and colleagues in a recent study investigating the preconditioning effect of diazoxide against OGD induced neuronal injury *in vitro*. Although diazoxide was able to protect neurons from injury in their experiments and although 5-HD reversed this effect the authors were not convinced that mKATP channels were involved (Kis et al., 2003): Their reasoning is based on their observations that the two additionally tested non-selective K_{ATP} channel openers cromakalim and nicorandil did not produce any protection in their experiments at all. Furthermore they found that glibenclamide, which is a K_{ATP} channel inhibitor that acts on both pK_{ATP} and mKATP channels, was ineffective in reversing diazoxide's effect. Since they also provide evidence that diazoxide might involve other pathways to induce preconditioning such as reactive oxygen species and protein kinases, and since there is evidence from other groups that 5-HD might prefer other targets as well (Hanley et al., 2002a; Lim et al., 2002; Das et al., 2003; Dzeja et al., 2003; Hanley et al., 2003), they question the role of mKATP channels in their model of pharmacological preconditioning. Therefore as explained above, results implying mKATP channel involvement in neuronal preconditioning should be viewed with some caution.

Nevertheless, the data of the present study demonstrating $K_{ir}6.2$/SUR1 type pK_{ATP} channel dependent preconditioning of diazoxide are in agreement with the literature since for instance Dabrowski and colleagues demonstrated high affinity activation of $K_{ir}6.2$/SUR1 by diazoxide (Dabrowski et al., 2003).

Another interesting aspect of the present study becomes obvious when the preconditioning concentration-response relationships of xenon and diazoxide are compared to the efficacy with which both were able to activate pK_{ATP} channels in the heterologous HEK293 cell expression system: One MAC of xenon was as sufficient a concentration to precondition neuronal glial cell co-cultures as it was to open pK_{ATP} channels in HEK293 cells. Furthermore channel opening with one MAC of xenon was equally efficient to diazoxide when the latter was given at its preconditioning concentration of 0.01mM. Finally, since the preconditioning doses of the two compounds xenon (1 MAC) and diazoxide (0.01mM) increase K^+

currents by 50% it can also be postulated that this level of K_{ATP} channel activity is enough to produce preconditioning.

Therefore the present study provides a clear link between the newly established property of xenon as a pK_{ATP} channel opener and xenon induced neuronal preconditioning. Moreover, both effects of xenon are achieved at anaesthetic or even sub-anaesthetic concentrations and thus can be accomplished with clinically safe doses.

Although the present study provides some mechanistic and functional data as regards to the involvement of pK_{ATP} channels in pharmacological, especially anaesthetic neuronal preconditioning some important problems are still elusive: For example the exact location of K_{ATP} channels in the preconditioning pathway(s) and therefore the question whether they represent triggers or effectors of protection remain as uncertain as the involved signalling cascades.

However, from the data presented here it seems feasible to hypothesise that K_{ATP} channels are triggers of preconditioning. This is deduced from the observations of the HEK293 cell experiments where xenon displays a short lasting effect, i.e. K_{ATP} channels close as soon as xenon is removed from the bath solution. In contrast, the preconditioning experiments of the present study show a lasting effect of xenon. These data taken together would suggest the presence of a signalling step between channel opening (trigger) and cellular protection (effector).

With the involvement of pK_{ATP} channels it seems that xenon mimics ischaemic preconditioning whereas sevoflurane might act on a different target (Figure 8.1). Whether this target is downstream from the activation of K_{ATP} channels, or involves an entirely independent pathway for preconditioning is unclear at present.

Nevertheless, sevoflurane's ability to precondition neuronal glial cell co-cultures against OGD-induced damage is supported by studies, which report neuronal protection induced by pre-treatment with nearly all modern volatile anaesthetics (for reviews see Clarkson (Clarkson, 2007) and Wang (Wang et al., 2008b)). Halothane and isoflurane preconditioning for example was shown to reduce injury in a rat model of focal brain ischaemia *in vivo* (Kapinya et al., 2002). A similar

observation was made in neuronal cultures *in vitro*: Less damage was found when neurons were exposed to sevoflurane preemptively (Kapinya et al., 2002; Kaneko et al., 2005). Furthermore, the potency of desflurane, halothane, isoflurane and sevoflurane to induce preconditioning was found to be linear correlated to the anaesthesia inducing potencies of those volatiles in rat cerebellar slices (Wang et al., 2007a).

Additionally, sevoflurane was shown to have preconditioning effects both *in vitro* and *in vivo*. Luo and colleagues for instance could demonstrate that sevoflurane pre-treatment protected neurons in a rat model of neonatal asphyxia. The same authors also reported sevoflurane preconditioning in primary neuronal as well as neuronal glial cell co-cultures (Luo et al., 2008). These results were in accordance with data provided by Payne as well as Wang and colleagues who showed reduced hippocampal injury by sevoflurane application against hypoxia-ischaemia both *in vivo* and *in vitro* (Payne et al., 2005; Wang et al., 2007b). However, the role of K_{ATP} channels in volatile -especially sevoflurane- induced neuronal preconditioning remained unclear until the present study.

Although reports by Kehl and colleagues as well as Wang and colleagues suggested an mKATP channel dependent mechanism, these authors did not exclude an involvement of pK_{ATP} channels (Kehl et al., 2004; Wang et al., 2006). The results of Kehl and Wang suggesting K_{ATP} channel involvement in sevoflurane preconditioning are in contrast not only to the results of the present study but also to findings of other groups with different inhalational anaesthetics. Zheng and colleagues for example were not able to reverse the effect of isoflurane preconditioning in cerebellar brain slices with K_{ATP} channel inhibitors (Zheng and Zuo, 2003). Additionally, Marinovic and colleagues found that preconditioning of cardiomyocytes with isoflurane did not involve plasmalemmal K_{ATP} channels (Marinovic et al., 2006).

Based on the results of the present study an involvement of K_{ATP} channels in volatile anaesthetic induced preconditioning seems highly unlikely, with the precise mechanisms conveyed by these agents still speculative.

Several recent reviews have discussed potential mediators and pathways of volatile anaesthetics preconditioning (De Hert, 2004; Tanaka et al., 2004;

Clarkson, 2007; Wang et al., 2008b). These signalling cascades can be broadly divided into cell membrane based and intracellular mechanisms. Amongst the membrane-based receptors that have been shown to be involved in protection are adenosine A1 and A3, glutamate as well as GABA receptors. Especially the interaction of anaesthetics with glutamatergic pathways have been investigated thoroughly, since glutamate represents the main excitatory amino acid of the body and is also involved in excitotoxicity (see introduction 5.5.3). For example halothane and isoflurane have recently been found to inhibit N-methyl-d-aspartate (NMDA) receptors likely via an interaction with the receptor's glycine binding site (Ogata et al., 2006; Dickinson et al., 2007). Also sevoflurane has been shown to reduce glutamate neurotransmission (Stucke et al., 2005). Intracellular mediators frequently associated with anaesthetics-induced protection are immediate early genes like c-fos, inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO), reactive oxygen species (ROS) and protein kinases such as MAPK, PKC, ERK (Wang et al., 2008b).
Whether these mediators also contributed to sevoflurane preconditioning has not been addressed in present study and remains therefore unclear. Also uncertain remain the cellular end-effectors of such preconditioning signalling cascades. However, in the following section a brief account on possible effectors is given when the K_{ATP} channel dependence of xenon preconditioning is put into context.

Although xenon preconditioning had been shown before in neurons and hearts (Ma et al., 2006; Luo et al., 2008; Weber et al., 2008) the obvious absolute dependence on plasmalemmal K_{ATP} channel opening in this study - as demonstrated by the complete reversal of the xenon effect by tolbutamide - was somehow surprising. Since xenon had previously been shown to be a N-methyl-d-aspartate (NMDA) receptor antagonist (Franks et al., 1998; Dickinson et al., 2007) and a two-pore K^+ channel opener (Gruss et al., 2004) one could have expected an involvement of these mechanisms as well. Partial instead of full antagonism of xenon's preconditioning effect with the pK_{ATP} channel blocker would have been therefore the most likely scenario. However, the present results are not unprecedented: In a clinical trial gavastinel, a NMDA receptor antagonist that like xenon inhibits the receptor via blocking of the glycine binding site

(Dickinson et al., 2007), had been shown ineffective in the treatment of acute ischaemic stroke in man (Sacco et al., 2001).

Whilst this study established xenon as an opener of plasmalemmal K_{ATP} channels and showed that one functional consequence of this property is neuroprotection, some questions remain unsolved: If pK_{ATP} channels are the trigger for xenon induced neuroprotection which are the downstream effectors responsible for the lasting effect? In the following part potential mechanisms are discussed and a concept introduced that could explain the seemingly divergent results of volatile anaesthetics and xenon-induced preconditioning.

In general K_{ATP} channels - derived from their intrinsic characteristics - can facilitate three different principles to trigger protection: The first principle is deduced from the attribute of K^+ channels to modulate neuronal membrane potentials and consequently cellular activity. The second principle is based on the hallmark feature of K_{ATP} channels, their regulation by ATP. As the third and final principle it can be hypothesised that the ion flow that is associated with K^+ channel opening might be of significance for regulation of cellular osmolarity and hence volume.

Once activated, K_{ATP} channels as members of the inward rectifier family of K^+ channels contribute to the stabilisation of the cell's resting membrane potential. They therefore are a counter-regulatory method that is set up to resist neuronal depolarisation and subsequently energy consuming excitation (Nichols et al., 1996; Ashcroft and Trapp, 2001). Since they are directly regulated by nucleotides, their opening mechanism is a function of the cell's metabolic state (Ashcroft and Trapp, 2001; Zhuo et al., 2005). At present it is hypothesised that although widely distributed in the CNS, pK_{ATP} channels come only into action once the cell has come under metabolic stress such as hypoxic-ischaemia or hypoglycaemia to help reduce neuronal activity and hence preserve energy (Wang et al., 2005; Zhuo et al., 2005). However, this principle can be assumed to be part of a quick adaptive mechanism probably designed to allow cells to respond rapidly to changes in their environment and substrate supply. Whether this principle participates also in ongoing protection remains to be determined as well as whether its implication, namely a potentially continuous down regulation of neuronal function, would be beneficial for the organism.

Therefore it is more likely that other mechanisms contribute to ongoing neuroprotection triggered by pK_{ATP} channel opening. As has been demonstrated in this study xenon opens pK_{ATP} channels most likely via interference with the capability of ATP to bind to the channels pore forming $K_{ir}6.2$ subunit. This in turn might lead to a temporary increase of the ATP concentration in the submembrane compartment.

Although a study by Gribble and colleagues found no gradient between the bulk cytosolic and submembrane ATP concentrations (Gribble et al., 2000) more recent publications support the hypothesis of energy compartmentalisation at least in cardiomyocytes (Selivanov et al., 2004; Zingman et al., 2007). Therefore the localised increase in ATP concentration might then either act as an intracellular second messenger or it might be released via connexin 43 hemichannels, which are present in neuronal cell membranes (Kang et al., 2008; Lin et al., 2008). Once in the extracellular space ATP could be metabolised to adenosine that subsequently mediates neuroprotection via binding to adenosine-A1 receptors (Heurteaux et al., 1995; Ballanyi, 2004). This 'extracellular ATP' hypothesis nicely explains the propagation of a protective impulse to neighbouring cells and seems to shift the focus away from K_{ATP} channels towards adenosine-A1 receptors and possibly other not yet undetermined cascades. However, given the fact that adenosine receptors not only stimulate G-protein coupled K^+ channels but also activate pK_{ATP} channels (Heurteaux et al., 1995), the central question of how pK_{ATP} channels mediate preconditioning remains unsolved.

The just discussed two potential protective mechanisms appear not very likely to be the underlying major principle of pK_{ATP} channel triggered preconditioning. Hence the question remains whether there is a pathway that is capable of facilitating a mechanism that relies more on the specific properties of K^+ channels. Loss of cell volume, or cell shrinkage, is a hallmark feature of programmed cell death (apoptosis). However, there is now an increasing body of evidence to suggest that cell volume reduction is not only an early morphological characteristic of apoptosis but also a triggering event for cell death pathways (Burg et al., 2006; Bortner and Cidlowski, 2007). Indeed it has not only been shown that experimental apoptotic stimuli induce early cell shrinkage but also that

cell shrinkage alone was able to induce apoptosis (Bortner and Cidlowski, 2007). Consequently this initial loss of cell volume at the beginning of an apoptotic process has been termed apoptotic volume decrease (AVD). AVD is an interesting concept when neuroprotection is discussed in the context of pK_{ATP} channel opening because it has been shown that the underlying process of AVD is the efflux of monovalent ions, especially K^+, out of the cell with subsequent reduction of the intracellular K^+ concentration. Therefore it has been postulated that physiological intracellular potassium concentrations are important not only for the cell's ionic and electrical homeostasis but also to suppress apoptotic mechanisms (Montague et al., 1999). There is indeed evidence that shows that cellular loss of potassium and hyperpolarisation due to K^+ channel opening induces a loss of cell volume and subsequently apoptosis (Burg et al., 2006; Bortner and Cidlowski, 2007).

Various K^+ channels have been linked to this process but there are also reports of a critical involvement of pK_{ATP} channels: Yu and colleagues for instance found that mouse neocortical neurons when exposed to an apoptotic stimulus like staurosporine showed early hyperpolarisation that was at least partially based on an ATP-sensitive K^+ current. A potential K_{ATP} channel involvement was further supported by their finding that sustained application of the K_{ATP} channel opener cromakalim also induced apoptosis (Yu et al., 1997).

Potassium efflux mediated AVD as a trigger for apoptosis seems not to be an exclusive phenomenon of toxin induced cell death. Instead there is indirect evidence to suggest that similar mechanisms are also involved in hypoxic-ischaemia induced events. For instance Fujimura and colleagues demonstrated increased transmembrane potassium flow and hyperpolarisation in rat hippocampal neurons after exposure to hypoxia (Fujimura et al., 1997). They and others also provide support for a critical role of K_{ATP} channels in this process (Trapp and Ballanyi, 1995; Fujimura et al., 1997; Ballanyi, 2004).

How then is xenon-induced preconditioning linked to K_{ATP} channel-triggered cell volume loss (AVD) and apoptosis?

If for biological systems the general rule is accepted that every mechanism is balanced to some extend by a counter-mechanism then one could assume the same

to be true for cellular death pathways. That means every stimulus that triggers apoptosis will ultimately also lead to the activation of protective anti-apoptotic pathways (Bortner and Cidlowski, 2007). As has been discussed above (see introduction 5.7) this forms the foundation for the concept of preconditioning. Preconditioning therefore is the exposure of cells to a minimal damaging stimulus that results in the induction of mechanisms that produce resistance against a second stronger insult (Murry et al., 1986). Based on this it could be hypothesised that the xenon induced K^+ efflux leads to a minor reduction of intracellular K^+ with subsequent cell shrinkage and induction of pro-apoptotic as well as in response to that anti-apoptotic pathways. Since the xenon effect on the K_{ATP} current is only mild, the same can be assumed for the induction of pro-apoptotic mechanisms. Therefore the protective pathways might be able to outweigh the damaging cascades thus shifting the ratio of damage to protection towards the latter.

How would this assumption fit with the K_{ATP} channel independent preconditioning of volatile anaesthetics? A first idea comes from the already discussed article from Wang and colleagues who found that pre-emptive application of sevoflurane protected neurones in hippocampal slices against hypoxic injury. They further identified hypoxic hyperpolarisations as part of the protective mechanism (Wang et al., 2007b). Since an involvement of pK_{ATP} channels in sevoflurane preconditioning was unlikely, the observed hyperpolarisation could have been the result of an activation of other K^+ channels by sevoflurane. This notion is supported by studies reporting opening of mainly two-pore K^+ (K_{2P}) channels through the volatile anaesthetics halothane, isoflurane and sevoflurane (Sirois et al., 2000; Gruss et al., 2004; Huneke et al., 2004; Putzke et al., 2007). This is the more important since Bortner and colleagues suggested potassium leak channels such as K_{2P} channels to play a central role in neuronal AVD and hence apoptotic pathways (Bortner and Cidlowski, 2007).

Therefore similar to what was said about the possible mechanism of xenon preconditioning volatile anaesthetics might also protect via potassium efflux induced cellular shrinkage. But other than xenon volatiles most likely trigger this through potassium channels other than K_{ATP} (see Figure 8.1). However, whether

and to what extent these mechanism participate in anaesthetic preconditioning needs to be determined by future research.

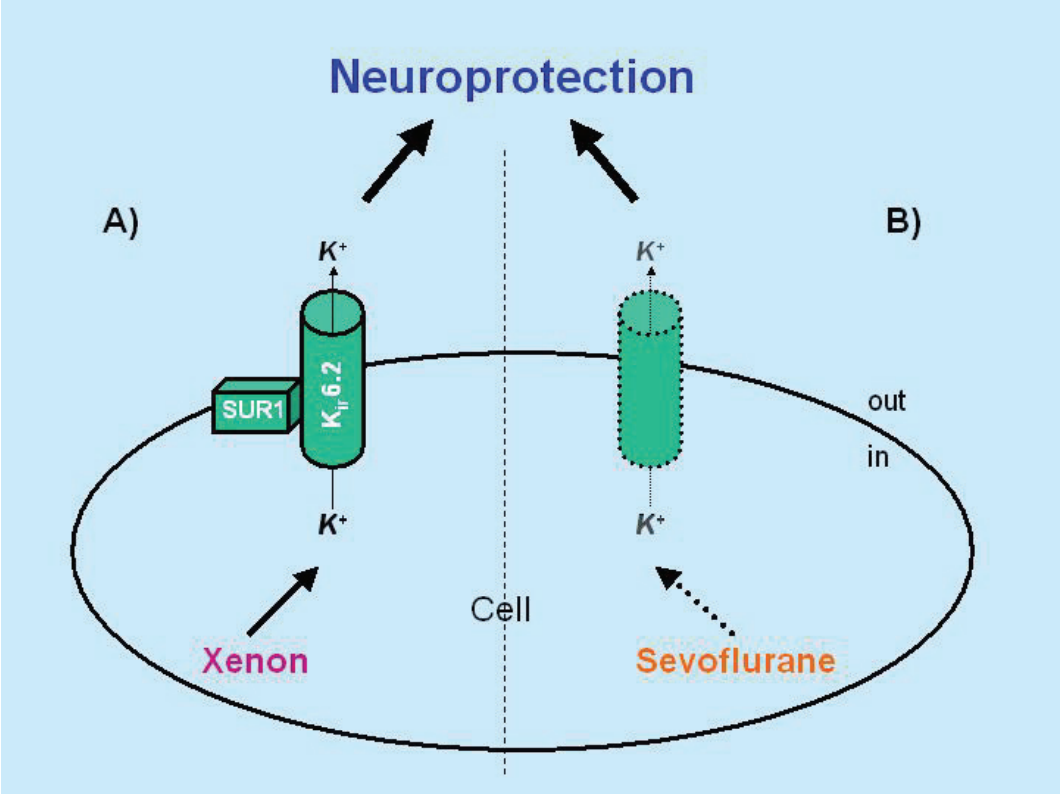


Figure 8.1: Suggested mechanisms of inhalational anaesthetics induced preconditioning

A) *Xenon exerts neuronal preconditioning via a mechanism that critically involves plasmalemmal K_{ATP} channels ($K_{ir}6.2/SUR1$).*

B) *Sevoflurane also preconditions neurons. This preconditioning effect does not involve K_{ATP} channels but might be mediated via other types K^+ channels.*

'In' denotes the inside of the cell, 'out' the outside.

8.3 Physiological Significance of K_{ATP} Channel Inhibition

This study provides evidence that halogenated anaesthetics inhibit plasmalemmal K_{ATP} channels. This should have very little effect under physiological circumstances in most tissues since K_{ATP} channels are closed under conditions where ATP is abundant. They would only open during conditions of metabolic stress, for instance in the course of thrombembolic stroke where hypoxia is present (see chapter 5.5). However, even under normal circumstances pancreatic β-cells use this channel characteristic to regulate the body's glucose homeostasis. At decreased blood glucose and hence low pancreatic ATP concentrations K_{ATP} channel open to hyperpolarize the membrane potential of the β-cell. Elevated glucose and ATP concentrations on the contrary lead to channel closure, which is followed by β-cell depolarisation and subsequent activation of voltage dependent Ca^{2+} channels. The increased intracellular Ca^{2+} concentration is then causing insulin release (Trapp and Ashcroft, 1997; Ashcroft and Gribble, 1999; Ashcroft, 2005). Consequently, K_{ATP} channel inhibition as a side-effect of drug therapy could potentially lead to unwanted hyperinsulinemia and hypoglycaemia that might even become critical if unnoticed - for example during the course of a general anaesthetic. Although scarce, there is indeed some evidence in the literature hinting towards a blood glucose lowering effect of volatile anaesthetics. For instance a recent case report described the occurrence of unexpected hypoglycaemia in a previously fit and well male patient during maxillary-facial surgery (Ackland et al., 2007). Moreover in a prospective trial including 154 patients Huang and colleagues intended to investigate the influence of glucose-free intravenous solutions on blood sugar levels during neurosurgery. They found in both adult and paediatric patients no evidence of hyperglycaemia but in about 25% of adult patients hypoglycaemia after prolonged eight to nine hours of surgery. In contrast, no hypoglycaemia was detected in children indicating a potential age difference (Huang et al., 2000). However, Loepke and colleagues found decreased blood sugar levels in neonatal mice anaesthetized with isoflurane (Loepke et al., 2006).

Additionally, K_{ATP} channel openers are or could be used in the near future in a variety of diseases such as heart disease, peripheral vascular disease, asthma,

bladder dysfunction or epilepsy (Jahangir and Terzic, 2005). Co-administration of drugs, which might antagonise K_{ATP} channel opening could potentially have disastrous consequences especially for patients where K^+ channel openers are given as anti-arrhythmics or anti-anginal medications (Reimann et al., 2001; Gribble and Reimann, 2003b). Despite the fact that the halogenated volatile anaesthetics tested here had inhibitory effects on K_{ATP} currents even at low concentrations, at present there are not sufficient data to allow a critical evaluation of potential interactions between those anaesthetics and K_{ATP} channel openers. Furthermore, since it so far remains unclear whether these drugs interact with the potassium channel (K_{ir}) subunit or the sulphonylurea receptor (SUR) as well as whether specific channel compositions are preferred (e.g. neuronal versus cardiac), more research is warranted on this potentially important topic. Since this study was concerned with the identification of pathways potentially involved in xenon preconditioning and since K_{ATP} channel opening rather than closure is the basic principle of pK_{ATP} channel mediated protection it seemed unlikely that preconditioning via volatile anaesthetics is mediated via pK_{ATP} channels. Therefore pathways involving these compounds were not further investigated in this study.

8.4 Physiological Consequence of K_{ATP} Channel Activation

One concern of the clinician intending to use K_{ATP} channel opening drugs for the prevention of stroke might be a potential interference with drugs used in the treatment of non-insulin dependent (type 2) diabetes mellitus. It could therefore be suggested that K_{ATP} channel opener antagonize the insulin releasing effect of sulphonylurea receptor antagonists like tolbutamide. This might be especially relevant in the perioperative period since diabetic patients per se carry an increased risk for the development of stroke after surgery (see also introduction chapters 5.3 and 5.4) (Bolognesi et al., 2008). They therefore represent a population likely benefiting from neuroprotective preconditioning strategies. Elevated blood glucose levels might offset this favourable effect of K^+ channel openers since perioperative hyperglycaemia has been shown to be significantly

associated with adverse outcomes after coronary bypass grafting (McAlister et al., 2003) as well as during intensive care treatment (van den Berghe et al., 2001). However, the findings of the present study do not support this notion because tolbutamide prevented the xenon induced opening of pK_{ATP} channels (Figure 7.11). These results argue on the contrary that treatment of type 2 diabetes mellitus might diminish the K_{ATP} channel dependent organ protective effects afforded by xenon. This concept is reminiscent of the interference of sulphonylurea drugs with the anti-anginal K_{ATP} channel opener nicorandil. A study by Reimann and colleagues demonstrated that the effect of nicorandil on cardiac and smooth muscle type pK_{ATP} channels was reversed by the action of the commonly used antidiabetic glibenclamide (Reimann et al., 2001).

Whilst the potential antagonism of K_{ATP} channel opening drugs by sulphonylureas is a valid worry for peripheral tissues, so far there is no evidence to support concerns about a similar interference in the central nervous system. To the contrary at present there is only data showing that tolbutamide does not easily partition into the brain (Takanaga et al., 1998; Murakami et al., 2000). Therefore xenon seems to be a viable choice as a neuroprotectant even for diabetic patients treated with tolbutamide.

Similarly to K_{ATP} channel inhibitors most K_{ATP} channel openers seem not to cross the blood-brain barrier under physiological conditions (Jahangir and Terzic, 2005). Nevertheless, studies have reported neuroprotective effects of these drugs *in vivo*. For instance, systemic application of diazoxide reduced infarct volume elicited by temporary occlusion of the middle cerebral artery in rats (Mayanagi et al., 2007). Similar results were obtained from a global ischaemia model in dogs: Intravenous administration of diazoxide improved neurological function and diminished histopathologically assessed brain injury (Shake et al., 2001). However, although the authors of these studies attributed the beneficial effects of diazoxide to a direct action on neurons, it is also possible that the observed outcome is secondary to an increased cerebral blood flow due to the vasodilatory effect of these drugs. This hypothesis is supported by a study reporting K_{ATP} channel opening in vascular smooth muscle cells in response to K_{ATP} channel activators such as diazoxide and pinacidil (Meisheri et al., 1990). Moreover,

experiments in human volunteers showed endothelial dysfunction - defined as reduced vasodilation in response to acetycholine - after 20 minutes of ischaemia-reperfusion. Ischaemic preconditioning as well as diazoxide were able to prevent this dysfunction whilst co-application of the K_{ATP} channel inhibitor glibenclamide restored it (Broadhead et al., 2004).

Likewise, xenon has been shown to stimulate cerebral blood flow and to dilate pial vessels (Gur et al., 1985; Junck et al., 1985; Hartmann et al., 1991; Fukuda et al., 2001). Additionally, the results of the present study indicate that xenon is able to precondition neuronal tissue directly via interaction with plasmalemmal K_{ATP} channels. However, if taken together with the studies mentioned above, which established xenon as a cerebral vasodilator, the present findings might be extrapolated to the hypothesis that xenon has no, or a reduced, tissue specificity. Therefore xenon might not only open neuronal but also other subtypes of plasmalemmal K_{ATP} channels such as the vascular smooth muscle type $K_{ir}6.1$/SUR2B channel. Since SUR confers relative tissue specificity to the channel and since xenon induced K_{ATP} channel opening without involving SUR1, this hypothesis is supported by the finding of the present study.

8.5 Possible Clinical Implications for the Use of Xenon as Preconditioner

Two recent studies have re-emphasised the risk of stroke and hence its clinical importance in the perioperative period – both for cardiac and non-cardiac surgical patients (Bateman et al., 2009; Li et al., 2009). Because of this clinical importance and because of the also remaining limited therapeutic options (see 5.6) clinicians now increasingly seek to either minimize the incidence of perioperative stroke by controlling contributing factors or to alleviate its effects on each individual patient (Caplan, 2009).

Based on current experimental evidence part of such a risk management strategy could be the pre-emptive administration of neuroprotecive drugs comparable to what has been shown to be effective in hearts (Landoni et al., 2009). However, the situation in the brain is slightly more complicated than that in cardiomyocytes: In hearts protection develops early (‘early phase’) after exposure to the

preconditioning stimulus. This is probably not the case in neuronal tissue. Here a preconditioner needs 24 hours to develop its effect ('late phase') (Kato et al., 1991; Barone et al., 1998), hence intraoperatively administered drugs are unlikely to protect brains on the day of surgery. Translated into the clinical situation with the use anaesthetics as preconditioners, this would require for patients to receive an anaesthetic twice during the course of a little bit more than a day – once as part of a preconditioning strategy and 24 hours later during surgery.

Possible concerns about such a clinical preconditioning paradigm might include logistic issues, the appropriate 'delivery' environment and unwanted drug effects on potentially seriously ill patients. Whilst rigorous planning and meticulous patient recruitment might solve the first aspect, the second appears less important given the fact that the delivery of an anaesthetic in a safe environment with trained doctors and nurses constitutes a standard treatment in every modern hospital. Preconditioning could therefore be expected to easily enrich the therapeutic spectrum of many trusts. However, the final concern about unwanted drug actions especially on the cardio-vascular system needs some additional considerations. In this context the potential for an acute cardiac decompensation during preconditioning should be as much limited as possible. This might be achieved through the choice of drug used for preconditioning. Although largely equipotent in their neuropotective effects, the currently available anaesthetic preconditioners display a considerable variability in their cardio-suppressive properties. At present the compound that comes closest to the ideal of being haemodynamically inert is xenon (Derwall et al., 2009). Therefore xenon might become the 'first-choice' neuronal preconditioning agent in critically ill patients or in patients with the highest risk of cardiac decompensation. This notion is based not only on its favourable side-effect profile but also supported by an increasing body of evidence in support for its preconditioning efficacy, including this study (Ma et al., 2006; Luo et al., 2008; Bantel et al., 2009).

However, to make xenon preconditioning applicable for use in clinical practice its best concentration as well as optimal exposure time and application-to-surgery interval in humans or higher mammals needs to be determined first. But based on the current evidence xenon might be worth the effort.

9. CONCLUSIONS

In summary, this study has established xenon as a novel plasmalemmal K_{ATP} channel opener and it has also demonstrated for the first time that xenon induced neuronal preconditioning is critically dependent on pK_{ATP} channel opening.

This study further provides insights into the mechanism of xenon-induced K^+-channel opening in demonstrating an interaction of xenon with the $K_{ir}6.2$ pore forming rather than the channel's regulatory SUR1 subunit. The channel opening effect of xenon was also shown to be critically dependent on the presence of ATP. Additional data even suggest a specific interference of xenon with ATP at the $K_{ir}6.2$ ATP binding site, which might follow the pattern of competitive antagonism. Therefore the results presented here support the idea that xenon opens neuronal plasmalemmal K_{ATP} channels by diminishing the block ATP normally confers to the channel.

This study also investigated the effects of the volatile anaesthetics halothane, isoflurane and sevoflurane on neuronal pK_{ATP} channels and found all of them to inhibit $K_{ir}6.2$/SUR1. The inability of volatile anaesthetics to open pK_{ATP} channels was confirmed in preconditioning experiments. Here sevoflurane, in contrast to xenon, preconditioned in a K_{ATP} channel-independent manner. Thus the data provided by the present study suggest two distinct pathways for inhalational anaesthetic induced preconditioning, one pK_{ATP} channel-dependent, and the other pK_{ATP} channel-independent.

Further studies are needed to gain more insight into the mechanism of xenon-induced K_{ATP} channel opening and also to reveal the differences in the protection afforded by the two preconditioning pathways. However, given xenon's favourable safety profile the results presented here might open new doors for the development of neuroprotective strategies especially in the peri-operative period.

10. PUBLICATIONS

10.1 Abstracts

Bantel C., Trapp S., Hossain M., Ma D., Maze M.
KATP channels and xenon preconditioning: A role for plasmalemmal rather than mitochondrial channels
American Society of Anesthesiologists Annual Meeting, Chicago, Illinois 2006

Bantel C., Maze M., Trapp S.
Effects of xenon and sevoflurane on plasmalemmal KATP channels in vitro: New insights into preconditioning
37th Annual Meeting of the Society for Neuroscience, San Diego, California 2007

Bantel, C., Maze, M., Trapp, S. (2008)
Preconditioning by volatile anaesthetics: involvement of plasmalemmal versus mitochondrial K_{ATP} channels.
Acta Physiologica Sinica 60, 236-237

10.2 Original Articles

Bantel, C., Maze, M., and Trapp, S. (2009)**
Neuronal preconditioning by inhalational anaesthetics - evidence for the role of plasmalemmal adenosine triphosphate-sensitive potassium channels. *Anesthesiology 110, 986-995.*

** This article is accompanied by an editorial comment by Bosnjak, Z.J., Sarantopoulos, C.D: Channels of preconditioning: potassium drain that protects the brain. *Anesthesiology. 2009 May;110(5):961-3.*

Original Article:

Anesthesiology 2009; 110:986-95 Copyright © 2009, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins, Inc.

Neuronal Preconditioning by Inhalational Anesthetics

Evidence for the Role of Plasmalemmal Adenosine Triphosphate-sensitive Potassium Channels

Carsten Bantel, M.D., F.R.C.A., Mervyn Maze, M.B., Ch.B., F.R.C.A., F.R.C.P., F.Med.Sci.,† Stefan Trapp, Ph.D.‡*

Background: **Ischemic preconditioning is an important intrinsic mechanism for neuroprotection. Preconditioning can also be achieved by exposure of neurons to K^+ channel–opening drugs that act on adenosine triphosphate–sensitive K^+ (K_{ATP}) channels. However, these agents do not readily cross the blood–brain barrier. Inhalational anesthetics which easily partition into brain have been shown to precondition various tissues. Here, the authors explore the neuronal preconditioning effect of modern inhalational anesthetics and investigate their effects on K_{ATP} channels.**

Methods: **Neuronal–glial cocultures were exposed to inhalational anesthetics in a preconditioning paradigm, followed by oxygen–glucose deprivation. Increased cell survival due to preconditioning was quantified with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction test. Recombinant plasmalemmal K_{ATP} channels of the main neuronal type (Kir6.2/SUR1) were expressed in HEK293 cells, and the effects of anesthetics were evaluated in whole cell patch clamp recordings.**

Results: **Both sevoflurane and the noble gas xenon preconditioned neurons at clinically used concentrations. The effect of sevoflurane was independent of K_{ATP} channel activation, whereas the effect of xenon required the opening of plasmalemmal K_{ATP} channels. Recombinant K_{ATP} channels were activated by xenon but inhibited by halogenated volatiles. Modulation of mitochondrial K-ATP channels did not affect the activity of K_{ATP} channels, thus ruling out an indirect effect of volatiles *via* mitochondrial channels.**

Conclusions: **The preconditioning properties of halogenated volatiles cannot be explained by their effect on K_{ATP} channels, whereas xenon preconditioning clearly involves the activation of these channels. Therefore, xenon might mimic the intrinsic mechanism of ischemic preconditioning most closely. This, together with its good safety profile, might suggest xenon as a viable neuroprotective agent in the clinical setting.**

This article is accompanied by an Editorial View. Please see: Bosnjak ZJ, Sarantopoulos CD: Channels of preconditioning: Potassium drain that protects the brain. ANESTHESIOLOGY 2009; 110:961-3.

* Clinical Fellow, † Sir Ivan Magill Professor of Anaesthetics, ‡ Senior Lecturer, Department of Anaesthetics, Pain Medicine and Intensive Care, Chelsea and Westminster Hospital, Imperial College London, and Biophysics Section, Blackett Laboratory, Imperial College London.

Received from Imperial College London, London, United Kingdom. Submitted for publication September 18, 2008. Accepted for publication December 17, 2008. Supported by the Medical Research Council, London, United Kingdom, and the Westminster Medical School Research Trust, London, United Kingdom. Presented at the Annual Meeting of the American Society of Anesthesiologists, Chicago, Illinois, October 17, 2006, and the Annual Meeting of the Society for Neuroscience, San Diego, California, November 6, 2007.

Address correspondence to Dr. Trapp: Biophysics Section, Blackett Laboratory, South Kensington Campus, Imperial College London, London SW7 2AZ, United Kingdom. s.trapp@imperial.ac.uk. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

EARLY ischemic stroke after heart surgery has a high incidence and is associated with substantial disability and mortality rates.[1-3] Although volatile anesthetics have been found to initiate early phase ischemic tolerance in neurons, models of focal brain ischemia suggest that it can be expected to take 24 h for the preconditioning to develop its full effectiveness.[4,5] Therefore, cardiac surgical patients are likely to be exposed to the greatest risk of cerebral ischemic damage in a phase where they are least protected. Consequently, neuroprotective strategies should be implemented well in advance of surgery to allow pathways to become fully activated. This stratagem is possible with the use of pharmacologic preconditioning.

The concept of preconditioning describes the phenomenon that an organ achieves protection against potentially lethal insults through preexposure to harmful stimuli.[6] The term gained wide recognition in the late 1980s after the observation that brief episodes of reduced myocardial perfusion before the extended, harmful ischemic period, diminished tissue damage, and preserved cardiac function.[7] Subsequently, preconditioning was also found in other organs, such as brain and kidneys, and this boosted clinical and scientific interest in this phenomenon.

Although a large body of studies thus far failed to unravel the entire cellular signaling pathways that lead to preconditioning, it became clear that adenosine triphosphate–sensitive K^+ (K_{ATP}) channels can play a critical role in the process.[6] This view received further support from recent studies demonstrating that genetic ablation of the pore-forming subunit Kir6.2 of the plasmalemmal K_{ATP} channel causes the loss of ischemic preconditioning of cardiac tissue[8-10] and the loss of neuroprotection against acute hypoxia.[11] Although these findings unequivocally demonstrated that the opening of plasmalemmal K_{ATP} channels is beneficial for cell survival, several drawbacks limit the scope for clinical exploration. For example, currently there is no convincing evidence that established K_{ATP} channel openers such as diazoxide readily cross the blood–brain barrier. This is different with anesthetics because penetration of the blood–brain barrier is one of the defining characteristics of this class of substances. Moreover, there is not only a vast amount of clinical experience in their safe use, but outcome parameters can also be directly monitored.

The noble gas xenon has been proposed as an alternative to classic anesthetics partly because of its suggested organ protective effects and its overall benign side effect profile.[12] However, its high production cost has so far stopped it from being used widely.[13]

A number of reports suggest that other inhalational anesthetics have preconditioning effects on cardiac tissues, and a few studies suggest they have neuroprotective properties, though there is no consensus about their mode of action.[14–16]

Those studies that explored the involvement of K_{ATP} channels in anesthetic preconditioning in the brain have focused almost exclusively on the role of mitochondrial K-ATP (mito K-ATP) channels[17,18]; no study has ever examined the involvement of plasmalemmal K_{ATP} channels in neuronal preconditioning induced by either of the newer inhalational anesthetics sevoflurane or xenon. Furthermore, the majority of studies have not investigated the effects of drugs directly on K_{ATP} currents but simply relied on the perceived specificity of the drugs used for channel subtypes.[19]

With the current study, we aim to clarify the role of K_{ATP} channels in preconditioning induced by the inhalational anesthetics xenon and sevoflurane. We hypothesize that the opening of plasmalemmal ATP-sensitive K^+ channels rather than mito K-ATP channels is required for neuronal preconditioning by these drugs. We also postulate that to verify the preconditioning action of these drugs *via* K_{ATP} channels, their direct stimulatory action on currents through these ion channels in a recombinant expression system must be evident.

Materials and Methods

Preconditioning Studies

Tissue culture neuronal–glial cell cocultures were prepared as described previously.[20] All procedures involving animals were performed in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the Home Office, London, United Kingdom. In brief, brains from 1- to 2-day-old BALB/c mouse pups were removed from the skull and microdissected to obtain whole cerebral neocortices. After trypsination and resuspension, cells were plated at a density of 6.25×10^4 cells/cm^2 on four-well plates (Nunc, Roskilde, Denmark). They were cultured in Eagle's minimum essential medium supplemented with 20 mM glucose, 26 mM $NaHCO_3$, 10% fetal bovine serum, 10% heat-inactivated horse serum, Antibiotic-Antimycotic Solution (AAS; Gibco, Paisley, United Kingdom), 2 mM glutamine (Sigma, Poole, United Kingdom), and 10 ng/ml murine epidermal growth factor (Gibco). Glial cells reached confluence approximately 5 days after plating. Using a similar procedure, cortical neuronal cells were obtained from fetal BALB/c mice at 14–16 days of gestation and were plated at a density of 1.25×10^5 cells/cm^2 onto the confluent monolayer of glial cells. Neuronal cells reached mature morphology within 10 days after plating, and cocultures were used for preconditioning experiments after a total of 15 ± 1 day. Cultures were assigned to one of the following protocols: preconditioning, oxygen–glucose deprivation (OGD) only, or naive control. Cultures in the preconditioning group were exposed to either 1 minimum alveolar concentration (MAC) xenon, 2 MAC sevoflurane, or 10 μM diazoxide for 2 h in the presence or absence of the K_{ATP} channel inhibitor tolbutamide (0.1 mM) or the inhibitor of mito K-ATP channels 5-hydroxy-decanoic acid (5-HD, 0.5 mM).

Preconditioning

Preconditioning was performed as described previously.[21] Anesthetic-enriched medium was obtained by bubbling xenon or sevoflurane for 15 min through experimental medium containing Eagle's minimal essential medium, 21.1 mM glucose, and 38 mM $NaHCO_3$ in a Drechsel bottle. Neuronal–glial cell cocultures were washed twice with HEPES-buffered solution (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 15 mM glucose, and 2.0 mM HEPES, titrated to pH 7.4 using 1 M NaOH) and then incubated with anesthetic-enriched medium or with experimental medium containing 10 μM diazoxide. Tolbutamide (0.1 mM) or 5-HD (0.5 mM) were added to some dishes as appropriate. Cocultures were then transferred to an airtight, temperature-controlled gas exposure chamber for 2 h. The gas composition in the chamber for the xenon experiments consisted of 20% O_2, 5% CO_2, and 75% xenon (approximately 1 MAC). The gas composition in the sevoflurane experiments was 20% O_2, 5% CO_2, 71.7% N_2, and 3.3% sevoflurane (2 MAC). The atmosphere in the chamber for diazoxide preconditioning consisted of 20% O_2, 5% CO_2, and 75% N_2. After 2 h, cultures were removed from the chamber, washed with HEPES-buffered solution, and finally incubated in culture medium until further use.

Cocultures of the OGD group underwent the same protocol as the cultures in the preconditioning group except that instead of being exposed to HEPES-buffered solution containing the preconditioning drugs, they were washed with HEPES-buffered solution only. Cultures of the naive control group received the same pattern of solution changes but were not exposed to the preconditioning drugs or OGD.

Oxygen–Glucose Deprivation

To model ischemic injury in the brain, neuronal cells were subjected to OGD.[22] Twenty-four hours after preconditioning, cultures were washed twice with HEPES-buffered solution and once with prewarmed OGD medium. OGD medium contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, and 26 mM $NaHCO_3$ and was bubbled with 5% CO_2 and 95% N_2 in a Drechsel bottle at 37°C. Cultures were then placed into an

airtight gas exposure chamber with an anaerobic environment consisting of 5% CO_2 and 95% N_2 at 37°C for 75 min. OGD was terminated by removing cultures from the chamber and changing the media back to the culture medium.

MTT Reduction Test

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction test is a quantitative measurement of cellular metabolic activity and therefore widely used for the assessment of cellular viability and cell proliferation.[23,24] In the presence of active mitochondrial and cytosolic redox systems, the tetrazolium salts of MTT are reduced to intensely blue-colored formazans, which can be quantified in a spectrophotometer. The measured optical density corresponds directly to the viability of the cells in culture. Cell viability is stable between 6 and 24 h after OGD[25]; therefore, the MTT test was performed on the following day. Neuronal-glial cocultures were incubated for 2 h in 600 μl phosphate-buffered saline with 0.5 mg/ml MTT (Merck, Darmstadt, Germany) to allow uptake of MTT into the cells. After removing MTT from cultures, cells were dissolved and homogenized in 1 ml dimethyl sulfoxide (DMSO). Then, 0.3 ml of each sample was transferred to one well of a 96-well plate. The optical density (OD) was measured in triplicate at 570 nm in an MRX Microplate Reader (Dynex Technologies, Chantilly, VA).

The OD readings were then converted into normalized cell viability readings using equation 1:

$$\%C_{via} = \frac{(OD_{drug} - OD_{OGD})}{(OD_{control} - OD_{OGD})} \times 100, \quad (1)$$

where % C_{via} is cell viability expressed in percent, OD_{drug} is the optical density reading of preconditioned cells, $OD_{control}$ is the optical density reading of untreated naive cells, and OD_{OGD} is the optical density reading of nonpreconditioned cells, which were exposed to OGD only.

Recombinant K_{ATP} Channel Studies

Tissue Culture. HEK293 cells were maintained in growth medium (minimum essential medium enriched with 10 ml per 500 ml penicillin-streptomycin solution, 5 ml per 500 ml minimum essential medium nonessential amino acids, 50 ml per 500 ml fetal bovine serum, and 2 mM L-glutamine) in 5% CO_2-95% O_2 in a humidified incubator at 37°C. When the cells were 80% confluent (after approximately 4 days), they were plated onto glass coverslips coated with poly-D-lysine (1 mg/ml), and after a 6-h resting period, transient transfection was performed using the calcium phosphate method.[26] Cells were transfected with pcDNA3 vectors containing the coding sequence of mouse Kir6.2 (Genbank D50581[27,28]), rat SUR1 (Genbank L40624[29]), and green fluorescent protein. Cells were then incubated at 3% CO_2-97% O_2 for 6-8 h. Subsequently, cells were washed twice with phosphate-buffered saline and incubated in growth medium in 5% CO_2-95% O_2 in a humidified incubator at 37°C for 24-96 h until use for electrophysiologic recordings.

Electrophysiology. Stock solutions of tolbutamide (50 mM), diazoxide (20 mM), and pinacidil (10 mM) were prepared in 100 mM NaOH. Solutions containing sevoflurane, isoflurane, or halothane were prepared as volume fractions of a saturated aqueous solution. The concentration of these saturated solutions was taken as 11.8, 15.3, and 17.5 mM, respectively.[30] Solutions for the xenon experiments were prepared by bubbling extracellular solution with pure gases (xenon, nitrogen, oxygen) at a rate of 30 ml/min for 20 min, producing saturated solutions for the individual gases.[31] The test solutions with fractional gas concentrations were then obtained by mixing adequate volumes of the individual solutions with maintaining 20% oxygen-saturated solution and the balance (80%) being made up by varying amounts of nitrogen- and xenon-saturated solutions. Immediately after mixing, these solutions were transferred to glass syringe barrels containing a polypropylene float.[31] All other drugs were added directly to the experimental solutions, which were prepared freshly each day. Xenon was obtained from Air Products (Basingstoke, United Kingdom), sevoflurane and isoflurane were obtained from Abbott (Queenborough, Kent, United Kingdom), and nitrogen and oxygen were obtained from BOC (Manchester, United Kingdom). All other chemicals were from Sigma (Poole, Dorset, United Kingdom).

For electrophysiologic recordings, a coverslip with transfected HEK cells was transferred into a recording chamber mounted under an upright microscope (Zeiss Axioskop 2FS; Carl Zeiss Ltd., Welwyn Garden City, United Kingdom) with epifluorescence. During the course of the experiment, cells were constantly superfused at 3-5 ml/min with extracellular solution of the following composition: 118 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 25 mM HEPES (pH adjusted to 7.4 with NaOH). Test drugs were added to this solution either directly or from stock solutions as described above. Only cells that were successfully transfected with green fluorescent protein as evident from green fluorescence (excitation: 395-440 nm, emission: 470-600 nm) were selected for electrophysiologic recordings.

Patch pipettes were pulled from thin-walled borosilicate glass (GC150TF; Harvard Apparatus, Edenbridge, Kent, United Kingdom) and had resistances of 3-5 MΩ when filled with pipette solution. The pipette solution contained 120 mM KCl, 1 mM NaOH, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 5 mM HEPES, and 0.3 mM K_2ATP (pH adjusted to 7.3 with KOH; final $[K^+]$ approximately 140 mM). Whole cell currents were recorded at a holding potential of −20 mV and at 20°-24°C. Currents were evoked by repetitive 700-ms hyperpolarizing voltage ramps from −20 mV to −120 mV every 15 s and re-

Table 1. Reversal Potential of the Drug-modulated Current in HEK293 Cells

	Reversal Potential, mV	No. of Observations
Xenon	−80 ± 3	10
Sevoflurane	−83 ± 3	22
Halothane	−76 ± 2	22
Isoflurane	−77 ± 2	14
Diazoxide	−77 ± 3	10
Tolbutamide	−84 ± 3	10
Washout	−80 ± 2	12

No significant difference was observed for any of the drugs tested.

corded using an EPC9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Recordings were digitized at 0.5 kHz and analyzed using Pulse/Pulsefit software (HEKA) running on a personal computer. To confirm that all effects on the holding current at −20 mV were indeed caused by modulation of K^+ currents, reversal potentials were determined for each experimental condition and compared (table 1). There was no statistically significant difference in the reversal potential between the groups.

Data Analysis

Concentration–response relations for the volatile anesthetics were obtained by alternating the control solution with a test concentration of anesthetic. The extent of inhibition by the anesthetic was then expressed as a fraction of the mean of the value obtained in the control solution before and after anesthetic application. Concentration–response curves were fitted to the Hill equation (equation 2):

$$\frac{I}{I_c} = \frac{1}{\left(1 + \left(\frac{[vol]}{k_i}\right)^h\right)}, \quad (2)$$

where I is the holding current at −20 mV in the presence of the drug, I_c is the current in the absence of the drug, [Vol] is the volatile anesthetic concentration, K_i is the anesthetic concentration at which inhibition is half maximal, and h is the slope factor (Hill coefficient).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). All data were expressed as mean ± SEM. To test for differences between groups, two-tailed unpaired *t* test or one-way analysis of variance followed by the Tukey multiple comparison test were used as appropriate. A value of $P < 0.05$ was considered significant.

Results

Xenon but Not Sevoflurane Preconditioning Requires Functional Plasmalemmal K_{ATP} Channels

The current study was designed to analyze the role of K_{ATP} channels in preconditioning induced by xenon and sevoflurane using OGD in neuronal–glial cocultures as an *in vitro* model of ischemic brain injury (fig. 1). In this model, 70–80 min of OGD was sufficient to maximally damage neurons, with glial cells being left largely unharmed. The extent of the injury was found to remain unchanged from 6 to 24 h after OGD, with no further evidence for either a delayed second burst of damage or repair mechanisms taking place.[32]

Exposure to the preconditioning anesthetics xenon or sevoflurane or to the potassium channel opener diazoxide 24 h before the OGD insult effectively prevented neuronal cell loss (survival: 80–100%; figs. 1B–D). The addition of the K_{ATP} channel inhibitor tolbutamide to the xenon group during preconditioning completely abolished the protective effect of xenon (fig. 1B). Xenon's protective effect was also slightly reduced by the addition of the mito K-ATP channel inhibitor 5-hydroxydecanoate (5-HD). Although statistically significant, the reversal was not complete, because cell viability remained high in cultures treated concomitantly with xenon and 5-HD (fig. 1B). In contrast, the preconditioning effect of sevoflurane could not be reversed with either the K_{ATP} channel inhibitor tolbutamide or the mito K-ATP channel inhibitor 5-HD (fig. 1C). Finally, diazoxide preconditioning was completely abolished by 5-HD and to a slightly lesser extent by tolbutamide (fig. 1D).

Diazoxide, at a concentration of 10 μM, is widely seen as specific for mito K-ATP channels. However, this is only true for cardiac tissue where K_{ATP} channels have a SUR2A subunit. The SUR1 subunit of neuronal K_{ATP} channels also responds to diazoxide. Therefore, it is not surprising that the preconditioning effect of diazoxide was sensitive to the K_{ATP} channel blocker tolbutamide as well as the mito K-ATP channel antagonist 5-HD, suggesting a role for both channels.

These results indicated that opening of plasmalemmal K_{ATP} channels, but not mito K-ATP channels, is essential for neuronal preconditioning by xenon, and demonstrated that sevoflurane preconditioning does not involve the opening of either plasmalemmal K_{ATP} channels or mito K-ATP channels.

Evaluation of the Effects of Volatile Anesthetics on K_{ATP} Currents

Plasmalemmal K_{ATP} channels exist in a wide variety of organs, but their subunit composition differs between tissues. For example, neurons and pancreatic β cells carry channels mostly composed of Kir6.2 and SUR1 subunits, whereas myocardial and skeletal muscle have channels made up of Kir6.2 and SUR2A.[33–35] Consequently, it is not easily possible to extrapolate functional data and physiologic pathways related to K_{ATP} channel activation from one organ to the other (*e.g.* heart to brain). Here, we used Kir6.2 and SUR1 as the components for K_{ATP} channels because these are the subunits

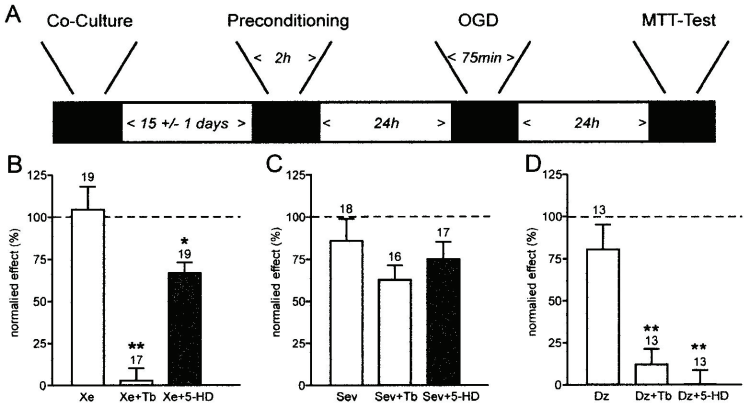


Fig. 1. Preconditioning by xenon but not sevoflurane involves adenosine triphosphate–sensitive K^+ (K_{ATP}) channels. *A* depicts the experimental timeframe adhered to. Neuronal and glial cells were cocultured for 2 weeks before a 2-h preconditioning stimulus with the anesthetics ± antagonists. After a further 24 h, cultures were exposed to oxygen–glucose deprivation (OGD) for 75 min, and a further 24 h later, damage was assessed using an 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. *B*, *C*, and *D* show the mean ± SEM data for preconditioning experiments. Cell viability was measured with MTT as optical density at 570 nm. The "normalized effect" describes the relative viability compared with maximal damage (OGD only, no preconditioning) and maximal survival (no OGD, naive cultures). Therefore, 100% cell viability represented the viability observed in naive cultures at the end of the protocol, whereas 0% cell viability indicated the viability found in cultures exposed to OGD only. *B* demonstrates that xenon preconditioning (Xe, 1 minimum alveolar concentration [MAC]) is effectively abolished by the K_{ATP} channel inhibitor tolbutamide (Xe + Tb, 0.1 mM), but not the mitochondrial K-ATP channel blocker 5-hydroxy-decanoic acid (5-HD, 0.5 mM). (*C*) In contrast, while sevoflurane (Sev, 2 MAC) effectively preconditions the cultures, this effect is not significantly reduced by tolbutamide (Tb) or 5-HD. (*D*) Preconditioning by the K_{ATP} channel opener diazoxide (Dz, 0.01 mM) is effectively blocked by either tolbutamide or 5-HD. Numbers (n) are given *above the bars*. *$P < 0.05$; **$P < 0.01$.

of K_{ATP} channels in most neurons. HEK293 cells were transiently transfected with Kir6.2 and SUR1 complementary DNA. In contrast to untransfected HEK293 cells, these cells exhibited a large standing outward current at −20 mV, had a resting potential more negative than −65 mV, and were sensitive to tolbutamide, consistent with the expression of K_{ATP} channels (fig. 2). During the first 5 min of whole cell voltage clamp recordings, an increase of outward current was observed consistent with the washout of adenosine triphosphate from the cytosol and subsequent progressive opening of plasmalemmal K_{ATP} channels. This was then followed by a stable holding current of 1.05 ± 0.12 nA (n = 30) in Kir6.2 and SUR1–transfected HEK293 cells (figs. 2A and B). This holding current was inhibited reversibly by the addition of the specific K_{ATP} channel blocker tolbutamide (100 μM; n = 17; figs. 2A and C). The zero-current potential for Kir6.2 and SUR1–transfected HEK293 cells was −77 ± 2

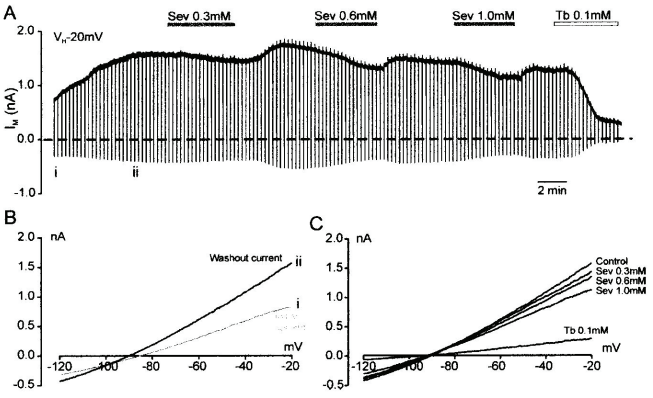


Fig. 2. Adenosine triphosphate–sensitive K^+ (K_{ATP}) channels expressed in HEK293 cells are mildly inhibited by sevoflurane. (*A*) Whole cell recording at a holding potential of −20 mV from an HEK293 cell transfected with Kir6.2 and SUR1. Sevoflurane (Sev) and tolbutamide (Tb) were bath applied as indicated by the *horizontal bars*. The *dashed line* indicates the zero-current level. *Vertical deflections* indicate the current response to a 700-ms voltage ramp from −20 mV to −120 mV. These current responses at points *i* and *ii* were plotted against the command voltage to obtain current–voltage relations (*B*). These demonstrate that the washout current has a reversal potential near the potassium equilibrium potential. (*C*) Current–voltage relations taken from *A* showing the concentration-dependent small inhibition of the K_{ATP} current by sevoflurane at clinically relevant concentrations, compared with the block by tolbutamide.

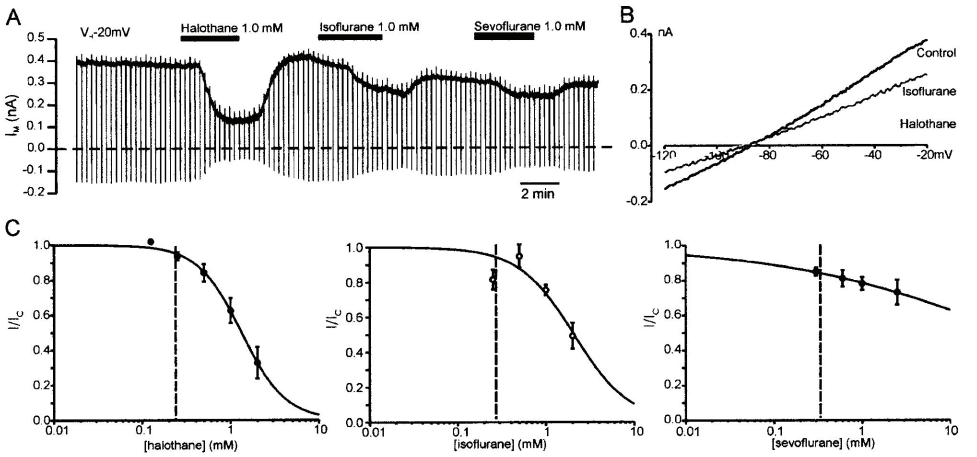


Fig. 3. Halogenated volatile anesthetics inhibit adenosine triphosphate–sensitive K^+ (K_{ATP}) channels. (*A*) Whole cell recording at a holding potential of −20 mV from an HEK293 cell transfected with Kir6.2 and SUR1. The *dashed line* indicates the zero-current level. Halothane, isoflurane, and sevoflurane (1 mM each) were bath applied as indicated by the *horizontal bars*. Each drug reversibly inhibited the whole cell K_{ATP} current. (*B*) Current–voltage relations taken from a recording as that shown in *A* demonstrating that the current inhibited by the volatile anesthetics has a reversal potential near E_K. (*C*) Concentration–response curves of the inhibitory effect of halothane (n = 4–8), isoflurane (n = 3–5), and sevoflurane (n = 3–8) on whole cell K_{ATP} currents. The current in presence of the drug (I) is expressed as a fraction of the current in its absence (I_C). The *solid lines* describe the best fit with a Hill equation using K_i = 1.32 mM and Hill coefficient (h) = 1.75 for halothane, K_i = 2.08 mM and h = 1.39 for isoflurane, and K_i = 47 mM and h = 0.34 for sevoflurane. In each case, it is assumed that complete inhibition of the K_{ATP} current could theoretically be achieved with high enough concentrations of the anesthetic. One minimum alveolar concentration is indicated by the *vertical dashed line* in each graph.

mV (n = 30), consistent with the fact that most of the whole cell conductance was made up by K_{ATP} channels. In contrast, untransfected HEK293 exhibited a zero-current potential of −40 ± 5 mV and a holding current of 22 ± 10 pA at −20 mV (n = 13). This current was not enhanced by the K^+ channel opener diazoxide (200 μM; n = 6), consistent with the absence of functional K_{ATP} channels in untransfected HEK293 cells.

Addition of sevoflurane to the superfusate reduced the K_{ATP} current reversibly in a concentration-dependent manner (figs. 2A and C). This lack of activation of K_{ATP} current by sevoflurane at clinically relevant concentrations (0.3–2.5 mM = 0.8–7.5 MAC) was consistent with the lack of effect of K_{ATP} channel inhibitors on the preconditioning effect of sevoflurane. Next, the effects of sevoflurane were compared with those of two other volatile anesthetics: halothane and isoflurane. At a concentration of 1 mM, halothane had the strongest inhibitory effect on whole cell K_{ATP} currents, followed by isoflurane, and the smallest effect by sevoflurane (figs. 3A and B). This difference in potency was also reflected in the concentration-response curves for the different drugs (fig. 3C). Halothane inhibited K_{ATP} currents with an IC_{50} of 1.3 ± 0.4 mM, isoflurane had an IC_{50} of 2.1 ± 0.6 mM, and the effect of sevoflurane was extrapolated to an IC_{50} of approximately 50 mM.

Next, the effects of xenon were tested on K_{ATP} currents. Xenon was applied at two concentrations: 50% and 80%. An 80% xenon solution is equivalent to 1 MAC or 3.4 mM xenon. Consequently, 50% xenon equates to 0.6 MAC and 2.1 mM. HEK293 cells transfected with Kir6.2 and SUR1 showed an increase in outward current by 19 ± 7% when superfused with a 50% xenon solution (n = 5) and by 44 ± 7% (n = 5) when exposed to 80% xenon solution as compared with control solution (80% N_2; fig. 4). The reversal potential of this current (fig. 4C and table 1) indicated it being a K^+ current. Furthermore, the current was inhibited by 0.1 mM tolbutamide (figs. 4C and D), and no increase in outward current in response to 80% xenon was observed in untransfected HEK293 cells (n = 5). These results strongly suggest that the xenon-induced K^+ current was due to the opening of Kir6.2/SUR1 channels. This activation of plasmalemmal K_{ATP} channels was consistent with the preconditioning effect seen in the neuronal-glial cocultures being K_{ATP} channel dependent.

Lack of Modulation of K_{ATP} Channels via *Mitochondrial K_{ATP} Channels or by Drugs that Act on These*

We next performed a series of experiments to demonstrate that the activity of K_{ATP} channels in HEK293 cells is not significantly modulated by mito K-ATP channels

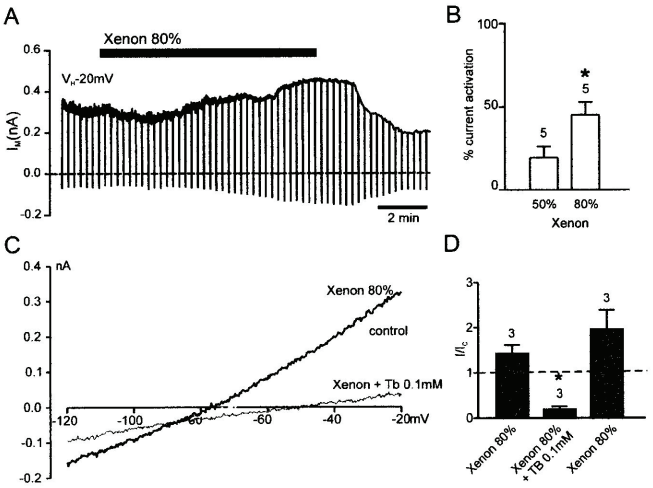


Fig. 4. Xenon activates adenosine triphosphate–sensitive K^+ (K_{ATP}) channels. (*A*) Whole cell recording from HEK293 cell transfected with Kir6.2 and SUR1 demonstrating reversible activation of the current by 80% xenon. (*B*) Mean effects of the two test concentrations of xenon on the holding current at −20 mV from K_{ATP} channel–transfected HEK293 cells. (*C*) Current–voltage relations in the absence and presence of 80% xenon and xenon + 0.1 mM tolbutamide. Xenon potentiated the K_{ATP} current, whereas tolbutamide completely inhibited the current, even in the presence of xenon. (*D*) Mean data from experiments as depicted in *C*. Mean holding current at −20 mV in the presence of the drug expressed as a fraction of that in the absence of any drug. Xenon led to an increase in holding current and tolbutamide blocked both the xenon-potentiated current and the current in the absence of xenon (*dashed line*). Numbers (n) are given *above the bars*. *$P < 0.05$.

under our experimental conditions. First, whereas the K_{ATP} channel antagonist tolbutamide decreased current amplitude dramatically, no significant reduction in whole cell current was observed using the mito K-ATP channel inhibitor 5-HD (fig. 5). Equivalent results were obtained when using K_{ATP} channel openers (fig. 6). Diazoxide and pinacidil have both been reported to open mito K-ATP channels,[36,37] but in addition, diazoxide activates K_{ATP} channels containing SUR1, and pinacidil activates K_{ATP} channels containing SUR2.[38] Consequently, diazoxide is expected to be mito K-ATP channel specific in systems that do not contain SUR1 (*e.g.*, heart muscle), and pinacidil would be specific for mito K-ATP channels in tissues not containing SUR2 (*e.g.*, most neurons). In HEK293 cells expressing Kir6.2 and SUR1, pinacidil did not increase the whole cell current significantly (figs. 6B and C). We took this as an indication that opening of mito K-ATP channels does not modulate K_{ATP} currents in these cells. In contrast, diazoxide concentration-dependently increased the whole cell current (figs. 6A and C) in accord with its direct effect on K_{ATP} channels containing SUR1. In fact, at a concentration of 10 μM, diazoxide was able to stimulate the K_{ATP} current to a similar extend as 80% xenon (fig. 6C). Because both of these drugs effectively precondition at these concentrations, one might postulate that an increase in K_{ATP} current by approximately 50% is sufficient to initiate preconditioning. However, because diazoxide is known as a potent activator of mito K-ATP channels, it was explored whether mito K-ATP channel activation and subsequent alterations in mitochondrial function might contribute significantly to the K_{ATP} current seen with 10 μM diazoxide. To test this hypothesis, the K_{ATP} current was enhanced by 10 μM diazoxide and then 5-HD was added. No significant reduction of the diazoxide-elicited current was observed under these conditions (fig. 6D). These results established that 10 μM diazoxide activates K_{ATP} channels independently from its effects on mito K-ATP channels. Taken together, these results provide strong evidence that the effects of volatile anesthetics on K_{ATP} whole cell currents observed in this study are not secondary to the interaction of these drugs with mito K-ATP channels.

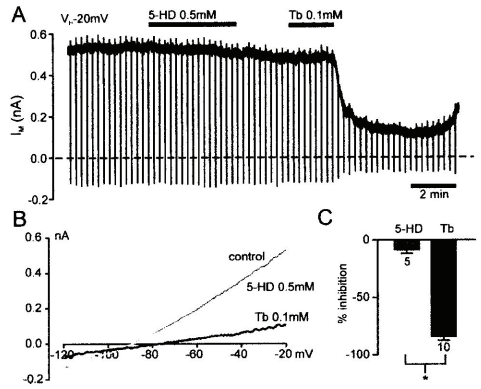


Fig. 5. Adenosine triphosphate–sensitive K^+ (K_{ATP}) currents in whole cell recordings are not affected by specific blockers of mitochondrial K_{ATP} (mito K-ATP) channels. (*A*) Whole cell recording demonstrating the lack of effect of the mito K-ATP channel blocker 5-hydroxy-decanoic acid (5-HD, 0.5 mM) on K_{ATP} channels made up of Kir6.2 and SUR1. In contrast, tolbutamide (Tb) strongly and reversibly inhibited the whole cell current. The drugs were applied as indicated by the *bars* above the recording. (*B*) Current–voltage relations in the absence (control) and presence of 0.5 mM 5-HD or 0.1 mM tolbutamide. (*C*) Mean data from experiments as that shown in *A*. *$P < 0.05$.

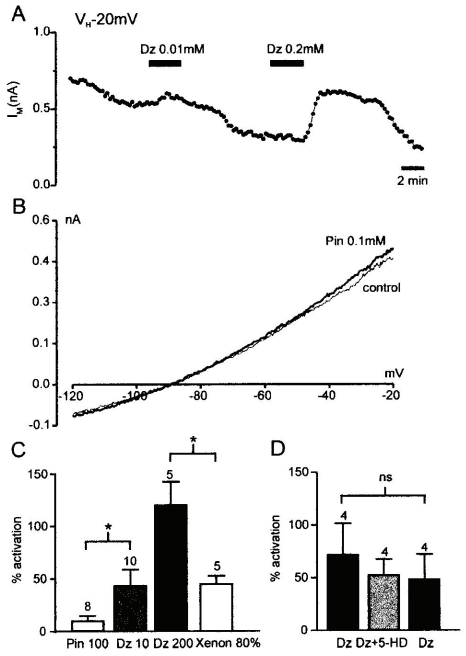


Fig. 6. Whole cell adenosine triphosphate–sensitive K^+ (K_{ATP}) currents are not affected by specific openers of mito K-ATP channel openers. (*A*) Whole cell recording from HEK293 cell expressing Kir6.2 and SUR1. Plotted is the mean holding current at −20 mV every 15 s. The SUR1-specific K^+ channel opener diazoxide (Dz) is applied as indicated by the *solid bars* and concentration-dependently potentiated the whole cell current. (*B*) Current–voltage relations demonstrating the lack of effect of the cardiac K^+ channel opener pinacidil (Pin, 0.1 mM) on Kir6.2/SUR1 currents in HEK293 cells. (*C*) Mean effects of the various K^+ channel openers on Kir6.2/SUR1 currents. (*D*) Diazoxide at 10 μM increases the plasma K_{ATP} current independently of its potential effect on mitochondrial K_{ATP} channels, as demonstrated by the lack of effect of the mitochondrial K_{ATP} channel inhibitor 5-hydroxy-decanoic acid (5-HD) on the diazoxide activation of the whole cell current. Numbers (n) are given *above the bars*. *$P < 0.05$.

Discussion

Neuronal Preconditioning by Anesthetics

The current study demonstrated that the mechanisms by which neuronal preconditioning is achieved can vary between individual inhalational anesthetics. More precisely, we were able to identify for xenon a pathway which was dependent on opening of plasmalemmal K_{ATP} channels. Conversely, sevoflurane preconditioning was plasmalemmal K_{ATP} channel independent. Importantly, neither of these anesthetics showed a substantial involvement of mito K-ATP channels in its preconditioning action. This lack of effect cannot be due to pathologic changes in our preparation because the control preconditioning with diazoxide, which acts in neurons on both plasmalemmal K_{ATP} channels and on mito K-ATP channels, was indeed impaired by both the K_{ATP} channel antagonist tolbutamide and the mito K-ATP channel blocker 5-HD. Given the dependence of ischemic preconditioning on functional plasmalemmal K_{ATP} channels, as demonstrated by the lack of this preconditioning in cardiac muscle of Kir6.2$^{-/-}$ mice,[8] it seems as if (1) xenon mimics ischemic preconditioning and (2) sevoflurane acts on a different target. Whether this target is downstream from the activation of K_{ATP} channels or involves an entirely independent pathway for preconditioning is unclear. A similar observation has been made in cerebellar brain slices, where blocking K_{ATP} channels had no effect on isoflurane preconditioning.[39] Similarly to these results, Marinovic *et al.* [40] reported that cardiac cytoprotection did not involve sarcolemmal K_{ATP} channel opening during the preconditioning phase with isoflurane.

Anesthetic Effects on Recombinant Neuronal Plasmalemmal K_{ATP} Channels

Contribution of Mitochondrial K-ATP Channels. Activation of mito K-ATP channels would be expected to dissipate the mitochondrial membrane potential, thus causing a reduction in adenosine triphosphate production.[37,41] With plasmalemmal K_{ATP} channels being archetypal adenosine triphosphate sensors,[38] it would be expected that this decrease in adenosine triphosphate production would lead to channel activation and an increase in outward current (*e.g.*, as seen with cyanide exposure). However, our observation that neither pinacidil nor 5-HD had any effect on the activity of plasmalemmal K_{ATP} channels indicates that modulation of mito K-ATP cannot influence plasmalemmal K_{ATP} channels in our system. Therefore, the observed effects of anesthetics on these channels cannot be explained by an indirect effect *via* mito K-ATP channels.

Direct Effects on Plasmalemmal K_{ATP} Channels. Probably the most interesting finding of this study was that the noble gas xenon acts as a K-channel opener. In previous studies that were primarily aimed at identifying the target for xenon's anesthetic action, this noble gas has been identified as an antagonist for the glycine binding site on *N*-methyl-D-aspartate receptors,[42] and it has been shown that xenon activates selective two-pore-domain potassium channels[31] (for review, see Preckel *et al.*[43]). Here, we have demonstrated that the tolbutamide-sensitive preconditioning with xenon is mirrored by the activation of plasmalemmal K_{ATP} channels by xenon at 1 MAC. At this concentration, xenon activated K_{ATP} channels as efficiently as the specific K_{ATP} channel opener diazoxide at its preconditioning concentration of 10 μM. Our results can be seen as indication that an increase in K_{ATP} channel activity by 50% is sufficient to trigger preconditioning responses. They also clearly demonstrate that the neuroprotective effect of xenon is achieved at

anesthetic or even subanesthetic concentrations and thus can be accomplished with clinically safe doses.

In contrast to xenon, sevoflurane did not precondition in a K_{ATP} channel-dependent manner, and sevoflurane also did not activate plasmalemmal K_{ATP} channels. In fact, slight inhibition was observed. Similar results were also found for isoflurane and halothane. Both of these inhibited the plasmalemmal K_{ATP} channel mildly at 1 MAC, but more substantially than sevoflurane at higher concentrations. Similarly, it has previously been shown that barbiturates inhibit neuronal K_{ATP} channels at supraclinical concentrations.[44]

A recent study investigating the acute effects of 4% sevoflurane on the membrane potential of hippocampal CA1 pyramidal cells also did not see an activation of K_{ATP} channels despite the fact that hypoxia elicited such a hyperpolarization, which was also enhanced after preexposure to sevoflurane.[45] These results probably reflect our finding that sevoflurane is able to precondition, but not in a K_{ATP} channel-dependent manner. Inhibition by halogenated anesthetics might be a general feature of inwardly-rectifying K^+ channels, because several members of this ion channel family were inhibited to varying degrees by these agents.[46] In the case of the K_{ATP} channel, the SUR subunit might have an additional modulatory influence on the anesthetic sensitivity, because Bienengraeber *et al.*[47] reported that the SUR2A subunit of cardiac sarcolemmal K_{ATP} channels conferred isoflurane activation of the K^+ current under acidic conditions.

Physiologic Significance of K_{ATP} Channel Inhibition

Here, we have shown that halogenated anesthetics inhibit plasmalemmal K_{ATP} channels. This should have little effect because K_{ATP} channels are closed under physiologic conditions in most tissues. They would only open under conditions of metabolic stress, such as ischemia. However, a notable exception is the pancreatic β cell, where K_{ATP} channel opening controls the glucose-dependent insulin release.[48] Consequently, inappropriate K_{ATP} channel inhibition could potentially lead to hyperinsulinemia and hypoglycemia. In addition, patients who take antianginal medication such as the K-channel opener nicorandil or antihypertensives such as cromakalim or pinacidil might lose some protection if exposed to drugs that inhibit K_{ATP} channels.[49,50]

The halogenated volatiles tested here had inhibitory effects on K_{ATP} currents at 1 MAC (fig. 3), but currently there are not sufficient data to allow a critical evaluation of potential interactions between anesthetics and K-channel openers. Furthermore, because it is unknown whether these drugs interact with the potassium channel subunit or the sulfonylurea receptor (SUR1 in this case), it is not clear whether the cardiac or the smooth muscle plasmalemmal K_{ATP} channel is affected at all.

Physiologic Consequence of K_{ATP} Channel Activation

Conversely, drugs that open K_{ATP} channels can potentially interfere with the sulfonylurea-induced insulin secretion in diabetic patients. However, our finding that tolbutamide prevents the opening of K_{ATP} channels by xenon argues that on the contrary diabetes treatment might interfere with the organ-protective effect afforded by xenon. This is reminiscent of the interference of sulfonylurea drugs with the antianginal drug nicorandil.[49] Although this is a valid concern for peripheral tissue, the fact that tolbutamide does not easily partition into the brain[51,52] makes xenon a viable choice even for diabetic patients.

Most K_{ATP} channel openers do not cross the blood-brain barrier under physiologic conditions. Nevertheless, studies have reported neuroprotective effects of these drugs *in vivo*. However, rather than exerting their effects directly on the neuronal plasma membrane, the observed effect might be secondary to increased cerebral blood flow due to the vasodilatory effect of these drugs. They open K_{ATP} channels in vascular smooth muscle cells.[53] Similarly, xenon has been shown to stimulate cerebral blood flow[54-56] and to dilate pial vessels.[57] This might indicate that xenon activates K_{ATP} channels of the smooth muscle subtype Kir6.1/SUR2B and raises the question of whether xenon differs from classic K_{ATP} channel openers by having no, or a reduced, tissue specificity and thus works *via* a novel mechanism.

In summary, we have established xenon as a novel plasmalemmal K_{ATP} channel opener (that can readily cross the blood-brain barrier) on both a molecular and a functional basis. Furthermore, we have demonstrated two distinct preconditioning pathways, one that is K_{ATP} channel dependent and one that is not. Further studies are likely to reveal subtle differences in the protection afforded by the K_{ATP} channel-dependent pathway that probably matches the naturally occurring ischemic preconditioning most closely, and the protection *via* the K_{ATP} channel-independent pathway. This might open new doors for the treatment of stroke patients. Finally, xenon, with its favorable safety profile, might be the drug of choice to preoptimize patients before surgery, especially for procedures that carry some risk of inducing ischemic stroke, such as heart surgery or carotid endarterectomy.

The authors thank Mahmuda Hossain, Ph.D. (Technician, Department of Anaesthetics, Pain Medicine and Intensive Care, Chelsea and Westminster Hospital, Imperial College London, London, United Kingdom), and Cleoper Paule, Ph.D. (Research Student, Department of Anaesthetics, Pain Medicine and Intensive Care, Chelsea and Westminster Hospital, Imperial College London), for their technical support.

References

1. Selim M: Perioperative stroke. N Engl J Med 2007; 356:706-13

2. Toumpoulis IK, Anagnostopoulos CE, Chamogeorgakis TP, Angouras DC, Kariou MA, Swistel DG, Rokkas CK: Impact of early and delayed stroke on in-hospital and long-term mortality after isolated coronary artery bypass grafting. Am J Cardiol 2008; 102:411-7

3. Lisle TC, Barrett KM, Gazoni LM, Swenson BR, Scott CD, Kazemi A, Kern JA, Peeler BB, Kron IL, Johnston KC: Timing of stroke after cardiopulmonary bypass determines mortality. Ann Thorac Surg 2008; 85:1556–62
4. Kitano H, Kirsch JR, Hurn PD, Murphy SJ: Inhalational anesthetics as neuroprotectants or chemical preconditioning agents in ischemic brain. J Cereb Blood Flow Metab 2007; 27:1108–28
5. Barone FC, White RF, Spera PA, Ellison J, Currie RW, Wang X, Feuerstein GZ: Ischemic preconditioning and brain tolerance: Temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. Stroke 1998; 29:1937–50
6. Hanley PJ, Daut J: K(ATP) channels and preconditioning: A re-examination of the role of mitochondrial K(ATP) channels and an overview of alternative mechanisms. J Mol Cell Cardiol 2005; 39:17–50
7. Murray CE, Jennings RB, Reimer KA: Preconditioning with ischaemia: A delay of lethal cell injury in ischaemic myocardium. Circulation 1986; 74:1124–36
8. Gumina RJ, Pucar D, Bast P, Hodgson DM, Kurtz CE, Dzeja PP, Miki T, Seino S, Terzic A: Knockout of Kir6.2 negates ischemic preconditioning-induced protection of myocardial energetics. Am J Physiol 2003; 284:H2106–13
9. Nakaya H, Miki T, Seino S, Yamada K, Inagaki N, Suzuki M, Sato T, Yamada M, Matsushita K, Kurachi Y, Arita M: Molecular and functional diversity of ATP-sensitive K+ channels: The pathophysiological roles and potential drug targets [in Japanese]. Nippon Yakurigaku Zasshi 2003; 122:243–50
10. Suzuki M, Sasaki N, Miki T, Sakamoto N, Ohmoto-Sekine Y, Tamagawa M, Seino S, Marban E, Nakaya H: Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. J Clin Invest 2002; 109:509–16
11. Yamada K, Ji JJ, Yuan H, Miki T, Sato S, Horimoto N, Shimizu T, Seino S, Inagaki N: Protective role of ATP-sensitive potassium channels in hypoxia-induced generalized seizure. Science 2001; 292:1543–6
12. Preckel B, Schlack W: Inert gases as the future inhalational anaesthetics? Best Pract Res Clin Anaesthesiol 2005; 19:365–79
13. Sanders RD, Maze M: Xenon: From stranger to guardian. Curr Opin Anaesthesiol 2005; 18:405–11
14. Payne RS, Akca O, Roewer N, Schurr A, Kehl F: Sevoflurane-induced preconditioning protects against cerebral ischemic neuronal damage in rats. Brain Res 2005; 1034:147–52
15. Ma D, Hossain M, Pettet GK, Luo Y, Lim T, Akimov S, Sanders RD, Franks NP, Maze M: Xenon preconditioning reduces brain damage from neonatal asphyxia in rats. J Cereb Blood Flow Metab 2006; 26:199–208
16. Wang L, Traystman RJ, Murphy SJ: Inhalational anesthetics as preconditioning agents in ischemic brain. Curr Opin Pharmacol 2008; 8:104–10
17. Kehl F, Payne RS, Roewer N, Schurr A: Sevoflurane-induced preconditioning of rat brain *in vitro* and the role of KATP channels. Brain Res 2004; 1021:76–81
18. Kaneko T, Yokoyama K, Makita K: Late preconditioning with isoflurane in cultured rat cortical neurones. Br J Anaesth 2005; 95:662–8
19. Xiong L, Zheng Y, Wu M, Hou L, Zhu Z, Zhang X, Lu Z: Preconditioning with isoflurane produces dose-dependent neuroprotection *via* activation of adenosine triphosphate-regulated potassium channels after focal cerebral ischemia in rats. Anesth Analg 2003; 96:233–7
20. Wilhelm S, Ma D, Maze M, Franks NP: Effects of xenon on *in vitro* and *in vivo* models of neuronal injury. ANESTHESIOLOGY 2002; 96:1485–91
21. Ma D, Hossain M, Rajakumaraswamy N, Franks NP, Maze M: Combination of xenon and isoflurane produces a synergistic protective effect against oxygen-glucose deprivation injury in a neuronal-glial co-culture model. ANESTHESIOLOGY 2003; 99:748–51
22. Goldberg MP, Choi DW: Combined oxygen and glucose deprivation in cortical cell culture: Calcium-dependent and calcium-independent mechanisms of neuronal injury. J Neurosci 1993; 13:3510–24
23. Berridge MV, Tan AS: Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 1993; 303:474–82
24. Takahashi S, Abe T, Gotoh J, Fukuuchi Y: Substrate-dependence of reduction of MTT: A tetrazolium dye differs in cultured astroglia and neurons. Neurochem Int 2002; 40:441–8
25. Rajakumaraswamy N, Ma D, Hossain M, Sanders RD, Franks NP, Maze M: Neuroprotective interaction produced by xenon and dexmedetomidine on *in vitro* and *in vivo* neuronal injury models. Neurosci Lett 2006; 409:128–33
26. Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 1987; 7:2745–52
27. Inagaki N, Inazawa J, Seino S: cDNA sequence, gene structure, and chromosomal localization of the human ATP-sensitive potassium channel, uKATP-1, gene (KCNJ8). Genomics 1995; 30:102–4
28. Sakura H, Ammala C, Smith PA, Gribble FM, Ashcroft FM: Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic beta-cells, brain, heart and skeletal muscle. FEBS Lett 1995; 377:338–44
29. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP IV, Boyd AE III, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA: Cloning of the beta cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. Science 1995; 268:423–6
30. Jenkins A, Franks NP, Lieb WR: Effects of temperature and volatile anesthetics on GABA(A) receptors. ANESTHESIOLOGY 1999; 90:484–91
31. Gruss M, Bushell TJ, Bright DP, Lieb WR, Mathie A, Franks NP: Two-pore-domain K+ channels are a novel target for the anesthetic gases xenon, nitrous oxide, and cyclopropane. Mol Pharmacol 2004; 65:443–52
32. Ma D, Hossain M, Rajakumaraswamy N, Arshad M, Sanders RD, Franks NP, Maze M: Dexmedetomidine produces its neuroprotective effect *via* the alpha 2A-adrenoceptor subtype. Eur J Pharmacol 2004; 502:87–97
33. Ashcroft FM, Gribble FM: New windows on the mechanism of action of K(ATP) channel openers. Trends Pharmacol Sci 2000; 21:439–45
34. Fujita A, Kurachi Y: Molecular aspects of ATP-sensitive K+ channels in the cardiovascular system and K+ channel openers. Pharmacol Ther 2000; 85:39–53
35. Liss B, Roeper J: Molecular physiology of neuronal K-ATP channels. Mol Membr Biol 2001; 18:117–27
36. Garlid KD, Paucek P, Yarov YV, Sun X, Schindler PA: The mitochondrial K-ATP channel as a receptor for potassium channel openers. J Biol Chem 1996; 271:8796–9
37. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A: Mitochondrial ATP-sensitive K+ channels modulate cardiac mitochondrial function. Am J Physiol 1998; 275:H1567–76
38. Trapp S, Ashcroft FM: A metabolic sensor in action: News from the ATP-sensitive K+-channel. News Physiol Sci 1997; 12:255–63
39. Zheng S, Zuo Z: Isoflurane preconditioning reduces Purkinje cell death in an *in vitro* model of rat cerebellar ischemia. Neuroscience 2003; 118:99–106
40. Marinovic J, Bosnjak ZJ, Stadnicka A: Distinct roles for sarcolemmal and mitochondrial adenosine triphosphate-sensitive potassium channels in isoflurane-induced protection against oxidative stress. ANESTHESIOLOGY 2006; 105:98–104
41. Ashcroft FM, Trapp S: The ATP-sensitive potassium channel: A metabolic sensor, Molecular Mechanisms of Metabolic Arrest: Life in Limbo. Edited by Storey KB. Oxford, BIOS Scientific Publishers, 2001, pp 43–76
42. Dickinson R, Peterson BK, Banks P, Simillis C, Martin JC, Valenzuela CA, Maze M, Franks NP: Competitive inhibition at the glycine site of the *N*-methyl-D-aspartate receptor by the anesthetics xenon and isoflurane: Evidence from molecular modeling and electrophysiology. ANESTHESIOLOGY 2007; 107:756–67
43. Preckel B, Weber NC, Sanders RD, Maze M, Schlack W: Molecular mechanisms transducing the anesthetic, analgesic, and organ-protective actions of xenon. ANESTHESIOLOGY 2006; 105:187–97
44. Ohtsuka T, Ishiwa D, Kamiya Y, Itoh H, Nagata I, Saito Y, Yamada Y, Sumitomo M, Andoh T: Effects of barbiturates on ATP-sensitive K channels in rat substantia nigra. Neuroscience 2006; 137:573–81
45. Wang J, Lei B, Popp S, Meng F, Cottrell JE, Kass IS: Sevoflurane immediate preconditioning alters hypoxic membrane potential changes in rat hippocampal slices and improves recovery of CA1 pyramidal cells after hypoxia and global cerebral ischemia. Neuroscience 2007; 145:1097–107
46. Yamakura T, Lewohl JM, Harris RA: Differential effects of general anesthetics on G protein- coupled inwardly rectifying and other potassium channels. ANESTHESIOLOGY 2001; 95:144–53
47. Bienengraeber M, Warltier DC, Bosnjak ZJ, Stadnicka A: Mechanism of cardiac sarcolemmal adenosine triphosphate-sensitive potassium channel activation by isoflurane in a heterologous expression system. ANESTHESIOLOGY 2006; 105:534–40
48. Ashcroft FM: ATP-sensitive potassium channelopathies: Focus on insulin secretion. J Clin Invest 2005; 115:2047–58
49. Reimann F, Ashcroft FM, Gribble FM: Structural basis for the interference between nicorandil and sulfonylurea action. Diabetes 2001; 50:2253–9
50. Gribble FM, Reimann F: Sulphonylurea action revisited: The post-cloning era. Diabetologia 2003; 46:875–91
51. Takanaga H, Murakami H, Koyabu N, Matsuo H, Naito M, Tsuruo T, Sawada Y: Efflux transport of tolbutamide across the blood-brain barrier. J Pharm Pharmacol 1998; 50:1027–33
52. Murakami H, Takanaga H, Matsuo H, Ohtani H, Sawada Y: Comparison of blood-brain barrier permeability in mice and rats using *in situ* brain perfusion technique. Am J Physiol Heart Circ Physiol 2000; 279:H1022–8
53. Quayle JM, Nelson MT, Standen NB: ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. Physiol Rev 1997; 77:1165–232
54. Gur D, Yonas H, Jackson DL, Wolfson SK Jr, Rockette H, Good WF, Maitz GS, Cook EE, Arena VC: Measurement of cerebral blood flow during xenon inhalation as measured by the microspheres method. Stroke 1985; 16:871–4
55. Junck L, Dhawan V, Thaler HT, Rottenberg DA: Effects of xenon and krypton on regional cerebral blood flow in the rat. J Cereb Blood Flow Metab 1985; 5:126–32
56. Hartmann A, Dettmers C, Schuler FJ, Wassmann HD, Schumacher HW: Effect of stable xenon on regional cerebral blood flow and the electroencephalogram in normal volunteers. Stroke 1991; 22:182–9
57. Fukuda T, Nakayama H, Yanagi K, Mizutani T, Miyabe M, Ohshima N, Toyooka H: The effects of 30% and 60% xenon inhalation on pial vessel diameter and intracranial pressure in rabbits. Anesth Analg 2001; 92:1245–50

Editorial Comment:

Anesthesiology 2009; 110:961-3 Copyright © 2009, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins, Inc.

Channels of Preconditioning

Potassium Drain that Protects the Brain

PERIOPERATIVE stroke and/or neurologic dysfunction, accompanied with significant mortality or incapacitating sequelae, not infrequently complicates cardiac, vascular, or neurologic surgery. Indeed, a recent study in ANESTHESIOLOGY showed a remarkably high incidence of perioperative stroke, greater than 1 per 200 such patients,[1] even after noncardiac or nonvascular surgery. Therefore, clinical strategies aimed at either protecting the brain or enhancing its tolerance to ischemic or anoxic insults are worthwhile from the clinical point of view. In an article published in this issue of the Journal, Bantel *et al.*[2] provide a fascinating preclinical series of studies examining the mechanisms of brain protection by anesthetics.

A particular mechanism of tolerance, by which cells become resistant to subsequent lethal events, is mediated by *preconditioning*, and within this context *ischemic preconditioning* develops when brief, sublethal episodes of ischemia activate endogenous protective mechanisms rendering organs resistant to subsequent, more severe ischemic events.[3] Mimicking ischemic preconditioning, *anesthetic preconditioning* is a similar protective mechanism whereby exposure to an inhaled anesthetic also renders a tissue tolerant to a subsequent ischemic insult. Anesthetic preconditioning for myocardial protection has been studied extensively and shown to be relevant in clinical situations as well.[4] Likewise, any benefits conferred by preconditioning strategies, including anesthetics, may be of value for protecting the brain or any neurologic tissue, even though such strategies have not been investigated in the brain as extensively as in the heart. However, several questions have been raised regarding feasibility of neuronal preconditioning, practical aspects, and its possible impact on perioperative outcome, especially after procedures with high incidence of perioperative stroke and adverse neuropsychiatric sequelae.[5-7]

Is neuronal preconditioning feasible? Several preclinical and clinical studies positively indicate that neurons can also be preconditioned against ischemia. Mechanisms of neuronal preconditioning have been recently reviewed by Gidday[8] and Obrenovitch.[9] It is evident that even without preconditioning, neurons naturally recruit endogenous countermeasures against ischemic injury; however, adaptive responses mobilized as a response to preconditioning are of a significantly different nature. The preconditioning cascade includes stimuli that, *via* sensors and transducers, activate downstream transcription factors, ultimately modulating gene expression. Then, novel proteins may act as effectors enhancing the resistance of neurons to ischemia, and/or preexisting proteins may be modulated by posttranslational modification acting as effectors, as well. By these cascades, a latent neuroprotective phenotype is established before ischemia, which, together with other specific cellular responses, eventually results in a reprogrammed brain or other neuronal tissue, capable of tolerating ischemia better.

The classic preconditioning stimuli are brief, sublethal episodes of ischemia, but other agents can trigger the preconditioning cascade in neurons, as well. These include molecules (such as glutamate, reactive oxygen species, inflammatory cytokines, and caspases) that in high concentrations are deleterious, but at lower concentrations initiate adaptive cascades. Of clinical interest is also the fact that drugs, such as anesthetics[6,10,11] or the adenosine triphosphate-sensitive potassium (K_{ATP}) channel openers,[12] may act on and be recognized by molecular sensors in a fashion that initiates the preconditioning signaling cascade. Molecular sensors include a variety of neurotransmitters, neuromodulators, cytokine receptors, ion channels, and redox-sensitive enzymes.[8] Of particular clinical and pharmacologic interest are the K_{ATP} channels, which, in addition to their role as sensors, may also have a parallel role as effectors of the phenotype that provides resistance to ischemia.[12]

Can anesthetics precondition the brain against ischemia? In the heart, preconditioning with volatile anesthetics mimics ischemic preconditioning in a fashion that involves K_{ATP} channel activation. The same is true in the brain: Preconditioning with inhaled anesthetics protects neurons from ischemia-induced death as well.[10,13,14] However, it is not really clear what preconditioning of the central nervous system is and how beneficial it really is. Could it simply be a period of hibernation? This would be beneficial in promoting survival of critical patients, but would not be beneficial for the quality of life. Although inhaled anesthetics may not be practically applicable in all clinical settings wherein neuroprotection is desirable, they nevertheless may be suitably used to optimize perioperative outcome after

This Editorial View accompanies the following article: Bantel C, Maze M, Trapp S: Neuronal preconditioning by inhalational anesthetics: Evidence for the role of plasmalemmal adenosine triphosphate-sensitive potassium channels. ANESTHESIOLOGY 2009; 110:986-95.

Accepted for publication December 22, 2008. Supported in part by National Institutes of Health grant Nos. GM066730 and HL034708 (to Dr. Bosnjak) and National Institute for Neurological Disorders and Stroke S049420A (to Dr. Sarantopoulos), Bethesda, Maryland. The authors are not supported by, nor maintain any financial interest in, any commercial activity that may be associated with the topic of this article.

surgical procedures associated with high-risk adverse neurologic sequelae. Studies of pertinent mechanisms may also lead to identification of novel and clinically more feasible means of neuronal preconditioning in clinical settings.[10]

Isoflurane, the most studied inhaled anesthetic, confers ischemic preconditioning in brain neurons of various species[10,13,14] in an age-specific and sex-specific fashion.[15] Preconditioning effects have also been shown for halothane[10,13,14] and sevoflurane.[16] In this issue of ANESTHESIOLOGY, Bantel *et al.*[2] provide further evidence supporting the role of plasmalemmal K_{ATP} channels as mediators and possibly effectors of neuronal preconditioning induced by the novel anesthetic gas xenon. Their study is also indicative of the multiple, diverse mechanisms by which neuronal preconditioning is attained. They show that xenon and sevoflurane at clinical concentrations precondition neurons, but *via* different pathways. Only xenon induced preconditioning *via* opening of plasmalemmal K_{ATP} channels; sevoflurane did not. Because K_{ATP} channels are proven "metabolic sensors," the agents activating these channels might initiate the preconditioning response in a way that most closely resembles that of the "physiologic" ischemic preconditioning. The next task is to elucidate which signal it is that the K_{ATP} channels generate to bring about longer-lasting cellular changes.

Many studies do not distinguish between mitochondrial K_{ATP} channels, neuronal K_{ATP} channels, and cardiac or vascular muscle K_{ATP} channels. These are clearly separate molecular entities with their own specific, but overlapping, pharmacologic profile. Bantel *et al.*[2] attempted to unambiguously distinguish between these, and one would hope that this will become a recurrent theme in future studies of neuronal preconditioning. K_{ATP} channels are octamers, consisting of four pore-forming (Kir6.x) coupled to four regulatory sulfonylurea receptor subunits (SURx). Three tissue-specific subtypes have been identified: Kir6.2/SUR1 (β-cell/neuroendocrine/neuronal), Kir6.2/SUR2A (cardiac and skeletal muscle type), and Kir6.2 (or 6.1)/SUR2B in smooth muscles.[17,18] In contrast to other types, neuronal K_{ATP} channels remain relatively underinvestigated, and their roles remain less well understood. Plasmalemmal Kir6.2/SUR1 channels are present in the brain, in areas such as hypothalamus, forebrain, and striatum.[12] These channels are activated by adenosine diphosphate, in the presence of Mg^{2+}, and are inhibited by high or physiologic intracellular adenosine triphosphate concentrations.[12,19] Oxygen depletion leads to anoxic depolarization, whereby opening of voltage-gated channels results in Ca^{2+} overload activating lethal cascades and promoting cell death.[12] However, parallel activation of K_{ATP} channels (from reduction of intracellular adenosine triphosphate) produces outward K^+ flux, membrane hyperpolarization, and reduced excitability. Concomitant limitation of transmembrane ionic fluxes results in energy conservation mainly by decreased activity of ion pumps that consume approximately half of the total energy used by the brain.[12] Activation of K_{ATP} channels also reduces excitatory neurotransmission, including glutamate release, thus limiting the extent of excitotoxic injury. The protective effects of K_{ATP} channels against neuronal death from ischemic insults have been confirmed by a number of studies,[18,20] including observations in mutant mice lacking Kir6.2.[19]

Mitochondrial K_{ATP} channels are also inhibited by adenosine triphosphate and mediate cytoprotective actions and preconditioning against cerebral and cardiac ischemia.[12] Both types of channels may act in a coordinated fashion,[12] but Bantel *et al.*[2] showed that this was not the case with xenon. Nevertheless, xenon, in contrast to halogenated anesthetics, at clinical concentrations independently enhanced plasmalemmal K_{ATP} channel activity by 50%, which is sufficient to trigger preconditioning. These findings should be verified in more complex systems: *e.g.*, is xenon preconditioning, but not sevoflurane preconditioning, lost in K_{ATP} knockout animals? The authors also implied that effective preconditioning may involve more than one diverse pathway in which K_{ATP} channels may or may not participate. These pathways may produce neuroprotection by converging to the same downstream effectors,[8] which will require further investigation. Further investigations are also needed to examine the role of K_{ATP} channels as mediators of preconditioning and protection against ischemic or anoxic injury in the spinal cord,[21] retina,[22] and peripheral nerves.[23]

Although there is no doubt that xenon may limit ischemic neuronal injury *via* a preconditioning effect,[24,25] controversy arises from the notion that it may exacerbate ischemic damage to brain neurons and aggravate neurologic sequelae by combined cardiopulmonary bypass and cerebral air emboli.[26] Therefore, further work is needed to ultimately document its efficacy and safety before indisputable, widespread acceptance for clinical use as a preconditioning and neuroprotective agent. The findings by Bantel *et al.*[2] are important and might stimulate the future investigations to combine systems-, cellular-, and molecular-level approaches. Clearly, in this challenging task, there is a need for more cross-talk between the different approaches taken by the large number of independent findings to make lasting contributions.

Zeljko J. Bosnjak, Ph.D.,* Constantine D. Sarantopoulos, M.D., Ph.D., D.E.A.A.† *Departments of Anesthesiology and Physiology, Medical College of Wisconsin, †Department of Anesthesiology, and Pharmacology & Toxicology, CJ Zablocki Veterans Affairs Medical Center Pain Management Center, Medical College of Wisconsin, Milwaukee, Wisconsin. zbosnjak@mcw.edu

References

1. Bateman BT, Schumacher HC, Wang S, Shaefi S, Berman MF: Perioperative acute ischemic stroke in noncardiac and nonvascular surgery: Incidence, risk factors, and outcomes. ANESTHESIOLOGY 2009; 110:231–8

2. Bantel C, Maze M, Trapp S: Neuronal preconditioning by inhalational anesthetics: Evidence for the role of plasmalemmal adenosine triphosphate-sensitive potassium channels. ANESTHESIOLOGY 2009; 110:000–000

3. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36

4. Julier K, da Silva R, Garcia C, Bestmann L, Frascarolo P, Zollinger A, Chassot PG, Schmid ER, Turina MI, von Segesser LK, Pasch T, Spahn DR, Zaugg M: Preconditioning by sevoflurane decreases biochemical markers for myocardial and renal dysfunction in coronary artery bypass graft surgery: A double-blinded, placebo-controlled, multicenter study. ANESTHESIOLOGY 2003; 98:1315–27

5. Hans P, Bonhomme V: The rationale for perioperative brain protection. Eur J Anaesth 2004; 21:1–5

6. Warltier DC, Kersten JR, Pagel PS, Gross GJ, Todd MM: Anesthetic preconditioning: Serendipity and science. ANESTHESIOLOGY 2002; 97:1–3

7. Warner DS: Perioperative neuroprotection: Are we asking the right questions? Anesth Analg 2004; 98:563–5

8. Gidday JM: Cerebral preconditioning and ischaemic tolerance. Nat Rev Neurosci 2006; 7:437–48

9. Obrenovitch TP: Molecular physiology of preconditioning-induced brain tolerance to ischemia. Physiol Rev 2008; 88:211–47

10. Kitano H, Kirsch JR, Hurn PD, Murphy SJ: Inhalational anesthetics as neuroprotectants or chemical preconditioning agents in ischemic brain. J Cereb Blood Flow Metab 2007; 27:1108–28

11. Stadnicka A, Marinovic J, Ljubkovic M, Bienengraeber MW, Bosnjak ZJ: Volatile anesthetic-induced cardiac preconditioning. J Anesth 2007; 21:212–9

12. Soundarapandian MM, Zhong X, Peng L, Wu D, Lu Y: Role of K_{ATP} channels in protection against neuronal excitatory insults. J Neurochem 2007; 103:1721–9

13. Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke KF, Isaev NK, Dirnagl U: Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent, Stroke 2002; 33:1889–98

14. Wang L, Traystman RJ, Murphy SJ: Inhalational anesthetics as preconditioning agents in ischemic brain. Curr Opin Pharmacol 2008; 8:104–10

15. Kitano H, Young JM, Cheng J, Wang L, Hurn PD, Murphy SJ: Gender-specific response to isoflurane preconditioning in focal cerebral ischemia. J Cereb Blood Flow Metab 2007; 27:1377–86

16. Payne RS, Akca O, Roewer N, Schurr A, Kehl F: Sevoflurane-induced preconditioning protects against cerebral ischemic neuronal damage in rats. Brain Res 2005; 1034:147–52

17. Aguilar-Bryan L, Bryan J: Molecular biology of adenosine triphosphate-sensitive potassium channels. Endocr Rev 1999; 20:101–35

18. Liss B, Roeper J: Molecular physiology of neuronal K_{ATP} channels. Mol Membr Biol 2001; 18:117–27

19. Sun HS, Feng ZP, Miki T, Seino S, French RJ: Enhanced neuronal damage after ischemic insults in mice lacking Kir6.2-containing ATP-sensitive K^+ channels. J Neurophysiol 2006; 95:2590–601

20. Ballanyi K: Protective role of neuronal K_{ATP} channels in brain hypoxia. J Exp Biol 2004; 207:3201–12

21. Caparrelli DJ, Cattaneo SM II, Bethea BT, Shake JG, Eberhart C, Blue ME, Marban E, Johnston MV, Baumgartner WA, Gott VL: Pharmacological preconditioning ameliorates neurological injury in a model of spinal cord ischemia. Ann Thorac Surg 2002; 74:838–44

22. Ettaiche M, Heurteaux C, Blondeau N, Borsotto M, Tinel N, Lazdunski M: ATP-sensitive potassium channels (K_{ATP}) in retina: A key role for delayed ischemic tolerance. Brain Res 2001; 890:118–29

23. Hoke A: Neuroprotection in the peripheral nervous system: Rationale for more effective therapies. Arch Neurol 2006; 63:1681–5

24. Ma D, Hossain M, Pettet GK, Luo Y, Lim T, Akimov S, Sanders RD, Franks NP, Maze M: Xenon preconditioning reduces brain damage from neonatal asphyxia in rats. J Cereb Blood Flow Metab 2006; 26:199–208

25. Wilhelm S, Ma D, Maze M, Franks NP: Effects of xenon on *in vitro* and *in vivo* models of neuronal injury. ANESTHESIOLOGY 2002; 96:1485–91

26. Jungwirth B, Gordan L, Blobner M, Schmehl W, Kochs EF, Mackensen GB: Xenon impairs neurocognitive and histologic outcome after cardiopulmonary bypass combined with cerebral air embolism in rats. ANESTHESIOLOGY 2006; 104: 770–6

11. REFERENCES

Abdulla EM and Campbell IC (1993) In vitro tests of neurotoxicity. *J Pharmacol Toxicol Methods* **29**:69-75.

Ackland GL, Smith M and McGlennan AP (2007) Acute, severe hypoglycemia occurring during general anesthesia in a nondiabetic adult. *Anesth Analg* **105**:553-554.

Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JPt, Boyd AE, 3rd, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J and Nelson DA (1995) Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* **268**:423-426.

Albers GW, Goldstein LB, Hall D and Lesko LM (2001) Aptiganel hydrochloride in acute ischemic stroke: a randomized controlled trial. *Jama* **286**:2673-2682.

Alberts MJ (2003) Update on the treatment and prevention of ischaemic stroke. *Curr Med Res Opin* **19**:438-441.

Antcliff JF, Haider S, Proks P, Sansom MS and Ashcroft FM (2005) Functional analysis of a structural model of the ATP-binding site of the KATP channel Kir6.2 subunit. *Embo J* **24**:229-239.

Argaud L, Rioufol G, Lievre M, Bontemps L, Legalery P, Stumpf M, Finet G, Itti R, Andre-Fouet X and Ovize M (2004) Preconditioning during coronary angioplasty: no influence of collateral perfusion or the size of the area at risk. *Eur Heart J* **25**:2019-2025.

Ashcroft FM (2005) ATP-sensitive potassium channelopathies: focus on insulin secretion. *J Clin Invest* **115**:2047-2058.

Ashcroft FM and Gribble FM (1998) Correlating structure and function in ATP-sensitive K+ channels. *Trends Neurosci* **21**:288-294.

Ashcroft FM and Gribble FM (1999) ATP-sensitive K+ channels and insulin secretion: their role in health and disease. *Diabetologia* **42**:903-919.

Ashcroft FM and Gribble FM (2000) Tissue-specific effects of sulfonylureas: lessons from studies of cloned K(ATP) channels. *J Diabetes Complications* **14**:192-196.

Ashcroft FM, Harrison DE and Ashcroft SJ (1984) Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* **312**:446-448.

Ashcroft FM and Trapp S (2001) The ATP-sensitive potassium channel: a metablic sensor, in *Molecular Mechanisms of Metabolic Arrest: Life in Limbo* (Storey KB ed) pp 43-58, BIOS Scientific Publishers Ltd, Oxford.

Astrup J, Siesjo BK and Symon L (1981) Thresholds in cerebral ischemia - the ischemic penumbra. *Stroke* **12**:723-725.

Babenko AP, Aguilar BL and Bryan J (1998) A view of sur/KIR6.X, KATP channels. *Annu Rev Physiol* **60**:667-687.

Back T (1998) Pathophysiology of the ischemic penumbra--revision of a concept. *Cell Mol Neurobiol* **18**:621-638.

Bains R, Moe MC, Larsen GA, Berg-Johnsen J and Vinje ML (2006) Volatile anaesthetics depolarize neural mitochondria by inhibiton of the electron transport chain. *Acta Anaesthesiol Scand* **50**:572-579.

Bajgar R, Seetharaman S, Kowaltowski AJ, Garlid KD and Paucek P (2001) Identification and properties of a novel intracellular (mitochondrial) ATP-sensitive potassium channel in brain. *J Biol Chem* **276**:33369-33374.

Ballanyi K (2004) Protective role of neuronal KATP channels in brain hypoxia. *J Exp Biol* **207**:3201-3212.

Bantel C, Maze M and Trapp S (2009) Neuronal preconditioning by inhalational anesthetics: evidence for the role of plasmalemmal adenosine triphosphate-sensitive potassium channels. *Anesthesiology* **110**:986-995.

Barone FC, White RF, Spera PA, Ellison J, Currie RW, Wang X and Feuerstein GZ (1998) Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* **29**:1937-1950.

Bateman BT, Schumacher HC, Wang S, Shaefi S and Berman MF (2009) Perioperative acute ischemic stroke in noncardiac and nonvascular surgery: incidence, risk factors, and outcomes. *Anesthesiology* **110**:231-238.

Baukrowitz T and Fakler B (2000) KATP channels gated by intracellular nucleotides and phospholipids. *Eur J Biochem* **267**:5842-5848.

Baukrowitz T, Schulte U, Oliver D, Herlitze S, Krauter T, Tucker SJ, Ruppersberg JP and Fakler B (1998) PIP2 and PIP as determinants for ATP inhibition of KATP channels. *Science* **282**:1141-1144.

Behne M, Wilke HJ and Harder S (1999) Clinical pharmacokinetics of sevoflurane. *Clin Pharmacokinet* **36**:13-26.

Berridge MV and Tan AS (1993) Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch.Biochem.Biophys.* **303**:474-482.

Bhandari S and Hunter M (2001) Biophysical effects of pore mutations of ROMK1. *Clin Sci (Lond)* **101**:121-130.

Bickler PE, Zhan X and Fahlman CS (2005) Isoflurane preconditions hippocampal neurons against oxygen-glucose deprivation: role of intracellular Ca2+ and mitogen-activated protein kinase signaling. *Anesthesiology* **103**:532-539.

Bienengraeber M, Warltier DC, Bosnjak ZJ and Stadnicka A (2006) Mechanism of cardiac sarcolemmal adenosine triphosphate-sensitive potassium channel activation by isoflurane in a heterologous expression system. *Anesthesiology* **105**:534-540.

Bliss TM and Sapolsky RM (2001) Interactions among glucose, lactate and adenosine regulate energy substrate utilization in hippocampal cultures. *Brain Res* **899**:134-141.

Boatright KM and Salvesen GS (2003) Mechanisms of caspase activation. *Curr Opin Cell Biol* **15**:725-731.

Bolognesi MP, Marchant MH, Jr., Viens NA, Cook C, Pietrobon R and Vail TP (2008) The impact of diabetes on perioperative patient outcomes after total hip and total knee arthroplasty in the United States. *J Arthroplasty* **23**:92-98.

Bortner CD and Cidlowski JA (2007) Cell shrinkage and monovalent cation fluxes: role in apoptosis. *Arch Biochem Biophys* **462**:176-188.

Bramlett HM and Dietrich WD (2004) Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. *J Cereb Blood Flow Metab* **24**:133-150.

Broadhead MW, Kharbanda RK, Peters MJ and MacAllister RJ (2004) KATP channel activation induces ischemic preconditioning of the endothelium in humans in vivo. *Circulation* **110**:2077-2082.

Burg ED, Remillard CV and Yuan JX (2006) K+ channels in apoptosis. *J Membr Biol* **209**:3-20.

Burke MA, Mutharasan RK and Ardehali H (2008) The sulfonylurea receptor, an atypical ATP-binding cassette protein, and its regulation of the KATP channel. *Circ Res* **102**:164-176.

Cahalan M and Neher E (1992) Patch clamp techniques: an overview. *Methods Enzymol* **207**:3-14.

Campagna JA, Miller KW and Forman SA (2003) Mechanisms of actions of inhaled anesthetics. *N Engl J Med* **348**:2110-2124.

Campbell JD, Sansom MS and Ashcroft FM (2003) Potassium channel regulation. *EMBO Rep* **4**:1038-1042.

Caparrelli DJ, Cattaneo SM, 2nd, Bethea BT, Shake JG, Eberhart C, Blue ME, Marban E, Johnston MV, Baumgartner WA and Gott VL (2002) Pharmacological preconditioning ameliorates neurological injury in a model of spinal cord ischemia. *Ann Thorac Surg* **74**:838-844.

Caplan LR (2009) Translating what is known about neurological complications of coronary artery bypass graft surgery into action. *Arch Neurol* **66**:1062-1064.

Caplan LR and Hennerici M (1998) Impaired clearance of emboli (washout) is an important link between hypoperfusion, embolism, and ischemic stroke. *Arch Neurol* **55**:1475-1482.

Carroll R, Gant VA and Yellon DM (2001) Mitochondrial K(ATP) channel opening protects a human atrial-derived cell line by a mechanism involving free radical generation. *Cardiovasc Res* **51**:691-700.

Cason BA, Shubayev I and Hickey RF (1994) Blockade of adenosine triphosphate-sensitive potassium channels eliminates isoflurane-induced coronary artery vasodilation. *Anesthesiology* **81**:1245-1255.

Cavero I, Djellas Y and Guillon JM (1995) Ischemic myocardial cell protection conferred by the opening of ATP-sensitive potassium channels. *Cardiovasc Drugs Ther* **9 Suppl 2**:245-255.

Cestelli A, Savettieri G, Salemi G and Di Liegro I (1992) Neuronal cell cultures: a tool for investigations in developmental neurobiology. *Neurochem Res* **17**:1163-1180.

Chen C and Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol.Cell Biol.* **7**:2745-2752.

Ciani S and Ribalet B (1988) Ion permeation and rectification in ATP-sensitive channels from insulin-secreting cells (RINm5F): effects of K+, Na+ and Mg2+. *J Membr Biol* **103**:171-180.

Claesson L, Gosman-Hedstrom G, Johannesson M, Fagerberg B and Blomstrand C (2000) Resource utilization and costs of stroke unit care integrated in a care continuum: A 1-year controlled, prospective, randomized study in elderly patients: the Goteborg 70+ Stroke Study. *Stroke* **31**:2569-2577.

Clark RS, Kochanek PM, Chen M, Watkins SC, Marion DW, Chen J, Hamilton RL, Loeffert JE and Graham SH (1999) Increases in Bcl-2 and cleavage of caspase-1 and caspase-3 in human brain after head injury. *Faseb J* **13**:813-821.

Clark WM, Raps EC, Tong DC and Kelly RE (2000) Cervene (Nalmefene) in acute ischemic stroke : final results of a phase III efficacy study. The Cervene Stroke Study Investigators. *Stroke* **31**:1234-1239.

Clarkson AN (2007) Anesthetic-mediated protection/preconditioning during cerebral ischemia. *Life Sci* **80**:1157-1175.

Clement JP, Kunjilwar K, Gonzalez G, Schwanstecher M and Panten (1997) Association and stoichiometry of K(ATP) channel subunits. *Neuron.* **18**:827-838.

Costa AD and Garlid KD (2008) Intramitochondrial signaling: interactions among mitoKATP, PKCepsilon, ROS, and MPT. *Am J Physiol Heart Circ Physiol* **295**:H874-882.

Craig TJ, Ashcroft FM and Proks P (2008) How ATP inhibits the open K(ATP) channel. *J Gen Physiol* **132**:131-144.

Crystal GJ, Gurevicius J, Salem MR and Zhou X (1997) Role of adenosine triphosphate-sensitive potassium channels in coronary vasodilation by halothane, isoflurane, and enflurane. *Anesthesiology* **86**:448-458.

Crystal GJ, Zhou X, Gurevicius J, Czinn EA, Salem MR, Alam S, Piotrowski A and Hu G (2000) Direct coronary vasomotor effects of sevoflurane and desflurane in in situ canine hearts. *Anesthesiology* **92**:1103-1113.

Cullen SC, Eger EI, 2nd, Cullen BF and Gregory P (1969) Observations on the anesthetic effect of the combination of xenon and halothane. *Anesthesiology* **31**:305-309.

Dabrowski M, Larsen T, Ashcroft FM, Bondo Hansen J and Wahl P (2003) Potent and selective activation of the pancreatic beta-cell type K(ATP) channel by two novel diazoxide analogues. *Diabetologia* **46**:1375-1382.

Dahlof B (2007) Prevention of stroke in patients with hypertension. *Am J Cardiol* **100**:17J-24J.

Das M, Parker JE and Halestrap AP (2003) Matrix volume measurements challenge the existence of diazoxide/glibencamide-sensitive KATP channels in rat mitochondria. *J Physiol* **547**:893-902.

Dave KR, Saul I, Prado R, Busto R and Perez-Pinzon MA (2006) Remote organ ischemic preconditioning protect brain from ischemic damage following asphyxial cardiac arrest. *Neurosci Lett* **404**:170-175.

De Hert SG (2004) Cardioprotection with volatile anesthetics: clinical relevance. *Curr Opin Anaesthesiol* **17**:57-62.

de Sousa SL, Dickinson R, Lieb WR and Franks NP (2000) Contrasting synaptic actions of the inhalational general anesthetics isoflurane and xenon. *Anesthesiology* **92**:1055-1066.

Derwall M, Coburn M, Rex S, Hein M, Rossaint R and Fries M (2009) Xenon: recent developments and future perspectives. *Minerva Anestesiol* **75**:37-45.

Devereaux PJ, Yang H, Yusuf S, Guyatt G, Leslie K, Villar JC, Xavier D, Chrolavicius S, Greenspan L, Pogue J, Pais P, Liu L, Xu S, Malaga G, Avezum A, Chan M, Montori VM, Jacka M and Choi P (2008) Effects of extended-release metoprolol succinate in patients undergoing non-cardiac surgery (POISE trial): a randomised controlled trial. *Lancet* **371**:1839-1847.

Dewey HM, Thrift AG, Mihalopoulos C, Carter R, Macdonell RA, McNeil JJ and Donnan GA (2001) Cost of stroke in Australia from a societal perspective: results from the North East Melbourne Stroke Incidence Study (NEMESIS). *Stroke* **32**:2409-2416.

Dickinson R, Peterson BK, Banks P, Simillis C, Martin JC, Valenzuela CA, Maze M and Franks NP (2007) Competitive inhibition at the glycine site of the N-methyl-D-aspartate receptor by the anesthetics xenon and isoflurane: evidence from molecular modeling and electrophysiology. *Anesthesiology* **107**:756-767.

Dirnagl U, Iadecola C and Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* **22**:391-397.

Dirnagl U, Simon RP and Hallenbeck JM (2003) Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci.* **26**:248-254.

Dwenger A, Pape HC, Bantel C, Schweitzer G, Krumm K, Grotz M, Lueken B, Funck M and Regel G (1994) Ascorbic acid reduces the endotoxin-induced lung injury in awake sheep. *Eur.J.Clin.Invest* **24**:229-235.

Dzeja PP, Bast P, Ozcan C, Valverde A, Holmuhamedov EL, Van Wylen DG and Terzic A (2003) Targeting nucleotide-requiring enzymes: implications for diazoxide-induced cardioprotection. *Am J Physiol Heart Circ Physiol* **284**:H1048-1056.

Edmands SD and Hall AC (2009) Role for metallothioneins-I/II in isoflurane preconditioning of primary murine neuronal cultures. *Anesthesiology* **110**:538-547.

Edwards DF, Hahn M, Baum C and Dromerick AW (2006) The impact of mild stroke on meaningful activity and life satisfaction. *J Stroke Cerebrovasc Dis* **15**:151-157.

Ehrlich JR (2008) Inward rectifier potassium currents as a target for atrial fibrillation therapy. *J Cardiovasc Pharmacol* **52**:129-135.

Enkvetchakul D, Loussouarn G, Makhina E, Shyng SL and Nichols CG (2000) The kinetic and physical basis of K(ATP) channel gating: toward a unified molecular understanding. *Biophys J* **78**:2334-2348.

Fakler B, Brandle U, Bond C, Glowatzki E, Konig C, Adelman JP, Zenner HP and Ruppersberg JP (1994) A structural determinant of differential sensitivity of cloned inward rectifier K+ channels to intracellular spermine. *FEBS Lett* **356**:199-203.

Fakler B, Brandle U, Glowatzki E, Weidemann S, Zenner HP and Ruppersberg JP (1995) Strong voltage-dependent inward rectification of inward rectifier K+ channels is caused by intracellular spermine. *Cell* **80**:149-154.

Fan Z and Makielski JC (1999) Phosphoinositides decrease ATP sensitivity of the cardiac ATP-sensitive K(+) channel. A molecular probe for the mechanism of ATP-sensitive inhibition. *J Gen Physiol* **114**:251-269.

Ferdinandy P, Schulz R and Baxter GF (2007) Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev* **59**:418-458.

Ferriero DM (2005) Protecting neurons. *Epilepsia* **46 Suppl 7**:45-51.

Fiehler J, von Bezold M, Kucinski T, Knab R, Eckert B, Wittkugel O, Zeumer H and Rother J (2002) Cerebral blood flow predicts lesion growth in acute stroke patients. *Stroke* **33**:2421-2425.

Fogelholm R, Palomaki H, Erila T, Rissanen A and Kaste M (2004) Blood pressure, nimodipine, and outcome of ischemic stroke. *Acta Neurol Scand* **109**:200-204.

Franks NP, Dickinson R, de Sousa SL, Hall AC and Lieb WR (1998) How does xenon produce anaesthesia? *Nature* **396**:324.

Fujimura N, Tanaka E, Yamamoto S, Shigemori M and Higashi H (1997) Contribution of ATP-sensitive potassium channels to hypoxic hyperpolarization in rat hippocampal CA1 neurons in vitro. *J Neurophysiol* **77**:378-385.

Fukuda S and Warner DS (2007) Cerebral protection. *Br J Anaesth* **99**:10-17.

Fukuda T, Nakayama H, Yanagi K, Mizutani T, Miyabe M, Ohshima N and Toyooka H (2001) The effects of 30% and 60% xenon inhalation on pial vessel diameter and intracranial pressure in rabbits. *Anesth Analg* **92**:1245-1250.

Fukushima T, Hirasaki A, Jones KA and Warner DO (1996) Halothane and potassium channels in airway smooth muscle. *Br J Anaesth* **76**:847-853.

Gahwiler BH, Capogna M, Debanne D, McKinney RA and Thompson SM (1997) Organotypic slice cultures: a technique has come of age. *Trends Neurosci* **20**:471-477.

Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA and Grover GJ (1997) Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circ Res* **81**:1072-1082.

Garlid KD, Paucek P, Yarov-Yarovoy V, Sun X and Schindler PA (1996) The mitochondrial KATP channel as a receptor for potassium channel openers. *J Biol Chem* **271**:8796-8799.

Geisen K, Hitzel V, Okomonopoulos R, Punter J, Weyer R and Summ HD (1985) Inhibition of 3H-glibenclamide binding to sulfonylurea receptors by oral antidiabetics. *Arzneimittelforschung* **35**:707-712.

Genestra M (2007) Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cell Signal* **19**:1807-1819.

Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ and Verdouw PD (1996) Myocardial protection by brief ischemia in noncardiac tissue. *Circulation* **94**:2193-2200.

Gidday JM (2006) Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci* **7**:437-448.

Glazner GW, Chan SL, Lu C and Mattson MP (2000) Caspase-mediated degradation of AMPA receptor subunits: a mechanism for preventing excitotoxic necrosis and ensuring apoptosis. *J Neurosci* **20**:3641-3649.

Glowatzki E, Fakler G, Brandle U, Rexhausen U, Zenner HP, Ruppersberg JP and Fakler B (1995) Subunit-dependent assembly of inward-rectifier K+ channels. *Proc Biol Sci* **261**:251-261.

Goldberg MP and Choi DW (1993) Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. *J.Neurosci.* **13**:3510-3524.

Green AR and Shuaib A (2006) Therapeutic strategies for the treatment of stroke. *Drug Discov Today* **11**:681-693.

Gribble FM and Ashcroft FM (2000) Sulfonylurea sensitivity of adenosine triphosphate-sensitive potassium channels from beta cells and extrapancreatic tissues. *Metabolism* **49**:3-6.

Gribble FM, Ashfield R, Ammala C and Ashcroft FM (1997a) Properties of cloned ATP-sensitive K+ currents expressed in Xenopus oocytes. *J Physiol* **498**:87-98.

Gribble FM, Loussouarn G, Tucker SJ, Zhao C, Nichols CG and Ashcroft FM (2000) A novel method for measurement of submembrane ATP concentration. *J Biol Chem* **275**:30046-30049.

Gribble FM and Reimann F (2003a) Differential selectivity of insulin secretagogues: mechanisms, clinical implications, and drug interactions. *J Diabetes Complications* **17**:11-15.

Gribble FM and Reimann F (2003b) Sulphonylurea action revisited: the post-cloning era. *Diabetologia* **46**:875-891.

Gribble FM, Tucker SJ and Ashcroft FM (1997b) The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide. *Embo J* **16**:1145-1152.

Gribble FM, Tucker SJ, Haug T and Ashcroft FM (1998a) MgATP activates the beta cell KATP channel by interaction with its SUR1 subunit. *Proc Natl Acad Sci U S A* **95**:7185-7190.

Gribble FM, Tucker SJ, Seino S and Ashcroft FM (1998b) Tissue specificity of sulfonylureas: studies on cloned cardiac and beta-cell K(ATP) channels. *Diabetes* **47**:1412-1418.

Gruss M, Bushell TJ, Bright DP, Lieb WR, Mathie A and Franks NP (2004) Two-pore-domain K+ channels are a novel target for the anesthetic gases xenon, nitrous oxide, and cyclopropane. *Mol.Pharmacol.* **65**:443-452.

Gumina RJ, Pucar D, Bast P, Hodgson DM, Kurtz CE, Dzeja PP, Miki T, Seino S and Terzic A (2003) Knockout of Kir6.2 negates ischemic preconditioning-induced protection of myocardial energetics. *Am J Physiol Heart Circ Physiol* **284**:H2106-2113.

Gur D, Yonas H, Jackson DL, Wolfson SK, Jr., Rockette H, Good WF, Maitz GS, Cook EE and Arena VC (1985) Measurement of cerebral blood flow during xenon inhalation as measured by the microspheres method. *Stroke* **16**:871-874.

Gutman GA, Chandy KG, Adelman JP, Aiyar J, Bayliss DA, Clapham DE, Covarriubias M, Desir GV, Furuichi K, Ganetzky B, Garcia ML, Grissmer S, Jan LY, Karschin A, Kim D, Kuperschmidt S, Kurachi Y, Lazdunski M, Lesage F, Lester HA, McKinnon D, Nichols CG, O'Kelly I, Robbins J, Robertson GA, Rudy B, Sanguinetti M, Seino S, Stuehmer W, Tamkun MM, Vandenberg CA, Wei A, Wulff H and Wymore RS (2003) International Union of Pharmacology. XLI. Compendium of voltage-gated ion channels: potassium channels. *Pharmacol Rev* **55**:583-586.

Haider S, Khalid S, Tucker SJ, Ashcroft FM and Sansom MS (2007a) Molecular dynamics simulations of inwardly rectifying (Kir) potassium channels: a comparative study. *Biochemistry* **46**:3643-3652.

Haider S, Tarasov AI, Craig TJ, Sansom MS and Ashcroft FM (2007b) Identification of the PIP2-binding site on Kir6.2 by molecular modelling and functional analysis. *Embo J* **26**:3749-3759.

Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391**:85-100.

Han J, Kim E, Ho WK and Earm YE (1996) Effects of volatile anesthetic isoflurane on ATP-sensitive K+ channels in rabbit ventricular myocytes. *Biochem Biophys Res Commun* **229**:852-856.

Hanley PJ and Daut J (2005) K(ATP) channels and preconditioning: a re-examination of the role of mitochondrial K(ATP) channels and an overview of alternative mechanisms. *J Mol Cell Cardiol* **39**:17-50.

Hanley PJ, Gopalan KV, Lareau RA, Srivastava DK, von Meltzer M and Daut J (2003) Beta-oxidation of 5-hydroxydecanoate, a putative blocker of mitochondrial ATP-sensitive potassium channels. *J Physiol* **547**:387-393.

Hanley PJ, Mickel M, Loffler M, Brandt U and Daut J (2002a) K(ATP) channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart. *J Physiol* **542**:735-741.

Hanley PJ, Ray J, Brandt U and Daut J (2002b) Halothane, isoflurane and sevoflurane inhibit NADH:ubiquinone oxidoreductase (complex I) of cardiac mitochondria. *J Physiol* **544**:687-693.

Hardingham GE, Cruzalegui FH, Chawla S and Bading H (1998) Mechanisms controlling gene expression by nuclear calcium signals. *Cell Calcium* **23**:131-134.

Hartmann A, Dettmers C, Schuier FJ, Wassmann HD and Schumacher HW (1991) Effect of stable xenon on regional cerebral blood flow and the electroencephalogram in normal volunteers. *Stroke* **22**:182-189.

Hebert SC, Desir G, Giebisch G and Wang W (2005) Molecular diversity and regulation of renal potassium channels. *Physiol Rev* **85**:319-371.

Hecker K, Baumert JH, Horn N and Rossaint R (2004) Xenon, a modern anaesthesia gas. *Minerva Anestesiol* **70**:255-260.

Heron-Milhavet L, Xue-Jun Y, Vannucci SJ, Wood TL, Willing LB, Stannard B, Hernandez-Sanchez C, Mobbs C, Virsolvy A and LeRoith D (2004) Protection against hypoxic-ischemic injury in transgenic mice overexpressing Kir6.2 channel pore in forebrain. *Mol Cell Neurosci* **25**:585-593.

Heurteaux C, Bertaina V, Widmann C and Lazdunski M (1993) K+ channel openers prevent global ischemia-induced expression of c-fos, c-jun, heat shock protein, and amyloid beta-protein precursor genes and neuronal death in rat hippocampus. *Proc Natl Acad Sci U S A* **90**:9431-9435.

Heurteaux C, Guy N, Laigle C, Blondeau N, Duprat F, Mazzuca M, Lang-Lazdunski L, Widmann C, Zanzouri M, Romey G and Lazdunski M (2004) TREK-1, a K+ channel involved in neuroprotection and general anesthesia. *Embo J* **23**:2684-2695.

Heurteaux C, Lauritzen I, Widmann C and Lazdunski M (1995) Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K+ channels in cerebral ischemic preconditioning. *Proc Natl Acad Sci U S A* **92**:4666-4670.

Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, Kanazirska MV and Hebert SC (1993) Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* **362**:31-38.

Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A and Terzic A (1998) Mitochondrial ATP-sensitive K+ channels modulate cardiac mitochondrial function. *Am J Physiol* **275**:H1567-1576.

Hopwood SE and Trapp S (2005) TASK-like K+ channels mediate effects of 5-HT and extracellular pH in rat dorsal vagal neurones in vitro. *J Physiol* **568**:145-154.

Horie M, Irisawa H and Noma A (1987) Voltage-dependent magnesium block of adenosine-triphosphate-sensitive potassium channel in guinea-pig ventricular cells. *J Physiol* **387**:251-272.

Hossmann KA (1994) Viability thresholds and the penumbra of focal ischemia. *Ann Neurol* **36**:557-565.

Hossmann KA (2006) Pathophysiology and therapy of experimental stroke. *Cell Mol Neurobiol* **26**:1057-1083.

Huang CL, Feng S and Hilgemann DW (1998) Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gbetagamma. *Nature* **391**:803-806.

Huang YC, Lui PW, Chu CC, Lur JY and Lee TY (2000) Effects of glucose-free maintenance solution on plasma glucose during anesthesia in patients undergoing long neurologic surgery. *Zhonghua Yi Xue Za Zhi (Taipei)* **63**:467-474.

Huneke R, Fassl J, Rossaint R and Luckhoff A (2004) Effects of volatile anesthetics on cardiac ion channels. *Acta Anaesthesiol Scand* **48**:547-561.

Huneke R, Jungling E, Skasa M, Rossaint R and Luckhoff A (2001) Effects of the anesthetic gases xenon, halothane, and isoflurane on calcium and potassium currents in human atrial cardiomyocytes. *Anesthesiology* **95**:999-1006.

Hussain M and Wareham AC (1994) Rundown and reactivation of ATP-sensitive potassium channels (KATP) in mouse skeletal muscle. *J Membr Biol* **141**:257-265.

Hussain MS and Shuaib A (2008) Research into neuroprotection must continue ... But with a different approach. *Stroke* **39**:521-522.

Iliodromitis EK, Georgiadis M, Cohen MV, Downey JM, Bofilis E and Kremastinos DT (2006) Protection from post-conditioning depends on the number of short ischemic insults in anesthetized pigs. *Basic Res Cardiol* **101**:502-507.

Inagaki N, Gonoi T, Clement JP, 4th, Namba N, Inazawa J, Gonzalez G, Aguilar Bryan L, Seino S and Bryan J (1995a) Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science* **270**:1166-1170.

Inagaki N, Gonoi T and Seino S (1997) Subunit stoichiometry of the pancreatic beta-cell ATP-sensitive K+ channel. *FEBS Lett* **409**:232-236.

Inagaki N, Inazawa J and Seino S (1995b) cDNA sequence, gene structure, and chromosomal localization of the human ATP-sensitive potassium channel, uKATP-1, gene (KCNJ8). *Genomics* **30**:102-104.

Inagaki N, Tsuura Y, Namba N, Masuda K, Gonoi T, Horie M, Seino Y, Mizuta M and Seino S (1995c) Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. *J.Biol.Chem.* **270**:5691-5694.

Inoue I, Nagase H, Kishi K and Higuti T (1991) ATP-sensitive K+ channel in the mitochondrial inner membrane. *Nature* **352**:244-247.

Isomoto S, Kondo C and Kurachi Y (1997) Inwardly rectifying potassium channels: their molecular heterogeneity and function. *Jpn J Physiol* **47**:11-39.

Jaburek M, Yarov-Yarovoy V, Paucek P and Garlid KD (1998) State-dependent inhibition of the mitochondrial KATP channel by glyburide and 5-hydroxydecanoate. *J Biol Chem* **273**:13578-13582.

Jahangir A and Terzic A (2005) K(ATP) channel therapeutics at the bedside. *J Mol Cell Cardiol* **39**:99-112.

Jenkins A, Franks NP and Lieb WR (1999) Effects of temperature and volatile anesthetics on GABA(A) receptors. *Anesthesiology* **90**:484-491.

Jenkinson DH (2006) Potassium channels-multiplicity and challenges. *Br J Pharmacol* **147 Suppl 1**:S63-71.

Jiang KW, Yu ZS, Shui QX and Xia ZZ (2005) Activation of ATP-sensitive potassium channels prevents the cleavage of cytosolic mu-calpain and abrogates the elevation of nuclear c-Fos and c-Jun expressions after hypoxic-ischemia in neonatal rat brain. *Brain Res Mol Brain Res* **133**:87-94.

Johnston SC (2004) Ischemic preconditioning from transient ischemic attacks? Data from the Northern California TIA Study. *Stroke* **35**:2680-2682.

Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN and Sollott SJ (2004) Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* **113**:1535-1549.

Junck L, Dhawan V, Thaler HT and Rottenberg DA (1985) Effects of xenon and krypton on regional cerebral blood flow in the rat. *J Cereb Blood Flow Metab* **5**:126-132.

Jung YS, Ryu BR, Lee BK, Mook-Jung I, Kim SU, Lee SH, Baik EJ and Moon CH (2004) Role for PKC-epsilon in neuronal death induced by oxidative stress. *Biochem Biophys Res Commun* **320**:789-794.

Kaiser-Family HJ (2007) U.S. Federal Funding for HIV/AIDS, in, The Henry J. Kaiser Family Foundation, Menlo Park, CA.

Kaku DA, Goldberg MP and Choi DW (1991) Antagonism of non-NMDA receptors augments the neuroprotective effect of NMDA receptor blockade in cortical cultures subjected to prolonged deprivation of oxygen and glucose. *Brain Res* **554**:344-347.

Kaneko T, Yokoyama K and Makita K (2005) Late preconditioning with isoflurane in cultured rat cortical neurones. *Br J Anaesth* **95**:662-668.

Kang J, Kang N, Lovatt D, Torres A, Zhao Z, Lin J and Nedergaard M (2008) Connexin 43 hemichannels are permeable to ATP. *J Neurosci* **28**:4702-4711.

Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke KF, Isaev NK and Dirnagl U (2002) Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent. *Stroke* **33**:1889-1898.

Karschin C, Ecke C, Ashcroft FM and Karschin A (1997) Overlapping distribution of K(ATP) channel-forming Kir6.2 subunit and the sulfonylurea receptor SUR1 in rodent brain. *FEBS Lett* **401**:59-64.

Karthik S, Grayson AD, Oo AY and Fabri BM (2004) A survey of current myocardial protection practices during coronary artery bypass grafting. *Ann R Coll Surg Engl* **86**:413-415.

Kato H, Liu Y, Araki T and Kogure K (1991) Temporal profile of the effects of pretreatment with brief cerebral ischemia on the neuronal damage following secondary ischemic insult in the gerbil: cumulative damage and protective effects. *Brain Res* **553**:238-242.

Kehl F, Payne RS, Roewer N and Schurr A (2004) Sevoflurane-induced preconditioning of rat brain in vitro and the role of KATP channels. *Brain Res.* **1021**:76-81.

Kersten JR, Lowe D, Hettrick DA, Pagel PS, Gross GJ and Warltier DC (1996) Glyburide, a KATP channel antagonist, attenuates the cardioprotective effects of isoflurane in stunned myocardium. *Anesth Analg* **83**:27-33.

Kidwell CS, Alger JR and Saver JL (2003) Beyond mismatch: evolving paradigms in imaging the ischemic penumbra with multimodal magnetic resonance imaging. *Stroke* **34**:2729-2735.

Kikura M, Takada T and Sato S (2004) Age- and sex-specific incidence, risk, and latency period of a perioperative acute thromboembolism syndrome (PATS). *Thromb Haemost* **91**:725-732.

Kin H, Zatta AJ, Lofye MT, Amerson BS, Halkos ME, Kerendi F, Zhao ZQ, Guyton RA, Headrick JP and Vinten-Johansen J (2005) Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. *Cardiovasc Res* **67**:124-133.

Kirino T (2002) Ischemic tolerance. *J Cereb Blood Flow Metab* **22**:1283-1296.

Kis B, Nagy K, Snipes JA, Rajapakse NC, Horiguchi T, Grover GJ and Busija DW (2004) The mitochondrial K(ATP) channel opener BMS-191095 induces neuronal preconditioning. *Neuroreport* **15**:345-349.

Kis B, Rajapakse NC, Snipes JA, Nagy K, Horiguchi T and Busija DW (2003) Diazoxide induces delayed pre-conditioning in cultured rat cortical neurons. *J Neurochem* **87**:969-980.

Kitagawa K, Matsumoto M, Kuwabara K, Tagaya M, Ohtsuki T, Hata R, Ueda H, Handa N, Kimura K and Kamada T (1991) 'Ischemic tolerance' phenomenon detected in various brain regions. *Brain Res* **561**:203-211.

Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K and et al. (1990) 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* **528**:21-24.

Kitano H, Young JM, Cheng J, Wang L, Hurn PD and Murphy SJ (2007) Gender-specific response to isoflurane preconditioning in focal cerebral ischemia. *J Cereb Blood Flow Metab* **27**:1377-1386.

Klijn CJ and Hankey GJ (2003) Management of acute ischaemic stroke: new guidelines from the American Stroke Association and European Stroke Initiative. *Lancet Neurol* **2**:698-701.

Kloner RA, Shook T, Antman EM, Cannon CP, Przyklenk K, Yoo K, McCabe CH and Braunwald E (1998) Prospective temporal analysis of the onset of preinfarction angina versus outcome: an ancillary study in TIMI-9B. *Circulation* **97**:1042-1045.

Kloner RA, Shook T, Przyklenk K, Davis VG, Junio L, Matthews RV, Burstein S, Gibson M, Poole WK, Cannon CP and et al. (1995) Previous angina alters in-hospital outcome in TIMI 4. A clinical correlate to preconditioning? *Circulation* **91**:37-45.

Knopp A, Thierfelder S, Doepner B and Benndorf K (2001) Mitochondria are the main ATP source for a cytosolic pool controlling the activity of ATP-sensitive K(+) channels in mouse cardiac myocytes. *Cardiovasc Res* **52**:236-245.

Kolominsky-Rabas PL, Sarti C, Heuschmann PU, Graf C, Siemonsen S, Neundoerfer B, Katalinic A, Lang E, Gassmann KG and von Stockert TR (1998) A prospective community-based study of stroke in Germany--the Erlangen Stroke Project (ESPro): incidence and case fatality at 1, 3, and 12 months. *Stroke* **29**:2501-2506.

Kopustinskiene DM, Toleikis A and Saris NE (2003) Adenine nucleotide translocase mediates the K(ATP)-channel-openers-induced proton and potassium flux to the mitochondrial matrix. *J Bioenerg Biomembr* **35**:141-148.

Koster JC, Sha Q and Nichols CG (1999) Sulfonylurea and K(+)-channel opener sensitivity of K(ATP) channels. Functional coupling of Kir6.2 and SUR1 subunits. *J Gen Physiol* **114**:203-213.

Krauter T, Ruppersberg JP and Baukrowitz T (2001) Phospholipids as modulators of K(ATP) channels: distinct mechanisms for control of sensitivity to sulphonylureas, K(+) channel openers, and ATP. *Mol Pharmacol* **59**:1086-1093.

Krolikowski JG, Weihrauch D, Bienengraeber M, Kersten JR, Warltier DC and Pagel PS (2006) Role of Erk1/2, p70s6K, and eNOS in isoflurane-induced cardioprotection during early reperfusion in vivo. *Can J Anaesth* **53**:174-182.

Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T and Tada M (1993) Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* **72**:1293-1299.

Kwok WM, Martinelli AT, Fujimoto K, Suzuki A, Stadnicka A and Bosnjak ZJ (2002) Differential modulation of the cardiac adenosine triphosphate-sensitive potassium channel by isoflurane and halothane. *Anesthesiology* **97**:50-56.

Landoni G, Bignami E, Oliviero F and Zangrillo A (2009) Halogenated anaesthetics and cardiac protection in cardiac and non-cardiac anaesthesia. *Ann Card Anaesth* **12**:4-9.

Langhorne P, Stott DJ, Robertson L, MacDonald J, Jones L, McAlpine C, Dick F, Taylor GS and Murray G (2000) Medical complications after stroke: a multicenter study. *Stroke* **31**:1223-1229.

Larach DR and Schuler HG (1993) Potassium channel blockade and halothane vasodilation in conducting and resistance coronary arteries. *J Pharmacol Exp Ther* **267**:72-81.

Lawson K (2000) Potassium channel openers as potential therapeutic weapons in ion channel disease. *Kidney Int* **57**:838-845.

Lebuffe G, Schumacker PT, Shao ZH, Anderson T, Iwase H and Vanden Hoek TL (2003) ROS and NO trigger early preconditioning: relationship to mitochondrial KATP channel. *Am J Physiol Heart Circ Physiol* **284**:H299-308.

Lee K, Dixon AK, Rowe IC, Ashford ML and Richardson PJ (1996) The high-affinity sulphonylurea receptor regulates KATP channels in nerve terminals of the rat motor cortex. *J.Neurochem.* **66**:2562-2571.

Lee WH and Bondy CA (1993) Ischemic injury induces brain glucose transporter gene expression. *Endocrinology* **133**:2540-2544.

Lees KR, Zivin JA, Ashwood T, Davalos A, Davis SM, Diener HC, Grotta J, Lyden P, Shuaib A, Hardemark HG and Wasiewski WW (2006) NXY-059 for acute ischemic stroke. *N Engl J Med* **354**:588-600.

Li Q, Zhu Y, Jiang H, Xu H and Liu H (2008a) Up-regulation of heme oxygenase-1 by isoflurane preconditioning during tolerance against neuronal injury induced by oxygen glucose deprivation. *Acta Biochim Biophys Sin (Shanghai)* **40**:803-810.

Li QF, Zhu YS and Jiang H (2008b) Isoflurane preconditioning activates HIF-1alpha, iNOS and Erk1/2 and protects against oxygen-glucose deprivation neuronal injury. *Brain Res* **1245**:26-35.

Li X and Eisenach JC (2005) Adenosine reduces glutamate release in rat spinal synaptosomes. *Anesthesiology* **103**:1060-1065.

Li Y, Walicki D, Mathiesen C, Jenny D, Li Q, Isayev Y, Reed JF, 3rd and Castaldo JE (2009) Strokes after cardiac surgery and relationship to carotid stenosis. *Arch Neurol* **66**:1091-1096.

Lieberman DN and Mody I (1994) Regulation of NMDA channel function by endogenous Ca(2+)-dependent phosphatase. *Nature* **369**:235-239.

Light PE, Bladen C, Winkfein RJ, Walsh MP and French RJ (2000) Molecular basis of protein kinase C-induced activation of ATP-sensitive potassium channels. *Proc Natl Acad Sci U S A* **97**:9058-9063.

Lim KH, Javadov SA, Das M, Clarke SJ, Suleiman MS and Halestrap AP (2002) The effects of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat heart mitochondrial volume and respiration. *J Physiol* **545**:961-974.

Limburg M, Wijdicks EF and Li H (1998) Ischemic stroke after surgical procedures: clinical features, neuroimaging, and risk factors. *Neurology* **50**:895-901.

Lin JH, Lou N, Kang N, Takano T, Hu F, Han X, Xu Q, Lovatt D, Torres A, Willecke K, Yang J, Kang J and Nedergaard M (2008) A central role of connexin 43 in hypoxic preconditioning. *J Neurosci* **28**:681-695.

Lin YF, Raab-Graham K, Jan YN and Jan LY (2004) NO stimulation of ATP-sensitive potassium channels: Involvement of Ras/mitogen-activated protein kinase pathway and contribution to neuroprotection. *Proc Natl Acad Sci U S A* **101**:7799-7804.

Lisle TC, Barrett KM, Gazoni LM, Swenson BR, Scott CD, Kazemi A, Kern JA, Peeler BB, Kron IL and Johnston KC (2008) Timing of stroke after cardiopulmonary bypass determines mortality. *Ann Thorac Surg* **85**:1556-1562; discussion 1562-1553.

Liss B and Roeper J (2001) Molecular physiology of neuronal K-ATP channels (review). *Mol Membr Biol* **18**:117-127.

Liu Y, Sato T, O'Rourke B and Marban E (1998) Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* **97**:2463-2469.

Loepke AW, McCann JC, Kurth CD and McAuliffe JJ (2006) The physiologic effects of isoflurane anesthesia in neonatal mice. *Anesth Analg* **102**:75-80.

Lopatin AN and Nichols CG (1996) [K+] dependence of open-channel conductance in cloned inward rectifier potassium channels (IRK1, Kir2.1). *Biophys J* **71**:682-694.

Lopatin AN and Nichols CG (2001) Inward rectifiers in the heart: an update on I(K1). *J Mol Cell Cardiol* **33**:625-638.

Lu Z (2004) Mechanism of rectification in inward-rectifier K+ channels. *Annu Rev Physiol* **66**:103-129.

Luengo-Fernandez R, Gray AM and Rothwell PM (2006) Population-based study of determinants of initial secondary care costs of acute stroke in the United Kingdom. *Stroke* **37**:2579-2587.

Luo Y, Ma D, Ieong E, Sanders RD, Yu B, Hossain M and Maze M (2008) Xenon and sevoflurane protect against brain injury in a neonatal asphyxia model. *Anesthesiology* **109**:782-789.

Ma D, Hossain M, Chow A, Arshad M, Battson RM, Sanders RD, Mehmet H, Edwards AD, Franks NP and Maze M (2005) Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia. *Ann Neurol* **58**:182-193.

Ma D, Hossain M, Pettet GK, Luo Y, Lim T, Akimov S, Sanders RD, Franks NP and Maze M (2006) Xenon preconditioning reduces brain damage from neonatal asphyxia in rats. *J Cereb Blood Flow Metab* **26**:199-208.

Ma D, Hossain M, Rajakumaraswamy N, Arshad M, Sanders RD, Franks NP and Maze M (2004) Dexmedetomidine produces its neuroprotective effect via the alpha 2A-adrenoceptor subtype. *Eur.J.Pharmacol.* **502**:87-97.

Ma D, Hossain M, Rajakumaraswamy N, Franks NP and Maze M (2003) Combination of xenon and isoflurane produces a synergistic protective effect against oxygen-glucose deprivation injury in a neuronal-glial co-culture model. *Anesthesiology* **99**:748-751.

Mandir AS, Poitras MF, Berliner AR, Herring WJ, Guastella DB, Feldman A, Poirier GG, Wang ZQ, Dawson TM and Dawson VL (2000) NMDA but not non-NMDA excitotoxicity is mediated by Poly(ADP-ribose) polymerase. *J Neurosci* **20**:8005-8011.

Marinovic J, Bosnjak ZJ and Stadnicka A (2006) Distinct roles for sarcolemmal and mitochondrial adenosine triphosphate-sensitive potassium channels in isoflurane-induced protection against oxidative stress. *Anesthesiology* **105**:98-104.

Matsuo M, Kimura Y and Ueda K (2005) KATP channel interaction with adenine nucleotides. *J Mol Cell Cardiol* **38**:907-916.

Matsuo M, Kioka N, Amachi T and Ueda K (1999) ATP binding properties of the nucleotide-binding folds of SUR1. *J Biol Chem* **274**:37479-37482.

Matsuo M, Tanabe K, Kioka N, Amachi T and Ueda K (2000) Different binding properties and affinities for ATP and ADP among sulfonylurea receptor subtypes, SUR1, SUR2A, and SUR2B. *J Biol Chem* **275**:28757-28763.

Mayanagi K, Gaspar T, Katakam PV and Busija DW (2007) Systemic administration of diazoxide induces delayed preconditioning against transient focal cerebral ischemia in rats. *Brain Res* **1168**:106-111.

McAlister FA, Man J, Bistritz L, Amad H and Tandon P (2003) Diabetes and coronary artery bypass surgery: an examination of perioperative glycemic control and outcomes. *Diabetes Care* **26**:1518-1524.

McKhann GM, Grega MA, Borowicz LM, Jr., Baumgartner WA and Selnes OA (2006) Stroke and encephalopathy after cardiac surgery: an update. *Stroke* **37**:562-571.

McNicholas CM, MacGregor GG, Islas LD, Yang Y, Hebert SC and Giebisch G (1998) pH-dependent modulation of the cloned renal K+ channel, ROMK. *Am J Physiol* **275**:F972-981.

Mehr EH and Lindeman KS (1993) Effects of halothane, propofol, and thiopental on peripheral airway reactivity. *Anesthesiology* **79**:290-298.

Meisheri KD, Dubray LA and Oleynek JJ (1990) A sensitive in vitro functional assay to detect K(+)-channel-dependent vasodilators. *J Pharmacol Methods* **24**:251-261.

Mellon RD, Simone AF and Rappaport BA (2007) Use of anesthetic agents in neonates and young children. *Anesth Analg* **104**:509-520.

Mergenthaler P, Dirnagl U and Meisel A (2004) Pathophysiology of stroke: lessons from animal models. *Metab Brain Dis* **19**:151-167.

Millar JA, Barratt L, Southan AP, Page KM, Fyffe RE, Robertson B and Mathie A (2000) A functional role for the two-pore domain potassium channel TASK-1 in cerebellar granule neurons. *Proc Natl Acad Sci U S A* **97**:3614-3618.

Misonou H, Mohapatra DP, Menegola M and Trimmer JS (2005) Calcium- and metabolic state-dependent modulation of the voltage-dependent Kv2.1 channel regulates neuronal excitability in response to ischemia. *J Neurosci* **25**:11184-11193.

Montague JW, Bortner CD, Hughes FM, Jr. and Cidlowski JA (1999) A necessary role for reduced intracellular potassium during the DNA degradation phase of apoptosis. *Steroids* **64**:563-569.

Moreau C, Prost AL, Derand R and Vivaudou M (2005) SUR, ABC proteins targeted by KATP channel openers. *J Mol Cell Cardiol* **38**:951-963.

Moustafa RR and Baron JC (2008) Pathophysiology of ischaemic stroke: insights from imaging, and implications for therapy and drug discovery. *Br J Pharmacol* **153 Suppl 1**:S44-54.

Mullenheim J, Ebel D, Bauer M, Otto F, Heinen A, Frassdorf J, Preckel B and Schlack W (2003) Sevoflurane confers additional cardioprotection after ischemic late preconditioning in rabbits. *Anesthesiology* **99**:624-631.

Murakami H, Takanaga H, Matsuo H, Ohtani H and Sawada Y (2000) Comparison of blood-brain barrier permeability in mice and rats using in situ brain perfusion technique. *Am J Physiol Heart Circ Physiol* **279**:H1022-1028.

Murry CE, Jennings RB and Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**:1124-1136.

Nakayama M, Fujita S, Kanaya N, Tsuchida H and Namiki A (1997) Blockade of ATP-sensitive K+ channel abolishes the anti-ischemic effects of isoflurane in dog hearts. *Acta Anaesthesiol Scand* **41**:531-535.

National-Cancer-Institute (2007) Cancer Trend Progress Report, in, U.S. National Institutes of Health.

Neagoe I and Schwappach B (2005) Pas de deux in groups of four--the biogenesis of KATP channels. *J Mol Cell Cardiol* **38**:887-894.

NHS-Direct (2009) Stroke, in (NHS ed), NHS-Choices.

Nichols CG (2006) KATP channels as molecular sensors of cellular metabolism. *Nature* **440**:470-476.

Nichols CG and Lopatin AN (1997) Inward rectifier potassium channels. *Annu Rev Physiol* **59**:171-191.

Nichols CG, Makhina EN, Pearson WL, Sha Q and Lopatin AN (1996) Inward rectification and implications for cardiac excitability. *Circ.Res.* **78**:1-7.

Noma A (1983) ATP-regulated K+ channels in cardiac muscle. *Nature* **305**:147-148.

O'Rourke B (2000a) Myocardial K(ATP) channels in preconditioning. *Circ Res* **87**:845-855.

O'Rourke B (2000b) Pathophysiological and protective roles of mitochondrial ion channels. *J Physiol* **529 Pt 1**:23-36.

Obal D, Dettwiler S, Favoccia C, Scharbatke H, Preckel B and Schlack W (2005) The influence of mitochondrial KATP-channels in the cardioprotection of preconditioning and postconditioning by sevoflurane in the rat in vivo. *Anesth Analg* **101**:1252-1260.

Obrist WD, Thompson HK, Jr., Wang HS and Wilkinson WE (1975) Regional cerebral blood flow estimated by 133-xenon inhalation. *Stroke* **6**:245-256.

Offner FF (1991) Ion flow through membranes and the resting potential of cells. *J Membr Biol* **123**:171-182.

Ogata J, Shiraishi M, Namba T, Smothers CT, Woodward JJ and Harris RA (2006) Effects of anesthetics on mutant N-methyl-D-aspartate receptors expressed in Xenopus oocytes. *J Pharmacol Exp Ther* **318**:434-443.

Ohtsuka T, Ishiwa D, Kamiya Y, Itoh H, Nagata I, Saito Y, Yamada Y, Sumitomo M and Andoh T (2006) Effects of barbiturates on ATP-sensitive K channels in rat substantia nigra. *Neuroscience* **137**:573-581.

Oldenburg O, Qin Q, Krieg T, Yang XM, Philipp S, Critz SD, Cohen MV and Downey JM (2004) Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoKATP channel opening and leads to cardioprotection. *Am J Physiol Heart Circ Physiol* **286**:H468-476.

Oppenheim RW (1999) Programmed Cell Death in *Fundamental Neuroscience* (Zigmont MJ, Bloom FE, Landis SC, Roberts JL and Squire LR eds) pp 581-610, Academic Press San Diego-London-Boston-New York-Sydney-Tokyo-Toronto

Palmer AJ, Valentine WJ, Roze S, Lammert M, Spiesser J and Gabriel S (2005) Overview of costs of stroke from published, incidence-based studies spanning 16 industrialized countries. *Curr Med Res Opin* **21**:19-26.

Pancrazio JJ (1997) Techniques of cellular electrophysiologic investigation, in *Anesthesia: Biologic Foundations* (Yaksh TL, Lynch C, Zapol WM, Maze M, Biebuyck JF and Saidman LJ eds) pp 337-352, Lippincott-Raven Publishers, Philadelphia-New York

Park CK, Nehls DG, Graham DI, Teasdale GM and McCulloch J (1988) The glutamate antagonist MK-801 reduces focal ischemic brain damage in the rat. *Ann Neurol* **24**:543-551.

Partiseti M, Collura V, Agnel M, Culouscou JM and Graham D (1998) Cloning and characterization of a novel human inwardly rectifying potassium channel predominantly expressed in small intestine. *FEBS Lett* **434**:171-176.

Paucek P, Mironova G, Mahdi F, Beavis AD, Woldegiorgis G and Garlid KD (1992) Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K+ channel from rat liver and beef heart mitochondria. *J Biol Chem* **267**:26062-26069.

Payne RS, Akca O, Roewer N, Schurr A and Kehl F (2005) Sevoflurane-induced preconditioning protects against cerebral ischemic neuronal damage in rats. *Brain Res* **1034**:147-152.

Peck TE and Williams M (2000) in *Pharmacology for Anaesthesia and Intensive Care* (Peck TE and Williams M eds) pp 81-100, Greenwich Medical Media, U.K., London.

Preckel B, Weber NC, Sanders RD, Maze M and Schlack W (2006) Molecular mechanisms transducing the anesthetic, analgesic, and organ-protective actions of xenon. *Anesthesiology* **105**:187-197.

Proks P and Ashcroft FM (2009) Modeling K(ATP) channel gating and its regulation. *Prog Biophys Mol Biol* **99**:7-19.

Proks P, Capener CE, Jones P and Ashcroft FM (2001) Mutations within the P-loop of Kir6.2 modulate the intraburst kinetics of the ATP-sensitive potassium channel. *J Gen Physiol* **118**:341-353.

Proks P, Gribble FM, Adhikari R, Tucker SJ and Ashcroft FM (1999) Involvement of the N-terminus of Kir6.2 in the inhibition of the KATP channel by ATP. *J Physiol* **514**:19-25.

Putzke C, Hanley PJ, Schlichthorl G, Preisig-Muller R, Rinne S, Anetseder M, Eckenhoff R, Berkowitz C, Vassiliou T, Wulf H and Eberhart L (2007) Differential effects of volatile and intravenous anesthetics on the activity of human TASK-1. *Am J Physiol Cell Physiol* **293**:C1319-1326.

Quast U, Stephan D, Bieger S and Russ U (2004) The impact of ATP-sensitive K+ channel subtype selectivity of insulin secretagogues for the coronary vasculature and the myocardium. *Diabetes* **53 Suppl 3**:S156-164.

Rabadi MH, Rabadi FM, Edelstein L and Peterson M (2008) Cognitively impaired stroke patients do benefit from admission to an acute rehabilitation unit. *Arch Phys Med Rehabil* **89**:441-448.

Radi R, Beckman JS, Bush KM and Freeman BA (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* **266**:4244-4250.

Rajakumaraswamy N, Ma D, Hossain M, Sanders RD, Franks NP and Maze M (2006) Neuroprotective interaction produced by xenon and dexmedetomidine on in vitro and in vivo neuronal injury models. *Neurosci Lett* **409**:128-133.

Raval AP, Dave KR, DeFazio RA and Perez-Pinzon MA (2007) epsilonPKC phosphorylates the mitochondrial K(+) (ATP) channel during induction of ischemic preconditioning in the rat hippocampus. *Brain Res* **1184**:345-353.

Rehni AK, Shri R and Singh M (2007) Remote ischaemic preconditioning and prevention of cerebral injury. *Indian J Exp Biol* **45**:247-252.

Reimann F, Ashcroft FM and Gribble FM (2001) Structural basis for the interference between nicorandil and sulfonylurea action. *Diabetes* **50**:2253-2259.

Reimann F, Ryder TJ, Tucker SJ and Ashcroft FM (1999) The role of lysine 185 in the kir6.2 subunit of the. *J-Physiol-Lond.* **520**:661-669.

Repunte VP, Nakamura H, Fujita A, Horio Y, Findlay I, Pott L and Kurachi Y (1999) Extracellular links in Kir subunits control the unitary conductance of SUR/Kir6.0 ion channels. *EMBO J* **18**:3317-3324.

Rezkalla SH and Kloner RA (2007) Preconditioning in humans. *Heart Fail Rev* **12**:201-206.

Riepe MW, Esclaire F, Kasischke K, Schreiber S, Nakase H, Kempski O, Ludolph AC, Dirnagl U and Hugon J (1997) Increased hypoxic tolerance by chemical inhibition of oxidative phosphorylation: "chemical preconditioning". *J Cereb Blood Flow Metab* **17**:257-264.

Riepe MW, Niemi WN, Megow D, Ludolph AC and Carpenter DO (1996) Mitochondrial oxidation in rat hippocampus can be preconditioned by selective chemical inhibition of succinic dehydrogenase. *Exp Neurol* **138**:15-21.

Riley B (2003) Acute cerebrovascular complications in *Oh's Intensive Care Manual* (Bersten AD, Soni N and Oh TE eds) pp 495-504, Butterworth Heinemann Edinburgh-London-New York-Sydney-Toronto.

Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M and Hong Y (2008) Heart disease and stroke statistics-2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **117**:e25-146.

Roscoe AK, Christensen JD and Lynch C, III (2000) Isoflurane, but not halothane, induces protection of human myocardium via adenosine A1 receptors and adenosine triphosphate-sensitive potassium channels. *Anesthesiology* **92**:1692-1701.

Rossaint R, Reyle-Hahn M, Schulte Am Esch J, Scholz J, Scherpereel P, Vallet B, Giunta F, Del Turco M, Erdmann W, Tenbrinck R, Hammerle AF and Nagele P (2003) Multicenter randomized comparison of the efficacy and safety of xenon and isoflurane in patients undergoing elective surgery. *Anesthesiology* **98**:6-13.

Roth S, Dreixler JC, Shaikh AR, Lee KH and Bindokas V (2006) Mitochondrial potassium ATP channels and retinal ischemic preconditioning. *Invest Ophthalmol Vis Sci* **47**:2114-2124.

Sacco RL, DeRosa JT, Haley EC, Jr., Levin B, Ordronneau P, Phillips SJ, Rundek T, Snipes RG and Thompson JL (2001) Glycine antagonist in neuroprotection for patients with acute stroke: GAIN Americas: a randomized controlled trial. *Jama* **285**:1719-1728.

Sakura H, Ammala C, Smith PA, Gribble FM and Ashcroft FM (1995) Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic beta-cells, brain, heart and skeletal muscle. *FEBS Lett.* **377**:338-344.

Sakura H, Trapp S, Liss B and Ashcroft FM (1999) Altered functional properties of KATP channel conferred by a novel splice variant of SUR1. *J Physiol* **521**:337-350.

Sanders RD, Franks NP and Maze M (2003) Xenon: no stranger to anaesthesia. *Br J Anaesth* **91**:709-717.

Sanders RD, Xu J, Shu Y, Januszewski A, Halder S, Fidalgo A, Sun P, Hossain M, Ma D and Maze M (2009) Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats. *Anesthesiology* **110**:1077-1085.

Sapolsky RM (2001) Cellular defenses against excitotoxic insults. *J Neurochem* **76**:1601-1611.

Sasaki N, Sato T, Marban E and O'Rourke B (2001) ATP consumption by uncoupled mitochondria activates sarcolemmal K(ATP) channels in cardiac myocytes. *Am J Physiol Heart Circ Physiol* **280**:H1882-1888.

Sato T, Nishida H, Miyazaki M and Nakaya H (2006) Effects of sulfonylureas on mitochondrial ATP-sensitive K+ channels in cardiac myocytes: implications for sulfonylurea controversy. *Diabetes Metab Res Rev* **22**:341-347.

Sato T, O'Rourke B and Marban E (1998) Modulation of mitochondrial ATP-dependent K+ channels by protein kinase C. *Circ Res* **83**:110-114.

Savitz SI and Fisher M (2007) Future of neuroprotection for acute stroke: in the aftermath of the SAINT trials. *Ann Neurol* **61**:396-402.

Schaller BJ (2007) Influence of age on stroke and preconditioning-induced ischemic tolerance in the brain. *Exp Neurol* **205**:9-19.

Schulte U and Fakler B (2000) Gating of inward-rectifier K+ channels by intracellular pH. *Eur J Biochem* **267**:5837-5841.

Schulz R, Cohen MV, Behrends M, Downey JM and Heusch G (2001) Signal transduction of ischemic preconditioning. *Cardiovasc Res* **52**:181-198.

Schwappach B, Zerangue N, Jan YN and Jan LY (2000) Molecular basis for K(ATP) assembly: transmembrane interactions mediate association of a K+ channel with an ABC transporter. *Neuron* **26**:155-167.

Sear JW, Giles JW, Howard-Alpe G and Foex P (2008) Perioperative beta-blockade, 2008: what does POISE tell us, and was our earlier caution justified? *Br J Anaesth* **101**:135-138.

SEER CS (2008) SEER Cancer Statistics Review 1975-2006; U.S. National Institutes of Health.

Seino S and Miki T (2003) Physiological and pathophysiological roles of ATP-sensitive K+ channels. *Prog Biophys Mol Biol* **81**:133-176.

Selim M (2007) Perioperative stroke. *N.Engl.J.Med.* **356**:706-713.

Selivanov VA, Alekseev AE, Hodgson DM, Dzeja PP and Terzic A (2004) Nucleotide-gated KATP channels integrated with creatine and adenylate kinases: amplification, tuning and sensing of energetic signals in the compartmentalized cellular environment. *Mol Cell Biochem* **256-257**:243-256.

Semaille CH, Downs AM, Alix JA and Hamers FF (2002) HIV case reporting in Western Europe: No evidence of increased HIV transmmission in the era of highly active anti-retroviral therapy, in *XIV International AIDS Conference* Barcelona, Spain.

Shake JG, Peck EA, Marban E, Gott VL, Johnston MV, Troncoso JC, Redmond JM and Baumgartner WA (2001) Pharmacologically induced preconditioning with diazoxide: a novel approach to brain protection. *Ann Thorac Surg* **72**:1849-1854.

Sharp FR, Butman M, Koistinaho J, Aardalen K, Nakki R, Massa SM, Swanson RA and Sagar SM (1994) Phencyclidine induction of the hsp 70 stress gene in injured pyramidal neurons is mediated via multiple receptors and voltage gated calcium channels. *Neuroscience* **62**:1079-1092.

Shindo T, Yamada M, Isomoto S, Horio Y and Kurachi Y (1998) SUR2 subtype (A and B)-dependent differential activation of the cloned ATP-sensitive K+ channels by pinacidil and nicorandil. *Br J Pharmacol* **124**:985-991.

Shuaib A, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, Diener HC, Ashwood T, Wasiewski WW and Emeribe U (2007) NXY-059 for the treatment of acute ischemic stroke. *N Engl J Med* **357**:562-571.

Shyng S and Nichols CG (1997) Octameric stoichiometry of the KATP channel complex. *J Gen Physiol* **110**:655-664.

Shyng SL, Barbieri A, Gumusboga A, Cukras C, Pike L, Davis JN, Stahl PD and Nichols CG (2000) Modulation of nucleotide sensitivity of ATP-sensitive potassium channels by phosphatidylinositol-4-phosphate 5-kinase. *Proc Natl Acad Sci U S A* **97**:937-941.

Shyng SL and Nichols CG (1998) Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science* **282**:1138-1141.

Sice PJA (2005) Depth of anaesthesia. *Pharmacology*:1.

Sigaut S, Jannier V, Rouelle D, Gressens P, Mantz J and Dahmani S (2009) The preconditioning effect of sevoflurane on the oxygen glucose-deprived hippocampal slice: the role of tyrosine kinases and duration of ischemia. *Anesth Analg* **108**:601-608.

Sigel E and Minier F (2005) The Xenopus oocyte: system for the study of functional expression and modulation of proteins. *Mol Nutr Food Res* **49**:228-234.

Sirois JE, Lei Q, Talley EM, Lynch C, 3rd and Bayliss DA (2000) The TASK-1 two-pore domain K+ channel is a molecular substrate for neuronal effects of inhalation anesthetics. *J Neurosci* **20**:6347-6354.

Small DL, Morley P and Buchan AM (1999) Biology of ischemic cerebral cell death. *Prog Cardiovasc Dis* **42**:185-207.

Soriano SG and Anand KJ (2005) Anesthetics and brain toxicity. *Curr Opin Anaesthesiol* **18**:293-297.

Sorimachi T and Nowak TS, Jr. (2004) Pharmacological manipulations of ATP-dependent potassium channels and adenosine A1 receptors do not impact hippocampal ischemic preconditioning in vivo: evidence in a highly quantitative gerbil model. *J Cereb Blood Flow Metab* **24**:556-563.

Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, Aupetit JF, Bonnefoy E, Finet G, Andre-Fouet X and Ovize M (2005) Postconditioning the human heart. *Circulation* **112**:2143-2148.

Stacey G and Viviani B (2001) Cell culture models for neurotoxicology. *Cell Biol Toxicol* **17**:319-334.

Steiger HJ and Hanggi D (2007) Ischaemic preconditioning of the brain, mechanisms and applications. *Acta Neurochir.* **149**:1-10.

Stowe DF, Rehmert GC, Kwok WM, Weigt HU, Georgieff M and Bosnjak ZJ (2000) Xenon does not alter cardiac function or major cation currents in isolated guinea pig hearts or myocytes. *Anesthesiology* **92**:516-522.

Stucke AG, Zuperku EJ, Tonkovic-Capin V, Krolo M, Hopp FA, Kampine JP and Stuth EA (2005) Sevoflurane depresses glutamatergic neurotransmission to brainstem inspiratory premotor neurons but not postsynaptic receptor function in a decerebrate dog model. *Anesthesiology* **103**:50-56.

Sugawara T, Fujimura M, Noshita N, Kim GW, Saito A, Hayashi T, Narasimhan P, Maier CM and Chan PH (2004) Neuronal death/survival signaling pathways in cerebral ischemia. *NeuroRx* **1**:17-25.

Sun HS, Feng ZP, Miki T, Seino S and French RJ (2006) Enhanced neuronal damage after ischemic insults in mice lacking Kir6.2-containing ATP-sensitive K+ channels. *J Neurophysiol* **95**:2590-2601.

Suzuki M, Kotake K, Fujikura K, Inagaki N, Suzuki T, Gonoi T, Seino S and Takata K (1997) Kir6.1: a possible subunit of ATP-sensitive K+ channels in mitochondria. *Biochem Biophys Res Commun* **241**:693-697.

Suzuki M, Sasaki N, Miki T, Sakamoto N, Ohmoto-Sekine Y, Tamagawa M, Seino S, Marban E and Nakaya H (2002) Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. *J Clin Invest* **109**:509-516.

Suzuki T, Ueta K, Sugimoto M, Uchida I and Mashimo T (2003) Nitrous oxide and xenon inhibit the human (alpha 7) 5 nicotinic acetylcholine receptor expressed in Xenopus oocyte. *Anesth Analg* **96**:443-448, table of contents.

Symon L, Branston NM, Strong AJ and Hope TD (1977) The concepts of thresholds of ischaemia in relation to brain structure and function. *J Clin Pathol Suppl (R Coll Pathol)* **11**:149-154.

Szeder V and Torbey MT (2008) Prevention and treatment of perioperative stroke. *Neurologist* **14**:30-36.

Szewczyk A and Marban E (1999) Mitochondria: a new target for K channel openers? *Trends Pharmacol Sci* **20**:157-161.

Takahashi S, Abe T, Gotoh J and Fukuuchi Y (2002) Substrate-dependence of reduction of MTT: a tetrazolium dye differs in cultured astroglia and neurons. *Neurochem Int* **40**:441-448.

Takanaga H, Murakami H, Koyabu N, Matsuo H, Naito M, Tsuruo T and Sawada Y (1998) Efflux transport of tolbutamide across the blood-brain barrier. *J Pharm Pharmacol* **50**:1027-1033.

Tallarida RJ (2000) *Drug Synergism and Dose-Effect Data Analysis*. Chapman & Hall, Boca Raton-London-New York-Washington.

Tanabe K, Tucker SJ, Matsuo M, Proks P, Ashcroft FM, Seino S, Amachi T and Ueda K (1999a) Direct photoaffinity labeling of the Kir6.2 subunit of the ATP-sensitive K+ channel by 8-azido-ATP. *J Biol Chem.* **274**:3931-3933.

Tanabe M, Mori M, Gahwiler BH and Gerber U (1999b) Apamin-sensitive conductance mediates the K(+) current response during chemical ischemia in CA3 pyramidal cells. *J Neurophysiol* **82**:2876-2882.

Tanaka K, Ludwig LM, Kersten JR, Pagel PS and Warltier DC (2004) Mechanisms of cardioprotection by volatile anesthetics. *Anesthesiology* **100**:707-721.

Taylor P (1990) Molecular Basis of Pharmacologic Selectivity, in *Principles of Drug Action* (Pratt WB and Taylor P eds) pp 1-102, Churchill-Livingstone Philadelphia-Edinburgh-London-Toronto-Montreal-Sydney-Tokyo.

Taylor P and Insel PA (1990) Molecular Basis of Pharmacologic Selectivity, in *Principles of Drug Action* (Pratt WB and Taylor P eds) pp 1-102, Churchill-Livingstone, Philadelphia-Edinburgh-London-Toronto-Montreal-Sydney-Tokyo.

Taylor TN, Davis PH, Torner JC, Holmes J, Meyer JW and Jacobson MF (1996) Lifetime cost of stroke in the United States. *Stroke* **27**:1459-1466.

The-Care-Quality-Commission (2006) Heart Surgery in the United Kingdom.

Thomas P and Smart TG (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins. *J Pharmacol Toxicol Methods* **51**:187-200.

Thrift AG, Dewey HM, Macdonell RA, McNeil JJ and Donnan GA (2001) Incidence of the major stroke subtypes: initial findings from the North East Melbourne stroke incidence study (NEMESIS). *Stroke* **32**:1732-1738.

Toumpoulis IK, Anagnostopoulos CE, Chamogeorgakis TP, Angouras DC, Kariou MA, Swistel DG and Rokkas CK (2008) Impact of early and delayed stroke on in-hospital and long-term mortality after isolated coronary artery bypass grafting. *Am J Cardiol* **102**:411-417.

Trapp S and Ashcroft FM (1997) A metabolic sensor in action: News from the ATP-sensitive K+-channel. *News-in-Physiological-Sciences* **12**:255-263.

Trapp S and Ballanyi K (1995) KATP channel mediation of anoxia-induced outward current in rat dorsal vagal neurons in vitro. *J Physiol* **487**:37-50.

Trapp S, Ballanyi K and Richter DW (1994) Spontaneous activation of KATP current in rat dorsal vagal neurones. *Neuroreport* **5**:1285-1288.

Trapp S, Haider S, Jones P, Sansom MS and Ashcroft FM (2003) Identification of residues contributing to the ATP binding site of Kir6.2. *Embo J* **22**:2903-2912.

Trapp S, Proks P, Tucker SJ and Ashcroft FM (1998) Molecular analysis of ATP-sensitive K channel gating and implications for channel inhibition by ATP. *J Gen Physiol* **112**:333-349.

Treger I, Shames J, Giaquinto S and Ring H (2007) Return to work in stroke patients. *Disabil Rehabil* **29**:1397-1403.

Trube G and Hescheler J (1984) Inward-rectifying channels in isolated patches of the heart cell membrane: ATP-dependence and comparison with cell-attached patches. *Pflugers Arch* **401**:178-184.

Tucker SJ and Ashcroft FM (1998) A touching case of channel regulation: the ATP-sensitive K+ channel. *Curr Opin Neurobiol* **8**:316-320.

Tucker SJ, Gribble FM, Proks P, Trapp S, Ryder TJ, Haug T, Reimann F and Ashcroft FM (1998) Molecular determinants of KATP channel inhibition by ATP. *Embo J* **17**:3290-3296.

Tucker SJ, Gribble FM, Zhao C, Trapp S and Ashcroft FM (1997) Truncation of Kir6.2 produces ATP-sensitive K+ channels in the absence of the sulphonylurea receptor. *Nature* **387**:179-183.

Tung CY, Granger CB, Sloan MA, Topol EJ, Knight JD, Weaver WD, Mahaffey KW, White H, Clapp-Channing N, Simoons ML, Gore JM, Califf RM and Mark DB (1999) Effects of stroke on medical resource use and costs in acute myocardial infarction. GUSTO I Investigators. Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries Study. *Circulation* **99**:370-376.

Ueda K, Inagaki N and Seino S (1997) MgADP antagonism to Mg2+-independent ATP binding of the sulfonylurea receptor SUR1. *J Biol Chem* **272**:22983-22986.

van den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, Vlasselaers D, Ferdinande P, Lauwers P and Bouillon R (2001) Intensive insulin therapy in the critically ill patients. *N Engl J Med* **345**:1359-1367.

Vanoye CG, MacGregor GG, Dong K, Tang L, Buschmann AS, Hall AE, Lu M, Giebisch G and Hebert SC (2002) The carboxyl termini of K(ATP) channels bind nucleotides. *J Biol Chem* **277**:23260-23270.

Velly LJ, Canas PT, Guillet BA, Labrande CN, Masmejean FM, Nieoullon AL, Gouin FM, Bruder NJ and Pisano PS (2009) Early anesthetic preconditioning in mixed cortical neuronal-glial cell cultures subjected to oxygen-glucose deprivation: the role of adenosine triphosphate dependent potassium channels and reactive oxygen species in sevoflurane-induced neuroprotection. *Anesth Analg* **108**:955-963.

Vyklicky L, Jr. (1993) Calcium-mediated modulation of N-methyl-D-aspartate (NMDA) responses in cultured rat hippocampal neurones. *J Physiol* **470**:575-600.

Wang C, Jin Lee J, Jung HH and Zuo Z (2007a) Pretreatment with volatile anesthetics, but not with the nonimmobilizer 1,2-dichlorohexafluorocyclobutane, reduced cell injury in rat cerebellar slices after an in vitro simulated ischemia. *Brain Res* **1152**:201-208.

Wang C, Wang K, Wang W, Cui Y and Fan Z (2002) Compromised ATP binding as a mechanism of phosphoinositide modulation of ATP-sensitive K+ channels. *FEBS Lett* **532**:177-182.

Wang J, Lei B, Popp S, Meng F, Cottrell JE and Kass IS (2007b) Sevoflurane immediate preconditioning alters hypoxic membrane potential changes in rat hippocampal slices and improves recovery of CA1 pyramidal cells after hypoxia and global cerebral ischemia. *Neuroscience* **145**:1097-1107.

Wang L, Kitano H, Hurn PD and Murphy SJ (2008a) Estradiol attenuates neuroprotective benefits of isoflurane preconditioning in ischemic mouse brain. *J Cereb Blood Flow Metab* **28**:1824-1834.

Wang L, Traystman RJ and Murphy SJ (2008b) Inhalational anesthetics as preconditioning agents in ischemic brain. *Curr Opin Pharmacol* **8**:104-110.

Wang Y, Haider HK, Ahmad N and Ashraf M (2005) Mechanisms by which K(ATP) channel openers produce acute and delayed cardioprotection. *Vascul Pharmacol* **42**:253-264.

Wang ZP, Zhang ZH, Zeng YM, Jiang S, Wang SQ and Wang S (2006) [Protective effect of sevoflurane preconditioning on oxygen-glucose deprivation injury in rat hippocampal slices: the role of mitochondrial K(ATP)channels.]. *Sheng Li Xue Bao* **58**:201-206.

Warach S, Kaufman D, Chiu D, Devlin T, Luby M, Rashid A, Clayton L, Kaste M, Lees KR, Sacco R and Fisher M (2006) Effect of the Glycine Antagonist Gavestinel on cerebral infarcts in acute stroke patients, a randomized placebo-controlled trial: The GAIN MRI Substudy. *Cerebrovasc Dis* **21**:106-111.

Weber NC, Frassdorf J, Ratajczak C, Grueber Y, Schlack W, Hollmann MW and Preckel B (2008) Xenon induces late cardiac preconditioning in vivo: a role for cyclooxygenase 2? *Anesth Analg* **107**:1807-1813.

Weber NC, Toma O, Wolter JI, Obal D, Mullenheim J, Preckel B and Schlack W (2005) The noble gas xenon induces pharmacological preconditioning in the rat heart in vivo via induction of PKC-epsilon and p38 MAPK. *Br J Pharmacol* **144**:123-132.

Wei H, Liang G and Yang H (2007) Isoflurane preconditioning inhibited isoflurane-induced neurotoxicity. *Neurosci Lett* **425**:59-62.

Weinberger JM (2006) Evolving therapeutic approaches to treating acute ischemic stroke. *J Neurol Sci* **249**:101-109.

White IL, Franks NP and Dickinson R (2005) Effects of isoflurane and xenon on Ba2+-currents mediated by N-type calcium channels. *Br J Anaesth* **94**:784-790.

Wiesinger H, Hamprecht B and Dringen R (1997) Metabolic pathways for glucose in astrocytes. *Glia* **21**:22-34.

Wilhelm S, Ma D, Maze M and Franks NP (2002) Effects of xenon on in vitro and in vivo models of neuronal injury. *Anesthesiology* **96**:1485-1491.

Won SJ, Kim DY and Gwag BJ (2002) Cellular and molecular pathways of ischemic neuronal death. *J Biochem Mol Biol* **35**:67-86.

Wong GY, Warner DO, Schroeder DR, Offord KP, Warner MA, Maxson PM and Whisnant JP (2000) Risk of surgery and anesthesia for ischemic stroke. *Anesthesiology* **92**:425-432.

Wu J, Piao H, Rojas A, Wang R, Wang Y, Cui N, Shi Y, Chen F and Jiang C (2004) Critical protein domains and amino acid residues for gating the KIR6.2 channel by intracellular ATP. *J Cell Physiol* **198**:73-81.

Xi Q, Cheranov SY and Jaggar JH (2005) Mitochondria-derived reactive oxygen species dilate cerebral arteries by activating Ca2+ sparks. *Circ Res* **97**:354-362.

Xie LH, Horie M and Takano M (1999a) Phospholipase C-linked receptors regulate the ATP-sensitive potassium channel by means of phosphatidylinositol 4,5-bisphosphate metabolism. *Proc Natl Acad Sci U S A*. **96**:15292-15297.

Xie LH, Takano M, Kakei M, Okamura M and Noma A (1999b) Wortmannin, an inhibitor of phosphatidylinositol kinases, blocks the MgATP-dependent recovery of Kir6.2/SUR2A channels. *J Physiol Lond* **514**:655-665.

Xu X, Kim JA and Zuo Z (2008) Isoflurane preconditioning reduces mouse microglial activation and injury induced by lipopolysaccharide and interferon-gamma. *Neuroscience* **154**:1002-1008.

Yamada K, Ji JJ, Yuan H, Miki T, Sato S, Horimoto N, Shimizu T, Seino S and Inagaki N (2001) Protective role of ATP-sensitive potassium channels in hypoxia-induced generalized seizure. *Science* **292**:1543-1546.

Yamada M, Isomoto S, Matsumoto S, Kondo C, Shindo T, Horio Y and Kurachi Y (1997) Sulphonylurea receptor 2B and Kir6.1 form a sulphonylurea-sensitive but ATP-insensitive K+ channel. *J Physiol Lond* **499**:715-720.

Yamakura T, Lewohl JM and Harris RA (2001) Differential effects of general anesthetics on G protein-coupled inwardly rectifying and other potassium channels. *Anesthesiology* **95**:144-153.

Yanamoto H, Nagata I, Niitsu Y, Zhang Z, Xue JH, Sakai N and Kikuchi H (2001) Prolonged mild hypothermia therapy protects the brain against permanent focal ischemia. *Stroke* **32**:232-239.

Yoneda Y, Uehara T, Yamasaki H, Kita Y, Tabuchi M and Mori E (2003) Hospital-based study of the care and cost of acute ischemic stroke in Japan. *Stroke* **34**:718-724.

Yoshida M, Nakakimura K, Cui YJ, Matsumoto M and Sakabe T (2004) Adenosine A(1) receptor antagonist and mitochondrial ATP-sensitive potassium channel blocker attenuate the tolerance to focal cerebral ischemia in rats. *J Cereb Blood Flow Metab* **24**:771-779.

Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, Tian M, Dugan LL and Choi DW (1997) Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science* **278**:114-117.

Yuan HB, Huang Y, Zheng S and Zuo Z (2004) Hypothermic preconditioning increases survival of purkinje neurons in rat cerebellar slices after an in vitro simulated ischemia. *Anesthesiology* **100**:331-337.

Zaugg M, Lucchinetti E, Uecker M, Pasch T and Schaub MC (2003) Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth* **91**:551-565.

Zaugg M, Schaub MC and Foex P (2004) Myocardial injury and its prevention in the perioperative setting. *Br J Anaesth* **93**:21-33.

Zerangue N, Schwappach B, Jan YN and Jan LY (1999) A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* **22**:537-548.

Zhang H, He C, Yan X, Mirshahi T and Logothetis DE (1999) Activation of inwardly rectifying K+ channels by distinct PtdIns(4,5)P2 interactions. *Nat Cell Biol* **1**:183-188.

Zhang M, Li WB, Geng JX, Li QJ, Sun XC, Xian XH, Qi J and Li SQ (2007) The upregulation of glial glutamate transporter-1 participates in the induction of brain ischemic tolerance in rats. *J Cereb Blood Flow Metab* **27**:1352-1368.

Zhao H (2007) The protective effect of ischemic postconditioning against ischemic injury: from the heart to the brain. *J Neuroimmune Pharmacol* **2**:313-318.

Zhao H, Sapolsky RM and Steinberg GK (2006) Interrupting reperfusion as a stroke therapy: ischemic postconditioning reduces infarct size after focal ischemia in rats. *J Cereb Blood Flow Metab* **26**:1114-1121.

Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA and Vinten-Johansen J (2003) Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* **285**:H579-588.

Zheng S and Zuo Z (2003) Isoflurane preconditioning reduces purkinje cell death in an in vitro model of rat cerebellar ischemia. *Neuroscience* **118**:99-106.

Zheng S and Zuo Z (2005) Isoflurane preconditioning decreases glutamate receptor overactivation-induced Purkinje neuronal injury in rat cerebellar slices. *Brain Res* **1054**:143-151.

Zhuo ML, Huang Y, Liu DP and Liang CC (2005) KATP channel: relation with cell metabolism and role in the cardiovascular system. *Int J Biochem Cell Biol* **37**:751-764.

Zingman LV, Alekseev AE, Hodgson-Zingman DM and Terzic A (2007) ATP-sensitive potassium channels: metabolic sensing and cardioprotection. *J Appl Physiol* **103**:1888-1893.

Zuo Z, Wang Y and Huang Y (2006) Isoflurane preconditioning protects human neuroblastoma SH-SY5Y cells against in vitro simulated ischemia-reperfusion through the activation of extracellular signal-regulated kinases pathway. *Eur J Pharmacol* **542**:84-91.