MECHANISMS BY WHICH VOLATILE ANESTHETICS IMPROVE THE RECOVERY OF HIPPOCAMPAL SLICE CA 1 PYRAMIDAL NEURONS AFTER HYPOXIA

The School of Graduate Studies

State University of New York

Downstate Medical Center

In partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Program in Neural and Behavioral Science

April 2013

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ACKNOWLEDGEMENT

I would like to thank all those people who gave me different kinds of help and encouraged me to complete my study here.

First and foremost, I would like to express my deep gratitude to my research advisor, Dr. Ira. Kass for giving me the opportunity to do the research and providing the support, guidance, advice which are all invaluable to me throughout this research and the thesis writing. His encouragement, enthusiasm, sincerity and motivation have deeply inspired me. He has taught me the right way to do the research and taught me how to present the work to others. Those will be useful in my whole life.

I would like to thank my thesis committee members: Dr. Robert K. S. Wong, Dr. Jenny Libien, Dr. Todd C. Sacktor and Dr. Riccardo Bianchi for their encouragement, insightful comments and hard questions.

I also would like to thank all my labmates in Kass lab: Dr. Jun Wang, Dr. Susanna Popp and Dr. Beiping Lei. I am especially grateful for Dr. Jun Wang for the wonderful collaboration and for teaching me the basic techniques in the lab. Susanna, one of my best friends in Downstate, gave me so much help in both life and research.

My sincere thanks also go to Mr. Andrew Tcherepanov, Drs. Matthew Taylor Kelly, Dezhi Tian, Yudong Yao and Wangfa Zhao. They all gave me a lot of technical help during my research. Without their help, I could not complete my thesis research. Whenever I need any kind of help, Andrew always said ok with smile. Dear Andrew, thank you very much!

I would like to thank Dr. James Cottrell for his suggestions and ideas concerning

the research, and thank Brooklyn Anesthesia Research for the financial support.

I would like to thank Dr. Mark G. Stewart, Dr. Susan Schwartz-Giblin and Dr. Peter Bergold for offering a wonderful study and research environment to us. And I also thank all the other staff of the Graduate School, Ed Throckmorton, Ellen Telesca, Denise Shears and Sharon Reid-Spencer for their kind help during my graduate study.

Last, but not least, I would like to express my profound gratitude from my deepest heart to my beloved parents, husband, and my two kids Kevin and Sarah for their love and continuous support – both spiritually and materially. I especially want to thank my parents-in-law for their help taking care of my little Sarah during the thesis writing.

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ABSTRACT

Isoflurane and sevoflurane are two volatile anesthetics that are commonly used in neurologic and cardiac surgery. Isoflurane is a more potent anesthetic and its maximal clinical concentration is approximately 2%; the equivalent concentration for sevoflurane is 4%. We studied the effects of isoflurane and sevoflurane on cornus ammonis 1(CA1) pyramidal cells in rat hippocampal slices subjected to 5 minutes or 10 minutes of hypoxia (95% nitrogen, 5% carbon dioxide). Both isoflurane (2% and 4%) and sevoflurane (2% and 4%) attenuated the changes in the intracellular concentrations of ATP, K^+ and Na^+ caused by the hypoxic insult, however only an absolute concentration of 4% for each agent attenuated the rise in intracellular Ca^{2+}. The effect of these concentrations of isoflurane and sevoflurane was not different for Na^+, K^+ or ATP concentrations at 10 minutes of hypoxia, the only difference at 5 minutes of hypoxia was that ATP was better maintained with 4% sevoflurane (2.2 vs. 1.3 nmol/mg). If the same absolute concentration (4%) of isoflurane and sevoflurane is compared then the cellular changes during hypoxia are similar for both agents and they both improve recovery. However if the same anesthetic potency and maximal clinical dose of sevoflurane (4%) and isoflurane (2%) are compared then sevoflurane better improves recovery and better attenuates the rise in Ca^{2+}. The mechanisms of sevoflurane-induced protection include delaying and attenuating the depolarization, attenuating the increase of cytosolic calcium and delaying the fall in ATP during hypoxia. The attenuation of $Na^+$and K^+ changes during hypoxia was not different between 2% isoflurane and 4% sevoflurane treatment and is therefore not sufficient to explain the protection, although it may be required in addition to the other effects of these drugs.

Anesthetic preconditioning occurs when a volatile anesthetic, such as sevoflurane, is administered before a hypoxic or ischemic insult; this has been shown to improve neuronal recovery after the insult. We found that sevoflurane-induced preconditioning in the rat hippocampal slice enhances the expression of PKMζ and this correlated with the altered electrophysiological effects and the improved recovery in the electrophysiological experiments from our lab. PKMζ is one of the atypical protein kinase C isoforms and it is mainly expressed in brain. We found that sevoflurane induces an increase of the new protein synthesis of PKMζ, and may be necessary for preconditioning induced protection of neurons after a short hypoxic insult. An inhibitor of this kinase, zeta inhibitory peptide (ZIP), which has been previously shown to block the altered electrophysiological effects and the improved recovery, also blocked the increase in the total amount of PKMζ protein and the amount of the activated form of this kinase, phospho-PKMζ (p-PKMζ). We conclude that sevoflurane increases PKMζ protein, which is constitutively phosphorylated to its active form, this pathway is likely one of the mechanisms by which sevoflurane-induced preconditioning improves recovery.

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LIST OF ABBREVIATIONS

3-NPA 3-nitropropionic acid

4E-BP1 Eukaryotic translation initiation factor 4E-binding protein 1

4E-BP2 Eukaryotic translation initiation factor 4E-binding protein 2

5-HD 5-hydroxydecanoate

aCSF artificial cerebrospinal fluid

AD Alzheimer's disease

AMPA 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid

AMPAR 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor

ANOVA One-way analysis of variance

APC anesthetic preconditioning

APD action potential duration

ATP Adenosine-5'-triphosphate

BCIP/NBT 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium

Bcl-2 B-cell lymphoma 2

Bcl-xL B-cell lymphoma-extra large

CA1 cornus ammonis 1

CA2 cornus ammonis 1

CA3 cornus ammonis 1

CNS central neural system

DAG diacylglycerol

DG dentate gyrus

eIF-4E eukaryotic translation initiation factor 4E.

eNOS endothelial NOS

EPO Erythropoietin

EPOR Erythropoietin Receptor

ERK the extracellular signal-related kinases

GABA Gamma-Aminobutryic Acid

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GluR1 glutamate receptor subunit 1

GluR2 glutamate receptor subunit 2

GluR3 glutamate receptor subunit 3

GluR4 glutamate receptor subunit 4

HIF Hypoxia-inducible factor

iNOS immunologic NOS()

IPC ischemic preconditioning

K_{ATP} ATP-sensitive potassium channels

LTP long-term potentiation

MAC minimum alveolar concentration

MAPKs the mitogen-activated protein kinases

mitoK_{ATP} Mitochondrial adenosine triphosphate-dependent potassium channels

mTOR mammalian TOR

NFTs neurofibrillary tangles

NMDA N-Methyl-D-aspartate

NMDAR N-Methyl-D-aspartate receptor

nNOS neuronal NOS

NO nitric oxide

NOSs Nitric oxide synthases

NSF N-ethylmaleimide-sensitive factor

nucK_{ATP} nuclear adenosine triphosphate-dependent potassium channels

P-PKMζ Phosphorylated Protein kinase M ζ

PDK-1 phosphoinositide-dependent protein kinase-1

PKC Protein kinase C

PKMζ Protein kinase M ζ

PLCγ phospholipase Cγ

PS phosphatidylserine

PSD-95 postsynaptic density 95

RACK the receptor of PKC

ROS reactive oxygen species

rTPA recombinant tissue plasminogen activator therapy

sarcK_{ATP} sarcolemmal adenosine triphosphate-dependent potassium channels

TOR the target of rapamycin

TPA phorbol esters

TSC1/TSC2 Tuberous sclerosis protein 1/ Tuberous sclerosis protein 2 complex

ZIP zeta inhibitory peptide

INTRODUCTION

Hypoxia in Brain

Hypoxia occurs when the whole body or one of the tissues is deprived of an adequate oxygen supply, this can lead to tissue damage and death (Lenihan and Taylor, 2013; Lutz, 1992; Simon et al., 2008). This can occur in patients due to lung pathology that interferes with gas exchange or iatrogenic gas supply problems. There are some technically clinical terms that may lead to confusion about hypoxia, anoxia, hypoxemia and ischemia; I will define these terms here. Hypoxia is defined as a condition in which the body or a region of the body does not get adequate oxygen supply (Robergs and Keteyian, 2003); anoxia refers to the condition when the oxygen supply is deprived completely (Grant and Adams, 2009); hypoxemia is when the pressure of oxygen in blood is very low(Grant and Adams, 2009); ischemia refers to blood flow to cells and organs that is not sufficient to maintain their normal function (Asai et al., 2007). Ischemia not only reduces the supply of oxygen and glucose but leads to a buildup of toxic waste metabolites since they cannot be washed out.

During cerebral hypoxia there is a decrease of the oxygen supply to the brain that may cause cellular injury to neurons within the central nervous system (Clarkson, 2007). There are many causes of cerebral hypoxia such as drowning, strangling, choking, suffocation, cardiac arrest, head trauma, carbon monoxide poisoning, and complications of general anesthesia. Brain cells are very sensitive to oxygen deprivation because of their high metabolic rate; the vulnerability is different for different neuronal

populations(Abe et al., 1995). The pyramidal neurons in the hippocampal CA1 region die after a brief period of global brain ischemia, other neurons, such as hippocampal CA3 pyramidal neurons, are less vulnerable(Kirino, 1982; Pulsinelli et al., 1982). When hypoxia lasts longer than 5 minutes, it can lead to brain death or a persistent vegetative state. This is characterized histologically by neuronal loss that is most prominent in the hippocampus, globus pallidus, cerebellum and inferior olive (Adams et al., 1997). Brain damage can occur both during and after a hypoxic insult. In response to hypoxic/ischemic insults, neurotrophic factors may regulate neuronal death by modulating apoptosis (Banasiak et al., 2000). There are other important damaging mechanisms which will be discussed later in the introduction. Although many studies have been performed, safe clinically effective drugs and methods to reduce hypoxic neuronal death have not been established. There are no clinically accepted methods to reduce neuronal damage from hypoxia other than reducing the hypoxia before the damage is permanent. Thus, further basic scientific research in this field that could lead to neuroprotective drugs and treatments is critically needed.

Diseases such as stroke and heart attack lead to oxygen deprivation and metabolic compromise (Siddiq et al., 2007). Stroke is the fourth leading cause of death in the United States after heart disease, cancer and chronic lower respiratory diseases(Duffis et al., 2013). Stroke also causes more serious long-term disability than any other disease. There are two major types of stroke: ischemic and hemorrhagic stroke(Donnan et al., 2008). Ischemic stroke occurs when the blood supply to the brain is blocked; cells will die within minutes(Doyle et al., 2008). About 80% of all strokes are ischemic stroke(Thrift et al., 2001).

Alzheimer's disease (AD) is the most common form of dementia and is characterized by memory loss and deterioration of higher cognitive function(Bazan et al., 2002). So far there is no evidence that shows a clinical association between hypoxia and AD, however, some research groups found that hypoxia promotes the formation of Aβ, which is the primary neurotoxic element of AD, (Peers et al., 2007) and increased ROS are also associated with aging and age-related degenerative disorders not only in AD, but also in Parkinson’s disease, arthritis and stroke (Nowicki et al., 1991). Thus hypoxia may interact with other neuropathology.

Anesthetics

Persistent anesthetic protection against ischemic damage is somewhat controversial; some studies have found improved recovery only for a few days after the ischemia (Elsersy et al., 2004; Inoue et al., 2004; Kawaguchi et al., 2004; Kawaguchi et al., 2000). When these studies examined damage at time periods greater than 1 week after ischemia the anesthetics alone did not improve recovery. Others have found prolonged protection after sevoflurane anesthesia and lidocaine treatment before, during and after ischemia (Cao et al., 2005; Engelhard et al., 2004; Engelhard et al., 1999; Lei et al., 2001; Lei et al., 2002; Pape et al., 2006). Most of the previous in vivo studies used a high dose of isoflurane (4%) to induce anesthesia until the animal could be intubated. Some other studies used other volatile anesthetic agents in the control and experimental groups before ischemia and only removed the anesthetics during and after the ischemia. Both of these types of studies would cause protection from anesthetic preconditioning in the control and experimental groups.

A short exposure to volatile anesthetic before hypoxia induces neuroprotection to the brain (Anesthetic preconditioning) and this preconditioning has an acute phase and delayed phase (Wang et al., 2007a). Anesthetic preconditioning was first reported in the heart (Murry et al., 1986) and has more recently been found to protect against cerebral ischemia (Codaccioni et al., 2009). Three volatile anesthetics are commonly used in the USA: isoflurane, sevoflurane and desflurane. These anesthetics share many properties and effects, such as inducing anesthesia, reducing cerebral metabolic rate and having a cerebral vasodilating effect (Matei et al., 2002). By 2000 sevoflurane had largely replaced the use of halothane in children because of the high the risk of halothane-induced hepatitis. Recently, studies examining preconditioning with volatile anesthetics before ischemic brain injury have focused on three agents, isoflurane, sevoflurane and halothane. Among them, isoflurane is the agent which attracts the majority of attention. Isoflurane induces neuroprotection in neonatal rats(Zhao and Zuo, 2004), but not in the hippocampal slice model at clinically usable levels(Kass et al., 1989). Halothane was first synthesized in 1954 and was first used clinically in 1956(Robinson and Toledo, 2012). Because of its severe side effect on the liver, interest in it has been reduced(Robinson and Toledo, 2012). Sevoflurane(2,2,2-trifluoro-1-[trifluoromethyl]ethyl fluoromethyl ether), which was initially introduced into clinical practice in 1990(Smith et al., 1996), is a volatile anesthetic used during surgical procedures for induction and maintenance of general anesthesia. Compared to other volatile anesthetics, sevoflurane has some advantages such as low solubility and rapid onset and recovery(Sakai et al., 2005). Clinically, it has been shown that sevoflurane causes less cardiac depression and tachycardia than some of the other volatile anesthetics

(Ghatge et al., 2003). Studies examining the long-term effects of sevoflurane on neurons show that sevoflurane increases the hippocampal concentration of the anti-apoptotic proteins Bcl-2 and Mdm-2 and inhibits the ischemia-induced up regulation of the proapoptotic protein Bax (Pape et al., 2006). These features make it an ideal general anesthetic. Currently sevoflurane is used more and more in modern anesthesiology together with desflurane. Sevoflurane is a liquid at room temperature and is administered via an anesthetic vaporizer attached to the anesthetic machine in the surgical suite.

Preconditioning with sevoflurane reduced the neuronal damage in vitro after hypoxia and in vivo after global cerebral ischemia (Codaccioni et al., 2009). Previous work in our lab (SUNY Downstate Medical Center) indicated that in rat hippocampal slices subjected to 4% sevoflurane 10 minutes before, during, and 10 minutes after hypoxia, the population spike demonstrated significantly better recovery after hypoxia in the sevoflurane treated group than the control group(Matei et al., 2002; Wang et al., 2006). However the molecular mechanisms of its protection are still unknown.

The minimum alveolar concentration (MAC) is the anesthetic concentration at which 50% of subjects do not make purposeful movements in response to a painful stimulus; it is also a widely used method to measure anesthetic potency (Quasha et al., 1980). In adult rats, the MAC value for sevoflurane is 1.84–2.1% (Orliaguet et al., 2001). Sevoflurane is approximately half as potent as isoflurane (MAC = 1.07–1.18%). Concentrations of sevoflurane as high as 8% have been used clinically for the induction of anesthesia but a more common level for the maintenance of anesthesia is 3 - 4 % (Ebert and Schmid, 2009). The maximum clinically used concentration of a volatile anesthetic is generally around two times its minimal alveolar concentration (2 MAC).

Hippocampus

The hippocampus is a horseshoe shaped sheet of neurons located within the temporal lobes and adjacent to the amygdala, one on each side of the brain(Knowles, 1992). It forms a part of the limbic system. The hippocampal formation includes the dentate gyrus, the *Cornus Ammonis* fields CA1-CA3, and the subiculum (Knowles, 1992). The CA1, CA2 and CA3 fields make up the hippocampus proper (Knowles, 1992). Pyramidal neurons are the principal cells located in the CA1 region of the rat hippocampus (Mizuseki et al., 2012). The granule cells are the principal cells in the dentate gyrus which receive stimuli from entorhinal cortex through the perforant-path and send axons to the large pyramidal cells in CA3 region via the mossy fiber system(Claiborne et al., 1990). The CA3 neurons transmit to the pyramidal cells in the CA1 subfield, via the Schaffer collateral system. Clinical and experimental studies have shown that the hippocampal formation and related structures in the medial temporal lobe are important for learning and memory (Zola-Morgan and Squire, 1990).

The most famous case to show the function of the hippocampus in learning and memory is H.M. In the early 1950s, the patient H.M had his hippocampus along with some other temporal lobe tissue removed; this caused anterograde amnesia, he lost the ability to create new memories after the event, while long-term memories formed before the event remained intact(Squire, 2009). Other patients who received similar surgery kept intact memories of their childhood and the years before the injury, but formed relatively few new memories. Further studies showed that the hippocampus is important for

converting short-term memory to long-term memory, and for recalling spatial relationships in the world. The hippocampus is a very vulnerable structure in the brain.

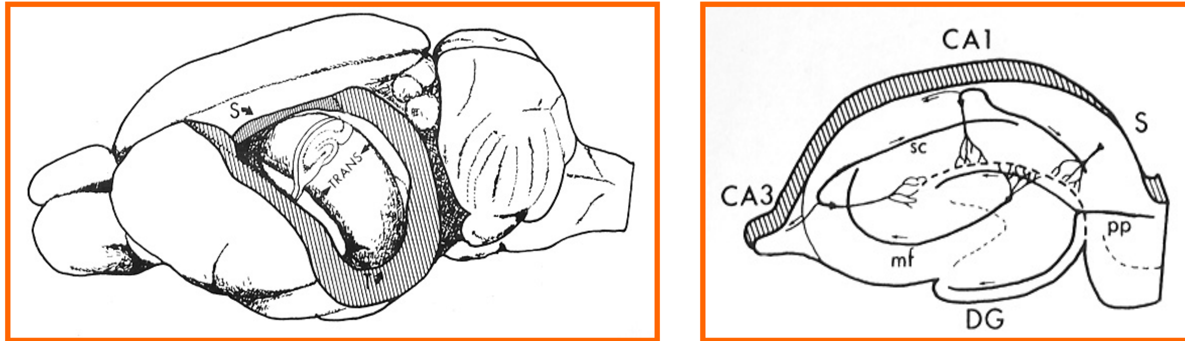


Figure 1. Brain slice containing the hippocampal formation show dentate gyrus (DG), CA3 and CA1 regions. Adapted from "The subiculum: a review of form, physiology and function", by O'Mara et al., 2001, Prog Neurobiol, 64: 129-55 (O'Mara et al., 2001).

Damage to the hippocampus is related to some neurologic and psychiatric illnesses such as Alzheimer's disease, schizophrenia, stress ,epileptic seizures etc. The relationship between anoxia/ischemia and hippocampal damage has received particular attention, especially since the 1920s (Kirino et al., 1985). The “pathoclisis theory ”of Vogt (Klatzo, 2002) believed that the selective intolerance of the hippocampus to ischemia was due to the difference in the physical or chemical characteristics; and the founders of “vascular theory” thought that the artery supplying the vasculature of the hippocampus were responsible for the hippocampal selective vulnerability problem. The selective hippocampal vulnerability to ischemia and preconditioning’s effects on it has been studied by Kitagawa et al (Kitagawa et al., 1990) who first described that a brief sublethal ischemia enhanced hippocampal neuron tolerance to subsequent more prolonged ischemia. This report drew wide attention because the protection of

hippocampal CA1 neurons afforded by their method was the most robust among neuroprotective measures ever reported (Smith et al., 1984). It is well known that the pyramidal neurons in the hippocampal CA1 region are very sensitive to ischemia/hypoxia; these neurons degenerate after ischemia of moderate duration (5 to 10 minutes), whereas CA3 and dentate gyrus neurons are preserved for longer periods of time (Bar-Am et al., 2004).

Preconditioning

Cerebral hypoxia and ischemia are important causes of death and disability, especially during surgical procedures (Wang J, 2012,J Physiology). Recent studies have found that short non-damaging ischemic episodes before a longer ischemic episode prevent the damage that would normally occur after the longer ischemia (Gidday, 2006; Malhotra et al., 2006; Roth et al., 2006). This is called ischemic preconditioning. The concept of preconditioning originated in 1986 when Dr. Murry discovered that pretreating myocardium with one or more brief episodes of ischemia protected it transiently against a sustained episode of ischemia (Marber and Yellon, 1996; Murry et al., 1986). Giving a short period of hypoxia or ischemia, which causes only minimal damage to the cell, can induce hypoxic and ischemic preconditioning protection. This is thought to activate protective mechanisms in the cell enabling it to be protected against a longer damaging period of hypoxia and/or ischemia (Chi and Karliner, 2004). However it is impossible to subject a compromised patient to a short non-damaging ischemia in order to protect them from a longer damaging ischemia; the compromised patient may have a lower threshold

for permanent neuronal damage. There also may be minor and currently unrecognized deleterious effects of these short ischemic periods (Tanay et al., 2006).

In addition to short episodes of ischemia, several preconditioning stimuli, such as hypothermia, hyperthermia, long-term hyperbaric oxygen, free radicals, toxins and cytokines, have been shown to be effective at providing neuroprotection via preconditioning (Wang et al., 2007a). There are two types of ischemic tolerance: the first, delayed tolerance (the original or classic type), requires new protein synthesis and induces neuronal protection one to three days after the preconditioning stimulus and the second, rapid ischemic tolerance, which has not previously been thought to require new protein synthesis and produces neuroprotection within 1 hour of the preconditioning event (Cadet and Krasnova, 2009). Though preconditioning stimuli, such as ischemic preconditioning, are capable of bringing about a beneficial result to the patients, even small changes in their intensity or duration can lead to serious injury. Thus, many preconditioning methods are difficult to put in clinical practice due to the dangers (such as toxins and cytokines) or the complex biological effects of the stimuli (such as hyperthermia and hypothermia) (Nishio et al., 1999).

Anesthesia is necessary for almost all kinds of surgeries. It is critical to choose an anesthetic that induces cerebral protection and subjects the patient to no additional risk, this is especially important for surgeries with a high risk of cerebral injury such as brain and thoracic surgery. Thus, identifying relatively safe and more effective anesthetics to precondition the central nervous system during the surgery will be important for potential clinical use. But it is still unclear what happens intracellularly and intercellularly during

preconditioning and following hypoxic injury and how preconditioning protects cells from death.

Mechanisms of ischemic/volatile anesthetic preconditioning

More and more studies have been focused on the mechanisms of ischemic and volatile anesthetic preconditioning in the heart and brain using different animal models. Evidence has accumulated during the past 20 years to suggest that multiple pathways are included in the protective mechanisms, these pathways involve reactive oxygen species (ROS), protein kinase C family enzymes, MAP kinases, mitochondrial K_{ATP} channels and NMDA/AMPA receptors. For delayed preconditioning, *de novo* protein synthesis is required. Each pathway is indispensable and they work together to complete the whole procedure for preconditioning protection. However, what is the relationship among these pathways and which are key for protection remains unclear.

Reactive oxygen species

Reactive oxygen species (ROS) refer to chemically-reactive molecules containing oxygen ions and peroxides with high reactivity due to the presence of unpaired valence shell electrons(Mageshwaran et al., 2012). They are normal by-products of oxygen metabolism and are over-expressed during the course of ischemia/reperfusion through a variety of mechanisms (Swamy et al., 2010). ROS can be both protective and deleterious

(Becker, 2004). Under the normal physiological condition, the tissues have an intrinsic tolerance to ROS. Large quantities of ROS induced by swelling, shrinkage and ischemia introduce tissue damage. While small quantities derived from mitochondria during a brief ischemic episode exert beneficial effects and produce preconditioning in the myocardium (Vanden Hoek et al., 1998). Free radical scavengers attenuated the protective function when they were administered during ischemic preconditioning (Baines et al., 1997). Further studies showed that isoflurane, a volatile anesthetic, induced the production of small quantities ROS and this is an essential part of the signaling pathway of anesthetic-induced preconditioning (Mullenheim et al., 2002; Tanaka et al., 2002). 3-nitropropionic acid (3-NPA), an agent that induces the production of ROS, was shown to promote tolerance to focal cerebral ischemia (Wiegand et al., 1999). The compound NS1619 induces neuronal preconditioning by increasing ROS production and mitochondrial depolarization (Gaspar et al., 2009). Pain (Pain et al., 2000) provided evidence that generation of free radicals is a trigger rather than a mediator of preconditioning-induced cardioprotection. As a consequence of K_{ATP} channel opening, the free radicals released from the mitochondria lead to a further amplified opening of K_{ATP} channels.

Mitochondrial adenosine triphosphate-dependent potassium (mitoK_{ATP}) channels

ATP-sensitive potassium channels (K_{ATP}) refer to the potassium permeable channels that open in response to low ATP levels (Du et al., 2010). These channels are composed of two types of subunits, $K_{ir}6.x$ subunits and sulfonylurea receptor (SUR)

subunits, along with additional components (Stephan et al., 2006) and are widely distributed in various cell types including pancreatic β-cells, skeletal muscle, smooth muscle, renal tubular cells, and neurons(Isomoto and Kurachi, 1997). Based on their location in cells, they are identified as sarcolemmal/plasmalemmal (sarcK_{ATP}), mitochondrial (mitoK_{ATP}), or nuclear (nucK_{ATP}). MitoK_{ATP} channels were first identified by Inoue et al in 1991 and shown to have similar characteristics to the surface K_{ATP} channels (Ardehali and O'Rourke, 2005). These channels are localized in the inner mitochondrial membrane in several tissues and brain mitochondria contain several times more mitoK_{ATP} channels than liver or heart mitochondria (Correia et al., 2010); this shows the importance of this channel in the brain . Diazoxide, a selective opener of mitoK_{ATP} channel decreases neuronal apoptosis and increases astrocyte survival and activation in the penumbral region of the ischemic cortex; this was abolished by 5-hydroxydecanoate (5-HD), a blocker of mitoK_{ATP} channels (Liu et al., 2002). Studies of cardiac myocytes showed that mitoK_{ATP} channels play a central role in ischemic preconditioning through oxygen radical production and activation of protein kinase C(Pain et al., 2000). The activation of mitoK_{ATP} channels seems to be a key event that elicits neuroprotection by preconditioning in the brain (Correia et al., 2010). One research group proposed the mechanism of the cell's K_{ATP} channel's reaction to hypoxia and ischemia; low levels of intracellular oxygen decrease the rate of metabolism and the intracellular NAD+/NADH ratio, block the electron transfer and activate phosphotidylinositol-3-kinase and extracellular signal-regulated kinases(Crawford et al., 2003). 5-HD administration before and during the preconditioning blocked the preconditioning protection induced by sevoflurane (Data unpublished from Dr. Kass'

lab). Raval and colleagues reported that opening of mitoK_{ATP} channels causes the influx of potassium to the mitochondrial inner matrix, which then induces the generation and release of ROS from the respiratory chain. ROS activates phospholipase C and PKC, which, in turn, might amplify the preconditioning stimulus (Raval et al., 2007).

Nitric oxide synthase

Nitric oxide synthases (NOSs) are a family of enzymes that catalyze the formation of nitric oxide (NO) from L-arginine (Weaver et al., 2002). There are three NOS isoforms that are named after the tissues from which they were first identified: neuronal NOS(nNOS), endothelial NOS(eNOS) and immunologic NOS(iNOS) (Parathath et al., 2007). nNOS plays a prominent role in the early stage of neuronal injury after cerebral ischemia; eNOS plays a role in the later stages of neuronal injury after cerebral ischemia, while iNOS plays a protective role in cerebral ischemia by maintaining cerebral blood flow (Samdani et al., 1997). NO is an important physiological messenger in the central nervous system, having a vital role in many biological processes such as cerebral blood flow and memory. All of these enzymes play a role in the neuronal injury after cerebral ischemia though they work on different functions. Under certain pathological condition, excessive activation of nNOS forms abundant NO that can induce mitochondrial dysfunction and cell death (Bolanos and Almeida, 1999). Evidence from transgenic mice showed that nNOS knockout mice are less vulnerable to hippocampal neuronal damage after transient or permanent ischemia in the MCA occlusion model (Huang et al., 1994), while eNOS-transgenic mice are highly vulnerable to cerebral ischemia (Lo et al., 1996).

Free cytosolic Ca^{2+} regulates the activity of nNOS through its interaction with calmodulin. When the concentration of free cytosolic[Ca^{2+}] released from intracellular stores were above 400nM, binding of calmodulin to nNOS activates the enzyme. When the concentration of Ca^{2+} decreased, the binding of calmodulin to nNOS was dissociated and the enzyme reduced its activity. Cerebral ischemia causes an excessive release of glutamate and subsequent activation of NMDA receptors which induces a massive influx of Ca^{2+} into the postsynaptic neuron and then triggers the activation of nNOS and overproduction of NO (Esplugues, 2002). Inhibition of NOS induces neuroprotection in animal models of stroke (Nowicki et al., 1991). The expression of iNOS was induced by inflammation and transient ischemia in various populations of cerebral cells. It is said that NO produced by iNOS exerts a detrimental effect on the ischemic brain, contributing to the progression of tissue damage and exacerbating glutamate neurotoxicity (Chao et al., 1992). Studies with iNOS knockout mice have confirmed the induction of iNOS contributes to the delayed neuronal damage following ischemia (Iadecola, 1997).

Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone produced by the kidney and liver, which controls red blood cell production. Both EPO and its receptor EPOR are expressed in the nervous system of rodents, primates and humans(Chong et al., 2002). Hypoxia induced the accumulation of EPO and EPOR in cultured hippocampal neurons (Lewczuk et al., 2000). Other evidence from in vivo animal models also demonstrated upregulation of EPO during the hypoxia. This upregulation is dependent on the hypoxia-inducible

factor 1 (HIF-1), a heterodimeric transcription factor containing two subunits, HIF-1 α and HIF-1β. Erythropoietin plays an important role in the brain's response to neuronal injury based on results from a number of independent groups. Administration of EPO (0.3units/ml) protects hippocampal cultures against hypoxia-induced neuronal death through activation of extracellular signal-regulated kinases ERK1, ERK2 and protein kinase Akt-1/protein kinase B (Siren et al., 2001). It was shown that the increase of intracellular calcium concentration induced by EPO is indicative of EPO's neuroprotective role after central neural system (CNS) related hypoxia or ischemia (Morishita et al., 1997).

Erythropoietin regulates the activity of Bcl-2(B-cell lymphoma 2) family proteins that modulate the induction of apoptosis and prevent the neuronal damage caused by ischemia/hypoxia. Administration of EPO induces the expression of antiapoptotic members Bcl-2 and Bcl-x_L through STAT5 activation (Klampfer et al., 1999). It is said that the MAPKs (the mitogen-activated protein kinases) were also involved in the protective mechanisms of EPO. EPO phosphorylates and increases the activity of ERK (the extracellular signal-related kinases, one of the MAPKs components) which block the proapoptotic pathways (Nagata and Todokoro, 1999).

mTOR pathway

The target of rapamycin (TOR), a conserved Ser/Thr kinase, was initially identified in the budding yeast *Saccharomyces cerevisiae* that was specifically inhibited by rapamycin (Heitman et al., 1991). Studies showed that TOR in yeast regulates cell

growth both in time and in space. The mammalian TOR (mTOR) is a 289-kDa-serine/threonine protein kinase that is a central regulator of protein synthesis whose activity is modulated by a variety of signals as stress and nutrient deprivation. Tang identified mTOR and its downstream targets or effectors, including eIF-4E, 4E-BP1, and 4E-BP2 in the adult rat hippocampal lysates (Tang et al., 2002). Their work also showed that the mTOR translational signaling pathway controlled de novo protein synthesis during late LTP. Cracco found that rapid activation of the mTOR pathway during LTP leads to new protein synthesis of PKMζ in synapses (Cracco et al., 2005). Disregulation of this pathway has been implicated as leading to neuronal damage (Hoeffer and Klann, 2010).

Hypoxia inhibits the activity of mTOR, though the mechanism of this is unclear. It is thought that mTOR inhibition by hypoxia requires the TSC1/TSC2 tumor suppressor complex and the hypoxia-inducible gene REDD1/RTP801(Brugarolas et al., 2004). Disruption of the TSC1/TSC2 complex through loss of TSC1 or TSC2 blocks the effects of hypoxia on mTOR, as measured by changes in the mTOR targets S6K and 4E-BP1, and results in abnormal accumulation of Hypoxia-inducible factor (HIF) (Brugarolas et al., 2004)，a transcriptional factor that contributes to cell adaption and survival in hypoxic environments and regulates oxygen homeostasis (Benizri et al., 2008) .

Protein kinase C

Protein kinase C (PKC) is a family of protein kinases consisting of ~10 isozymes. Based on their second messenger requirements, they are divided into three subfamilies: conventional (or classical), novel, and atypical(Sokolova O, Vieth M, Naumann M. 2012).

Conventional (c)PKCs are Ca^{2+} dependent via their C2 domains and are activated by phosphatidylserine (PS), diacylglycerol (DAG) and phorbol esters (TPA or PMA) through their cysteine-rich C1 domains. Novel PKCs are Ca^{2+} independent but are still regulated by PS, DAG and TPA. Atypical PKCs are Ca^{2+}independent and do not require PS, DAG or TPA for their activation (Salamanca & Khalil, 2005). The term "protein kinase C" usually refers to the entire family of isoforms.

Protein kinase C is a ubiquitous enzyme found in a variety of mammalian tissues and is especially highly enriched in brain and lymphoid organs (Yoshida et al., 1988). Members of PKC family are a single polypeptide including an N-terminal regulatory region (approximately 20-40 kDa) and a C-terminal catalytic region (approximately 45 kDa). There are four conserved domains: C1-C4. Each domain is a functional module. Biochemical and mutational analysis established the function of the domains: the C1 domain contains a Cys-rich motif that forms the diacylglycerol/phorbol ester binding site (Figure 2); this domain is immediately preceded by an autoinhibitory pseudosubstrate sequence; the C2 domain contains the acidic lipid recognition site and the Ca-binding site in some isozymes. The C3 and C4 domains form the ATP- and substrate-binding lobes of the kinase core (Newton, 1995).

Protein kinase C is an important component of the signal transduction pathway used by cell to respond to a variety of extracellular stimuli(Nishizuka, 1992). More and more evidence shows its importance on regulation of contractile function, gene expression, and /or tolerance to ischemia in cardiac tissue(Steinberg et al., 1995). Studies also have indicated that the activation and phosphorylation of several PKC isoforms, such as the novel PKC epsilon, contribute to protection in the heart and in the hippocampal

slice after ischemic preconditioning (Jia et al., 2007). PKC is activated by translocating from the cytosolic fraction of the cell to membrane (Kraft and Anderson, 1983a).

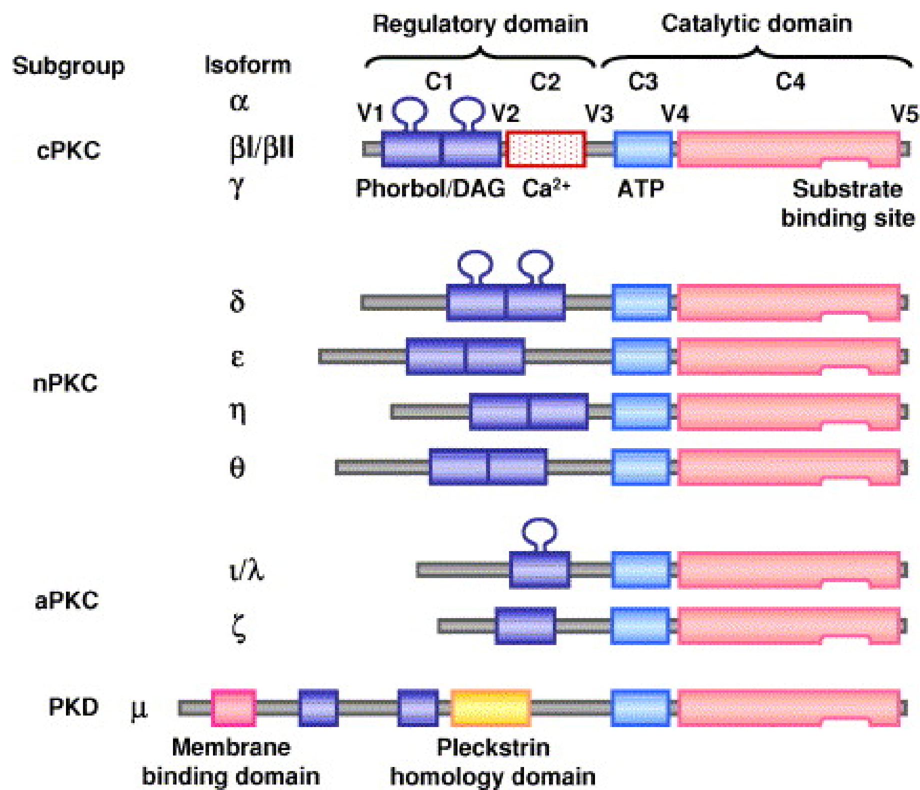


Figure 2. Structure of PKC isoforms. PKC is composed of four conserved (C1–C4) and five variable (V1–V5) regions. C1 region contains binding sites for DAG, phorbol ester, phosphatidylserine and the PKC antagonist calphostin C. C2 region contain the binding site for Ca^{2+}. C3 and C4 regions contain binding sites for ATP, some PKC antagonists and different PKC substrates. The PKC molecule folds to bring the ATP binding site into proximity with the substrate-binding site. Binding of an endogenous or exogenous pseudosubstrate peptide sequence to the catalytic domain prevents PKC from phosphorylating the true substrate. Figure and legend are adapted from" Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension" by Salamanca and Khalil, 2005, Biochem Pharmacol, 70: 1537-47. (Salamanca and Khalil, 2005).

PKC and volatile anesthetic precondition

Volatile anesthetic agent exposure before the ischemic period increased the resistance of the tissue to a subsequent insult; this is defined as anesthetic preconditioning (APC). It is a cellular protective mechanism and has been found in cardiac and neurologic tissue (Zvara et al., 2006). Bhardwaj and colleagues demonstrated that rats exposed (less than one hour) to halothane immediately before focal ischemia had a reduced cerebral infarction (Bhardwaj et al., 2001). Kapinya and colleagues exposed rats to 3h of 1 MAC isoflurane anesthesia and subsequently subjected them to focal ischemia immediately, 12 h or 24 h post-isoflurane exposure. The results showed that isoflurane reduced cerebral infarction in animals (Kapinya et al., 2002).

Some research findings show that the cellular and molecular mechanisms of APC appear to mimic those of ischemic preconditioning (IPC) (Stadnicka et al., 2007). Evidence from cardiac tissue indicates that IPC involves a complex cascade of intracellular events, beginning with receptor activation by an ischemic stimulus (Figure3). Ischemic injury stimulates some sarcolemmal receptors (e.g. adenosine A1, [alpha]-adrenergic, muscarinic M2, and bradykinin B2), and then activates G proteins (Gi/o). The G proteins activate the adenosine triphosphate-regulated potassium channel (K_{ATP}) channel or stimulate PKC directly. PKC subsequently phosphorylates K_{ATP} channels (Toller et al., 2000) and other protein kinases. Opening of sarcolemmal K_{ATP} channels enhances potassium efflux and decreases intracellular calcium content and ATP is preserved. Opening of mitochondrial K_{ATP} channels increases mitochondrial potassium influx, reduces the calcium concentration in mitochondria, and causes matrix swelling.

These actions decrease ATP synthesis and accelerate mitochondrial respiration (Toller et al., 2000).

In both neuronal and heart tissue PKC activation has been implicated as a factor for ischemic preconditioning. In the cardiac tissue, activation of PKCδ during reperfusion induces cell death through the regulation of mitochondrial function and induction of apoptosis and necrosis; in contrast, activation of PKCε before ischemia protects mitochondrial function and diminishes apoptosis (Churchill and Mochly-Rosen, 2007). To determine whether PKCε was also involved during NMDA-induced preconditioning, Raval et al (Raval et al., 2003) administered the PKCε inhibitor (εV1-2 peptide, 0.02 μM) to hippocampal organotypic slices during the NMDA preconditioning treatment. This treatment resulted in significant inhibition of protection compared with IPC and NMDA preconditioning.

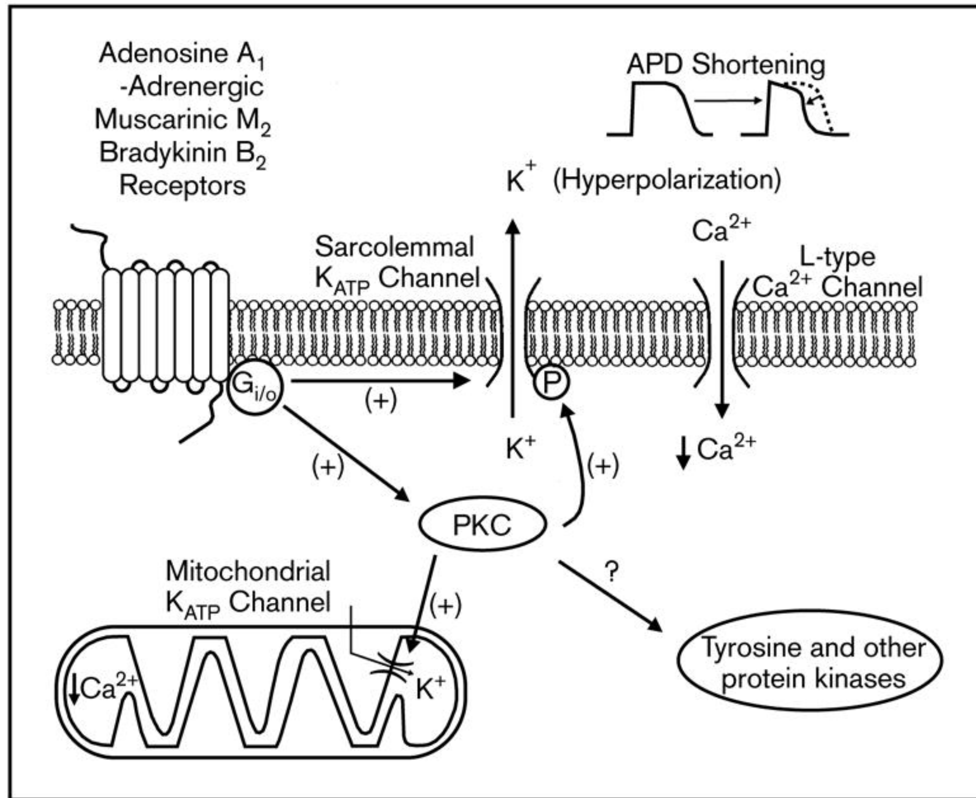


Figure 3. Illustration of the proposed signal transduction pathway for ischemic preconditioning in heart. Ischemia stimulates sarcolemmal receptors, activates G proteins that either directly activate the adenosine triphosphate-regulated potassium channel (K_{ATP}) or stimulate protein kinase C (PKC). PKC subsequently activates other protein kinases and phosphorylates sarcolemmal and mitochondrial K_{ATP} channels. Openings of sarcolemmal K_{ATP} channels result in enhanced potassium efflux during phase 3 of the action potential and membrane hyperpolarization, shortening of action potential duration (APD), and blockade of L-type calcium channels. Thus, intracellular calcium content decreases and ATP is preserved. Opening of mitochondrial K_{ATP} channels enhances mitochondrial potassium influx and results in depolarization of the mitochondrial membrane and reduction of intramitochondrial calcium, and causes matrix swelling. These actions decrease ATP synthesis and accelerate mitochondrial respiration. Figure and legend are adapted from " Ischemic preconditioning, myocardial stunning and anesthesia" by Toller et al., 2000, Curr Opin Anaesthesiol, 13: 35-40. (Toller et al, 2000).

NMDA receptor and AMPA receptor

Glutamate is one of the key excitatory neurotransmitters in the central nervous system that can activate both ion-channel-forming (ionotropic) and G-protein-coupled (metabotropic) glutamate receptors (GluRs)(Nusser, 2000). NMDA receptors and AMPA receptors are the two major ionotropic glutamine receptors, which play important roles in synaptic plasticity and synaptic transmission (Voglis and Tavernarakis, 2006).

The NMDA receptor is a heterotetramer ion channel made of two obligatory NR1 subunits and two other subunits from NR2 subfamily (2A, 2B, 2C and 2D) or NR3 subfamily (3A or 3B) (Ulbrich and Isacoff, 2008). The extracellular domain contains a modulatory domain and a ligand-binding domain binding glycine and glutamate respectively. The membrane domain contributes residues to the channel pore and is responsible for calcium permeability and voltage-dependent magnesium block. The cytoplasmic domain contain protein kinase interaction sites and protein phosphatase interaction sites , as well as adaptor, and scaffolding protein binding sites. Studies with electron microscopic autoradiography and conventional immunocytochemistry show that the majority of NMDA receptors are present at glutamatergic synaptic junctions (Nusser, 2000). NMDA receptors are associated with the influx of Ca^{2+} and increased excitability post-synaptically (Purves et al., 2001). Activation of NMDA receptors results in the opening of an ion channel that is permeable to Na^{+}, K^{+} and Ca^{2+}. During LTP, the increase of the PKMζ level began 10 min after the tetanic stimulation and persisted for at least 2 hours, paralleling the persistence of LTP. During the maintenance phase of LTD, the level of cytosolic PKMζ was reduced. NMDA-receptor antagonists blocked the loss

of cytosolic PKMζ during the LTD (Hrabetova and Sacktor, 1996), and the increase of PKMζ during LTP (Osten et al., 1996). It is reported that NMDA receptor antagonists also alter the development of ischemic tolerance induced by brief repeated ischemic insults in gerbil brains (Kato et al., 1992), by oxygen glucose deprivation in mouse cortical cultures (Grabb and Choi, 1999), and by hypoxia induced damage in hippocampal slices (Cadet and Krasnova, 2009; Kasischke et al., 1996). The activation of protein kinase C is associated with potentiation of NMDA receptor function in hippocampal neurons largely through an increase in the probability of channel opening (Xiong et al., 1998). Intracellular application of PKM to both cultured and isolated CA1 hippocampal neurons potentiated NMDA-evoked currents (Xiong et al., 1998). PKMζ was found to be critical for the maintenance of the late phase of long-term potentiation (LTP) (Yao et al., 2008). PKMζ inhibitory peptide (ZIP) blocks the electrophysiological changes and improved neuronal recovery during and after hypoxia induced by 4% sevoflurane preconditioning (Wang et al., 2007b). Studies from in vitro and in vivo brain ischemia models showed that ischemic preconditioning require activation of NMDA receptors (Lange-Asschenfeldt et al., 2004). The NMDA receptor is involved in both ischemic neuronal injury and ischemic tolerance (Grabb and Choi, 1999). The noncompetitive NMDA receptor antagonist dizocilpine (MK-801) attenuates ischemic cell death and blocks the development of ischemic tolerance in gerbils and in cultured cortical neurons (Chen et al., 2008).

The AMPA receptors mediate fast excitatory synaptic neurotransmission in the central nervous system (Santos et al., 2012). This heterotetrameric receptor includes four types of subunits GluR1, GluR2, GluR3 and GluR4 (Dingledine et al., 1999). The second

membranous domains from the four subunits form the ion-permeable pore of the receptor. It has been shown that AMPA receptors play an integral role in the process of LTP and phosphorylation by protein kinases regulates its localization and function for LTP. LTP has two necessary components: presynaptic glutamate release and postsynaptic depolarization. When the presynaptic cells were stimulated, glutamate is released and induces depolarization of the postsynaptic cells by activating AMPA receptors. During LTP maintenance, PKMζ regulates the AMPAR trafficking to favor receptor insertion into postsynaptic site by modifying the interaction of *N*-ethylmaleimide-sensitive factor (NSF) and glutamate receptor subunit 2 (GluR2) (Yao et al., 2008). Further work found that the activity of PKMζ blocked the GluR2-dependent pathway that removes the postsynaptic AMPA receptors to persistently promote increased levels of GluR2-containing AMPA receptors at postsynaptic sites (Migues et al., 2010). This is likely the mechanism by which PKMζ maintains LTP.

mGlutamate Receptors

Another class of Glutamate receptors is the metabotropic receptors (mGluR). mGluRs are a family of G protein-coupled receptors that are widely distributed within the nervous system (Ferraguti and Shigemoto, 2006). They are around eight genes that encode the different subtypes of mGluRs (Pin and Duvoisin, 1995). Based on their sequence homology, G-protein coupling and ligand activity, mGluRs are divided into three subfamilies: mGluR group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3) and Group III (mGluR4, 6, 7 and 8) (Niswender and Conn, 2010). It was shown that activation of each group of the mGluR system offers cytoprotection for the

cerebrovascular endothelial cells against anoxia injury (Lin and Maiese, 2001). During ischemia and hypoxia, the K_{ATP} channel is activated by the fall of intracellular [ATP]. The activity of K_{ATP} channel was differently regulated by the group I and group II mGluRs; the agonists of group I mGluRs ((S)-3,5-dihydrophenylglycine (DHPG), trans-azetidine-2,4-dicarboxylic acid (t-ADA)) inhibited the activiation of K_{ATP} channel, and the agonists of group II mGluRs (N-acetyl-l-aspartyl-l-glutamic acid (NAAG), (2S,1′S,2′S)-2-carboxycylcopropylglycine (CCG-I)) enhancedits activity (Mironov and Richter, 2000). The selective agonist of group III mGluRs (R, S)-4-phosphonophenylglycine [(R, S)-PPG] increased the recovery of neurons in brain tissue from adult animals after hypoxic or hypoglycaemic insults (Sabelhaus et al., 2000).

Protein kinase M zeta

Protein kinase M was originally purified from bovine cerebellum. The name of this kinase came from its dependence on the Mg^{2+} (Nishizuka, 2003). The enzyme was neither inhibited by protein inhibitor (75 mM Mg^{2+}) nor by the regulatory subunit of cyclic AMP-dependent protein kinase (Takai et al., 1977). At that time, research from the same lab supposed that the enzyme was produced from its precursor protein by a limited proteolytic reaction (Inoue et al., 1977). In 1987, a cyclic AMP and calcium-independent protein kinase had been identified and purified from pig brain that was isolated in an inactive form, and its activation required ATP and Mg^{2+} (Yang et al., 1987). This was the first discovery of the PKM in the mammalian animals. Although some isozymes of PKM

were detected in the brain like PKMα, PKMβI and PKMδ in minor amounts, PKMζ was found to distribute at higher concentrations in rostral structures, such as cerebral cortex, striatum, and hippocampus, and lower levels in caudal structures, cerebellum, and medulla (Naik et al., 2000).

PKCζ is an atypical form of PKCs, its gene has two promoters. One promoter from the exons encodes the full-length protein kinase Cζ including a regulatory domain and a catalytic domain. In the brain, an internal promoter within the protein kinase C, zeta gene (*PRKCZ)* produces a PKMζ mRNA that encodes a ζ catalytic domain without a regulatory domain (Hernandez et al., 2003). The unusual structure of this kinase endows it with a unique feature that makes it independent of second messengers and it is a constitutively active isoform of PKC. However, in Aplysia, PKM is formed by the cleavage of PKC induced by calcium induced calpain activation; calpain inhibitors such as calpain inhibitor V (Bougie et al., 2009) block this. PKMζ is specially expressed in neural tissue and is the main form of PKCζ in the hippocampus in rodent. The constitutive activity of this kinase is caused by its lack of the regulatory domain's autoinhibition and therefore leads to its continual phosphorylation of downstream proteins. This perpetuates the maintenance of long-term potentiation (LTP) and thereby the long-term memory traces (Sacktor, 2011). Tian et al found that the total level of the PKM ζ and phosphorylated-PKMζ (Thr560) significantly increased 24 hours following IPC in the hippocampus, suggesting that levels of active PKM ζ increased following IPC at time when neurons were protected (Tian et al., 2008). Recently it has been found that PKMζ is critical for the maintenance of late LTP in hippocampal slices(Ling et al., 2002) (Bliss et al., 2006). Inhibition of PKMζ by zeta inhibitory protein (ZIP), a selective

membrane-permeant peptide that minics the autoregulatory domain of PKMζ and thus acts as an inhibitor (Bliss et al., 2006), erased long-term memory (Pastalkova et al., 2006); ZIP also blocks the electrophysiological changes and the improved neuronal recovery during and after hypoxia induced by 4% Sevoflurane preconditioning (Wang et al., 2007b).

A physiological action of PKCζ is to mediate the insulin-stimulated transport of glucose (Bandyopadhyay et al., 2002). PKM ζ appears necessary for the maintanance the late phase of LTP (Ling et al., 2002). Overexpression of protein kinase Mζ enhances consolidated long-term memory, even memory formed long before the enzyme was overexpressed in the Neocortex of rat (Shema et al., 2011). In primary neuronal cultures, the overexpression of protein kinase Mζ affects the morphology and function of cortical neurons (Ron et al., 2012); this may be one of the mechanisms by which it allows for the maintenance of long-term memory.

PKMζ activity was recently found in the spinal dorsal horn, which has similar mechanisms as the brain for LTP. The maintenance of persistent nociceptive sensitization requires the activity of PKMζ (Asiedu et al., 2011). In the central visual pathway, phospholipase Cγ (PLCγ) and PKMζ mediate the process of palmitoylation of postsynaptic density 95(PSD-95) by regulating of BDNF/TrkB pathway (Yoshii et al., 2011). The palmitoylation of PSD-95 is necessary for the rapid movement of PSD-95 from visual neuron soma to the synapse and it is triggered by opening of the eye. Alzheimer's disease (AD) is the most common form of dementia and causes many social problems in the world. More and more governmental and non-governmental funds are used to find a cure for this disease. PKMζ accumulated in a subset of neurofibrillary

tangles (NFTs) restricted to limbic or medial temporal lobe structures (i.e. hippocampal formation, entorhinal cortex, and amygdala) in the patients with AD (Crary et al., 2006). It is suggested that aggregation of PKMζ disrupts glutamatergic synaptic transmission and this may contribute to the memory impairment in AD(Crary et al., 2006). Understanding the mechanisms of PKMζ aggregation in AD might lead to better treatment and prevention of this disorder.

Following ischemic preconditioning (IPC), the level of PKMζ was increased to a similar degree as during LTP(Tian et al., 2008). It is known that IPC protects neurons from a subsequent lethal ischemic insult. The effect of PKMζ on Na/K ATPase activity has been shown to play a role in IPC neuroprotection (Tian et al., 2008).

Electrophysiological recovery

Concurrent electrophysiological results by my colleagues in the lab showed that untreated neurons demonstrate a hyperpolarization of the membrane potential after the onset of hypoxia; this is followed by a slow depolarization and then a rapid and complete depolarization. In slices subjected to 10 minutes of hypoxia without sevoflurane treatment none of the CA1 pyramidal cells recovered their resting or action potentials after hypoxia. When 4% sevoflurane is present for 15 minutes followed by a 5 minutes washout before hypoxia, 90% of the neurons recovered their resting and action potentials 1 hour after the hypoxia (Figure 4; Wang et al. 2007). This evidence indicates that sevoflurane improves recovery even if it is not present during or after the hypoxia. Chelerythrine, a protein kinase C inhibitor, when present 10 minutes before and during

the sevoflurane application, significantly attenuated the sevoflurane induced improvement of recovery to 10%. Sevoflurane-induced preconditioning enhances the hypoxic hyperpolarization (6.2 vs. 3.1mV), delays and attenuates the hypoxic depolarization (–36 vs. –0.4mV), and increases the number of CA1 pyramidal neurons in the rat hippocampal slice that recover their resting and action potentials after hypoxia (88 vs. 0%). The selective inhibitor of this kinase, ZIP, blocked the altered electrophysiological effects and the improved recovery (0 vs 88%). The electrophysiological changes and the improved recovery due to sevoflurane-induced preconditioning were also blocked by rapamycin and cyclohexamide (0, 0 vs 83%) (Wang et al. 2012). The results support the hypothesis that sevoflurane induces activation of the mTOR mitogenic pathway increasing the new protein synthesis of PKMζ which is constitutively phosphorylated to its active form. The increased pPKMζ enhances the hypoxic hyperpolarization, delays and attenuates the hypoxic depolarization thereby improving the recovery of neurons following hypoxia. Thus sevoflurane may be acting via a metabotropic pathway to improve recovery following hypoxia.

My current work focuses on two aims. The first aim is to compare effective concentrations of isoflurane and sevoflurane for protection against hypoxia-induced damage and to examine the mechanisms by which these volatile anesthetics may protect against neuronal damage. The second aim is to measure the translocation and phosphorylation of PKC isoforms and PKMζ to see if the PKC/PKM pathway is involved in the sevoflurane-induced preconditioning. Based on this work, we will obtain more evidence to explain the mechanisms of anesthetic protection against hypoxic injury to neurons.

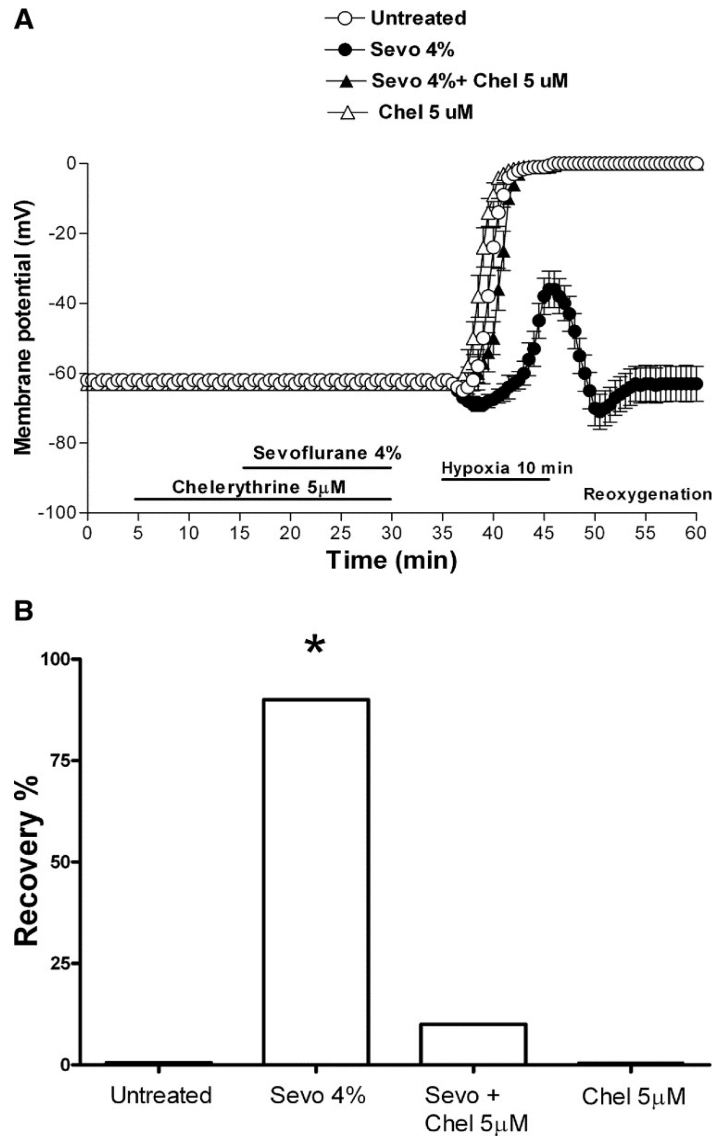


Figure 4. Electrophysiological changes after hypoxia *in vitro*. Intracellular recordings were made from CA1 pyramidal cells in rat hippocampal slices. The slices were subjected to 10 min of hypoxia and either untreated, preconditioned with 4% sevoflurane for 15 min, treated with sevoflurane preconditioning and chelerythrine (5 μM) or subjected to chelerythrine alone. (**A**) The mean membrane potential (±standard error of the mean) is plotted during the experiment. (**B**) The number of CA1 pyramidal neurons that recover their resting and action potentials after hypoxia; sevoflurane was significantly different (*$P<0.05$) from the untreated, sevoflurane plus chelerythrine or chelerythrine alone. Figure and legend are adapted from "Metabotropic actions of the volatile anesthetic sevoflurane increase protein kinase M synthesis and induce immediate preconditioning protection of rat hippocampal slices" by Wang et al., 2007, Neuroscience, **145:** 1097-1107. (Wang et al., 2007).

CHAPTER 1

ANESTHETICS BEFORE AND DURING HYPOXIA

<u>Specific aims:</u> *Examine the effect of sevoflurane and isoflurane treatment before and during hypoxia on rat hipppocampal CA1 neurons after a short period of hypoxia*.

During hypoxia and ischemia, the fall of intracellular ATP concentrations induce the opening of K_{ATP} channels on the cell membrane that will directly change the flux of K^+ ions and indirectly affect Na^+ and Ca^{2+}. At the same time, the fall of intracellular ATP deceases the activity of Na/K ATPase that is the major pathway to maintain the normal intracellular Na^+ and K^+ levels. We measure the effect of sevoflurane and isoflurane treatment before and during hypoxia on the changes of the ions and ATP concentration to evaluate the mechanisms by which anesthetics may improve the recovery of neurons from hypoxia.

<u>Methods</u>

Water-bath experiments for the assay of ATP, sodium and potassium

All the animal experiments were carried out in accordance with the National Institute of Health Guide on the Care and Use of Laboratory Animals and were approved by the

Institutional Animal Care and Use Committee of SUNY Downstate. Male Sprague–Dawley rats (100–120 days old) were anesthetized with 2% isoflurane for 2 min, decapitated, and their brains were quickly removed and placed into chilled (2–4 °C) artificial cerebrospinal fluid (aCSF) (in mmol/L: NaCl, 126; KCl, 3; KH_2PO_4, 1.4; $NaHCO_3$, 26; $MgSO_4$, 1.3; $CaCl_2$, 1.4; glucose 4; pH 7.4). We sectioned the hippocampus into 500μm slices using a Stoelting tissue chopper with a microtome-advance stage. The slices were kept at 4° C in oxygenated aCSF solution. Slices from the same rat were placed on each grid for an experiment. This method allowed us to control the variability between animals by directly comparing the response in normoxic, hypoxic and drug-treated slices from the same animals. Each grid was placed in the 20-ml beaker filled with 15ml standard aCSF that was aerated with 95% O_2 and 5%CO_2 and the beakers were covered with rubber stoppers. After 30minutes of incubation at room temperature in the water-bath, the heater was turned on and the water-bath was allowed to slowly heat up to 37°C (it takes 30 min to come up to temperature). After 2 hours of incubation, the slices were subjected to anesthetics for 10 minutes and then 5 or 10minutes of hypoxia. Sevoflurane or isoflurane was added to the gas mixture using a calibrated agent specific vaporizer (Penlon Ltd., Abingdon, UK) before it aerated the aCSF. The control slices were treated identically except that sevoflurane or isoflurane was not administered. The anesthetic- treated groups received 10 minutes of anesthetic treatment, then 5 or 10 minutes of hypoxia with anesthetic. The untreated group received 10 minutes without anesthetic, then 5 or 10 minutes of hypoxia.

ATP measurement

For the ATP assay, grids containing the slices were removed from the vials at the end of the water-bath experiments and immediately frozen in liquid N_2. The slices from the different groups (normoxia, hypoxia, 2/4% isoflurane treated groups and 2/4% sevoflurane treated groups) were then lyophilized, the CA1 area including the region from the stratum radiatum to the alveus including the CA1 pyramidal cell layer were dissected and weighed (dry weight). ATP was extracted from the hippocampal tissue by homogenizing in 3N ice- cold perchloric acid and measured, after neutralization, using the firefly luciferin-luciferase assay (Kass et al., 1992).

Sodium and potassium measurements

To measure sodium and potassium concentrations, at the end of the experiment the grids were removed from the vials and placed in a beaker containing ice-cold isotonic sucrose for 10min in order to wash ions from the extracellular space.

The slices were then removed from the grid, and the CA1 regions of the slices were micro dissected. CA1 regions from different slices of the same grid/beaker were pooled, so that there was enough tissue, to measure sodium and potassium and then placed in a pre-weighed microtube, weighed, dried at 85 °C for 48 hours and re-weighed. After dilute nitric acid (0.1 N) was added, the tissue was shaken for 18hours and the supernatant assayed in a flame photometer. Ten minutes in the sucrose solution permitted the washout of sodium and potassium from the extracellular space with only a small

effect on intracellular sodium and potassium levels. The extract was assayed in a flame photometer (Amorim et al., 1999; Kass et al., 1992).

Calcium imaging

We sectioned the hippocampus into 300µm slices using a vibratome with chilled aCSF (4-6°C). Slices were placed in a small beaker containing 6 ml aCSF, 9 umol/l Fura-2 AM (Molecular Probes; Eugene, OR, USA), 0.01% pluronic acid and 50 ul of DMSO to incubate 45 minutes. They were then washed in fresh aCSF and incubated for an additional 45 minutes to remove extracellular Fura-2 and allow the AM moiety of intracellular Fura-2AM to hydrolyze. The slices were maintained at 33°C from the initial incubation until they were placed in a tissue chamber on the microscope stage; maintenance at this temperature improves dye loading. The slices were maintained at 37 °C in the tissue chamber on the microscope stage throughout the experiment. An Incyt Im2 dual wavelength imaging system and its associated software were used for measuring Ca^{2+} (Intracellular Imaging Inc., Cincinnati, OH, USA). A long working distance Nikon Plan Fluor 20X UV objective (n.a. 0.5) and a Nikon TMS inverted microscope were attached to a filter changer (340 nm and 380 nm filter), a 300 W Xenon light source and a low light level CCD camera for image acquisition.

We used Ca buffers in solution for calibration, all values represent Ca^{2+} concentrations corrected for background fluorescence in time matched, unlabeled (no Fura-2) slices subjected to hypoxia. This was done to account for the increase in background fluorescence due to the increase in NADH levels during hypoxia.

Statistics

One-way analysis of variance (ANOVA) followed by a post hoc Neuman-Keul test was used to analyze the significant difference between different experimental groups. All the data are expressed as mean ± S.E.M. The difference between different groups was considered significant if $p < 0.05$.

Results

Anesthetics, such as sevoflurane and isoflurane, have been shown to reduce neuronal damage during hypoxia and ischemia. Previous work in our lab established that sevoflurane, when present 10 minutes before and during 10 minutes of hypoxia improved the recovery of resting and action potentials after ischemia. Anesthetics are known to act directly on ligand gated ion channels, such as the glutamate and GABA (Gamma-Aminobutryic Acid) channels as well as on second messenger systems. The experiments in this chapter examine anesthetics before and during hypoxia and do not distinguish between primary ionotropic effects and secondary metabotropic effects.

ATP levels

We measured the intracellular concentrations of ATP after a short hypoxic insult

with or without anesthetics before and during hypoxia to see if isoflurane and sevoflurane inhibit the changes of ATP concentration induced by hypoxia. ATP provides energy for many cellular processes; cells in which ATP falls below a certain level will die. Hypoxia and ischemia lead to a reduction in intracellular [ATP]. Under normal physiological condition, one molecule of glucose produces 38 ATP with oxygen. Only two ATP are synthesized from one molecule of glucose without oxygen. ATP is required for protein synthesis, ion pumping and intracellular signaling; during hypoxia and ischemia many cellular processes are inhibited. The magnitude of the decrease of ATP levels indicates the severity of the hypoxia. If anesthetics can inhibit the fall of ATP during hypoxia, this may be a mechanism by which they protect physiological function.

Based on the electrophysiological results from our lab, we tested the effect of isoflurane and sevoflurane before and during hypoxia on ATP levels. We found that ATP fell to 18% of its normoxic concentration after 5 minutes of hypoxia and to 10% of its normoxic concentration after 10 minutes of hypoxia. Isoflurane and sevoflurane significantly attenuated the fall in ATP concentrations during hypoxia (Figure 5). At 5 minutes of hypoxia the ATP concentration fell to only 25% and 24% of its normoxic concentration in the 2% and 4% isoflurane groups respectively and 29% and 44% of its normoxic concentration in the 2% and 4% sevoflurane groups respectively. At 10 minutes of hypoxia the ATP concentrations fell to 18% and 22% of its normoxic concentration in the 2% and 4% isoflurane groups respectively and 21% and 22% of its normoxic concentration in the 2% and 4% sevoflurane groups respectively. The high concentration of sevoflurane (4%) significantly attenuated the fall in ATP compared with 2 and 4% isoflurane and 2% sevoflurane after 5 minutes hypoxia; there was no significant

difference between any of the agents after 10 minutes hypoxia.

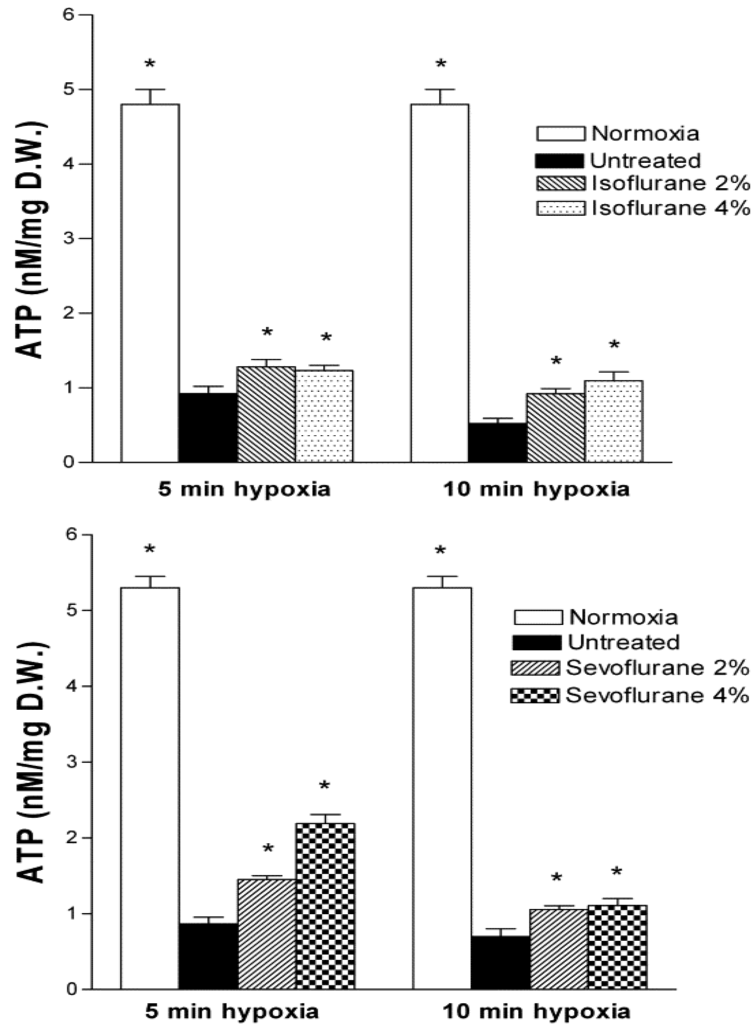


Figure 5. The effect of isoflurane and sevoflurane on ATP concentrations (nM/mg dry weight [D.W.]) during hypoxia. The treatment of slices 10 min before and during hypoxia with either isoflurane or sevoflurane significantly attenuated the fall in ATP at 5 and 10 min of hypoxia compared with the untreated hypoxic group. Data are the mean±S.E.M., Newman-Keuls multiple comparison test, $^{*}P<0.05$; $n\geq13$ for each group (Wang et al., 2006).

The results show that both isoflurane and sevoflurane attenuated the fall in ATP levels at 5 and 10 minutes of hypoxia when compared to the untreated hypoxic group. This is likely one of the mechanisms by which anesthetics protect neurons.

Intracellular Na^+ and K^+ concentration

We compared the changes of intracellular Na^+ and K^+ concentrations with and without sevoflurane/isoflurane treatment before and during hypoxia. The fall of ATP due to hypoxia lowers the activity of the Na-K pump, which is important for directly maintaining the balance of the cellular ion concentrations of Na^+, and K^+ and indirectly for Ca^{2+}. The Na^+ concentration in the CA1 region of the hippocampus increased to 160% of its concentration in normal oxygen at 5 minutes of hypoxia and 183% at 10 minutes hypoxia. Isoflurane (2%) significantly attenuated this increase at 5 and 10 minutes of hypoxia to 133% and 140% respectively (Figure 6). A higher concentration of isoflurane (4%) reduced the 5 and 10 minutes hypoxic increase of Na^+, to 132% and 149% respectively, these were not significantly difference from the attenuation found with 2% isoflurane. Sevoflurane (2%) significantly attenuated the 5 and 10 minutes increase to 138% and 155% respectively (Figure 6). A higher concentration of sevoflurane (4%) attenuated the increase at 5 minutes hypoxia to 122% and at 10 minutes to 144%. Thus both sevoflurane and isoflurane attenuate the increase in Na^+ during hypoxia, however there is no significant dose or agent dependent difference.

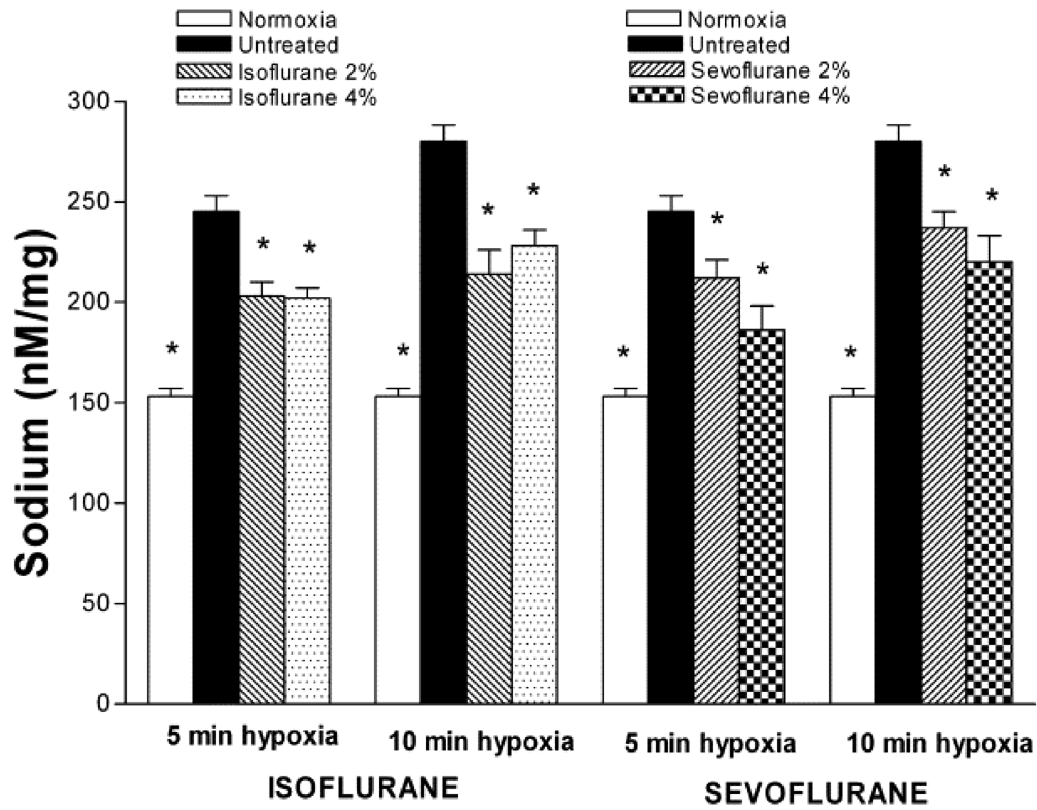


Figure 6. The effect of isoflurane and sevoflurane on sodium concentrations (nM/mg dry weight) during hypoxia. The treatment of slices 10 min before and during hypoxia with either isoflurane or sevoflurane significantly attenuated the increase in intracellular sodium at 5 and 10 min of hypoxia compared with the untreated hypoxic group. Data are the mean±S.E.M., Newman-Keuls multiple comparison test, $^{*}P<0.05$; $n=6$ for each group (Wang et al., 2006).

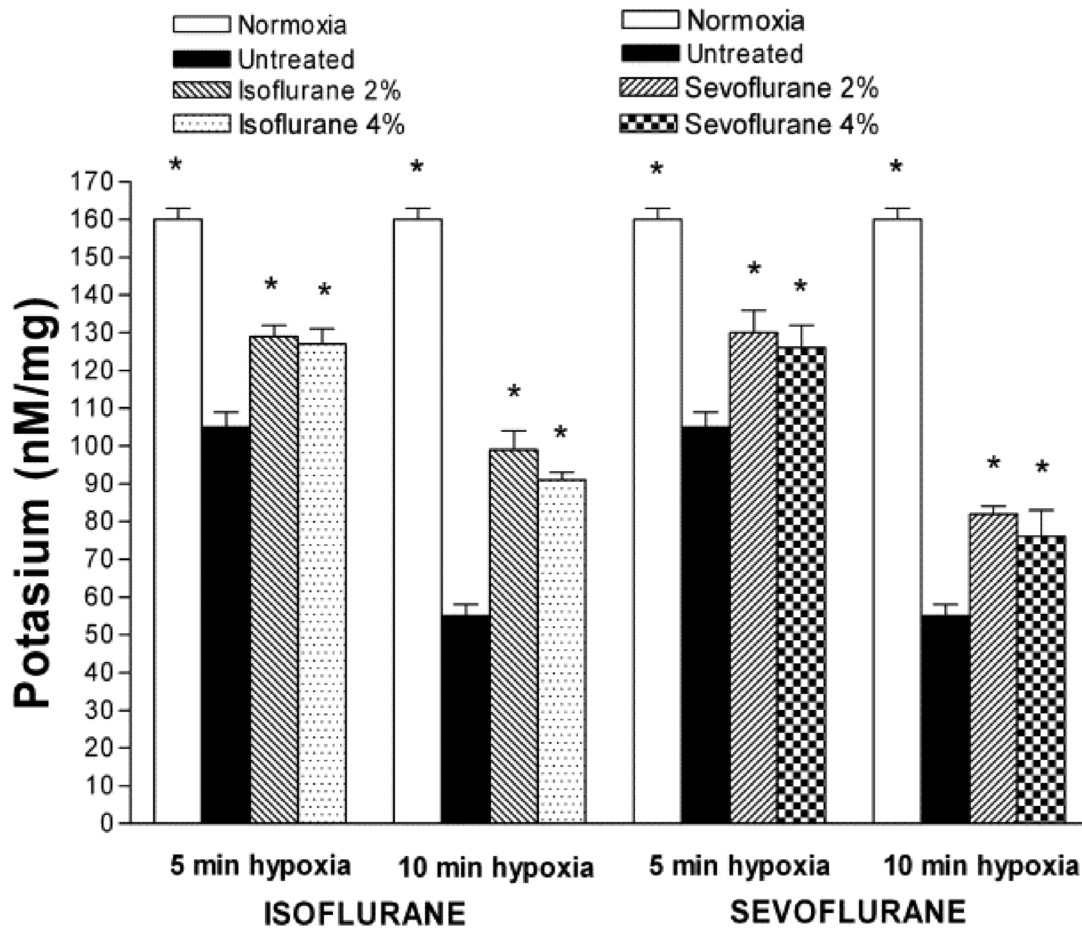


Figure 7. The effect of isoflurane and sevoflurane on potassium concentrations (nM/mg dry weight) during hypoxia. The treatment of slices 10 min before and during hypoxia with either isoflurane or sevoflurane significantly attenuated the decrease in intracellular potassium at 5 and 10 min of hypoxia compared with the untreated hypoxic group. Data are the mean±S.E.M., Newman-Keuls multiple comparison test, $^{*}P<0.05$; $n=6$ for each group (Wang et al., 2006).

The K^+ concentration in the CA1 region of the hippocampus decreased to 66% of its concentration in normal oxygen at 5 minutes of hypoxia and 34% at 10 minutes hypoxia. Isoflurane (2%) significantly attenuated the decrease at 5 and 10 minutes of

hypoxia to 81% and 62% respectively (Figure 7). A higher concentration of isoflurane (4%) reduced the 5 minutes hypoxic decrease of K^+ to 79% and the 10 minutes decrease to 56%, these were similar to those found with 2% isoflurane. Sevoflurane (2%) significantly attenuated the 5 and 10 min decrease to 81% and 51% respectively (Figure. 7). A higher concentration of sevoflurane (4%) attenuated the decrease at 5 minutes hypoxia to 79% and at 10 minutes hypoxia to 48%.

The results indicate that both concentrations of isoflurane and sevoflurane significantly attenuated the increase in Na^+ and the fall in K^+ concentrations induced by hypoxia to a similar extent; the anesthetic effect on Na^+ and K^+ concentrations was neither agent nor concentration dependent too.

Intracellular Ca^{2+} concentration

Ca^{2+} is involved in multiple pathways that influence important physiological functions in the CNS. We examined the intracellular Ca^{2+} concentrations with and without sevoflurane/isoflurane treatment before and during hypoxia to determine if attenuating the increase in cytosolic Ca^{2+} is one of the mechanisms of anesthetic protection. During hypoxia there is a rapid increase in intracellular Ca^{2+} concentrations (Figure 8). A high concentration (4%) of either isoflurane or sevoflurane significantly attenuated the increase in intracellular Ca^{2+} during hypoxia compared with untreated hypoxic slices (231% and 211% vs. 309%, respectively) and delayed the onset of its rapid increase (384±42 s and 432±60 s vs. 288±36 s, respectively). The low concentration (2%)

of isoflurane or sevoflurane had no significant effect on the latency or magnitude of the increase in intracellular Ca^{2+} during hypoxia.

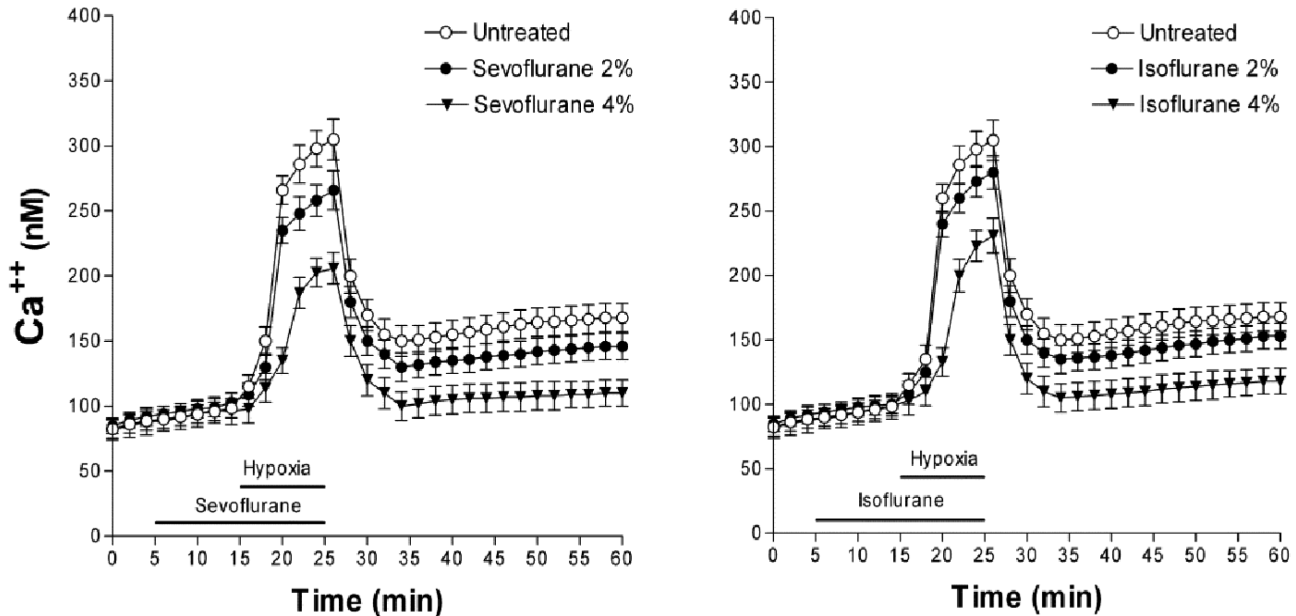


Figure 8. The effect of isoflurane and sevoflurane on calcium concentrations during hypoxia. The treatment of slices 10 min before and during hypoxia with either 4% isoflurane or 4% sevoflurane significantly attenuated the peak increase in intracellular calcium during hypoxia compared with the untreated hypoxic group. Data are the mean±S.E.M., Newman-Keuls multiple comparison test on peak calcium concentration, $P<0.05$; $n=8$ for each group, except $n=10$ for the untreated group which are the same data plotted on each graph (Wang et al., 2006).

Since 2% isoflurane and 4% sevoflurane are equipotent as anesthetics, the results indicate that the protective effects of sevoflurane and isoflurane are independent of their anesthetic potency and that other properties, such as their ability to delay the hypoxic depolarization and reduce Ca^{2+} influx explain anesthetic protection from hypoxic

damage. The 4% sevoflurane and isoflurane concentrations reduce Ca levels during hypoxia, however sevoflurane is less potent as an anesthetic and is used clinically at a higher dose. Thus 4% sevoflurane is used clinically but isoflurane is never used at a dose of 4%, the maximal dose of isoflurane is approximately 2%. At clinically usable concentrations sevoflurane, but not isoflurane reduces Ca levels during hypoxia. Therefore we focused the remainder of our work on Sevoflurane since it was effective at a clinically used concentration.

CHAPTER 2

SEVOFLURANE PRECONDITIONING

<u>Specific aims</u>: *Examine the activity of sevoflurane preconditioning on the protein kinase C and the protein kinase Mζ pathway before during and after hypoxia.*

The PKC pathway is an important pathway in ischemic preconditioning of cardiac tissue (Churchill EN et al, 2007; Xuan YT et al, 2007; Hassouna A et al, 2004; Wolfrum S et al, 2002; Liu GS et al, 1999). For example, activation of PKCδ in the heart during reperfusion after a short period of ischemia induces cell death through the regulation of mitochondrial function and the triggering of apoptosis and necrosis; however, activation of PKCε before ischemia protects mitochondrial function and diminishes apoptosis and necrosis (Churchill EN et al, 2007). PKC has also been shown to be important for ischemic preconditioning in neural tissue. PKCε is required for the induction of tolerance by ischemic preconditioning in organotypic hippocampal slice cultures (Raval AP et al, 2003). Our lab's previous work also found that some PKC isoforms (PKCα and PKCε) are involved in the mechanism of magnesium neuroprotection against neuronal hypoxic insults (Libien et al, 2005). We plan to measure the activity of isoforms that have been previously implicated in ischemic preconditioning, PKCα, PKCε and PKCδ, by monitoring their expression level with western blots in rat hippocampal CA1 neurons after 0, 4 and 10min of hypoxia with and without sevoflurane pre-treatment.

The structure of PKMζ is identical to the catalytic domain of PKCζ. PKMζ is a PKC isoform, synthesized from a PKMζ mRNA encoding the PKCζ catalytic domain without a regulatory domain (Hernandez AI et al, 2003). Recently it has been found that PKMζ is critical for the maintenance of late LTP in hippocampal slices (Ling et al., 2002). Inhibition of PKMζ by zeta inhibitory protein (ZIP) erased long-term memory (Pastalkova E et al, 2006); ZIP also blocked the protection of hippocampal CA1 neurons with sevoflurane preconditioning (Wang J et al, 2012). We plan to examine the change of PKMζ activation before and after hypoxia with sevoflurane preconditioning by detecting the expression of phosphorylated PKMζ with Western Blot analysis.

Methods

Water-bath experiments for the assay of and PKCα and PKCε translocation

All the animal experiments were carried out in accordance with the National Institute of Health Guide on the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of SUNY Downstate. Male Sprague–Dawley rats (100–120 days old) were anesthetized with 2% isoflurane for 2 min, decapitated, and their brains were quickly removed and placed into chilled (2–4 °C) artificial cerebrospinal fluid (aCSF) (in mmol/L: NaCl, 126; KCl, 3; KH_2PO_4, 1.4; $NaHCO_3$, 26; $MgSO_4$, 1.3; $CaCl_2$, 1.4; glucose 4; pH 7.4). We dissected the hippocampi, sectioned them into 500μm slices, and pooled them together in order to get enough tissue for each experiment. The slices were kept in 4° C oxygenated aCSF solution. 6-8slices were placed on each grid and one experiment included four grids (control group, hypoxia

group, sevoflurane pretreated hypoxia group and sevoflurane pretreated normoxia group). This method allowed us to control the experimental time and variability between animals by directly comparing the response in normoxic, hypoxic and drug-treated slices from the same animals. Each grid was placed in the 20ml beaker filled with 15ml standard aCSF that was aerated with 95% O_2 and 5% CO_2 and the beakers were covered with rubber stoppers. After 30minutes of incubation at room temperature in the water-bath, the heater was turned on and the water-bath was allowed to slowly heat up to 37°C (it takes 30 minutes to come up to temperature). After 2 hours incubation, the slices were subjected to sevoflurane (4%) for 15minutes followed by a 5minutes washout and then 10minutes of hypoxia. Sevoflurane was added to the gas mixture before it aerated the aCSF using a calibrated sevoflurane vaporizer (Penlon Ltd., Abingdon, UK). The control slices were treated identically except that sevoflurane was not administered.

Slice chamber incubation for the phosphorylation assay of PKCα, PKCε, PKCδ and PKMζ.

The slices for these experiments were prepared in a similar way to the methods used for the intracellularly recorded physiology experiments. Male Sprague–Dawley rats (100–120 days old) were anesthetized with 2% isoflurane for 2 min, decapitated, and their brains were quickly removed and placed into chilled (2–4 °C) aCSF solution. Hippocampal slices of 400μm thickness were sectioned in chilled aCSF (4–6 °C) using a vibratome. The slices were stored in a beaker containing aCSF saturated with 95% O_2 and 5% CO_2, and remained there for approximately 2 hours at 25 °C. Slices were

transferred to a tissue chamber and maintained at 37 °C in the chamber. Hypoxia was generated by switching the gas to 95% N_2–5% CO_2.

The hippocampal slice was submerged in the recording chamber and perfused with aCSF at a rate of 3.0 ml/min. The untreated group received 25 minutes of perfusion, then 10 minutes of hypoxia. The sevoflurane-treated groups received 5 minutes of perfusion, 15 minutes of sevoflurane treatment, 5 minutes washout and then 4minutes or 10 minutes of hypoxia (no anesthetic). ZIP (5 µM; Anaspec, San Jose, CA) was added 10 minutes before and during sevoflurane pretreatment and washed out 5 minutes before hypoxia. The samples were removed after 0 minute, 4 minutes and 10 minutes of hypoxia and after 60 minutes of reperfusion;-, then the CA1 region was dissected (see below). These experiments exactly mimic the conditions of the electrophysiological experiments that demonstrated improved recovery after sevoflurane preconditioning (Wang et al., 2007b).

The sample preparation and Western Blot analysis

The CA1 regions were dissected under a microscope on powdered dry ice and then were homogenized with 15 strokes of Teflon-glass Potter–Elvehjem tissue grinder at 4 °C in 300 µl of buffer (50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, aprotinin (kallikrein units/ml), 5 mM benzamidine, and 0.1 mM leupeptin. At this point there are 2 separate procedures: (A) for the water bath experiments and (B) for the slice chamber experiments.

A). For the samples from the water-bath experiments, the homogenate was centrifuged at 3000 × *g* for 5 minutes and the resulting supernatant (S1) centrifuged at

100,000 × *g* for 30 minutes to produce a cytosolic fraction (S2) and a membrane-particulate pellet (P2), which was resuspended with a syringe in 300μl of homogenization buffer.

B). For the slice chamber experiments to measure the phosphorylation of PKC/PKM, the homogenates do not need to be separated by high-speed centrifugation. After centrifuging at 3000× *g* for 5 minutes, the S1 solution was used as the total protein in the following experiments. Protein concentrations were determined by the Bicinchoninic acid method (BCA, Pierce).

The samples were boiled in SDS-polyacrylamide gel sample buffer **(**50 mM Tris-HCl pH 6.8, 2%SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue) at 95°C for 10 minutes, and then 10ug total protein from each sample loaded onto adjacent lanes of 8% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE gels). Following electrophoresis, the proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA)**,** and blocked for 90 minutes in 1% bovine serum albumin and 1% hemoglobin in Tris-HCl, pH 7.5 (10 mM), NaCl (150 mM), and non ionic detergent (0.2%) (TBSN) (Unless otherwise stated, reagents were from Sigma, St. Louis, MO). The nitrocellulose membrane was incubated in primary antisera overnight at 4°C. The membrane was washed in TBSN and then incubated for 90 minutes with secondary antibody coupled to alkaline-phosphatase (1:2000). The blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT)(KPL Inc., Gaithersburg, Maryland), scanned, and analyzed with NIH Image. PKC isozymes were assayed by Western blots with optimized concentrations of affinity-purified rabbit/mouse antisera to the carboxy-terminal of PKCα (1:1000), PKCδ (1:1000), PKCε

(1:2000) and PKMζ (1:100), and phosphorylated PKMζT560 antiserum (pT560, 1:100). The equal amounts of total protein from the fractions of hippocampal slices were loaded on the gel to compare the levels of PKC isozymes from the different groups. The antibodies were obtained from the following sources: rabbit anti-p-PKMζ pT560 from Epitomics Inc., rabbit anti-PKCδ and the phosphorylated primary antibodies (p-PKCα (Ser657), p-PKCε (Ser729) and p-PKCδ (Ser645)) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA), all other antibodies used were obtained from and made in Dr. T. Sactor's laboratory. The densities of the protein bands on the Western membrane were in the linear range of detection as determined with NIH Image software after membranes were scanned with a ZRS 6cx Scanner (OmniMedia, Torrance, CA). Two housekeeping proteins, α-tubulin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were chosen as the internal loading controls. Recent results indicate that GAPDH gives better results (the protein band is more stable) and we have switched to only examining this enzyme to standardize our samples.

For each Western blot an untreated nonhypoxic control is run on the same gel and used as the normalization standard; its density is set to 1 and all other bands are a ratio of this density. Six CA1 regions from tissue treated in the same chamber are from one single animal and are microdissected and pooled to run in a single lane. GAPDH is not affected by hypoxia and used to normalize the values for total protein within each lane. Each bar represents the mean and standard error of the mean from 10 animals; the individual value for each animal is the average of 2 Western blots. Sevoflurane-induced preconditioning significantly increased the amount of PKMζ protein before, during, and after hypoxia. ZIP reduced the amount of PKMζ protein at these time points. The before hypoxia,

untreated value is used for normalization of the other values and set to 1; this was also done for the 10 minutes hypoxia and 60 minutes reperfusion values but is not shown (this value is always in the absence of hypoxia).

Statistics

A one-way analysis of variance (ANOVA) followed by a post hoc Neuman-Keul test was used to analyze the difference between the experimental groups. All the data are expressed as mean ± S.E.M. The difference between different groups was considered significant if $p < 0.05$.

Results

Anesthetic preconditioning is thought to be a procedure that reduces neuronal damage during hypoxia and ischemia. In this thesis we examine the potential mechanisms of sevoflurane preconditioning. Work by others in the lab established that sevoflurane, when present for 15 minutes starting 20 minutes before the hypoxia improved the recovery of resting and action potentials after hypoxia. Since the anesthetic is not present during the hypoxia it is acting on a mechanism that improves recovery even when the anesthetic is not continuously present. Thus sevoflurane is probably activating a metabotrophic pathway that remains active after the anesthetic is washed off. We examined the effect of sevoflurane on the protein kinase C second messenger system

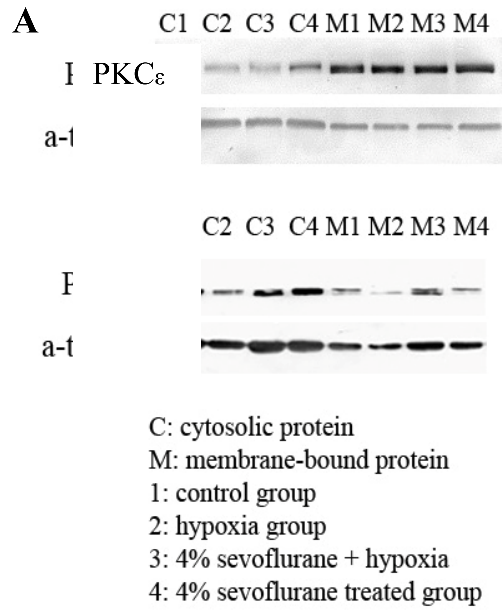
since physiological experiments indicated that blockade of this pathway with pharmacological agents abolished the preconditioning protection (Wang J, 2007). Though both 4% sevoflurane and 4% isoflurane showed protection of the neurons when administrated before and during the hypoxic insults, we focused our preconditioning research on sevoflurane for two main reasons: 1) The maximum clinically used concentration of a volatile anesthetics is around two times the MAC of that agent. 4% sevoflurane is approximately the 2 MAC and this concentration can be and is used clinically. However, 4% isoflurane is almost 4 MAC, which is never used clinically. 2) Sevoflurane is one of the most commonly used volatile anesthetics; it has the highly desirable property of rapid onset of action combined with rapid reversal of anesthesia after washout.

Translocation of PKCα and PKCε measured after 10minutes hypoxia

We examined the effect of sevoflurane on the response of PKCα and PKCε isoforms to a short hypoxic insult using the western blot method. Upon activation, members of the PKC family translocate from the cytosol to the plasma membrane of the cells for activation (Kraft et al., 1982). Dr. Libien's work found a transient translocation of PKCα and PKCε to the membrane fraction in aCSF solution with a high concentration of magnesium (10mM) during hypoxia in hippocampus slices (Libien et al., 2005), while this did not happen in the normal aCSF solution during the hypoxia. This indicates that the mechanisms of magnesium neuroprotection may include the activity of PKC. The results from my experiments show that there were no significant changes in the levels of

PKCα and PKCε in the cytosolic and membrane fractions between the hypoxic group and the normoxic control group. The levels of cytosolic PKCα and PKCε after 10 minutes hypoxia were lower than the same isoforms of PKC levels in the normoxic control slices (0.79 vs 1 and 0.54 vs 1, no significant differences). In the membrane-bound fraction, the levels of PKCα and PKCε after 10 minutes hypoxia were a little bit higher than the controls (1.09 vs 1 and 1.15 vs 1), but these changes were not significant. 15minutes of sevoflurane (4%) pretreatment before the hypoxic insult did not alter PKCα and PKCε levels under the hypoxic and normoxic conditions. PKCα and PKCε levels in the cytosol after pretreatment with 15minutes sevoflurane under normoxic conditions were 1.12 vs 1 and 1.26 vs 1, respectively, compared to levels in normoxic slices without sevoflurane. In the membrane fraction after hypoxia, PKCα and PKCε levels were 0.85 vs 1 and 1.59 vs 1 compared to levels in normoxic slices. Under the sevoflurane pretreated hypoxic conditions, the levels of PKCα were 0.86 vs 1 and PKCε was 0.75 vs 1 in the cytosol. In the membrane-bound protein, the level of PKCα was 1.14 vs 1 and PKCε was 1.27 vs 1(Figure 9).

We did not find any significant changes among the different groups. These results indicate that PKCα and PKCε are not critical for the mechanism of sevoflurane induced preconditioning on the neurons in the CA1 area of rat hippocampus slices.



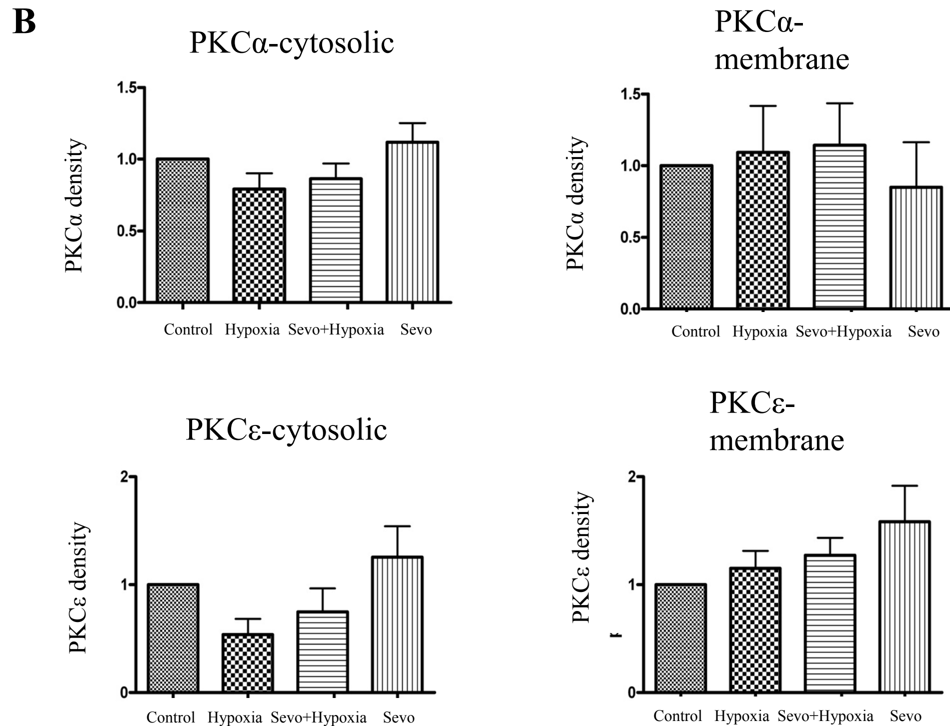


Figure 9. Translocation of PKCα and PKCε after 10min hypoxia. (**A**) Examples of Western blots of the cytosolic and membrane-bound PKCαand PKCε after 10min hypoxia with or without pretreatment with 4% sevoflurane. (**B**) Levels of PKCα and PKCε in the cytosolic and membrane fractions from CA1 regions of hippocampal slices after 10min hypoxia with or without pretreatment with 4% sevoflurane. The results show that there are no significant differences among the groups. Data are the mean±S.E.M., Newman-Keuls multiple comparison test, $P<0.05$; $n=6$ for each group.

Phosphorylation of PKCα, PKCε, PKCδ and PKMζ after 4 minutes hypoxia

They are important time points where the fates of some neurons are changed. In the electrophysiological experiment, the rapid depolarization started after about 4minutes hypoxia. Although translocation is indicative of PKC activation, a more direct measure of PKC activation is phosphorylation of PKC. We used Western blot analysis to measure the phosphorylation of certain PKCs after 4minutes hypoxia to see if the PKC pathways were activated shortly after the stimulus. 4minutes hypoxia did not change the levels of PKCα (0.78 vs. 1), PKCε (0.94 vs. 1) (Figure 10), PKCδ (1.31 vs. 1) and PKMζ (1.29 vs. 1)(Figure 11) compared to the normoxic group (set to 1). The slices were pretreated with 15minutes sevoflurane (4%) followed by 4minutes of hypoxia. The level of PKCα was1.39 vs 1, PKCε was 1.41 vs 1(Figure 10), PKCδ was 1.56 vs 1and PKMζ was 2.23 vs 1(Figure 11). The total PKMζ was increased significantly in the 4minute hypoxic group with sevoflurane pretreatment compared to control (normoxic group)($P<0.05$). The total levels of PKCα, PKCε, PKCδ and PKMζ were not affected by 4minutes hypoxia without sevoflurane pretreatment. 15 minutes of sevoflurane (4%) preconditioning enhanced the PKMζ level under hypoxic condition, but did not change the levels of PKCα, PKCε and PKCδ.

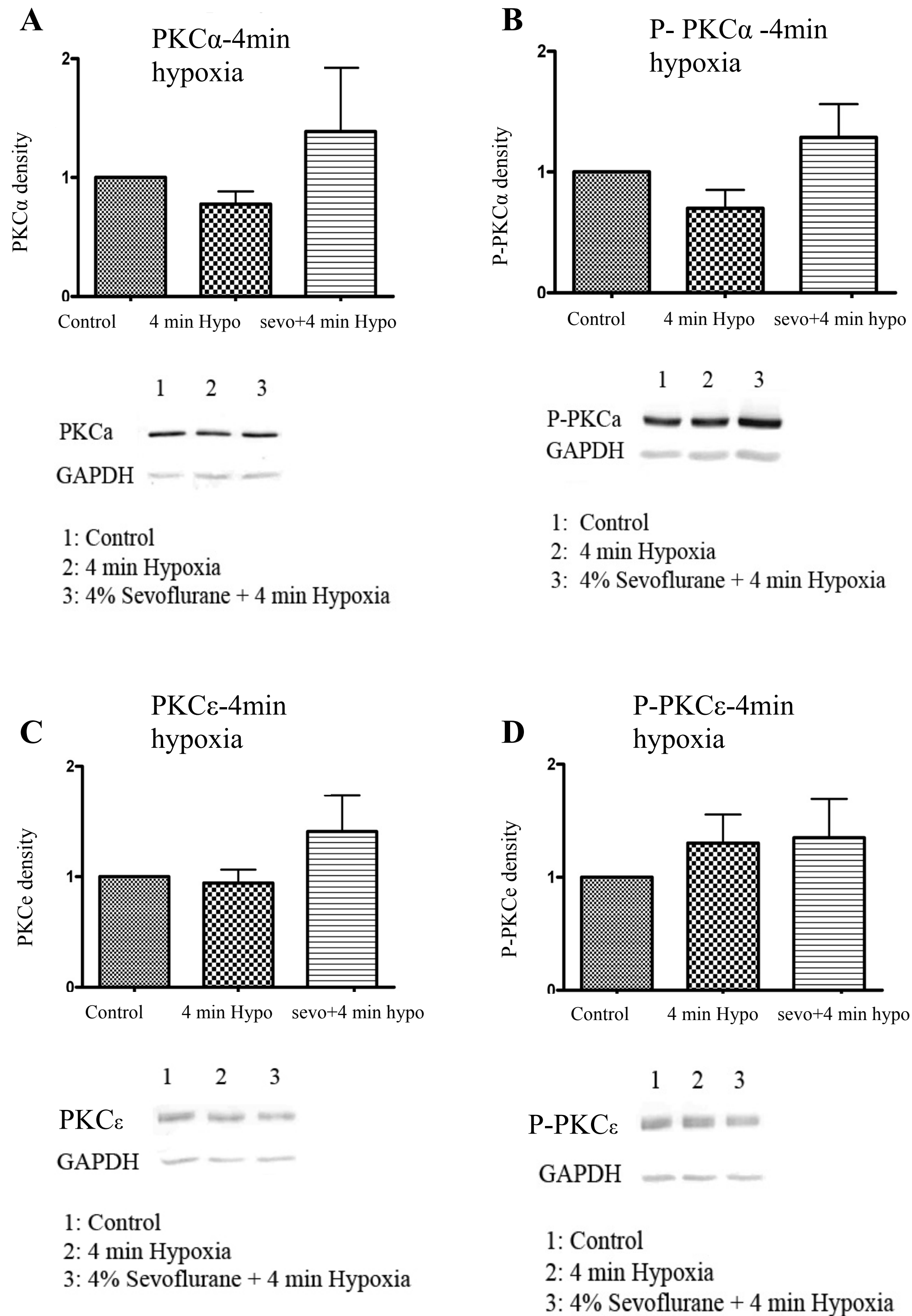


Figure 10. Phosphorylation of PKCα and PKCε after 4 min hypoxia with and without pretreatment with 4% sevoflurane. (**A, B, C and D**) Western blots show that after 4 min hypoxia, the density of the PKCα, PKCε, P-PKCα and P-PKCε are not significantly different between the control group, 4min hypoxia group and 4% sevoflurane pretreated groups. Data are the mean±S.E.M., Newman-Keuls multiple comparison test, $^{*}P<0.05$; $n=6$ for each group.

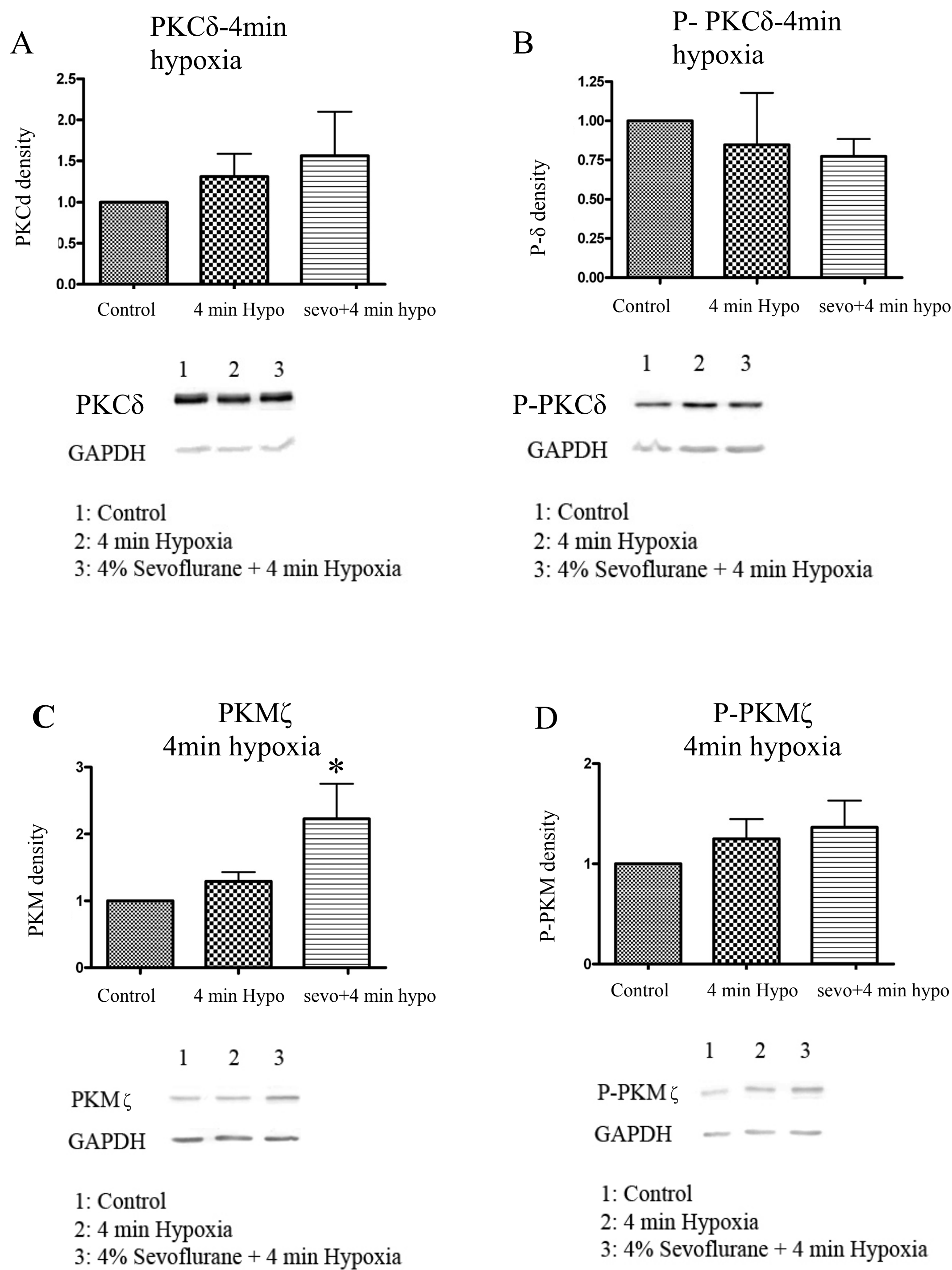


Figure 11. Phosphorylation of PKCδ and PKM after 4 min hypoxia with or without pretreatment with 4% sevoflurane. (**A, B, and D**) Western blots show that after 4 min hypoxia, the density of PKCδ, P- PKCδ and P-PKM are not significantly different between the control group, 4 min hypoxia group and 4% sevoflurane pretreated groups. (**C**) PKM was increased significantly in the 4 min hypoxic group with 4% sevoflurane pretreatment compared to the control group (normoxic group)(P<0.05)(C). Data are the mean±S.E.M., Newman-Keuls multiple comparison test, $^{*}P<0.05$; $n=6$ for each group.

After 4 minutes hypoxia, the levels of phosphorylated PKCα (P-PKCα) was 0.70 vs 1, P-PKCε was 1.30 vs 1(Figure 10), P-PKCδ was 0.85 vs 1and P-PKMζ was 1.25 vs 1(Figure 11). In slices pretreated for 15minutes with sevoflurane (4%) the level of P-PKCα was 1.33 vs 1, P-PKCε was 1.35 vs1 (Figure 10), P-PKCδ was 0.77vs 1 and PKMζ was 1.36 vs 1(Figure 11). The results showed that 15 minutes sevoflurane (4%) preconditioning followed by 5 minutes washout did not affect the phosphorylation level of P-PKCα, P- PKCε, and P-PKCδ and P-PKMζ after the 4 minutes hypoxia compared with untreated hypoxic group and normoxic group.

Effect of sevoflurane and PKMζ inhibitor on the expression of PKMζ and phosphorylated PKMζ before, during and after 10 minutes hypoxia.

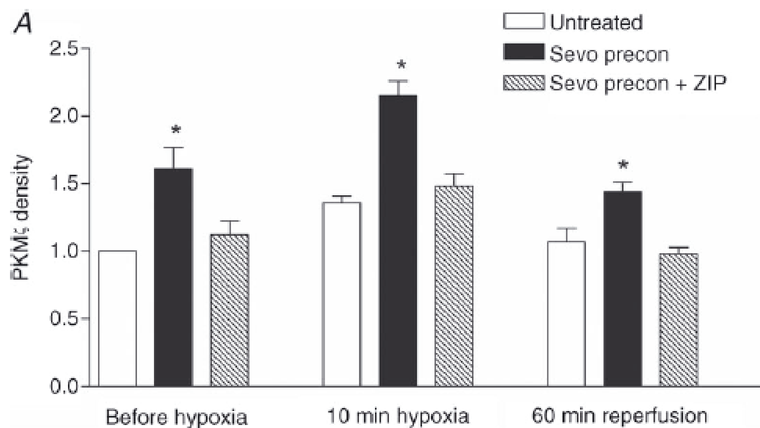
The physiology experiments found that chelerythrine blocked sevoflurane induced preconditioning (Wang J, 2007) and we did not find any significant changes with the PKCs we examined, we decided to examine the effect of sevoflurane-induced preconditioning on PKMζ, the constitutively active catalytic part of PKCζ. Sacktor et al (Hrabetova and Sacktor, 1996) has shown that chelerythrine blocks PKMζ and PKMζ is important for LTP/LTD.

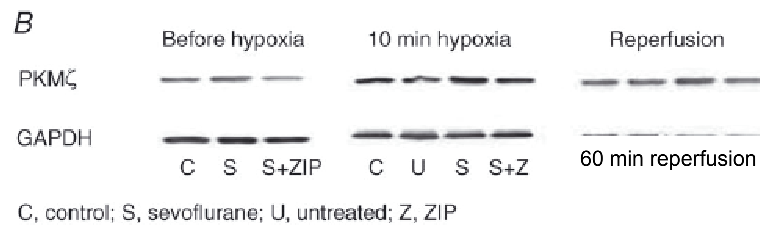
Total PKMζ protein was measured by Western blots on untreated and sevoflurane-treated tissue, before hypoxia, at 10 minutes of hypoxia and at 60 minutes after hypoxia (figure 12 a, b). When sevoflurane (4%) was present for 15 minutes and then washed out for 5 minutes, the PKMζ levels increased significantly compared to the group that did not get sevoflurane (1.6 vs 1). The PKMζ inhibitor ZIP (5μM) attenuated the increase of

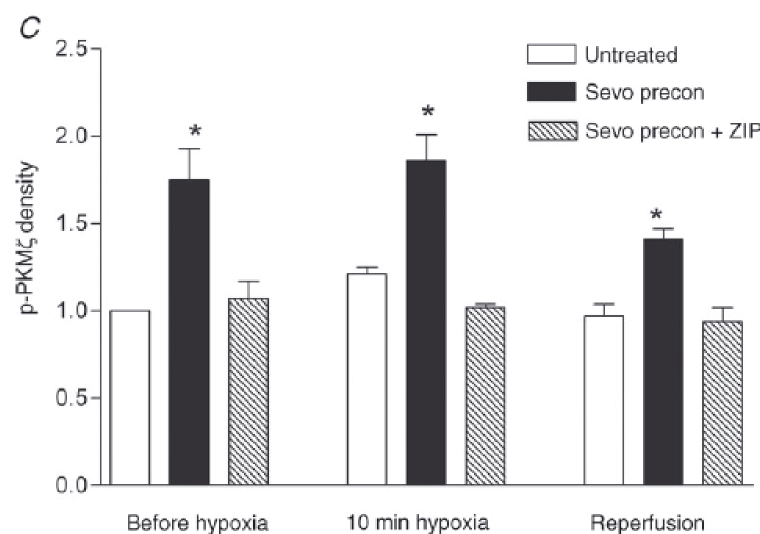
PKMζ protein expression with sevoflurane (1.1 vs. 1.6), such that the PKMζ level between the non-hypoxic untreated group and the sevoflurane plus ZIP treated group was not significantly different. PKMζ levels were measured at the end of 10 minutes of hypoxia, hypoxia alone increased the level of PKMζ compared to the non-hypoxic control (1.4 vs. 1); sevoflurane preconditioning increased this further (2.2), however the level with sevoflurane plus ZIP was less than that with sevoflurane alone during hypoxia. Thus, 10 minutes of hypoxia stimulated the expression of PKMζ; sevoflurane pretreatment with hypoxia increased the level over hypoxia alone and sevoflurane alone without hypoxia. The sevoflurane-pretreated neurons recovered their physiological activity after the end of hypoxia; we therefore measured PKMζ levels 60 minutes after the end of hypoxia. Sevoflurane preconditioning not only enhanced PKMζ levels before and during hypoxia, the enhancement was maintained for at least 60 minutes after the end of hypoxia. Neither the untreated nor the sevoflurane treated with ZIP slices showed enhanced PKMζ levels 60 minutes following hypoxia.

PKMζ is active in its phosphorylated state, under normal conditions it is rapidly phosphorylated and all of it exists in the activated state. We measured the phosphorylated, activated PKMζ form to confirm this, especially during hypoxia when ATP for phosphorylation could be limited. Sevoflurane significantly increased the level of phosphorylated PKMζ before hypoxia when compared to the level in control untreated non-hypoxic tissue; ZIP blocked this increase. At 10 minutes of hypoxia and at 60 minutes reoxygenation there were maintained increases in p-PKMζ in the sevoflurane pretreatment group; ZIP blocked the effects of sevoflurane. The untreated hypoxia group had a small but significant increase in p-PKMζ at 10 minutes hypoxia that was not

sustained with reoxygenation. Thus the results with the p-PKMζ mirrored those of PKMζ indicating that most of the PKMζ was constitutively phosphorylated as it is under normal conditions. This similar increase suggests that the increase in p-PKMζ was due to newly synthetized PKM zeta and not just phosphorylation of preformed PKMζ. In the sevoflurane pretreated followed by 4 minutes hypoxia group, we only found an increase of the total concentration of PKMζ; the P-PKMζ was not increased significantly. In separate experiments with sevoflurane pretreated tissue at the end of 10 minutes hypoxia, both of total PKMζ and P-PKMζ were increased significantly. The reason for this is unclear and will require further studies.







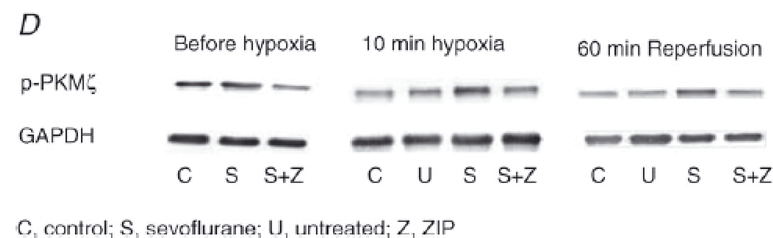


Figure 12. Effect of sevoflurane and ZIP on the expression of PKMζ and p-PKMζ. **A**) Effect of ZIP and sevoflurane-induced preconditioning on protein kinase M zeta (PKMζ) protein levels before, during, and after 10 min of hypoxia. Slices are either untreated, subjected to sevoflurane-induced preconditioning or ZIP 10 min before sevoflurane-induced preconditioning. **B**) Representative Western blots from each experiment measuring PKMζ and GAPDH. **C**) Effect of ZIP and sevoflurane-induced preconditioning on phosphorylated protein kinase M zeta (p-PKMζ) protein levels before, during, and after 10 min of hypoxia. Sevoflurane-induced preconditioning significantly increased the amount of p-PKMζ

protein before, during, and after hypoxia. ZIP reduced the amount of p-PKMζ protein at these time points. **D**) Representative Western blots from each experiment measuring p-PKMζ and GAPDH.

DISCUSSION

Hypoxia and ischemia are important causes of neuronal damage and neurological dysfunction following stroke, cardiac arrest, traumatic brain injury and surgical procedures that may lead to a temporary block of the blood supply to the brain (Lipton P, 1999). These events lead to the reduction of energy availability in the cell and the loss of essential functions such as ion pumping and protein synthesis (Kass and Lipton, 1982; Lipton, 1999; Raley-Susman et al., 2001). The cell is then depolarized which triggers damaging pathways and eventually leads to cell death. An increase of extracellular [K^+] was found in both rat cerebral cortex *in vivo* and nerve cells *in vitro* (Hansen, 1977, 1978). In both preparations during ischemia or hypoxia there was a similar initial slow rate of rise in [K^+] followed by a rapid rise (Hansen et al., 1982). In the *in vivo* models, there was a slow increase of extracellular [K^+] during the first 2 minutes of ischemia without a significant changes of extracellular [Na^+] and [Cl^-], this was followed by a rapid increase of extracellular [K^+] and rapid decrease of extracellular [Na^+] and [Cl^-] (Hansen and Zeuthen, 1981). It is thought that during the initial period of ischemia, the [ATP] in the neurons is high enough to preserve [Na^+] and [Cl^-] gradients across the cell membrane. When the homeostatic status of the neurons fails, the fall of [ATP] leads to an inability to maintain ions gradients due to reduced Na-KATPase pump activity and cellular depolarization. The depolarization of the membrane increases the overall ionic permeability of the cell membrane (Hansen, 1985) inducing the cell death. The

homeostasis of neurons is critical for maintaining cell viability. In chapter 1 of my thesis, I measured the changes of intracellular [Na^+], [K^+], [Ca^{2+}] and [ATP] at two key time points of hypoxia, 5 minutes and 10 minutes. I examined the effects of sevoflurane and isoflurane when applied before and during hypoxia. The 5 minutes time point corresponds to the approximate time of the rapid depolarization in the neurons with in vitro (Wang et al., 2006) and in vivo hypoxia / ischemia (Hansen, 1977, 1978). The 10 minutes time point corresponds to the maximum change in these values at the end of hypoxia. The aim of this study is to determine if a clinical concentration of sevoflurane can improve ionic homeostasis during a hypoxic insult.

Sevoflurane is a commonly used anesthetic for surgery. The electrophysiological data from our lab showed that 2% and 4% sevoflurane significantly increased the recovery of the neurons when applied before and during a short hypoxic period (Wang et al., 2006). Based on these findings, we examined the effects of sevoflurane on the [ATP], [Na^+], [K^+] and [Ca^{2+}], these parameters are important for maintaining the physiological status of neurons. Attenuating the intracellular changes of [ATP], [Na^+], [K^+] and [Ca^{2+}] during hypoxia may be one of the mechanisms by which sevoflurane protects against hypoxic damage to neurons.

The effects of Isoflurane and Sevoflurane on ATP and ions changes during hypoxia

We examined the effect of sevoflurane and isoflurane on ions and ATP concentrations in rat hippocampal slices. Since previous studies have found that the protective efficacy of an anesthetic agent did not correlate well with its anesthetic potency, distinct reaction sites may contribute to anesthesia and protective effects

respectively (Wang et al., 2006). ATP sensitive K^+ (K_{ATP}) channels are activated by low [ATP] and therefore would be activated under hypoxic but not under normoxic conditions. They are unlikely to play a role in inducing the anesthetic state but are likely to be important for anesthetic protection, indeed, we found that blockade of the K_{ATP} channel abolished the protective effect of sevoflurane (Wang et al., 2006). There is a complex interaction between [ATP], [Na^+] and [K^+]. While ATP provides the energy to power the Na, K-ATPase pump and thereby maintain the [Na^+] and [K^+] gradients, the leakage of Na^+ into the neuron due to enhanced conductance increases the Na-K pump rate and uses ATP at a faster rate. The rate of the Na-K pump is primarily increased by increased cytosolic [Na^+]. So the pump has the beneficial effect of maintaining the ionic gradients and reducing the slope of the slow depolarization, but the disadvantage of increasing the rate of ATP utilization. Once the rapid depolarization is triggered, the ion fluxes will be too rapid for the pump to maintain ion gradients, even though the pump is operating rapidly and using large amounts of ATP; this would worsen the energy status of the neurons. Sevoflurane has independent effects on the neurons; one potential effect is the enhancement of mitochondrial K_{ATP} channels leading to the preservation of mitochondrial function and improved ATP concentrations during hypoxia.

The 4% sevoflurane concentration, by attenuating the Na^+ influx and delaying the rapid depolarization, improved ATP concentrations and contributed to the improved recovery. We propose that the fall in ATP is sufficient to activate the K_{ATP} channels and that the 4% sevoflurane, which improves physiologic recovery and increases the amplitude and the duration of the hypoxic hyperpolarization, modulates the K_{ATP} channel by enhancing its activation, open time and/or conductance. It is important that the fall in

the ATP concentration during hypoxia was attenuated by the anesthetics. This better maintenance of ATP would preserve Na–K pumping and thereby prolong the maintenance of cytosolic [K^+]. This is a potential explanation for the prolonged initial hyperpolarization by anesthetics. ATP, Na^+ and K^+ concentrations were closer to their normoxic levels at 10 minutes hypoxia in the tissue treated with sevoflurane and isoflurane than in the untreated hypoxic tissue; however there was no difference between the different anesthetic concentrations even though some anesthetic concentrations improved recovery while others did not. Thus the effect of anesthetics on [Na^+] and [K^+] did not correlate with the protection. A reduction in Na^+ and K^+ changes by anesthetics could be permissive and required, however they may not be sufficient in and of themselves to lead to improved recovery.

Hypoxia induced a slow increase in [Ca^{2+}] followed by a large rapid increase. Only the higher concentrations (4%) of sevoflurane and isoflurane attenuated the increase in [Ca^{2+}] during hypoxia and delayed the onset of this rapid increase. The onset of the rapid increase in [Ca^{2+}] induced by hypoxia was well matched with the onset of the rapid depolarization. These results suggest that the marked increase in [Ca^{2+}] is associated with the rapid depolarization. We demonstrate that 4% sevoflurane and isoflurane delayed the onset and attenuated the magnitude of the [Ca^{2+}] increase; this correlated with electrophysiological recovery and may be part of the mechanism of anesthetic protection from damage.

Changes of intracellular Na^+, K^+ and Ca^{2+} concentrations induced by hypoxia are due to a reduction in ATP followed by depolarization. Hypoxia induces an initial hyperpolarization, followed by a slow depolarization and then a rapid depolarization in

hippocampal CA1 pyramidal neurons (Raley-Susman et al., 2001). The fall of ATP levels inhibits the Na-K pump and induces a dramatic increase of intracellular [Na] and a decrease of intracellular [K]. The depolarization of the membrane increases the permeability to ions and a rise in intracellular [Ca^{2+}].

I compared the protective effects of isoflurane and sevoflurane at the same concentrations but different anesthetic potencies. The results indicate that 4% sevoflurane (≈2 MAC) and 4% isoflurane (≈4 MAC), but not 2% isoflurane (2 MAC), attenuated the increase in cytosolic Ca^{2+} during hypoxia. While all concentrations of these agents attenuated the fall in ATP and K^+ and the rise in Na^+ there was no significant difference between the agents. The results indicate that the protective effects of sevoflurane and isoflurane are independent of their anesthetic potency and that other properties, such as their ability to delay the hypoxic depolarization and reduce Ca^{2+} influx explain anesthetic protection from hypoxic damage.

The mechanisms of sevoflurane preconditioning

A short ischemic period before a longer ischemic period has been shown to make the neurons more resistant to damage from the longer period of ischemia; this has been termed ischemic preconditioning and is thought to involve the synthesis of new proteins (Gidday, 2006; Malhotra et al., 2006; Roth et al., 2006; Schurr et al., 1986; Xu et al., 2002). The problem with ischemic preconditioning as a therapeutic tool is that it can also cause damage in certain cases (Tanay et al., 2006). Therefore there has been a search for drugs that reduce damage when applied before the longer ischemia; volatile anesthetics,

such as sevoflurane, have been shown to do this in heart tissue(Stowe and Kevin, 2004; Tanaka et al., 2002). This is called anesthetic-induced (or sevoflurane-induced) preconditioning. Recently, more and more research has focused on the mechanism of anesthetic preconditioning in the brain using different models from in vitro neurons and slices to in vivo animal studies. Sevoflurane preconditioning enhanced the recovery after hypoxia of CA1 pyramidal cell in rat hippocampal slices (Wang et al., 2007b). Moreover, in an in vivo global ischemic model, sevoflurane preconditioning improved CA 1 pyramidal cell survival 1 and 6 weeks after ischemia (Wang et al., 2007b). Others have found that sevoflurane enhances long-term survival of neurons in vivo by altering biochemical pathways normally induced by ischemia (Pape et al., 2006).

Previously, we demonstrated that 4% sevoflurane, when present only before but not during hypoxia, improved recovery of the resting and action potentials after hypoxia. This improved recovery was blocked by the protein kinase inhibitor chelerythrine; indicating that sevoflurane was activating a metabotrophic response to improve recovery (Wang et al., 2007b). In previous studies we found that both 2 and 4% sevoflurane induced preconditioning protection but 2% required 60 minutes to establish its effect (Wang et al., 2007b); we used the 2 MAC of Sevoflurane (4%) to study the mechanism here because its shorter onset of preconditioning protection allowed others in our lab to carry out intracellular experiments that would have been more difficult over longer time periods.

The mechanisms by which either ischemic or volatile anesthetic preconditioning improves recovery after ischemia are not completely known. Sevoflurane is capable of inducing preconditioning in rat hippocampus slices, the preconditioning protection was

blocked by tolbutimide, glibenclamide and 5-HD, blockers of the K_{ATP} channel (Kehl et al., 2004; Wang et al., 2007b; Wang et al., 2012). In addition to the plasma membrane K_{ATP} channel, the mitochondrial K_{ATP} channel is likely to be important since 5-HD blocks protection with sevoflurane and it is thought to be a selective blocker of the mitochondrial K_{ATP} channel. The protective effects of sevoflurane (4%) were also blocked by chelerythrine, a selective inhibitor of PKC and PKM. Activation of PKC causes transient phosphorylation activity, while PKM provides persistent phosphorylation of its substrates (Serrano et al., 2005). In neuronal tissue and heart tissue PKC activation has been implicated as a factor for ischemic preconditioning (Roth et al., 2006). High Mg during 10minutes of hypoxia has been shown to improve electrophysiological recovery and increase the activity of PKC in rat hippocampal brain slices (Libien et al., 2005). Both PKC and PKM may be involved in anesthetic preconditioning; the separation of the actions of these two closely related kinases is difficult.

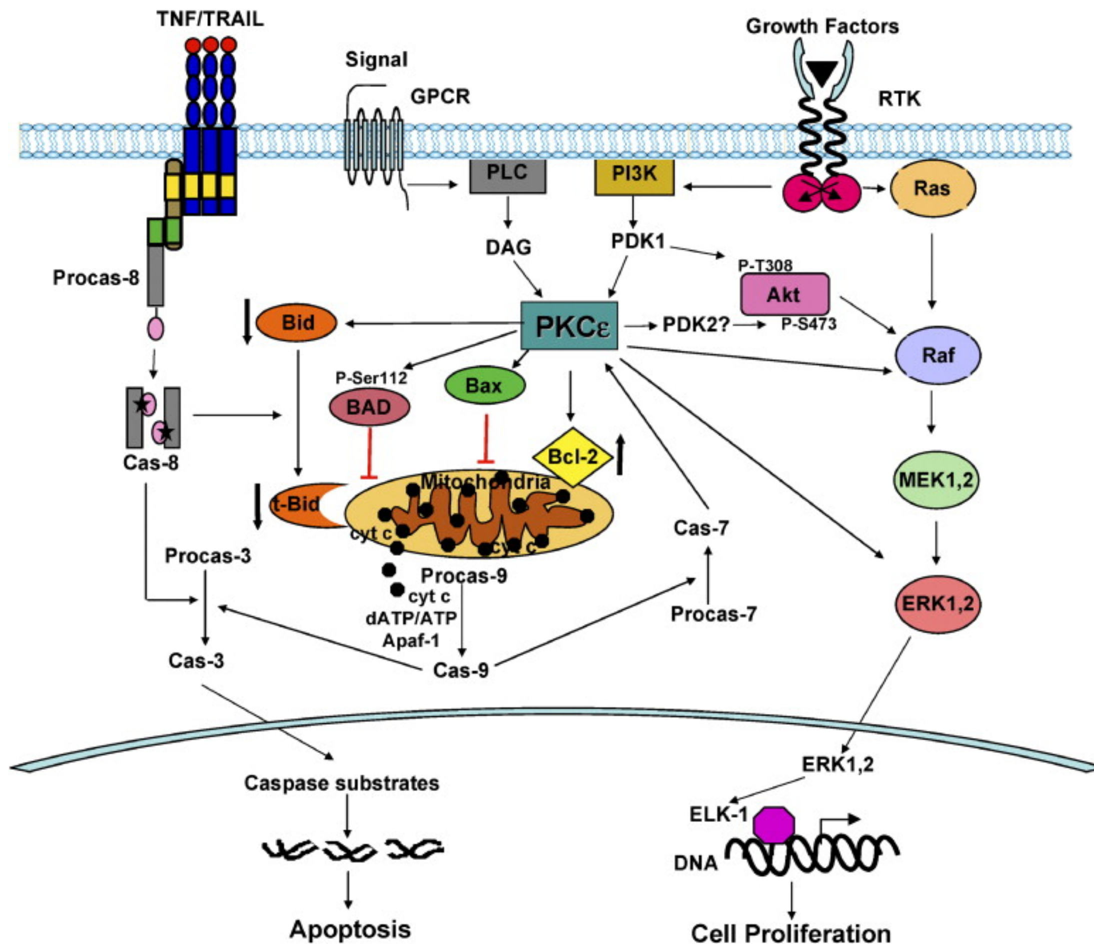


Figure 13. Regulation of cell proliferation and cell death by PKCε. PKCε has been shown to increase the levels of antiapoptotic Bcl-2. It can interact with Bax and inhibit its activation, dimerization and translocation to mitochondria. It can also phosphorylate Bad and prevents its translocation to the mitochondria. An increase in the ratio of anti- to proapoptotic Bcl-2 family members in the mitochondria inhibits release of cytochrome c from the mitochondria and prevents activation of procaspase-9. PKCε can also inhibit receptor-initiated cell death pathway by decreasing the level of Bid and thus decreasing the amount of truncated Bid in the mitochondria. PKCε is a substrate for caspase-7. In response to apoptotic stimuli, full-length PKCε is cleaved at the hinge region to generate the C-terminal catalytic domain, which is active in the absence of cofactors. Figure and legend are adapted from " Protein kinase C epsilon makes the life and death decision" by Basu and Sivaprasad, 2007, Cell Signal, 19: 1633-42. (Basu and Sivaprasad, 2007).

PKC

The PKCs belong to the family of Ser/Thr kinases (Liu, 1996). They are two ways to monitor the activity of PKCs: directly measuring kinase activity against a specific PKC substrate or examining the translocation of specific isoforms from the soluble cytosolic fraction to the particulate membrane fraction (Kraft and Anderson, 1983b). It is known that PKC must be phosphorylated before it is capable of phosphorylating its substrate (Keranen et al., 1995). The phosphorylation of PKCα at Ser^{657} is required for its full catalytic activation and stabilizes the binding between the enzyme to the membrane (Gysin and Imber, 1996). For PKCε, the phosphorylation of the Ser^{729} residue in the hydrophobic motif at the C-terminus makes the PKCε ready for translocation (Shinmura et al., 2008). The phosphorylation of PKCδ at Ser^{645} (Rybin et al., 2009) is an autophosphorylation site which can be used to test the activity of the PKCδ.

PKC activity is regulated by many factors including different lipid cofactors, phosphorylation events and/or specific binding proteins (Meier and King, 2000). It is known that PKC combines with diacylglycerol and different membrane phospholipids, is translocated from the cytosolic fraction to the membrane fraction of the cell and interacts with the receptor of PKC (RACK) in the membrane and is then phosphorylated by phosphoinositide-dependent protein kinase-1 (PDK-1) which is a universal PKC kinase (Le Good et al., 1998). PKCε has been shown to regulate cell proliferation and cell death (Figure 14)(Basu and Sivaprasad, 2007).The role played by PKCs on the cascade of events after the ischemic insult is still unclear (Calabresi et al., 2001). Even in the same

tissue, PKC activation mediates both protective and harmful effects (Reshef et al., 1997). These discrepancies may be explained by the different PKC isoforms activated by different signals, which may play contrasting roles in the development of neuronal damage (Selvatici et al., 2003).

Accumulated evidence showed that the activity of PKC phosphorylates the mitoK_{ATP} channels and that this reduces the affinity of ATP to these channels, lowers the threshold for mitoK_{ATP} opening during intracellular ATP depletion (de Ruijter et al., 2003). Magnesium treatment during the anoxia resulted in a transient translocation of PKCα and PKCε to the membrane fraction, which improved recovery from neuronal anoxia in the rat hippocampus slices (Libien et al., 2005; Xu et al., 2004). Sevoflurane preconditioning induces a delayed neuroprotection including the translocation of PKCε from the cytosol to the plasma membrane at 6h after reperfusion and the opening of K_{ATP} channel (Zheng and Zuo, 2004).

In the current work, we used Western Blot analysis, a widely used protein analytical technique, to detect the translocation of PKCα and PKCε from the cytosolic fraction to the membrane-bound fraction of the neurons in the hippocampical CA1 area of rat brain after 10minutes hypoxia. The results show that both the cytosolic and the membrane-bound PKCα, PKCε are not affected by 10minutes hypoxia with or without 15minute sevoflurane (4%) preconditioning. The difference between our results and the work of others work may be due to the different experimental conditions and different time windows. We only tested the changes of PKCα and PKCε at the time point immediately after the insults. The phosphorylated PKCs, which work on their substrate, were the activated forms of this kinase family. The antibodies to the phosphorylated

PKCs (α, ε and δ) were used directly in the Western Blot assay as well as antibodies to phosphorylated PKMζ, because chelerythrine also inhibits the function of PKMζ. In previous electrophysiological experiments, 15minutes of sevoflurane (4%) pretreatment protected the neurons against the hypoxic insult. We found that the rapid depolarization started around 4minutes hypoxia. Although 4minutes hypoxia does not damage the neurons, it is a critical time point where chemical and physiological changes occur that may trigger the irreversible damage. Immediately after 4minutes hypoxia, we measured the phoshporylation of PKC α, δ, ε and PKMζ. The results show that the levels of P-PKCα, P-PKCε, P-PKCδ and P-PKMζ were not affected by sevoflurane pretreatment when measured at 4minutes hypoxia. However the total level of PKMζ increased significantly in the sevoflurane group compared to the control group (fig 11). Since PKMzeta is constitutively phosphorylated it was surprising that we did not find an increase in P-PKMzeta (fig 11). It is possible that the Ca increase during hypoxia activated phosphatases such as calcineurin to dephosphorylate PKMzeta. However subsequent experiments done at different hypoxic time points (fig 12) did show an increase in in PKMzeta and P- PKMzeta. We cannot explain this discrepancy between the 2 experiments but it is possible that the different hypoxic sample times in the 2 experiments influenced the results.

PKMζ

The phosphorylation sites of PKMζ of threonine-410 at the activation loop and threonine-560 at the autophosphorylation site are the major phosphorylation sites for this kinase. Both of them are required for full activation of PKC-ζ (Standaert et al., 2001).

Therefore the amount of Thr-560 autophosphorylation can be used to measure the activity of PKMζ (Tian et al., 2008).

Protein kinase Mζ is a unique and interesting kinase whose synthesis is associated with LTP in hippocampal brain slices and learning and memory in animals (Pastalkova et al., 2006; Sacktor, 2011; Yao et al., 2008). Its activity is blocked by chelerythrine (Ling et al., 2002). LTP, a prolonged enhancement in synaptic transmission has been shown to be dependent on the activity of PKMζ, blocking PKMζ synthesis and activation with PKMζ inhibitory peptide ZIP, blocks the formation and reverses the potentiation with LTP (Pastalkova et al., 2006). Moreover, when ZIP was infused into the hippocampus of animals that had learned a specific task, the animals could no longer do that task even after the ZIP was washed out. However, those animals could learn a new task after the ZIP was removed, indicating that the animals still had the capacity to learn but the memory of the old task was lost (Pastalkova et al., 2006). Our data indicate that sevoflurane-induced preconditioning protection also requires the activation of PKMζ. Since PKMζ is mainly expressed in brain tissue it represents a unique pathway for sevoflurane-induced preconditioning cerebral protection (Hernandez et al., 2003). While our current results demonstrate that PKMζ is necessary for sevoflurane-induced preconditioning, it may not be sufficient, as other metabolic pathways have been shown to be important for volatile anesthetic induced preconditioning (Bickler et al., 2005; Sanders et al., 2010; Zheng and Zuo, 2004). Recent unpublished results (Sacktor Lab) indicate that ZIP may also block the closely related atypical PKC/M iota/lambda; thus we cannot exclude the possibility that sevoflurane preconditioning requires this isofom in

addition to PKMζ. However we did measure a blockade of PKMζ with ZIP; we have not examined PKM iota lambda.

We found that sevoflurane increased the amount of PKMζ and phospho-PKMζ in the CA1 pyramidal cells, this increase was measured before the hypoxia even though it was only 20 minutes after its application. ZIP, a selective blocker of PKMζ, prevented the increase in PKMζ and phospho-PKMζ and blocked the protective effect of sevoflurane-induced preconditioning. This indicates that synthesis and activation of PKMζ is necessary for sevoflurane- induced preconditioning. It is unlikely that an increase in phosphorylation of preformed PKMζ accounts for the increase in phospho-PKMζ and it is likely that the increase in PKMζ and phospho-PKMζ is due to new protein synthesis. There are 3 main reasons for this conclusion: 1) almost all PKMζ is rapidly and constituatively phosphorylated under basal conditions in hippocampal slices(Kelly et al., 2007); 2) PKMζ has been shown to enhance its own synthesis and blocking its activity reduces PKMζ levels during LTP and 3) we found an increased level of total PKMζ that paralleled the increase in phospho-PKMζ, similar to that observed with LTP. The 100kb mRNA of PKMζ needs approximately 50-100minutes to finish transcription (1~2kb/min)(Hernandez et al, 2003). However, we observed an increase of PKMζ and P-PKMζ within 20 to 90minutes. For LTP, one hypothesis is that there is increased translation PKMζ protein of pre-existing PKM ζ   mRNA localized in the dendrites (Hernandez et al., 2003). Additionally, PKM ζ does not need the activation by second-messengers that is necessary for the other PKC isoforms. The synthesis of preexisting mRNA and constitutive activation of PKMζ dramatically shorten the time until its activity is expressed. We measured the level of phospho-PKMζ after sevoflurane

pretreatment in the presence of the protein synthesis inhibitor cycloheximide, it was found that cyclohexamide blocked the increase in phospho- PKMζ with sevoflurane and also blocked sevoflurane preconditioning protection(Wang et al., 2012). These results support the importance of de novo synthesis of PKMζ protein for preconditioning protection with sevoflurane.

The current study of anesthetic induced preconditioning examines the mechanisms by which sevoflurane when present before, but not during hypoxia, improves neuronal recovery after the hypoxia. Our studies have focused on immediate preconditioning where the protection from ischemia starts shortly after the treatment (Wang et al., 2007b), other studies have examined delayed preconditioning which is expressed starting 12 hours after the ischemia (Bickler et al., 2005; Payne et al., 2005; Sanders et al., 2010; Xiong et al., 2003; Zheng and Zuo, 2004). While previous studies have recognized the importance of protein synthesis and gene activation for delayed preconditioning, the importance of protein synthesis was not recognized for immediate preconditioning because of the time constraints. Gene expression and mRNA formation in the nucleus and protein synthesis and transport to the axons and dendrites would take too long to allow immediate preconditioning within one hour. However recent experiments examining LTP in dendrites indicate new protein can be synthesized rapidly from mRNA located in the dendrites (Bazan et al., 2002; Casadio et al., 1999; Cracco et al., 2005; Hernandez et al., 2003; Kelly et al., 2007; Muslimov et al., 2004; Sacktor, 2011; Yao et al., 2008). Thus immediate preconditioning indeed requires protein synthesis, others have demonstrated this in the lab using cycloheximide a protein synthesis inhibitor.

The mTOR pathway is rapidly activated during LTP and leads to new protein synthesis of PKMζ in synapses (Cracco et al., 2005); this mitogenic pathway has also been implicated in hypoxia and the signaling pathways induced by hypoxia (Arsham et al., 2003; Martin and Hall, 2005). The regulation of the mTOR pathway has been extensively studied (Hay and Sonenberg, 2004) and it has been shown to be important for synaptic plasticity (Casadio et al., 1999; Kelleher et al., 2004; Tang et al., 2002). Disregulation of this pathway has been implicated for neuronal damage (Hoeffer and Klann, 2010). Our lab tested whether this mitogentic pathway is important for sevoflurane-induced preconditioning (Wang et al, 2012). Rapamycin, a specific blocker of this protein synthesis activating pathway blocked protection by sevoflurane induced preconditioning and also blocked the formation and activation of PKMζ. Thus it appears that sevoflurane is activating the mTOR pathway and this is leading to the synthesis of PKMζ. It is currently unclear how sevoflurane activates these pathways. It is indicated that sevoflurane is not enhancing glutamate NMDA receptors and thereby Ca^{2+} influx to activate these pathways (Wang et al., 2012), as occurs during LTP. However, sevoflurane has been shown to increase cytosolic Ca^{2+} levels by enhancing the release of Ca^{2+} from the endoplasmic reticulum and it is possible that the increased cytosolic Ca^{2+} can activate the mTOR pathway; this will need to be examined carefully in future experiments (Bickler et al., 2009; Wei and Xie, 2009; Zhao et al., 2010).

In order to confirm the effect of agents on the expression of preconditioning my colleague in the lab, Dr Jun Wang altered the protocol of the electrophysiological methods to include a 30 minutes instead of a 5 minutes washout; he also extended the sevoflurane application to 30 minutes. The results showed that 30 minutes of 4%

sevoflurane followed by a 30 minutes washout enhanced the hypoxic hyperpolarization delayed the depolarization and improved recovery following hypoxia. Tolbutamide, a K_{ATP} channel blocker, or ZIP, an inhibitor of PKMζ, prevented these preconditioning-induced changes when the agents were applied after the initiation and expression of preconditioning(Wang et al., 2012). Thus the continual activity of PKMζ and K_{ATP} channel conductance is required for the expression of sevoflurane-induced preconditioning.

Volatile anesthetics are known to alter ionotropic neuronal transmission; they have been shown to activate $GABA_A$ and inhibit NMDA and AMPA glutamatergic transmission (Alkire et al., 2008). Previous investigators have examined the role of these pathways in protecting against hypoxic and ischemic damage (Bickler et al., 2005; Head and Patel, 2007). Anesthetics, however, only affect these pathways when they are continually present, and this may not be possible during ischemia, which limits the efficacy of these agents. Our study focuses on metabotropic actions of the volatile anesthetic sevoflurane. These metabotropic actions are prolonged and maintained even when the anesthetic is no longer present. The current experiments provide evidence to support only parts of the following putative pathway: Sevoflurane causes the release of Ca^{2+} from the endoplasmic reticulum; this increase in cytosolic Ca^{2+}activates the mTOR pathway (we provide evidence that blocking mTOR pathway with rapamycin prevents preconditioning (Wang et al, 2012)), which in turn induces the synthesis of the constitutively active protein kinase PKMζ (blocking PKMζ synthesis and activation with either cycloheximide or ZIP prevents preconditioning); this causes an enhanced activity or increased number of open K_{ATP} channels in the membrane (blocking the K_{ATP} channels

with glibenclamide blocks the hypoxic hyperpolarization and the preconditioning protection); this hypoxic hyperpolarization delays the hypoxic / ischemic depolarization and reduces damage. The importance of delaying and attenuating the spread of the depolarization during hypoxia and ischemia for reducing the core of a cortical lesion has been demonstrated in vivo (Higuchi et al., 2002; Kobayashi et al., 2007; Sasaki et al., 2009). The parts of this putative pathway that require further work to substantiate are the sevoflurane-induced increase in cytosolic $Ca^{2+}$and Ca^{2+} induced PKMζ synthesis and activation. These steps have been shown by others to be present in studies not examining hypoxia or ischemia (Sacktor, 2011; Wei and Xie, 2009; Zhao et al., 2010).

SUMMARY

In chapter 1, I examined the effects of isoflurane and sevoflurane on hypoxic damage when the anesthetic is present both before and during the hypoxia; sevoflurane may have direct effects on ion channels during the hypoxic period. Both of the concentrations of isoflurane and sevofurane (2% and 4%)attenuated the fall in [ATP] and [K^+] and the rise in [Na^+] though there was no significant difference between the anesthetic treated groups. Isoflurane and sevoflurane (4% but not 2%) attenuated the increase in cytosolic [Ca^{2+}] during hypoxia. The results indicate that the protective effects of isoflurane and sevoflurane are independent of their anesthetic potency and those other properties, such as their ability to delay the hypoxic depolarization and reduce [Ca^{2+}] influx, may explain anesthetic protection from hypoxic damage. Since the effective concentration of isoflurane (4%) was higher than its clinically usable concentration we focused on sevoflurane (4%).

In the second chapter I examined the mechanisms of sevoflurane preconditioning. I measured isoforms of the PKC family including PKMζ in order to determine how sevoflurane induces preconditioning protection. I found that PKC α, δ, and ε were not affected by preconditioning but that PKMζ was increased. Sevoflurane preconditioning leads to increased PKMζ levels. Thus the protection with sevoflurane preconditioning appears to require an increase in the synthesis of protein kinase M zeta. This is supported by the blockade of preconditioning protection and PKMζ synthesis by ZIP, however a caveate of this is that ZIP may also block other atypical PKMs. The later will have to be further examined.

CONCLUSION

In conclusion, the present study demonstrates that the protective effects of sevoflurane and isoflurane when present before and during hypoxia are independent of their anesthetic potency and that reduced Ca^{2+} influx may explain anesthetic protection from hypoxic damage. Sevoflurane-induced preconditioning enhances PKMζ synthesis and activation. This pathway leads to improved recovery from short periods of hypoxia in the neurons of the hippocampus CA1 subfield.

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