PKMζ Regulation of Na/K ATPase: A Potential Endogenous Neuro-protective Mechanism of Ischemic Preconditioning

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Abstract

In ischemic preconditioning, a sublethal ischemic insult protects neurons from subsequent ischemia. We studied ischemic preconditioning in organotypic hippocampal slice cultures, in which a preconditioning 5-minute hypoxia-hypoglycemia treatment protected against a 10-minute experimental ischemic (EI) treatment of hypoxia-hypoglycemia. Whereas the preconditioning treatment protected against EI given 24 hours later, it did not prevent neuronal loss when EI given 2 hours later. This model was used to identify two regulators of ischemic preconditioning; the atypical PKC isoform PKMζ, and the Na/K ATPase. Two hours following preconditioning, when there was no neuroprotection, Na/K ATPase activity was unchanged from basal level. In contrast, Na/K ATPase activity as measured by ^{86}Rb uptake was significantly increased 24 hours after the preconditioning treatment. Elevated Na/K ATPase activity 24 hours following preconditioning was accompanied by increased surface expression of the Na/K ATPase α_1 and α_2 isoforms. Similarly, levels of active PKMζ were increased at 24 hours, but not 2 hours, after preconditioning. To examine the possibility of PKMζ regulation of Na/K ATPase, pharmacological occlusion experiments were performed, using marinobufagenin to inhibit α_1, dihydroouabain to inhibit $\alpha_{2/3}$ and a ζ-pseudosubstrate peptide to inhibit PKMζ. These experiments showed that PKMζ regulated both the activity and surface expression of the α_1 isoform of the Na/K ATPase. Finally, we used marinobufagenin, dihydroouabain and ζ-

pseudosubstrate peptide to determine if PKMζ or the α_1 and α_2 Na/K ATPase isoforms protected neurons. All three compounds blocked neuroprotection following ischemic preconditioning. These data indicate key roles of PKMζ and Na/K ATPase in neuroprotection following ischemic preconditioning.

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List of Abbreviations

IPC	Ischemic preconditioning
GLT	Glutamate transporter
PKC	Protein kinase C
LTP	Long term potentiation
EBSS	Earle’s balanced salt solution
EI	Experimental ischemia
DG	Dentate gyrus
PI	Propidium iodide
TPA	Phorborl 12-myristate 13-acetate
DN	Dominant negative
ZIP	ζ-peptide
DHO	Dihydroouabain
NMDA	*N*-methyl-D-aspartic acid
NFATc4	Nuclear factor of activated T-cells family members
CREB	cAMP-response-element-binding
HIF	Hypoxia inducible factor
CNS	Central nervous system

Background and significance

The pathophysiology of stroke

Stroke is the nation's third leading cause of death and leading cause of disability (Caplan, 1984). There are relatively few therapies to limit ischemic injury. A better understanding of stroke pathophysiology will assist in the neuroprotective therapies. The brain is a high risk for ischemia due to its constant need of oxygen and glucose supply for its function. Loss of glucose and oxygen supply rapidly causes cell death and functional impairment.

Animal models have been developed to study how these processes are involved in the pathophysiology of stroke. There are two major types of stroke, focal ischemia and global ischemia. Since focal ischemia is a more common event in stroke, most of the following exposition is based on the findings from focal ischemia models.

Ischemia is triggered by a reduction of blood flow. In focal ischemia, there is an infarct and a penumbra. Cerebral blood flow supply of the core of the infarct is usually lower than 10ml/100g/min, which causes cell damage to outweigh endogenous protection mechanisms and is sufficient to cause cell necrosis. At the periphery of the core infarct, the supply of blood flow is between 10ml/100g/min and 20ml/100g/min (Wise et al., 1983). It causes electric failure but not permanent cell damage because endogenous protection still continuants

with cell damage signals. This zone has been referred as ischemic penumbra and stands between the death and survival (Garcia and Anderson, 1989;Astrup et al., 1981). Most clinical therapies are trying to rescue cells at the penumbra (Memezawa et al., 1992). The strategy is either limiting the damage reaction or promoting endogenous protection. Cell damage is more apparent and masks the endogenous protection phenomenon. Most early studies of stroke focused on the mechanism of cell damage.

Within minutes after onset of ischemia, oxidative phosphorylation of the cell is inhibited and the anaerobic metabolism is increased, causing a local rise in lactate production and acidification of intracellular pH. At the same time, the ATP production drops drastically. Ion homeostasis across the plasma membranes is progressively impaired. This impairment is due, in part, to energy failure that inactivates the Na/K ATPase. During ischemia, there is a massive efflux of K ions and an influx of Na ions. This results in reversal of the normal ion gradients. Both neurons and glia depolarize as a result the loss of ion gradients (Katsura et al., 1994). This depolarization triggers Ca influx through voltage-gated Ca channels, which further depolarizes the membrane (Budd, 1998). The reversed Na gradient causes Ca influx through Na/Ca exchanger which further aggravates the Ca overload. The reversed Na gradient also causes reversed transport of protons through Na/H exchanger and intensifies intracellular acidosis (Garcia and Anderson, 1989). The depolarized neuron releases synaptic vesicles containing excitatory amino acids into the extracellular space. Vesicle release has a smaller

effect on the rise in extracellular glutamate than the reversal of Na-dependent glutamate uptake that pumps glutamate out of the neuron (Collins et al., 1989;Mattson et al., 1993). The net effect of non-synaptic and synaptic glutamate release is prolonged, excitotoxic activation of glutamate receptors and Ca influx. Ca overload profoundly damages cell function, leading to activation of lipolysis, which produces potentially neurotoxic free fatty acids, arachidonic acid; and facilitate free radical production, which is extremely toxic to DNA, proteins, lipids and components of the extracellular matrix; Ca overload also damages mitochondria, resulting in formation of the permeability transition pore and cytochrome c release. Cytochrome C release activates caspases that leads to apoptosis. All of these direct and indirect effects cause and aggravate the damage of the cell (Dirnagl et al., 1999).

These studies of how neurons are damaged suggest particular strategy to interfere with ischemic injury. For example, glutamate receptor antagonists have been tested in clinical trials to inhibit the ischemic damage. There are numerous cellular processes that damage the brain, and effective therapeutics will need to interfere with many, if not all, of them. An alternative approach is to study endogenous protection mechanisms to prevent the ischemic damage. The study of ischemic preconditioning, which activates endogenous protection mechanisms, may provide the insight for new therapeutic interventions.

Ischemic preconditioning

In 1986, Murry found that brief non-lethal ischemia protected the heart from a subsequent lethal ischemia (Murry et al., 1986). This phenomenon was termed as ischemic preconditioning (IPC). IPC was also found in brain in 1991 (Kitagawa et al., 1991). Since IPC provides the rare opportunity to study neuroprotection that is not “masked” behind tissue damage, numerous studies have been conducted to identify potential molecules involved in this endogenous protection mechanism.

Mild ischemia induces neuroprotection, while more severe ischemia damages the brain despite the activation of endogenous neuroprotection. If mild ischemia precedes severe ischemia by hours to days, endogenous protection mechanisms have had time to be established. In this case endogenous neuroprotection can counter the neurotoxicity of ischemia. In the study of ischemia, many different cellular or molecular changes can be identified; yet it is hard to distinguish which changes belong to the damage or protection. In IPC, in which there is no tissue damage, the protection mechanisms are more apparent. Furthermore, we can study the quantitative effects of some cellular responses because many responses are beneficial at a physiological level but malicious at over activated level.

Two types of IPC exist: rapid and delayed IPC. Rapid IPC develops within several minutes after brief ischemia and lasts a few hours (Schurr et al., 1986;Perez-Pinzon et al., 1997a). Rapid IPC has only been described in the heart. In delayed IPC, protection slowly develops over 24 hours and lasts several days. IPC in the brain belongs to the delayed type (Kitagawa et al., 1991).

Despite many years of investigation, the mechanism of IPC still remains to be elucidated. Broadly speaking, potential mechanisms of preconditioning include alteration of preexisting protein by posttranslational modification or synthesis of new proteins. These changes may directly inhibit apoptosis and prevent inflammation. Alternatively, IPC may strengthen the effects of survival factors. Newly synthesized stress proteins may increase health capacity of cells by unfolding misfolded proteins and disposing denatured proteins. In addition, neurons may up-regulate the activity of glucose transporter or other proteins which enhance neuronal metabolism and promote ATP formation (Kirino, 2002). The mechanism described in this thesis, which is up-regulation of Na/K ATPase, potentially protects by supporting the maintenance of ion gradients across the membrane.

A model of ischemic injury and neuroprotection using young adult hippocampal slice cultures

Both *in vivo* (Kitagawa et al., 1991;Kato et al., 1992;Perez-Pinzon et al., 1997b) and *in vitro* (Bruer et al., 1997;Grabb and Choi, 1999;Khaspekov et al., 1998) animal models have been used to study ischemia and IPC in the brain. This thesis studies the endogenous neuroprotective mechanisms using an *in vitro* model that uses hippocampal slice cultures. Due to the biological complexity of ischemia and various methods to induce ischemia, *in vivo* results from different experiments are hard to compare. Ischemic preconditioning has also been modeled *in vitro* in cell lines or disassociated embryonic neurons. A caveat of these models is that these neurons are isolated from embryonic or perinatal animals and the susceptibility to ischemic injury is well known to be age-dependent. In addition they do not model neuronal-glial interactions that are likely to be important in both ischemic injury and protection by IPC. Research in ischemic injury and IPC has also been conducted in primary glia cells and glial cell line. These studies have implicated the induction of glutamate uptake (Stanimirovic et al., 1997b). However, in pure glial cultures, it is hard to determine if the alteration of glutamate uptake contributes to glutamate excitotoxicity.

For these reasons, hippocampal slice cultures have emerged as a more physiological *in vitro* model. The hippocampus is a brain region that is frequently damaged following ischemia. The laminar arrangement of neurons and glia make it easy to assay neuronal damage. Slice cultures can be isolated from rodents as early as P0 and as late as P30. Above P30, slice can be cultured, but the plating

efficiency of the cultures is much lower. The difficulty in getting large numbers of cultures hinders their experimental use. A comparison of slices isolated from P6-P10 rats to cultures isolated from P20-30 rats show that age-dependent susceptibility to ischemic injury was maintained *in vitro*. As a result, studies utilizing slice cultures from P6-P10 rats displayed inherent hypoxia resistance from seen in young animals (Bonde et al., 2003;Xu et al., 2002). This thesis utilized P20-P25 rats. These cultures from P20-25 rats are substantially more susceptible to ischemic injury than cultures from P6-10 rats. Slice cultures from P20-25 day old rats can be utilized to study neuronal or glial death during ischemia more accurately than previous models. The use of slice cultures from P20-25 rats allows a large number of samples to be treated and analyzed in one experiment. Slice cultures provide greater access to the neurons before and after preconditioning; thus allowing for optical, biochemical and electrophysiological studies that would not be feasible *in vivo*. *In vitro* models also provide a more controlled environment for the testing of a neuroprotective drugs. It is especially useful for screening of potential drugs on neuroprotection before expensive *in vivo* animal model drug screening. In general, this *in vitro* organotypic slice ischemic preconditioning model provides a unique system for the study of neuroprotection following IPC (Hassen et al., 2004).

Neuroprotection by Na/K ATPase

One of the earliest events after the onset of ischemia is the energy failure and loss of ion membrane gradients. The Na/K ATPase is the major molecule

that maintains the ion homeostasis through membrane. The Na/K ATPase likely plays a key role during ischemia.

Na/K ATPase restores ionic gradients following action potentials as well as powering a large number of sodium-dependent membrane transporters. During ischemia, energy failure during ischemia leads to loss of Na, K, and Ca gradients (Lipton, 1999;Martinez-Sanchez et al., 2004;Mergenthaler et al., 2004). Loss of ion gradients leads to membrane depolarization, cell swelling, glutamate release and cell death (Tanaka et al., 1997a;Lipton, 1999;Breder et al., 2000;Therien and Blostein, 2000;Sheldon et al., 2004). The Na/K ATPase restores the sodium and potassium gradients when perfusion returns (Tanaka et al., 1997a;Lipton, 1999;Tanaka et al., 2002).

In the heart, IPC preserves Na/K ATPase activity in the early phase of ischemia and reduces infarct size. Inhibition of Na/K ATPase activity reduces the neuroprotective effect of IPC. Preservation of Na/K ATPase activity increases Na/Ca exchanger activity and reduced accumulation of intracellular Ca. Other studies in the heart also support the idea that the Na/K ATPase regulates the Ca ion homeostasis after IPC (Nawada et al., 1997;Elmoselhi et al., 2003;Yorozuya et al., 2004). In brain, preconditioning may also preserve Na/K ATPase activity following ischemia(de Souza Wyse et al., 2000). This study, however, did not test whether maintenance of Na/K ATPase is neuroprotective. A caveat of all above studies is that total activity of Na/K ATPase was assayed. As discussed further

below, a large amount of Na/K ATPase is intracellular. These intracellular Na/K ATPases do not control ion gradients across the plasma membrane. Measurement of total Na/K ATPase activity may not reflect cell surface pumping. Finally, previous studies did not address how IPC increased in total Na/K ATPase activity.

Na/K ATPase

This thesis examines the neuroprotective role of the Na/K ATPase. Na/K ATPase is a highly conserved membrane protein throughout all higher eukaryotes. By hydrolysis of the terminal phosphate bond of ATP, Na/K ATPase actively transports sodium out of the cell and potassium into the cell. In each cycle of transport, three Na ions are transported out and two K ions are transported in the cell. The Na/K ATPase is a heterodimer consisting of α and β subunits. The α subunit is the ion transport unit. The α subunit is a membrane protein with a molecular mass about 110 kDa that has ten transmembrane segments (Jewell and Lingrel, 1991). The β unit stabilizes α unit conformation and targets the protein to the cell surface (McDonough et al., 1990;Chow and Forte, 1995). The Na/K ATPase can also associate with a set of proteins termed γ-subunits. γ–subunits are a family of small transmembrane proteins that specifically associate with α-subunits but are not an integral part of the enzyme complex (DeTomaso et al., 1993;Scheiner-Bobis and Farley, 1994).

Alpha subunit isoforms are expressed by four genes. Though all alpha isoforms utilize the same mechanism to fulfill their function, they differ in protein sequence. The α_1, α_2, α_3 isoforms share 87% amino acid similarity. The highest amino acid variability is at the N-terminus which contains the binding site for ouabain and ouabain like compounds (Blanco and Mercer, 1998). The role of these compounds in Na/K ATPase action will be discussed below. Three of four genes for individual alpha subunits have been knocked out. Homozygous mutant animals display developmental lethality suggesting an essential function for each isoform (Dobretsov and Stimers, 2005). In addition, there appears to be no complementation among α subunit isoforms. This non-overlapping function of each isoform is not well understood, yet it may relate to the isoform specific tissue distribution. The α_1 isoform is present in all cell types suggesting a housekeeping function. The α_2 isoform is located in the muscle cells and glia. The α_3 isoform is expressed in neurons and has a lower sodium ion affinity that responds to the extra demand for sodium ion homeostasis in neurons. The α_4 isoform is expressed in sperm (Kaplan, 2002). In brain, the α_1 and α_3 are major isoforms in neurons and α_1 and α_2 are major isoforms in astrocytes.

Na/K ATPase isoforms also differ in the affinity for Na, K, and ATP. By expressing individual α isoforms in Sf-9 insect cells (Blanco and Mercer, 1998), Mercer et al compared the dose response of various Na/K ATPase isozymes toward Na, K and ATP. The affinity for Na varies with a rank order of $\alpha_2>\alpha_1>\alpha_3$, for K $\alpha_1>\alpha_2>\alpha_3$, and for ATP $\alpha_3=\alpha_2>\alpha_1$. These differences in affinity likely reflect

specific functions in the brain. In neurons, α_1 and α_2 isoforms maintain basal ion gradients. The α_3 isoform, the most abundant form in neurons, has a lower affinity to Na and K but higher affinity to ATP and may function during repeated action potential firing when ATP maybe locally depleted along with elevated intracellular Na and extracellular K. In glia, α_2 functions at steady state to efficiently restore extracellular K resulting from neuronal activity. Restoration of the potassium gradient prevents both further depolarization and spreading depression. The α_2 may also be important for regulation of glutamate transport.

Na/K ATPase and brain energy usage

The human brain accounts for 20% of resting metabolism of our body, though it is only 2% of body weight. Under normoxic conditions, 40% brain ATP is consumed by Na/K ATPase (Hochachka, 1998). Much of this ATP is used by Na/K ATPase for maintenance of the ion fluxes needed for excitatory postsynaptic potentials and action potentials.

The Na/K ATPase not only restores the ion gradient following action potentials but also establishes the membrane resting membrane potential (Glitsch, 2001). Because the Na/K ATPase pumps 3 Na ions out for every 2 K ions, it transfers net charge across the membrane. Inhibition of the Na/K ATPase results in a slow depolarization of the membrane since pumping counters the

leak of Na and K across the membrane down their electrochemical potential gradients.

The Na ion gradient established by Na/K ATPase also stores potential energy that is utilized for active transport. The cell imports all key metabolites against their concentration gradients by utilizing the energy gained by co-transport with the Na down its electrochemical gradient.

During ischemia, the increased glutamate release and decreased glutamate uptake play a key role to initiate the excitotoxic neuronal loss. Under normal conditions, glutamate is essential for the excitatory synaptic transmission. After interacting with glutamate receptors, clearance of glutamate is necessary for proper function. In the brain, a family of glutamate transporters are responsible the clearance of extracellular glutamate. There are five different type transporters, GLT, GLAST, EAAT3, EAAT4, and EAAT5. GLT and GLAST mostly expressed in glia while EAAT3, EAAT4, EAAT5 are specifically expressed in neurons (Danbolt, 2001). Recent studies have implicated GLT and GLAST as the most important glutamate transporters to clear glutamate.

All glutamate transporters co-import one sodium ion per glutamate. The Na gradient established by Na/K ATPase provides the energy for Na-dependent glutamate transport. During ischemia, both the sodium gradient and the direction of glutamate transporter are reversed, which increases glutamate and worsens

excitotoxicity. However, a single report studying glial cell culture suggested that sublethal ischemia stimulates glutamate uptake and Na/K ATPase activity (Stanimirovic et al., 1997a). Recent evidences showed that GLT and GLAST co-localized with α2 isoform of Na/K ATPase in perisynaptic areas surrounding glutamatergic synapses. These data suggest functional coupling of Na/K ATPase and glutamate transporter. These data also underscore how the Na/K ATPase may facilitate glutamate uptake and repress excitotoxicity following ischemia.

Other similar transporters that utilize the Na gradient include the Na/Ca exchanger and Na/H exchanger. The Na/Ca exchanger imports two Na ions and exports one Ca ion. It is a key molecule to maintain the local Ca homeostasis. The Na/H exchanger prevents intracellular acidification by exporting protons. These transporters play indispensable roles for the proper cellular function. The Na ion gradient is also central in maintaining osmotic balance of the cell because excess intracellular Na influx triggers cell swelling.

Under anoxia, 75% of cellular ATP is utilized by the Na/K ATPase (Buck and Hochachka, 1993). This reflects its importance in the restoration of the Na and K gradients. During ischemia, there is a large influx of Na, Ca, H and a large efflux of K. The Na/K ATPase directly restores the Na gradient. Neurons use the Na gradient to re-establish the Ca and H gradients. These observations show the essential role of Na/K ATPase in normal cellular function and following ischemia.

Regulation of Na/K ATPase

Cell utilizes many mechanisms to regulate Na/K ATPase activity. These include allosteric regulation by intracellular Na or extracellular K, membrane associated components, phosphorylation by protein kinase A, protein kinase C and tyrosine kinases (Therien and Blostein, 2000;Al-Khalili et al., 2003).

Pump activity is significantly stimulated by increased of intracellular Na. Pump activity is also increased by elevated extracellular K. The Na/K ATPase is also regulated by the direct or indirect interaction with membrane associated components including γ-subunit and cytoskeleton proteins such as spectrin, ankyrin, actin, and adducin. The spectrin-ankyrin complex is required for transport of pumps from ER to Golgi apparatus (Devarajan et al., 1997). Adducin stimulates pump activity by increasing the apparent affinity for ATP (Ferrandi et al., 1999). γ-subunit regulates pump activity by increasing ATP affinity (Therien et al., 1999). These regulators may be important in the response of Na/K ATPase during anoxia.

Protein phosphorylation also regulates Na/K ATPase activity. The two kinases most studied that regulate Na/K ATPase are PKA and PKC. The effects of PKA and PKC phosphorylation on Na/K ATPase activity differ depending upon the cell type being studied. Both regulate the pump by phosphorylation of the α subunit. The phosphorylation site of PKA lies at ser-943 (Fisone et al., 1994).

PKA phosphorylation may regulate the exit of Na/K ATPase from the ER. The phosphorylation site for PKC is at ser-18 (Chibalin et al., 1998a). PKC phosphorylation is thought to be responsible for endocytosis of Na/K ATPase from plasma membrane. PKC phosphorylation recruits PI3K binding to the N-terminal of α subunit which expose binding site for the clathrin associated protein AP-2 (Yudowski et al., 2000). PI3K binding changes the membrane phosphoinositide composition which promotes the development of clathrin-coated pits. Phosphorylated Na/K ATPase is internalized and membrane pump activity is reduced.

The internalized pump may be recycled back to plasma membrane. This membrane trafficking may be critical for Na/K ATPase function since a large amount of Na/K ATPase is sequestered in a cytoplasmic pool (Chibalin et al., 1998b). Plasma membrane Na/K ATPase activity can increase due to enhanced transfer from intracellular pool to the plasma membrane pool. Alternatively, surface activity can increase by inhibition of pump endocytosis. This thesis will demonstrate that atypical PKC activity regulates Na/K ATPase trafficking.

Tyrosine phosphorylation of Na/K ATPase is a newly emerging field. Protein tyrosine kinases are involved in a number of physiological and pathological functions including cell growth, proliferation, cell cycle, and cell survival. In skeletal muscle, insulin stimulation increases Na/K ATPase activity by translocation of Na/K ATPase molecules to the plasma membrane following

phosphorylation of the _-subunit on both serine/threonine and tyrosine residues (Al-Khalili et al., 2003). *In vitro* phosphorylation experiments showed that the _-subunit of Na/K ATPase from skeletal muscle was a substrate for the tyrosine-specific protein kinase c-Src. Other studies suggest a direct link between Src kinases and the Na/K ATPase protein, as a pathway to transmit ouabain-triggered signals into intracellular compartments via Src kinases (Xie and Xie, 2005). These results suggests that Na/K ATPase activity is regulated by specific Src tyrosine kinases via a protein-protein mechanism, which in turn regulates the Na/K ATPase trafficking.

Atypical PKC and ischemic preconditioning

Protein kinase C (PKC) belongs to the family of serine-threonine kinases. PKC is a family of isozymes that is divided into three subfamilies based on their homology and sensitivity to activators (Bright and Mochly-Rosen, 2005). Members of the classical or cPKC subfamily, — α, βI, βII, and γPKC, —contain four homologous domains (C1, C2, C3, and C4) interspaced with isozyme-specific (variable or V) domains. Classical PKCs require calcium, phosphotidyl serine, and diacylglycerol for activation. Members of the novel or nPKC subfamily, —δ, ε, η, and θ, —lack the C2 homologous domain. They require phosphotidyl serine, and diacylglycerol, but do not require calcium for activation. Finally, members of the atypical or aPKC subfamily, ζ and ι, lack both the C2 and one-half of the C1 domains and are insensitive to diacylglycerol, and calcium.

Phorbol esters mimic diacylglycerol and are potent stimulators of cPKCs and nPKCs, but have no effect on aPKC activation. The brain expresses an additional aPKC isoform, PKMζ. PKMζ is expressed as an alternative mRNA from the PKCζ gene. PKMζ lacks the negative regulatory domain found in other PKC isoforms (Hernandez et al., 2003). The lack of a negative regulatory domain suggests that PKMζ is constitutively active.

Atypical PKCs also has an unusual ATP binding site with the sequence $GXGXXAX_{16}K$ that has a higher ATP affinity than other PKC family members that contain the binding site $GXGXXGX_{16}K$. Thus the affinity of aPKCs to ATP is higher. In the one example in which it was directly compared, PKCι [$K_{m(ATP)}$=13.5± 4.25μM] has a higher ATP affinity than cPKCs or nPKCs [$K_{m(ATP)}$= 38.8μM] (Spitaler et al., 2000). This difference in ATP affinity suggests that aPKCs better retain activity at low ATP level than cPKCs or nPKCs. In addition, inhibitors at the ATP binding site differentiate between aPKCs and cPKCs and nPKCs. Two such inhibitors are used in this thesis, Go6850 and staurosproine. Another kinase inhibitor used in this thesis is derived from the protein sequence of substrate binding domain. This pseudosubstrate peptide prevents the substrate binding and has been extensively used as specific inhibitors of aPKCs.

This thesis identifies PKMζ as a key regulator of Na/K ATPase. PKMζ has two features that make it an excellent candidate to mediate IPC. It lacks the negative regulatory domain of other PKCs suggesting high constitutive kinase

activity in the absence of second messenger activation. Second messengers are typically short-lived molecules that require ATP for their synthesis. As result, during the energy failure that accompanies ischemia or IPC, most kinases will be inactive since second messenger synthesis will be inhibited. Secondly, protein levels of PKMζ and other atypical PKCs remain unchanged following ischemia while conventional and novel PKC isoforms are downregulated by proteolysis (Libien et al., 2005). The substrates of PKMζ have not been well studied in brain. In thyroid cells and in vascular smooth muscle, PKCζ induces Na/K ATPase activity (Marsigliante et al., 2003). Na/K ATPase in brain may be regulated by PKMζ, since PKMζ has an identical catalytic domain as PKCζ.This thesis examines whether PKMζ is neuroprotective after ischemic preconditioning by regulating Na/K ATPase.

Regulation of Na/K ATPase by endogenous ouabain molecules

Cardiotonic steroids such as ouabain and ouabain like compounds such as marinobufagenin, have been used to treat congestive heart failure for more than 200 years. For decades it has been thought that their only action is inhibition of Na/K ATPase. The evolutionary retention of ouabain binding site has been suggested that endogenous ouabain like molecules regulate the Na/K ATPase. In the past few years has it become apparent that mammals naturally produce a family of compounds similar to ouabain. How ouabain, and other steroids that have an effect on heart function has recently been elucidated. As a result, the

physiological role for endogenous ouabain and ouabain like compounds has recently become a very active area of research (Schoner, 2002).

The extracellular domain α isoform of Na/K ATPase contains the ouabain binding site. Different α isoforms have greatly differing affinity to ouabain. In the rat, these isoforms bind the inhibitor ouabain with a rank order of $\alpha_3 > \alpha_2 > \alpha_1$. The identification of endogenous ouabain has utilized the role of ouabain as a hypertensinongenic factor. Prolonged exposure of rats to small doses of ouabain leads to hypertension (Manunta et al., 1994). In addition, administration of a ouabain-binding antibody significantly lowers the arterial blood pressure (Hamlyn et al., 1998). Genetic studies suggest that the ouabain binding site of α isoform is a key regulator of blood pressure (tanic-Larson et al., 2005). However, the dose response of the hypertensinogenic effect of ouabain does not correlate with its ability to inhibit the Na/K ATPase because ouabain-like molecules that inhibit pumping with lower affinity are potent inducerss of hypertension (Manunta et al., 2001). This has led to the hypothesis that ouabain and ouabain like molecules do not simply act as pump inhibitors, rather they act as endogenous hormones and use Na/K ATPase as a receptor that activates intracellular signals.

Additional evidence shows that ouabain functions both in pump inhibition and signal transduction using the pump as a receptor. As a Na/K ATPase inhibitor, high levels of exogenous ouabain specifically inhibit pump activity. Endogenous ouabain like compounds never reach the high concentration of

exogenous doses of ouabain needed to inhibit pumping. At it low endogenous levels, ouabain acts through signal transduction in cardiac myocytes. Ouabain binding leads to the conformation change of Na/K ATPase, which activates the tyrosine kinase Src that is bound to the pump. Src phosphorylates epidermal growth factor receptor which induces signals through ERK kinases or Ras. Both pathways signal the nucleus to modify gene expression (Xie and Xie, 2005). Although this pathway has been elucidated in myocytes, the role of ouabain and ouabain like compounds to signal in the brain has not been studied. However, all the signal molecules in myocyte are present in neurons suggesting that the brain could use a similar signal transduction pathway.

Ouabain like compounds also has different binding affinities for pump isoforms than ouabain. For example the rat α_1 isoform binds ouabain at low affinity, but binds ouabain like compound marinobufagenin (Doris, 2001) with higher affinity. The ouabain derivative dihydroouabain binds with a higher affinity to α_2 and α_3 isoforms (Vaillend et al., 2002). These ouabain like compounds signal through the pump at low concentrations but inhibit pumping at high concentrations. In this thesis, ouabain like compounds will be used to test the role of individual Na/K ATPase isoforms.

Ischemic preconditioning and synaptic plasticity

IPC may share similar mechanisms with one form of synaptic plasticity called long-term potentiation (LTP). Induction of LTP requires the activation of the NMDA type of glutamate receptor and consequential increase in intracellular Ca. LTP has been divided into an induction phase that lasts for 30 minutes and a maintenance phase that lasts hours to days. The maintenance phase of LTP requires the atypical PKC isoform PKMζ (Sacktor et al., 1993;Ling et al., 2002).

LTP produces persistent changes in the brain. Neuroprotection following preconditioning also requires many hours to develop and persists for many days. A preconditioning stimulus occluded LTP formation (Kawai et al., 1998). Preconditioning-like stimuli also produce a persistent increase in synaptic efficacy termed anoxic LTP (Crepel et al., 1993). Tetanic stimulation of afferent axons produces LTP as well as protecting against a subsequent lethal ischemia (Youssef et al., 2001). All these studies suggest that similar mechanisms underlie LTP and IPC. The atypical PKC isoform PKMζ mediates of the long-term maintenance of tetanic LTP (Sacktor et al., 1993;Ling et al., 2002). The higher ATP affinity, lack of regulatory domains and resistance to proteolysis induced by ischemia suggest PKMζ has a role in precondioning. Therefore the involvement of PKMζ in IPC was investigated for the persistent neuroprotection following ischemic preconditioning.

Methods

Hippocampal slice culture

Hippocampal slices from 18-25 day old Sprague Dawley rat were isolated and cultured. Rats were anesthetized with halothane followed with Ketamine (8mg/kg) and decapitated. The brain was isolated and transferred to ice-chilled dissection solution (in mM): KCl, 4.96; KH_2PO_4, 0.2; NaCl, 136.9; $NaHCO_3$, 2.71; Na_2HPO_4, 1; $CaCl_2$, 1.54; $MgCl_2$, 11.03; $MgSO_4.7H_2O$, 0.57; Glucose 33.2; HEPES, 2,PH 7.2. The brain remained in solution for 30 minutes continuously bubbled with 95%O_2, 5% CO_2. The hippocampus was then carefully isolated and cut into 400μm slice using McIllwain Tissue Chopper (Brinkman Instruments, Westbury, NY). Slices with intact neuronal cell layers were plated into Millicell CM filters (Millipore, Bedford, MA). Slices were incubated in 1ml high-potassium, high serum media: 50% (V/V) BME, 25% EBSS, 25% horse serum; Glutamine, 1mM; and HEPES, 25 mM; pH 7.2. 3 days later, slices were then transferred to low-potassium, high serum media: 50%BME, 20%horse serum; 30% homemade salt solution ($CaCl_2$, 1.8mM; $MgSO_4$, 4 mM; NaCl, 200mM; $NaHCO_3$, 31mM; Na_2HPO_4, 0.7mM); glutamine, 1mM; and HEPES, 25mM; pH 7.2. After 2 days, the slice were changed to low-potassium, low serum media: 50% (V/V) BME, 30% HMSS, 5% horse serum; Glutamine, 1mM; and HEPES, 25 mM; pH 7.2. Once in low-potassium, low serum media, the cultures were fed with 1ml this media twice per week. All the cultures were maintained at 32^0C in a 20% O_2,

$5\%CO_2$ atmosphere and for at least two weeks prior to the beginning of all experiments. One day before an experiment, the cultures were shifted to a 37^0Cincubator in a 20% O_2, 5% CO_2 atmosphere.

Experimental ischemia (EI)

For ischemic preconditioning, slice cultures were submerged in Earles Balanced Salt solution (EBSS) without glucose for 5 minutes and bubbled vigorously with 95% N_2, 5% CO_2. A mock ischemic preconditioning group were submerged in EBSS with 5 mM glucose for 5 minutes and bubbled vigorously with 20% O_2, 75% N_2, 5% CO_2. Twenty-four hours later, both groups received a potentially neurotoxic 10-minute treatment of EBSS without glucose and bubbled vigorously with 95% N_2, 5% CO_2. Propidium iodide (4ug/ml) staining was used to assay neuron loss.

NeuN staining

NeuN staining was used to confirm that ischemic preconditioning induced neuroprotection. A monoclonal NeuN antibody (clone A60, Chemicon, Temecula, CA) specifically reacts with the antigen NeuN, which is present in nuclei of neurons in hippocampal slice cultures. Seven days following the 10-min EI treatment, slice cultures were chilled to 4 °C and washed three times with phosphate buffered saline (PBS) and fixed with 4% (w/v) paraformaldehyde overnight at 4 °C. The cultures were carefully removed from their Millipore-CM filter insert using a paint brush and incubated, free-floating, in PBS containing

0.5% (v/v) Triton X-100 at room temperature. The slices were then incubated in 10% (v/v) horse serum in PBS for 1 h at 4 °C. NeuN antibody (diluted 1:100 in PBS containing 0.5% (v/v) Triton X-100) was added and the cultures incubated overnight at 4 °C. The slices were washed three times for 10 min with PBS. Alexa 488 labeled goat anti-mouse IgG (Molecular Probes, diluted 1:200 in PBS containing 0.5% Triton X-100) was added and incubated for 2 h at room temperature in the dark. The slices were washed three times for 10 min with PBS, dried and mounted. NeuN immunofluorescence was analyzed using a Bio-Rad MRC 1024 ES scanning confocal laser mounted on an Olympus LX70 microscope.

ATP measurement

Slices were immediately frozen on dry ice. ATP was extracted in 100μl 3N perchloric acid and 300μl 1mM EDTA with tissue homogenizer. Extracts were neutralized with 150μl 2M $KHCO_3$ followed by 8μl 2M Tris-OH. ATP content was determined using photometric luciferin-luciferase assay. Samples were analyzed in duplicate in Tricine buffer containing 25 mM Tricine, 5mM $MgSO_4$, 0.5mM EDTA, 0.5mM DTT with firefly luciferase (Sigma, St. Louis, MO). A standard 1μM ATP sample was assayed fro re-calibration purposes every 6 samples. The fluorescence was read with Fluoro-colorimeter (SLM, Urbana, IL). Slice culture protein was determined using the BCA assay (Pierce, Rockford, IL).

^{86}Rb uptake in slice cultures

The slice cultures were shifted from 32^0C to 37^0C incubator overnight in 5% CO2 atmosphere. The culture media was changed with 2.3mM K EBSS and at the same time, ^{86}Rb 1μCi was added to the solution and incubated 30 minutes at 37^0C. The reaction was stopped with ice-chilled 2.3 mM EBSS and extracellular ^{86}Rb removed with 3 washes of 2.3 mM EBSS. Then the slices were homogenized with 1ml 0.1N NaOH. A 200 μl aliquot of the homogenized sample was added to 10 ml of Ecolume scintillation fluid (ICN Biomedicals, Irvine, CA). Samples were counted on a Beckman LS6000IC scintillation counter (Beckman Instruments, Fullerton, CA). Protein content was measured using Pierce BCA assay (Rockford, IL). Values are expressed as Mean (pmol/ug/min) ±SEM.

Biotinylation of slice culture cell surface proteins

Slice cultures were washed with ice-chilled 2.3 mM EBSS (pH 7.4) three times after pharmacology administration or other treatment. Samples were biotinylated in EBSS containing 1.0 mg/ml EZ-Link sulfossuccinimidobiotin (sulfo NHS-S-S-biotin; Pierce) for 40 minutes at 4°C on a roller system. The size of biotin cross linker is about 5nm and it can penetrate the slice and label almost all the membrane protein. After labeling, samples were washed one time with ice-chilled EBSS containing 0.1M glycine (wt/vol) at 4°C to quench the free unreacted sulfo NHS-S-S-biotin. Next the samples were washed with EBSS two more times. Then the samples were lysed with lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, and protease inhibitor cocktail). After that, the samples were sonicated and solubilized 2 hours

on a roller system. The supernatants were collected from samples after 13,000rpm 20 minutes centrifuge. Biotinylated proteins were precipitated in the presence of streptavidin-agarose beads (Immunopure immobilized streptavidin; Sigma) diluted in lysis buffer and incubated overnight at 4°C on a roller system. Then the beads were washed twice with lysis buffer. After resuspension in sample buffer, samples were processed for SDS-PAGE and Western blotting.

^{32}P labeling of hippocampal slice cultures

Slice cultures were transferred from 32^0C to 37^0C and the media was changed to with 2.3mM K EBSS. The slice was then incubated with 250 mCi of [^{32}P] orthophosphoric acid in 1 ml of the same buffer (without Na_2HPO_4) at 37°C for 120 min to radiolabel the intracellular ATP pool. Then the drug-treatment was performed for another 1 hour in the same media. At the end of the labeling, the buffer was removed, and the ^{32}P-labeled hippocampal slice was rinsed twice with 2 ml of fresh buffer. The samples were lysed in 200ul of cold lysis buffer (50 mM Tris hydrochloride, 120 mM NaCl, 1 mM EDTA, 1% NP-40, and ; pH 7.4) containing 30 mM NaF, 1 mM EGTA, 25 mM benzamidine, 0.1 mM PMSF, 20 µg/ml leupeptin, 20 µg/ml antipain, 5 µg/ml pepstatin A, and 5 µg/ml chymostatin. Aliquots from each sample containing equal amounts of protein were used for immunoprecipitation.

Immunoprecipitation

Lysates were subsequently incubated at 4°C overnight on a rotatory shaker with 60 µl of α6F antibody. The resultant immunocomplexes were incubated with protein G-agarose beads at 4°C for 4 h. The beads were collected by centrifugation and washed at 4°C first with 1 ml of lysis buffer, then three times with 1 ml of a buffer containing 50 mM Tris · HCl, 120 mM NaCl, 1 mM EDTA, 1% NP-40 (pH 7.4); three times with 1 ml of a buffer containing 50 mM Tris · HCl, 500 mM NaCl, 1% NP-40 (pH 7.4); and finally with 1 ml of a buffer containing 50 mM Tris · HCl (pH 7.4). After the final wash, the beads were re-suspended in 25-30 µl of 2X Laemmli sample buffer and incubated at 65°C 15-20 minutes. The supernatants were collected by centrifugation. The supernatants were resolved by electrophoresis on 7.5% SDS-polyacrylamide gel (Bio-rad). Gels were dried and subjected to autoradiography. ^{32}P-labeled immunoprecipitated Na/K ATPase α_1-subunit was identified by phosphoimager analysis (Molecular Dynamics).

Immunoblotting

Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fifteen ug/cm-lane of total protein was loaded on the gels. The SDS-PAGE was run 1 hour with 160volts. Then the SDS-PAGE was transferred

to nitrocellulose and blocked for 1 hour in 5% powdered milk in 10 mM Tris-HCl, 150mM NaCl, 0.1% Triton-X100. The nitrocellulose was incubated overnight in primary antibody (1:1000), and then 1 hour in secondary antibody coupled to alkaline-phosphatase (1:2000, Sigma). Development was performed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (KPL). Isoform-specific antibodies against Na/K ATPase alpha subunit isoforms, α_1 (1:1000, Developmental studies hybridoma bank, Univ. Iowa), α_2 (1:1000, kindly provided from Dr. T. Pressely, Texas Tech Univ(Pressley, 1992). α_3 (1:1000, Affinity Bioreagents, Golden, CO), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:100,000, Sigma). Immunoreactive bands were scanned and quantified using Image J 1.30 software (NIH).

Virus infection

Recombinant sindbis virus vectors were constructed and grown by the method of Xiong (Xiong et al., 1989). These recombinant virus vectors were provided by T. Sacktor, SUNY Downstate Medical Center. The two constructs used in this study were pSinRep5-HA-EGFP and pSinRep5-HA-PKMζ. The pSinRep5-HA-EGFP and pSinRep5-HA-PKMζ virus vectors were constructed using a sindbis expression kit (Invitrogen). The fragments coding for EGFP and PKMζ were subcloned into the SinRep5 to generate pSinRep5-HA-EGFP and pSinRep5-HA-PKMζ respectively. Capped mRNAs from these constructs were in vitro transcribed using the InvitroScript^{TM}Cap SP6 Kit and cotransfected with the sindbis DH (26S) helper RNA (invitrogen) into baby hamster kidney cells. The

virus particles were collected from the supernatant of cell lysates and concentrated by differential centrifugation. The pSinRep5-HA-EGFP and pSinRep5-HA-PKMζ viruses were injected into the CA1 pyramidal cell area of slice cultures. The injection was performed with autoclaved 20uM diameter glass micropipettes under the control of Nanoliter 2000 injector (World Precision Instruments, Fl). Multiple 100nl injections were performed in a single CA1 pyramidal cell area. After the injections were complete, the slices were incubated at 37^0C in a 20% O_2, 5% CO_2 atmosphere. Six hours after incubation, the CA1 area of slices was microdissected and ^{86}Rb uptake was measured.

Results

Establishment of *in vitro* ischemic preconditioning model using hippocampal slice cultures

In ischemic preconditioning, a short, sublethal episode of EI protects against a longer, neurotoxic episode (Dirnagl et al., 2003). Ischemic injury also shows sharp time thresholds: hence, a small increase in the duration converts non-toxic EI to neurotoxic EI. To determine their threshold for ischemic injury, young adult slice cultures received an EI treatment for either 5 or 10 min (Figure 1). Control cultures received 10 min of mock-EI. PI fluorescence was assayed daily for 8 days. PI fluorescence was determined separately in the CA1 and CA3 pyramidal cell layers and the dentate granule cell layer. PI fluorescence did not increase in cultures receiving a 10-min mock-EI treatment or a 5-min EI treatment (Figure 1A). After a 10-min EI treatment, PI fluorescence did significantly increase (Figure 1). In CA3 and the granule cell layer, PI fluorescence remained elevated until day eight. In the CA1 pyramidal cell layer PI fluorescence remained elevated at day 8. To confirm that PI was measuring neuronal death, slice cultures were fixed and stained with Nissal staining. Mock-EI-treated cultures showed the characteristic pyramidal and granule cell layers. Virtually no Nissal staining was observed in cultures receiving the 10-min EI treatments. These data suggest that slice cultures show high sensitivity and a sharp threshold to injury following EI.

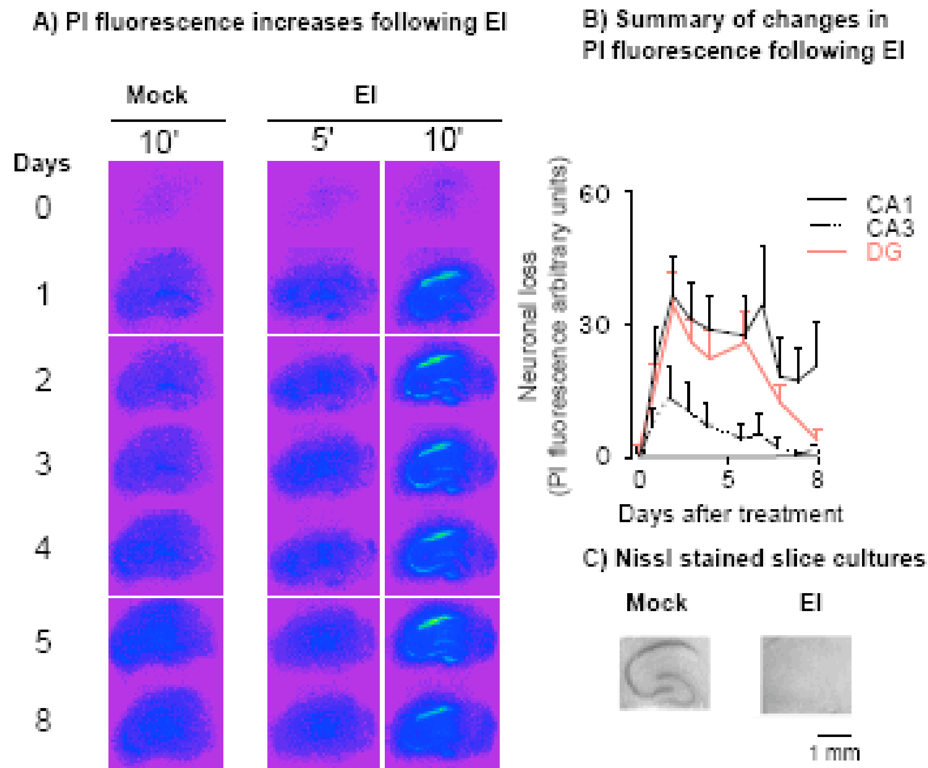
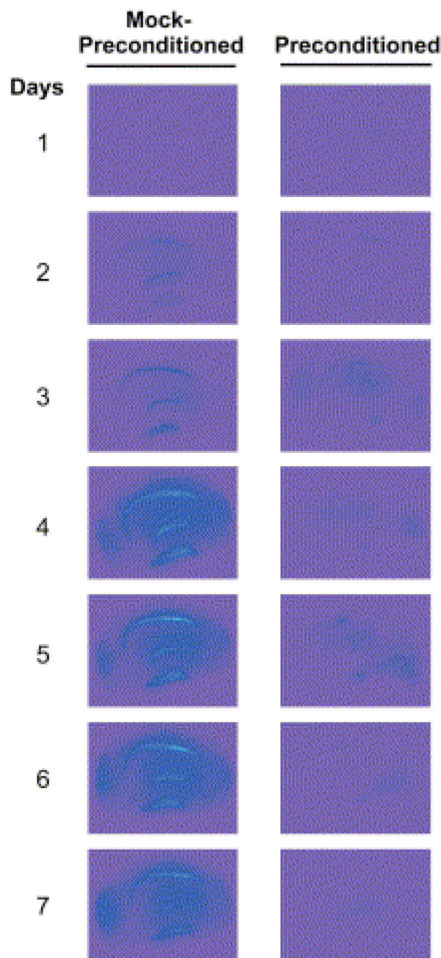


Figure 1. A 10-min EI treatment is neurotoxic to young adult slice cultures. Panel A: Changes in propidium iodide (PI) fluorescence. False colored PI fluorescent images were recorded from individual slice cultures immediately before and after 10 min of mock-treatment, and 5 or 10 min of EI. Fluorescence images were recorded at 1-day intervals for ten days. Magnification 6_. Panel B: Summary of the changes in PI fluorescence. PI fluorescence increased in CA1 and CA3 following 10 min of EI. Values are mean arbitrary fluorescence units±S.E.M. The change in PI fluorescence in CA3 was significantly smaller than in CA1 (n=6, ANOVA, F=4.5296, p<0.0001). Panel C: Neuronal loss following the 10-min EI treatment. Following the last PI assay, cultures were stained with Nissal staining. Mock-treated cultures showed prominent pyramidal and granule cell layers, while cultures treated with 10 min of EI showed few neurons remaining. DG: Dentate gyrus.

We then tested if the sublethal 5-min EI treatment protected against a subsequent, neurotoxic 10-min EI treatment (Figure 2). Slice cultures were divided into two groups. The IPC group received a 5-min EI treatment while the mock-IPC group received a 5-min mock-EI treatment. One day later, both the groups received a neurotoxic 10-min EI treatment. PI fluorescence was assayed daily for 7 days. For the first 2 days following the 10-min EI treatment, PI fluorescence was similar between the IPC or mock-IPC groups. On day 3, PI fluorescence was significantly decreased in the IPC as compared to the mock-IPC group. This difference persisted until day 6. These data suggest that 5 min of EI reduced neuronal loss following a subsequent 10-min EI. To confirm that neurons were protected in the IPC group, cultures in the IPC and mock-IPC groups were stained with an antiserum specific for the neuron-specific nuclear antigen, NeuN. Numerous NeuN-stained cells were observed in the CA1 pyramidal cell layer of the IPC group, whereas few NeuN-stained cells was observed in the mock-IPC group (Figure 3). A similar retention of NeuN-stained cells of CA3 pyramidal cell layer and dentate granule cell was seen in the IPC group (data not shown). Retention of NeuN staining as well as the lack of PI staining suggests that the 5-min EI treatment was neuroprotective against the 10-min EI treatment.

A large and rapid drop in [ATP] has been hypothesized to trigger ischemic preconditioning (Dirnagl et al., 2003). We tested whether a 5-min preconditioning



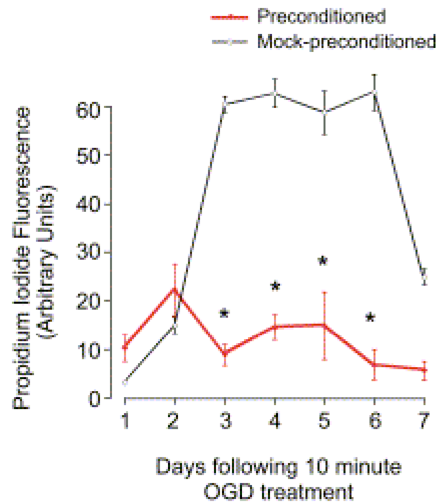


Figure 2. Ischemic preconditioning in young adult slice cultures. Panel A: PI fluorescence following preconditioning. Slice cultures were either mock-preconditioned with a 5-min mock-preconditioning treatment (Mock-preconditioned, left) or preconditioned with a 5-min EI treatment (Preconditioned, right). The cultures were returned to the incubator for 24 h. Both groups received a 10-min EI treatment. Shown is PI fluorescence at daily intervals starting at 1 day following the 10-min EI treatment. PI fluorescence was seen most prominently in the mock-preconditioned group in the dentate gyrus and the CA1 pyramidal cell layer. Panel B: Summary of changes in PI fluorescence following preconditioning. The amount of propidium iodide fluorescence in the CA1 pyramidal cell region of mock-preconditioned (n=4) or preconditioned (n=4) slice cultures was assayed at daily intervals. Values are mean arbitrary fluorescence units±S.E.M. Beginning at day 3 following the 10-min EI treatment, the amount of PI fluorescence in the preconditioned group was significantly less than in the mock-preconditioned group (ANOVA, F=48.363, p<0.0001; *p<0.01, Student–Newman–Keul's post hoc test).

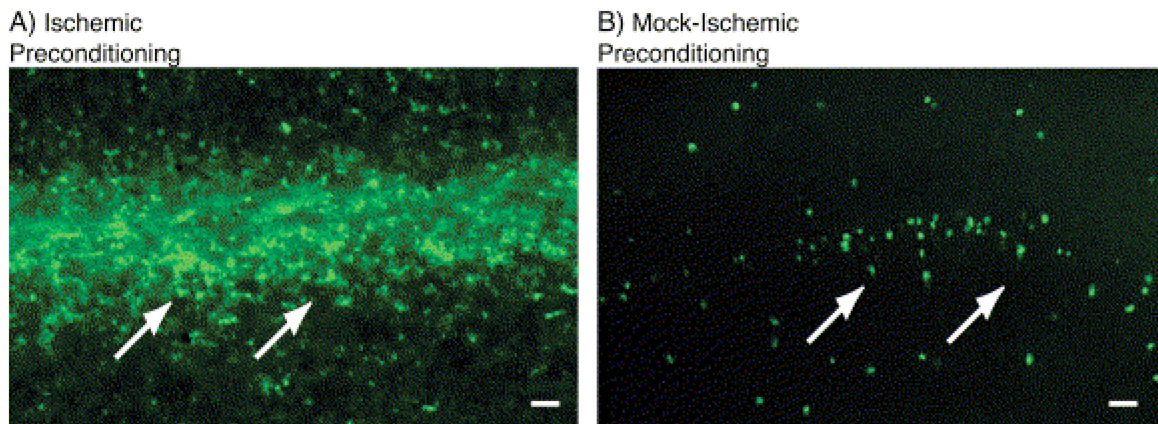


Figure 3. Neuroprotection following ischemic preconditioning in slice cultures. Slice cultures were fixed 7 days following a 10-min EI treatment that had been preconditioned (panel A) or mock-preconditioned (panel B). The fixed cultures were stained with an antibody against the neuronal-specific nuclear antigen NeuN. Prominent anti-NeuN staining was observed in the CA1 pyramidal cell layer of preconditioned cultures that was absent in mock-preconditioned cultures (arrows). Scale bars, 10 _M.

EI treatment depleted ATP in slice culture. Slice cultures received 5 min of either mock-treatment or EI. Basal [ATP] was assayed in an additional set of cultures that received no treatment. Immediately after or 2 h after the end of the treatment, the cultures were snap-frozen, ATP extracted and measured (Table 1). [ATP] in mock-treated slice cultures was similar to untreated cultures. In contrast, [ATP] was of 7.0% of control in cultures receiving a 5-min EI treatment. Two hours later, [ATP] was unchanged in the mock-EI group, but was recovered to 74.2% of the control value in the EI group. Thus, the 5-min EI treatment reversibly induced energy failure in slice cultures.

Table 1.Energy failure following a 5-min EI treatment

Hours after treatment	Mock (pg ATP/ μg)	EI (pg ATP/ μg)
0	647.2±40.1 (5)	45.9±10.1** (5)
2	514.9±72.5 (5)	362.6±55.6** (6)
24	1182.5±199.8 (6)	1044.1±296.0 (6)

At the indicated times, ATP was measured following mock-EI or EI. Basal slice culture ATP is 758.2±131.9 pg ATP/_g protein (n=5). Values are means±S.E.M. The number of times the experiment was done is indicated in parentheses. ATP levels immediately after and 2 h after EI were significantly lower than mock-EI (ANOVA, F=6.3278, P<0.0005; Student–Newman–Keul's post hoc test, **p<0.001).

A preconditioning stimulus slowly develops neuroprotection in hippocampal slice cultures.

Neuroprotection following ischemic preconditioning may arise rapidly within minutes or may slowly arise within one day. In slice cultures, neuroprotection following ischemic preconditioning has been typically assayed 24 hours following the preconditioning stimulus. We previously showed that a 5-min ischemic preconditioning (IPC) treatment protected against an experimental ischemia (EI) treatment administered 24 hours later. We examined whether IPC also provided protection if EI given 2 hours after the IPC treatment. Slice cultures received either IPC or mock-IPC. EI was administered, 2 or 24 hours following treatment, (Figure 4). Neuronal loss was assayed using propidium iodide (PI). EI induced equivalent amounts of PI staining when EI was given 2 hours following IPC or mock-IPC treatment indicating that IPC in hippocampal slice cultures does not rapidly induce neuroprotection. In contrast, PI

fluorescence was less in the IPC group as compared to mock-IPC when EI was given 24 hours after treatment (Figure 5).

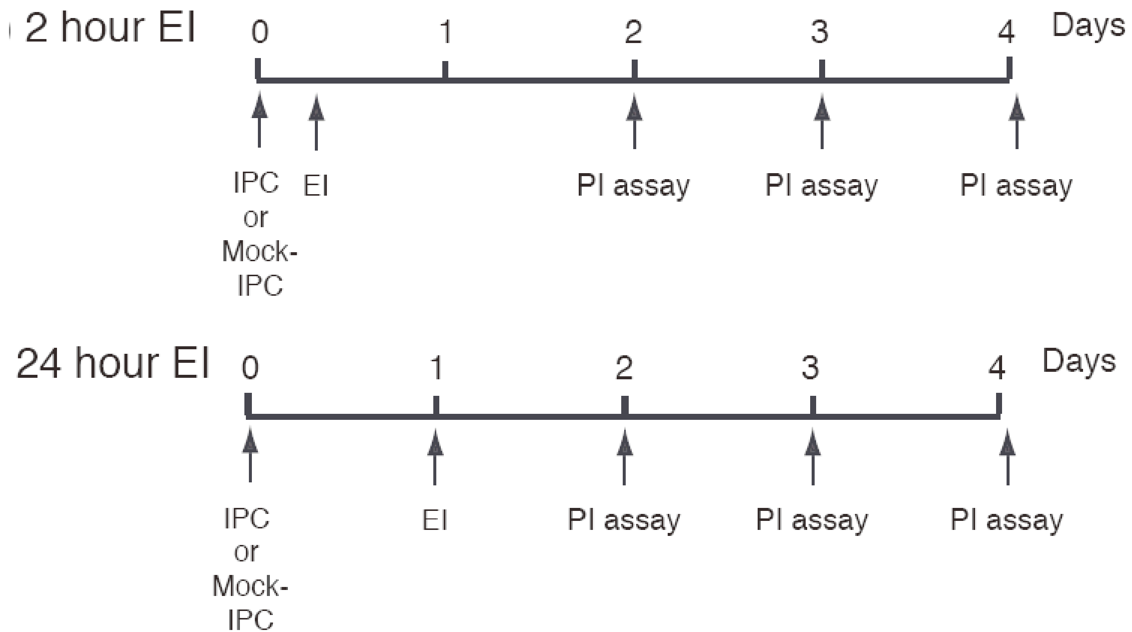
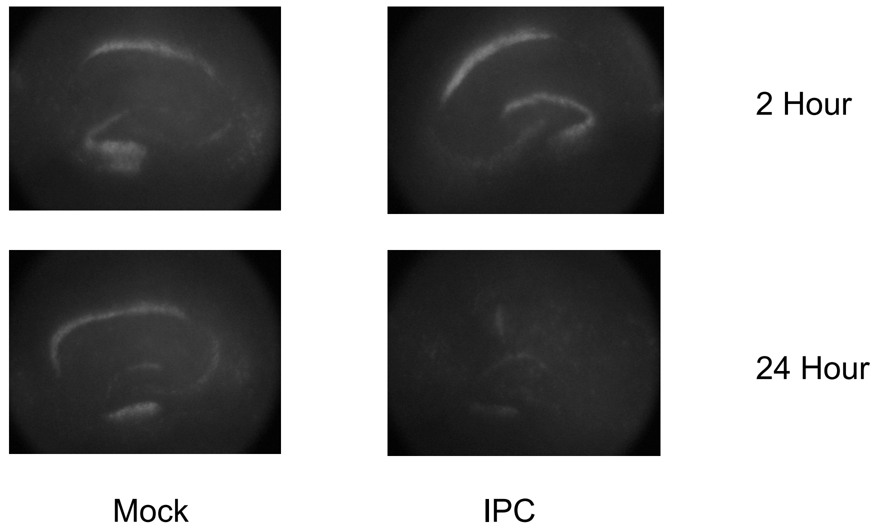


Figure 4. Schematic of the experimental design. On day 0, slice cultures received mock-IPC or IPC and were divided into two groups. One group received EI two hours after mock-IPC or IPC (EI at 2 hours, top). A second group received EI on day 1 (EI at 24 hours. bottom). Neuronal loss in both groups was assayed using PI on days 2-4.

A



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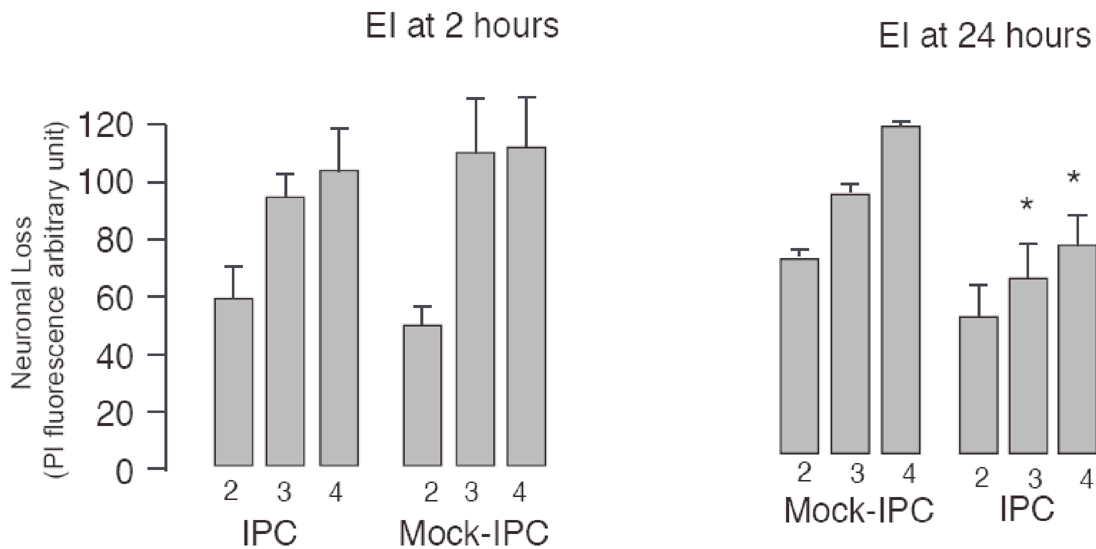

Figure 5. Neuroprotection slowly develops after a preconditioning in slice cultures. Panel A, 2 hours or 24 hours after ischemic preconditioning Shown is PI fluorescence on day 2 of experiment following EI. PI fluorescence was seen most prominently in the mock-preconditioned group in the CA1 pyramidal cell layer. **Panel B, Summary of neuronal loss.** In the group that received EI 2 hours after IPC or mock-IPC, equivalent PI fluorescence was observed. In the group that received EI 24 hours after IPC or mock-IPC, PI fluorescence was significantly less in the cultures receiving IPC. (ANOVA, $F = 11.354$, $p < 0.0001$; *$p<0.01$ Student Neuman Keuls post-test) These data suggest that neuroprotection developed between 2 and 24 hours after IPC.

Ischemic preconditioning elevates Na/K ATPase activity and increases surface expression of the α_1 and α_2 isoforms

Na/K ATPase activity is needed for preconditioning in the heart. We therefore examined Na/K ATPase activity following an IPC treatment. Two time points were chosen, 2 hours after IPC which does not protect neurons; and at 24 hours when IPC protects. Na/K ATPase activity was assayed by ouabain-sensitive ^{86}Rb uptake. Na/K ATPase is expressed both intracellular and on the cell surface, ^{86}Rb uptake in intact slice cultures assays only cell surface pump activity. ^{86}Rb uptake was assayed 2 and 24 hours after mock-IPC or IPC treatment (Figure 6). Two hours after treatment, Na/K ATPase activity was similar in the mock-IPC and IPC groups. Twenty-four hours after treatment, however, Na/K ATPase activity was significantly greater in the IPC group. These data suggest increased Na/K ATPase activity when neurons are protected against EI.

Elevated surface Na/K ATPase activity may result from an enhanced pumping rate or an increase number of surface pumps. Three Na/K ATPase isoforms (α_1, α_2 and α_3) are expressed in hippocampus and in slice cultures (Figure 7A). Surface expression of Na/K ATPase isoforms was assayed by selective biotinylation of surface proteins, isolation of biotinylated proteins, followed by immunoblot with isoform-specific antisera. Total expression and

surface expression of the α_1, α_2 or α_3 isoforms were assayed 2 and 24 hours after mock-

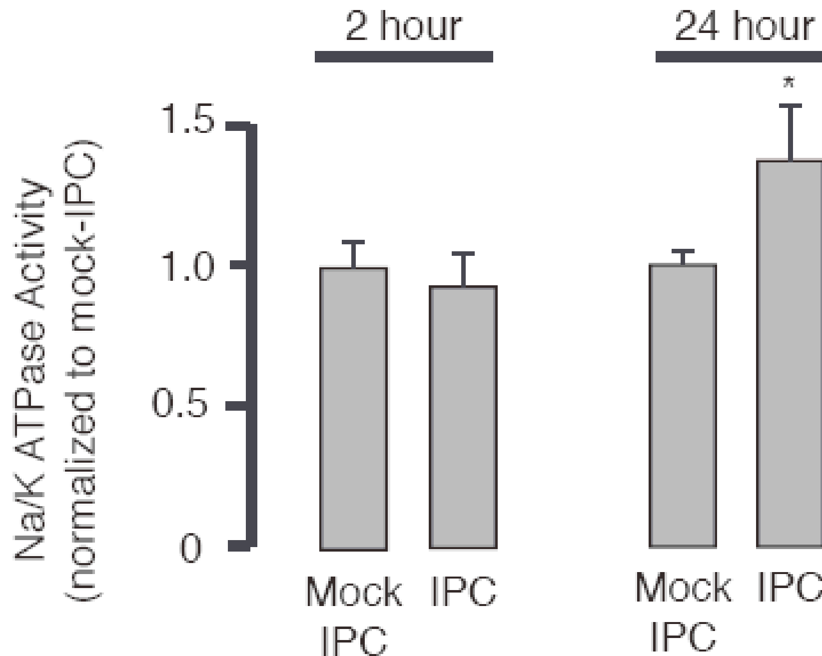

Figure 6. Increased Na/K ATPase activity following IPC Cell surface Na/K ATPase activity was assayed using ^{86}Rb uptake. Na/K ATPase activity did not significantly differ at 2 hours following IPC or mock-IPC. Pumping had significantly increased 24 hours following IPC but not mock-IPC, (ANOVA, $F=5.0812$, $p<0.01$; *$p<0.05$, Student Newman Keul's post hoc test; n=6, mock-IPC 2 hours; n=7, IPC 2 hours; n=6, mock-IPC 24 hours; n=5, IPC 24 hours).

IPC or IPC treatment. At 2 hours following treatment, similar levels of surface expression of the α_1 and α_2 isoforms were observed with in the mock-IPC and IPC groups (Figure 7A). Surface expression of α_3 was lower in the IPC group (figure 8A). In contrast, 24 hours after treatment, surface expression of the α_1

and α_2 had significantly increased in the IPC group, while expression of α_3 was unchanged (Figure 7A). This increased surface α_1 and α_2 expression may be

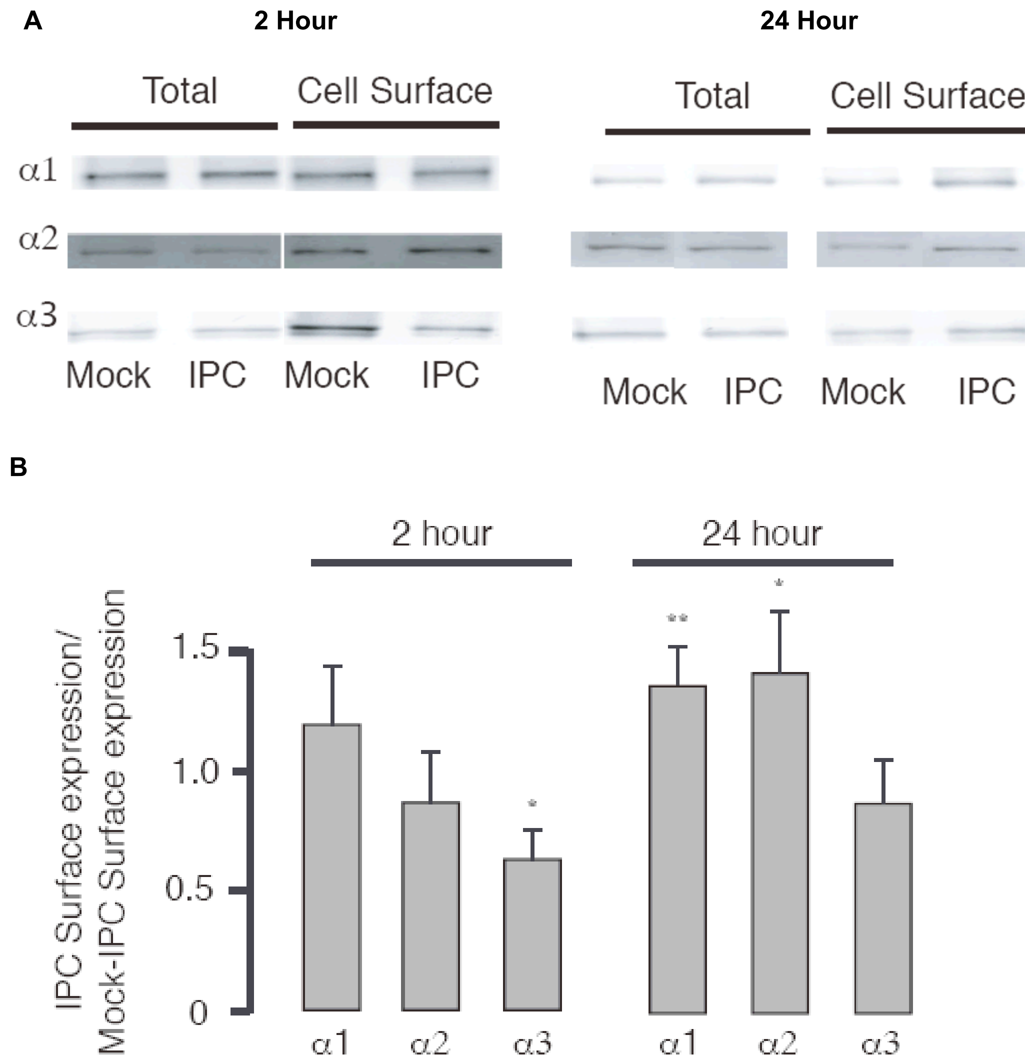

Figure 7. IPC Increased surface expression of Na/K ATPase isoforms following IPC Panel A Slice cultures received either IPC or mock-IPC. Two or 24 hours following treatment, biotinylated surface proteins were isolated and analyzed by immunoblot using antisera specific for individual Na/K ATPase α isoforms. **Panel B**, At 2 hours after IPC, surface expression of α_1 and α_2 isoforms were remained with depressed levels of α_3 (2hours, n=6 for all isoforms). At 24 hours after IPC surface expression of α_1 and α_2 isoforms were significantly elevated with no change in $\alpha 3$ (n=6 for all isoforms; α_1, **$p<0.005$, α_2, *$p<0.05$; Student's t test). Values are the mean of

ratio of the expression of mock-IPC and IPC for each isoform ± SEM. Surface expression is derived from the ratio of surface amount versus total amount.

responsible for increased Na/K ATPase activity observed 24 hours after IPC treatment.

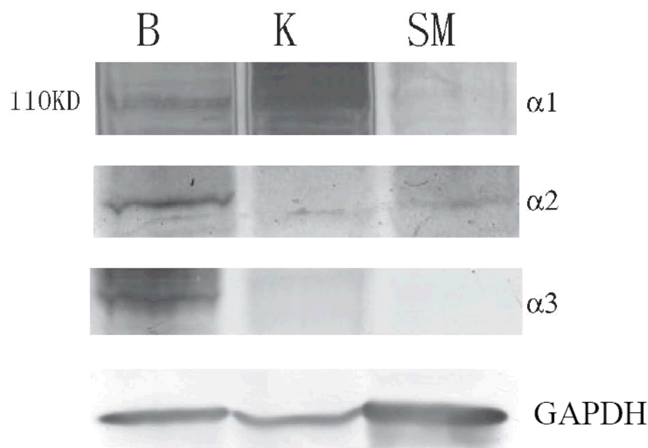

Figure 8. Confirmation of the tissue specific distribution of Na/K ATPase isoforms using isoform-specific antibodies. Proteins were extracted from rat brain, kidney, smooth muscle and analyzed by immunoblot using antisera specific for α1, α2, α3 Na/K ATPase isoforms. The same extracts were reacted with an antibody against GAPDH that ensured that an equal amount of protein was loaded in each lane. Isoform-specific antibodies against the α subunit of Na/K ATPase showed patterns of immunoreactive bands that were consistent with the known distribution of Na/K ATPase isoforms. Brain (B) expressed all three isoforms of Na/K ATPase, while kidney (K) expressed α1 isoform as a major isoform and smooth muscle (SM) expressed α1 and α2 isoform as major isoforms.

The specificity of the isoform-specific antibodies against Na/K ATPase α subunits was tested on protein extracts from brain, kidney and muscle. These tissues express specific patterns of Na/K ATPase isoforms. If these antibodies detect only one isoform, they should yield a similar pattern of tissue specific expression (Gick et al., 1993). The α_1 antibody detected an 110KD

immunoreactive band in brain, kidney and smooth muscle. This is consistent with ubiquitous expression of the α_1 isoform. The α_2 antibody detected expression of an 110KD immunoreactive protein in brain and smooth muscle, but not kidney while the anti-α_3 antibody only detected an 110KD immunoreactive protein in brain. (Figure 8). These data suggest that the isoform specific antibodies detect patterns of Na/K ATPase expression that fits the known expression pattern of each isoform. These data further suggest that the antibodies directed against Na/K ATPase α subunits are specific for a single isoform.

Preconditioning increases active PKMζ

PKCζ increases Na/K ATPase activity in thyroid cells and in vascular smooth muscle. PKMζ levels increases during the maintenance phase of LTP. PKMζ must be phosphorylated on threonine-560 to be active (Kanoh et al., 2000;Shirai and Saito, 2002). We therefore examined levels of PKMζ and threonine-560 phosphorylation following IPC (Figure 9). Slice cultures received either a mock-IPC or IPC treatment. PKMζ levels and threonine-560 (Thr560) phosphorylation were examined by immunoblot 2 and 24 hours after a mock-IPC or IPC treatment. At two hours, both the mock-IPC and IPC groups had similar levels of PKMζ and Thr560 phosphorylation suggesting no change. At 24 hours, however, PKMζ levels and Thr560 phosphorylation had significantly increased in the IPC group. These data suggest that active PKMζ increases following IPC a similar time when as Na/K ATPase activity.

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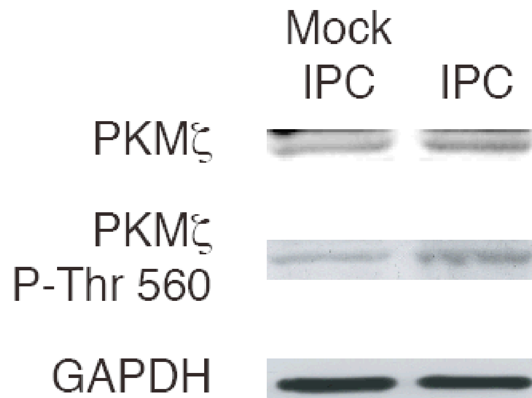

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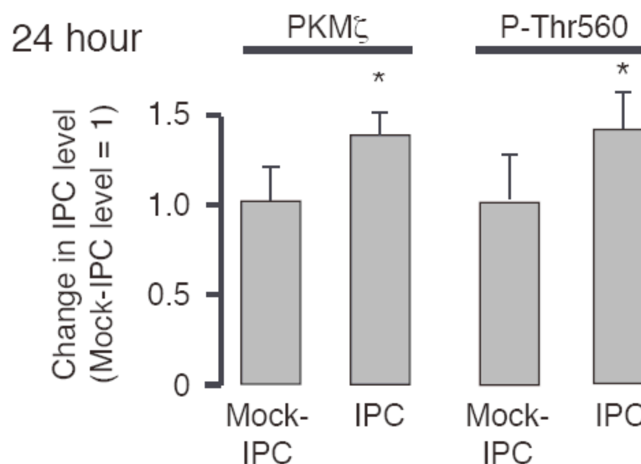

Figure 9. Ischemic preconditioning increases levels of active PKMζ Panel A, Active PKMζ levels are increased 24 hours after IPC. Slice cultures received mock-IPC or IPC, maintained for 24 hours and immunoblotted with antisera against total PKMζ, phosphorylated PKMζ (PKMζP-Thr 560) or glyceraldehyde-3-dehydrogenase (GAPDH). **Panel B Summary of the change in activated PKMζ after IPC.** Slice cultures received mock-IPC or IPC, maintained for 24 hours and immunoblotted with antisera against total PKMζ, PKMζ phosphorylated on theronine 560 or glyceraldehyde-3-dehydrogenase (GAPDH). At 24 hours following IPC, levels of total PKMζ and phosphorylated PKMζ were significantly increased (n=6, *p<0.05 student's t test) Values are from densitometry scans with the level of mock-IPC normalized to 1.

PKMζ regulates the Na/K ATPase

A variety of kinases regulate Na/K ATPase. In contrast to most kinases, PKMζ has a high basal activity without second messenger activation because it lacks the negative regulatory domain found in other PKC isoforms. This suggests that PKMζ regulates Na/K ATPase at basal condition. Basal Na/K ATPase activity was assayed in serum-free Earle's balanced salt solution (EBSS) which contains 2.3 mM potassium. Myristoylated ζ-pseudosubstrate peptide (ζ-peptide) was used as a specific inhibitor of PKMζ. To examine PKMζ regulation of Na/K ATPase, slice cultures were either mock-treated or treated with ζ-peptide for 30 minutes, followed by ^{86}Rb uptake assay of surface Na/K ATPase. ζ-peptide treatment significantly inhibited ^{86}Rb uptake (Figure 10). To test whether PKMζ regulated Na/K ATPase under conditions of elevated pumping, slice cultures were incubated in serum-free Earle's BSS containing 5.5 mM potassium. Na/K ATPase activity was measured in slice cultures that were mock-treated or treated with ζ-peptide. Pumping was stimulated by 5.5 mM potassium, and ζ-peptide treatment decreased pumping (Figure 10). These data suggest that PKMζ regulates Na/K ATPase in conditions of basal or stimulated pumping.

A variety of other protein kinases regulate Na/K ATPase activity including: conventional and novel PKCs, the cAMP-dependent protein kinase, cGMP-dependent protein kinase, and tyrosine kinases. A panel of well-characterized kinase inhibitors was used to examine if kinases other than PKMζ regulate basal

Na/K ATPase activity (Figure 11). Staurosporine (100nM) inhibits most major protein kinases expressed in brain, including conventional and novel PKCs (IC_{50} = 5nM), cAMP-activated protein kinase (IC_{50} = 8nM), Ca calmodulin kinase II (IC_{50} = 20nM), and cGMP-activated protein kinase (IC_{50} = 9nM). Staurosporine

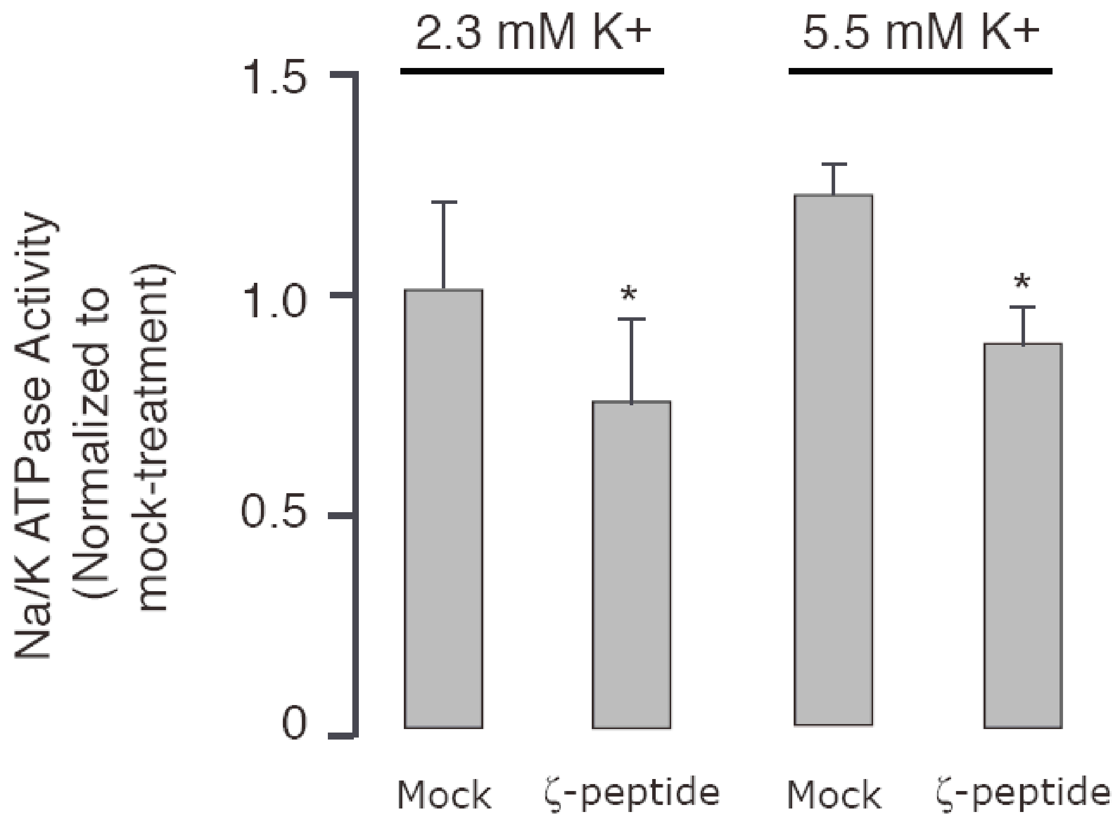

Figure 10. PKMζ regulates Na/K ATPase activity Slice cultures were either mock-treated (mock) or treated with myrisylated PKMζ-pseudosubstrate peptide (ζ-peptide, 80μM) for 30 minutes. Pumping was assayed in 2.3 mM (left) or 5.5 mM (right) potassium. Mock group was treated with ^{86}Rb (1μCi) or and ζ-peptide group was treated with ^{86}Rb plus ouabain (2mM) the cultures incubated for 30 minutes. Ouabain-sensitive potassium uptake in mock-treated cultures (n=6) is significantly higher than in peptide-treated (n=6) cultures (ANOVA, $F=5.8376$, $p<0.0001$, $^*p< 0.01$, Student Neumann Keul's post-hoc test).

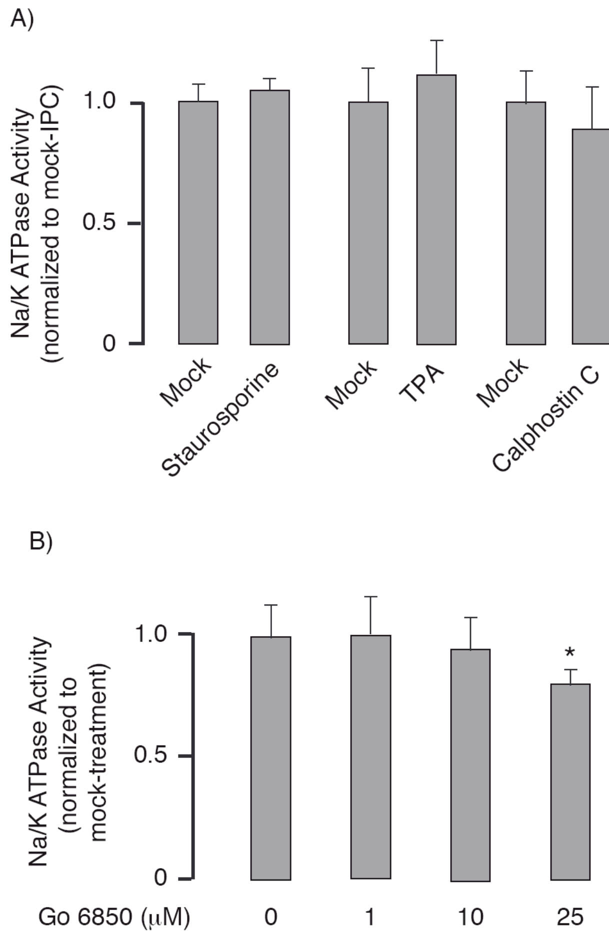

Figure 11. Atypical PKC regulates Na/K ATPase activity Panal A, Slice cultures were pretreated for 30 minutes with staurosporine (100nM), TPA (phorbol 12-myristate 13-acetate, 400nM), or calphostin C (500nM). Mock-treated cultures were treated with DMSO vehicle. ^{86}Rb uptake was assayed for 30 minutes and ouabain dependent potassium uptake determined. No significant difference was observed between vehicle-treatment and treatment with staurosporine, TPA, or calphostin C (ANOVA, F= 0.3492). **Panel B**, Atypical PKC regulates basal Na/K ATPase activity. Slice cultures received Gö6850 (1μM, 10μM and 25μM) or were mock-treated. Na/K ATPase assayed by ^{86}Rb uptake. Slice cultures treated with Gö6850 (1μM, 10μM) had Na/K ATPase activity no different than mock-treatment, but a significant decrease in Na/K ATPase activity was observed in cultures treated with Gö6850 (25μM) (n=6 for each group; ANOVA, 5.2824, p<0.01; Student Neumann Keul's post-hoc test; *p<0.05). The dose–response of slice culture Na/K ATPase activity to Gö6850 is consistent with regulation by atypical PKC.

(100nM), however, has no effect on PKMζ activity. Basal Na/K ATPase activity was unaffected by staurosporine (100nM) treatment (Figure 11A). TPA (phorbol

12-myristate 13-acetate, 400nM) is a potent activator of conventional and novel PKCs. Na/K ATPase activity was similar in mock-treated or PMA treated cultures. Calphostin C (IC_{50} = 50nM) irreversibly inhibits PKC by binding to their regulatory domain. Calphostin C (500 nM) has no effect on basal Na/K ATPase activity (Figure 10A). An additional PKC inhibitor, Gö6850, was used to further examine a role for PKC isozymes to regulate Na/K ATPase (Figure 11B). Gö6850 (1μM) inhibits conventional and novel PKCs but not atypical PKCs. Gö6850 (25μM), inhibits all three classes of PKCs. Whereas Gö6850 (1μM) had no affect on basal Na/K ATPase activity, Gö6850 (25μM) significantly reduced pumping (Figure 11B). Inhibition of basal Na/K ATPase by Gö6850 (25μM), combined with the absence of an effect with staurosporine, TPA, and Gö6850 (1μM) provides additional evidence that basal Na/K ATPase activity is regulated by aPKCs.

Over expression of PKMζ increases basal Na/K ATPase activity

Inhibition of PKMζ by ζ-peptide suggests that PKMζ regulates basal Na/K ATPase activity. However, the lowering of Na/K ATPase activity may due to effects of ζ-peptide other than PKMζ inhibition. We therefore used sindbis virus gene transfer to examine whether PKMζ regulates Na/K ATPase activity. The pSinRep5-HA-PKMζ virus vector was constructed that directs expression of PKMζ under the control of a sindbis viral promoter. As a control for effects of pSinRep5-HA-PKMζ vector other than PKMζ expression, slice cultures were also

injected with pSinRep5-HA-EGFP, a sindbis viral vector that directs expression of EGFP. Infection of dissociated hippocampal neuronal cultures with pSinRep5-HA-PKMζ virus showed that this expressed PKMζ protein (Rochna Sondhi, Todd Sacktor unpublished data).

To see if PKMζ increased Na/K ATPase activity, 100nl of either pSinRep5-HA-PKMζ or pSinRep5-HA-EGFP were injected 7-10 times to the CA1 pyramidal cell layer of slice cultures. EGFP expressing cells in the CA1 pyramidal cell layer were first detected 1 day after injection using fluorescence microscopy. These data suggested that the vectors infected neurons. Na/K ATPase activity was also assayed at 6, 24 and 72 hours after microinjection of pSinRep5-HA-PKMζ or pSinRep5-HA-EGFP. Six hours after the injection, Na/K ATPase activity was significantly increased in pSinRep5-HA-PKMζ virus vector infected cultures as compared with the pSinRep5-HA-EGFP virus vector infected cultures. The Na/K ATPase activity increase induced by pSinRep5-HA-PKMζ virus vector infection was fully blocked by ζ-peptide (80uM) treatment (Figure 12). At 24 hours and 72 hours, Na/K ATPase activity did not significantly change when compared with the control group. These results suggested that the exogenous expressed PKMζ regulates the basal Na/K ATPase activity.

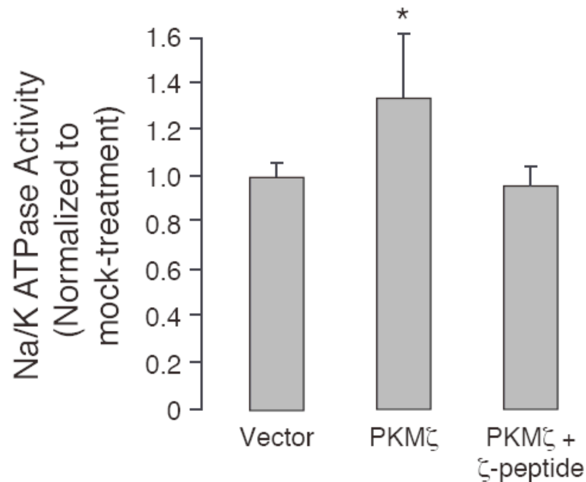

Figure 12. Over expression of PKMζ induces Na/K ATPase activity at CA1 area. The CA1 pyramidal cell area of the hippocampal slices cultures was infected with pSinRep5-HA-EGFP virus vector (n=6) or pSinRep5-HA-PKMζ virus vector (n=6). ^{86}Rb uptake was measured 6 hours after infection on micro-dissected CA1 area. ζ-peptide (80uM) (n=5) was applied 30 minutes before ^{86}Rb uptake assay. The pSinRep5-HA-PKMζ virus vector infected group has a significant higher Na/K ATPase activity than pSinRep5-HA-EGFP virus vector infected group. But the ζ-peptide (80uM) treatment block this activity induction ($F= 6.6626$, $p<0.01$; Student Neuman Keuls,* $p<0.01$).

PKMζ regulates the α_1 isoform of the Na/K ATPase

Regulation of Na/K ATPase by PKMζ was further tested using Na/K ATPase inhibitors that are specific for the α_1 isoform or for the α_2 and α_3 isoforms. Marinobufagenin is an endogenous ouabain-like compound that specifically inhibits α_1 (α_1, IC_{50} = 2.1 nM; α_3, IC_{50} = 50nM). Slice cultures were either mock-treated or treated with differing concentrations of marinobufagenin (1-5 nM) and surface Na/K ATPase activity assayed by ^{86}Rb uptake assay. Marinobufagenin (2.1nM) inhibited 23.7 ± 8.3% of Na/K ATPase activity (Figure

13A). Higher marinobufagenin concentrations (2-5 nM) did not change the amount of inhibition (data not shown). The partial inhibition of Na/K ATPase by marinobufagenin is likely due to selective inhibition of the α_1 isoform. Slice cultures were treated with marinobufagenin and ζ-peptide to test if PKMζ regulated the α_1 isoform. If marinobufagenin and PKMζ both act on the α_1, they should occlude; there would be no added effect when a combined ζ-peptide and marinobufagenin (2.1nM) treatment are compared to the individual treatments. Inhibition of Na/K ATPase by the combined treatment of ζ-peptide and marinobufagenin (2.1nM) was no different than marinobufagenin (2.1nM) alone (Figure 13A). The absence of an additive effect of ζ-peptide and marinobufagenin suggests that they both act on the α_1 isoform of Na/K ATPase.

The low-affinity ouabain analog, dihydroouabain (20μM) specifically inhibits the α_2 and α_3 isoforms of Na/K ATPase. If PKMζ acts on α_1, the inhibitory effect of dihydroouabain and ζ-peptide on Na/K ATPase activity should not occlude, inhibition by the combined treatment would be the sum of the individual treatments. Slice cultures were either mock-treated or individually treated with dihydroouabain (20μM), or ζ-peptide and Na/K ATPase activity assayed by ^{86}Rb uptake (Figure 13B). Pumping was inhibited by 27.2% dihydroouabain and 26.1% by ζ-peptide. The combined treatment of dihydroouabain and ζ-peptide inhibited pumping 52.2%. Inhibition by the combination of dihydroouabain and ζ-peptide (52.2%) was very similar to the sum of the inhibition (53.3%) of the two

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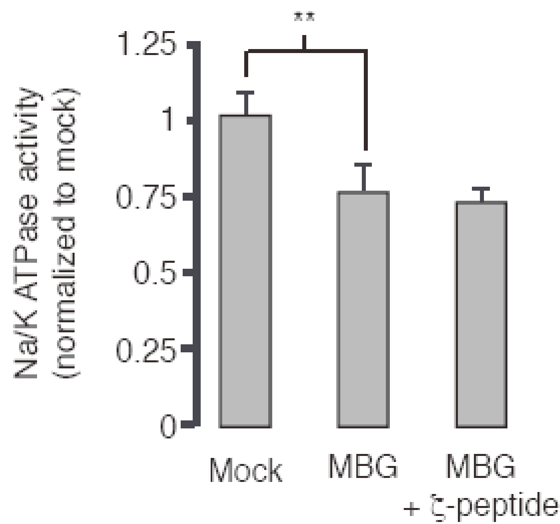

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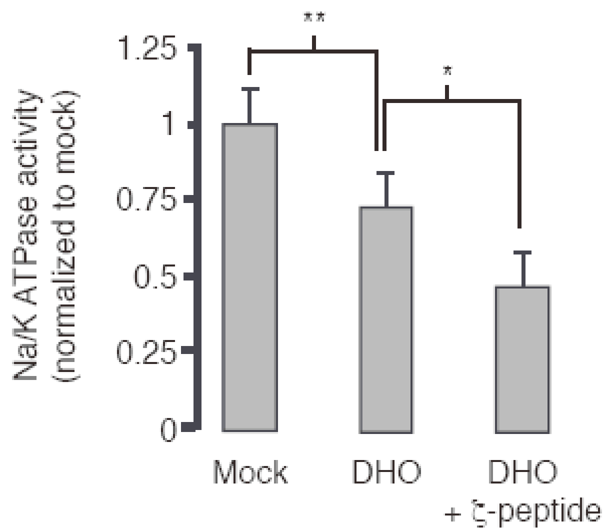


Figure 13. PKMζ regulates the Na/K ATPase α_1 isoform. **Panel A, Occlusion of ζ-peptide and marinobufagenin (MBG)**. Slice cultures were treated either mock-treated, or treated with MBG (2.1nM) or MBG and ζ-peptide (80μM). MBG significantly inhibited Na/K ATPase activity (ANOVA, $F=5.515$, $p<0.005$; *$p<0.01$, Student Neuman Kuels). Na/K ATPase inhibition was not increased by the combined treatment of ζ-peptide and MBG suggesting that they occlude. **Panel B, Absence of occlusion between ζ-peptide and dihydroouabain (DHO).** Slice cultures were treated either mock-treated, or treated with DHO (20μM) or DHO and ζ-peptide. DHO significantly inhibited Na/K ATPase activity and the combined treatment of DHO and ζ-peptide was significantly greater than dihydroouabain alone (ANOVA, $F=16.26$, $p<0.0005$; **$p<0.01$,

*p<0.05, Student Neuman Kuels). These data suggest the absence of occlusion between ζ-peptide and DHO.

compounds applied individually. These data suggest that PKMζ and dihydroouabain act on different isoforms of pumps with PKMζ regulating α_1 and dihydroouabain regulating α_2 and α_3.

IPC increased Na/K ATPase activity, surface expression of the α_1 and α_2 isoforms and active PKMζ (Figure 7). We therefore tested the increase in Na/K ATPase surface expression following IPC was regulated by PKMζ. After mock-treatment or treatment with ζ-peptide, biotinylated slice culture surface proteins were isolated and analyzed by immunoblot using Na/K ATPase isoform specific antisera (Figure 14). ζ-peptide-treatment lowered α_1 surface expression but did not alter surface expression of the α_2 and α_3 isoforms. These data suggest PKMζ maintains Na/K ATPase activity through surface expression of α_1 following IPC.

PKMζ regulates the α_1 isoform of the Na/K ATPase not by direct phosphorylation

Protein kinase transfers a phosphate group to its substrate by using ATP. We tested whether PKMζ regulates Na/K ATPase by direct phosphorylation. Slice cultures was labeled with P^{32} orthophosphoric acid 2 hours. Then slice cultures were either mock-treated or treated with ζ-peptide for 1 hour. Total

protein was collected for immunoprecipitation with α_1 Na/K ATPase isoform specific antisera. ζ-peptide treatment did not change the phosphorylation status

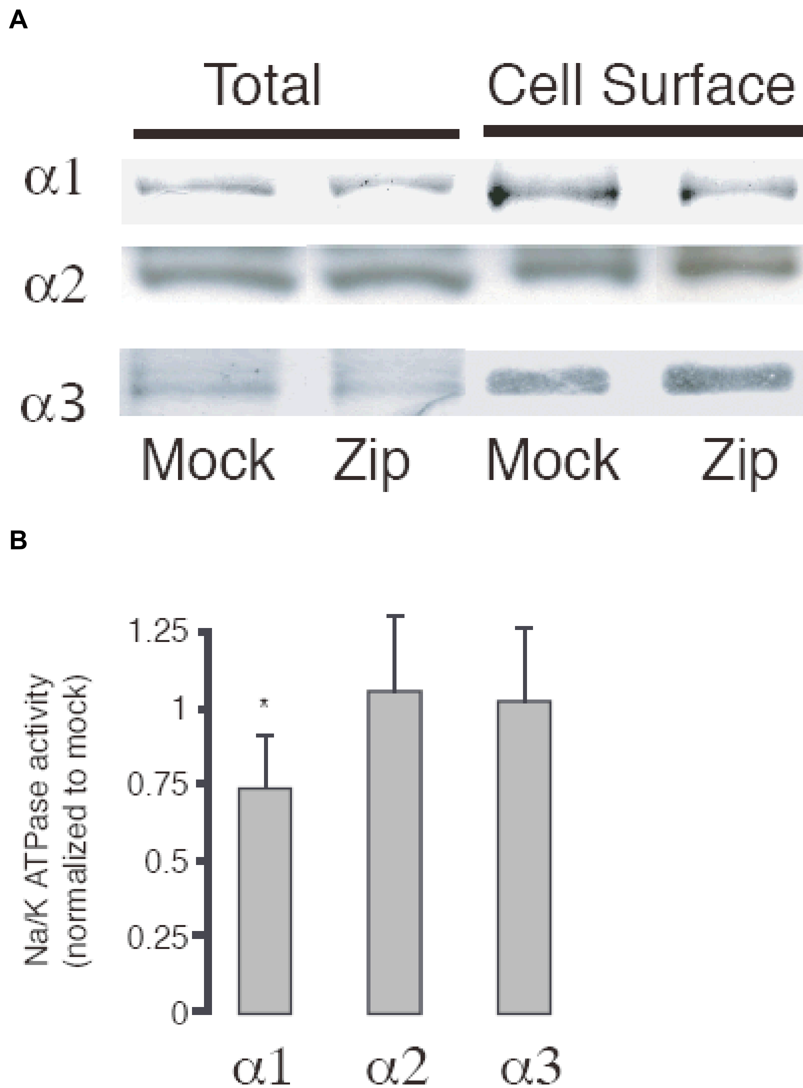


Figure 14. PKMζ regulates surface distribution of the α_1 isoform of the Na/K ATPase. Panel A, Slice cultures were either mock-treated or treated with ζ-peptide (80um) for 1 hours. Surface proteins were biotinylated, isolated on avidin beads and immunoblotted with Na/K ATPase isoform-specific antisera. Panel B, Summary of surface biotinylation experiments. Data was normalized to mock treatment. Surface expression of the α_1 isoform was lowered by ζ-peptide, despite the absence of change in its total expression (α_1, n=5; α_2, n=4; α_3, n=5; Student's t test, $p<0.01$).

of α_1 isoform of Na/K ATPase (Figure15). These data suggest that PKMζ regulates Na/K ATPase activity through phosphorylating other substrate.

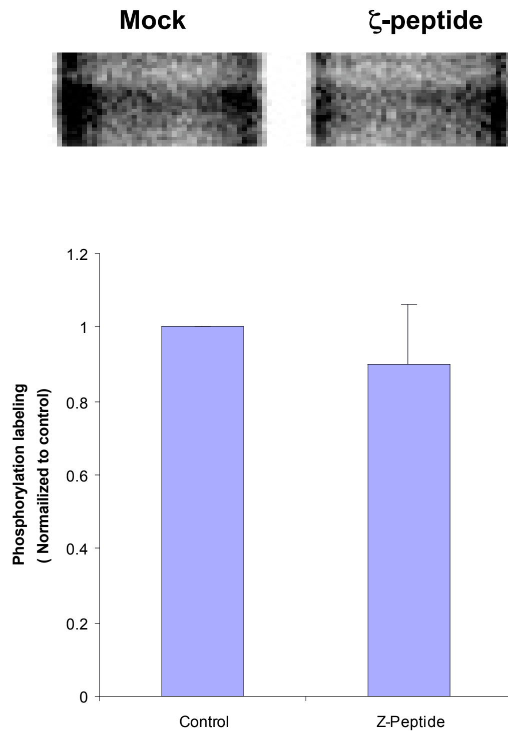


Figure 15 PKMζ does not change the phosphorylation status of the α_1 isoform of the Na/K ATPase Slice cultures were labeled with [^{32}P] orthophosphoric acid for 2 hours. Then the slice cultures were either mock-treated or treated with ζ-peptide (80um) for 1 hour. Total proteins were collected for immunoprecipitation with α1 Na/K ATPase isoform-specific antisera. Data were normalized to mock treatment. ζ-peptide doesn't change the phosphorylation status of α_1 isoform (α_1, n=3; Student's t test, $p>0.05$).

PKMζ and Na/K ATPase activity are needed for neuroprotection following ischemic preconditioning

IPC increased Na/K ATPase activity, surface expression of α_1 and α_2 isoforms, and levels of active PKMζ. We tested whether these increases mediated neuroprotection. Slice cultures received either mock-IPC or IPC and returned to the incubator for 24 hours. IPC group was further sub-divided into 4 groups that were either mock-treated, or treated with marinobufagenin (2.1nM), dihydroouabain (20μM), ζ-peptide. After 60 minutes of treatment, all groups received EI. Neuronal loss was assayed at one and three days following EI by PI assay (Figure 16. PI fluorescence was significantly less in the IPC group than mock-IPC group suggesting neuroprotection (Figure 17). PI fluorescence in the marinobufagenin (2.1uM) group or the dihydroouabain group was similar to the mock-IPC group suggesting that both the activity of α_1 or α_2 protected neurons. Dihydroouabain (20μM), does not distinguish between the α_2 and α_3 isoforms of the Na/K ATPase suggesting a protective role for the α_3 isoform as well.

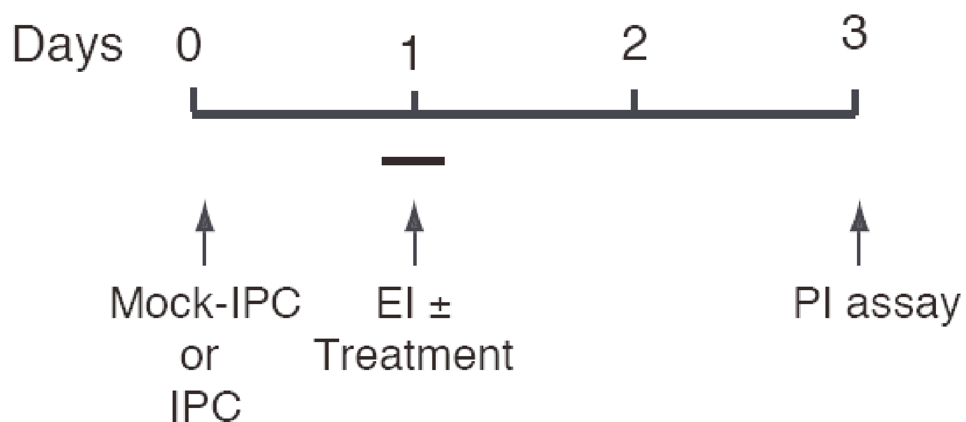
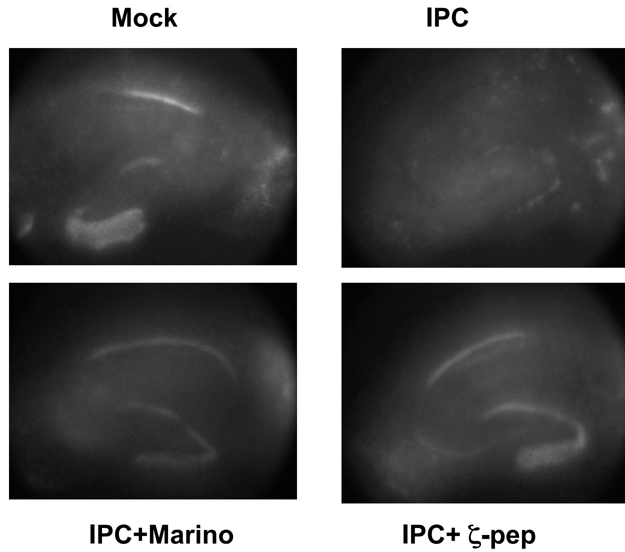


Figure 16 Experimental design. Slice cultures received either mock-IPC or IPC and were returned to the incubator for 1 day. Thirty minutes before EI treatment, slice cultures were either

mock-treated or treated with ζ-peptide, marinobufagenin (2.1nM) or dihydroouabain (20μM). Thirty minutes following EI treatment, the drugs were washed out. Three days later, cell loss was assayed by PI.



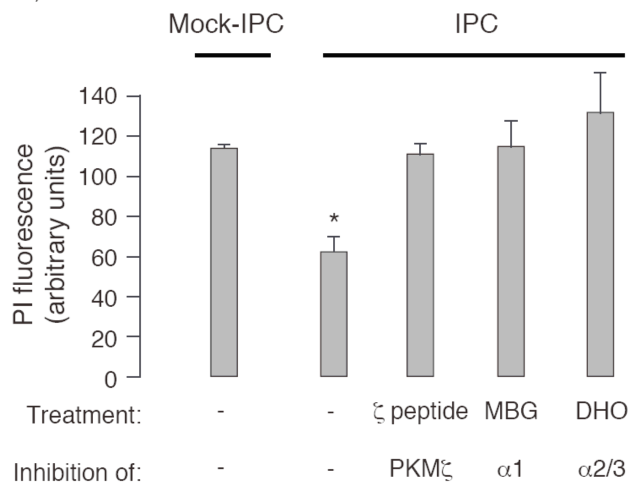


Figure 17 PKMζ and Na/K ATPase protect neurons following ischemic preconditioning. Panel A, Shown is PI fluorescence at 3 day following the EI. PI fluorescence was seen most prominently in the mock-preconditioned group, preconditioned with marinobufagenin treatment group and preconditioned with ζ-peptide treatment group in the dentate gyrus and the CA1 pyramidal cell layer. **Panel B**, summary of neuronal loss 3 days following EI. PI fluorescence the IPC group was significantly less than PI fluorescence in cultures that received mock-IPC or IPC

plus ζ-peptide, marinobufagenin or dihydroouabain (ANOVA, $F=7.932$, $p<0.0005$; $^*p<0.01$, Student Neuman Keuls).

Discussion

Summary

This thesis describes a new model of IPC using hippocampal slice cultures. This model was used to identify two key mediators of IPC, PKM ζ and the Na/K ATPase. The major findings of this thesis are: (1) In young adult slice cultures, a 5-min EI treatment was non-neurotoxic, while a 10-min treatment was highly neurotoxic (Figure 1). This result suggests that young slice cultures faithfully model the high sensitivity and sharp thresholds for ischemic injury *in vivo* (Hassen et al., 2004). (2) Preconditioning is present in slice cultures since a 5-min EI treatment protected against a subsequent 10-min EI treatment. The 5-min EI treatment used is of similar duration to the interruption of blood flow that provides neuroprotection *in vivo* (Figure 2). (3) The 5-min EI treatment induced a rapid and reversible energy failure in the slice cultures (Table 1). Energy failure occurs during ischemic injury and is thought to be a key event in the induction of ischemic preconditioning (Dirnagl et al., 2003). (4) The 10-min EI treatment remained neurotoxic 2 hours after preconditioning suggesting that neuroprotection was slow to develop (Figure 5). The examination of the mechanism of endogenous neuroprotection in slice cultures showed important neuroprotective roles for PKMζ and Na/K ATPase. (5) The activity of Na/K ATPase and surface expression of α_1 and α_2 isoforms increase 24 hours after a

preconditioning stimulus (Figure 6,7), (6) PKMζ levels also increase 24 hours after a preconditioning stimulus (Figure 9). (7) PKMζ regulates basal Na/K ATPase activity and surface expression of the α_1 isoform (Figure 11 and 13), (8) The changes in PKMζ and Na/K ATPase occur at the time window when ischemic preconditioning protects neurons (Figure 7, 9). (9) activity of the α_1 isoform and PKMζ are needed for neuroprotection (Figure 17), and (10) activity of the α_2 and/or α_3 are also neuroprotective (Figure 17). These data provide strong evidence that PKMζ and Na/K ATPase activity protect neurons.

Ischemic preconditioning in young adult hippocampal slice cultures

Ischemic preconditioning in slice cultures from young adult rats provides a valuable addition to pre-existing *in vivo* and *in vitro* models of ischemic preconditioning (Dirnagl et al., 2003). The large advantage using these slice cultures as compared to other *in vitro* models is that slice neurons are protected or damaged using oxygen-glucose deprivations that are similar in duration to those protect or damage neurons *in vivo*. *In vivo* models more closely mimic the pathophysiology of human preconditioning than *in vitro* models (Hassen et al., 2004). However, *in vivo* models have the disadvantages of that they are time consuming, expensive, and use many numbers of animals. *In vitro* models yield more uniform data because: (1) a larger number of experiments are performed simultaneously, (2) temperature and oxygen partial pressure are better regulated and (3) the application and removal of potentially neuroprotective drugs is better controlled. In addition to slice cultures, ischemic preconditioning *in vitro* has been

modeled in cell lines or disassociated embryonic neurons. A caveat of these models is that the neurons are isolated from embryonic or perinatal animals and susceptibility to ischemic injury is well known to be age-dependent. Therefore, neuroprotection is being studied in immature neurons that have intrinsic resistance to EI. As a result, excessively prolonged episodes of EI are needed for preconditioning or neuronal loss (Bruer et al., 1997;Grabb and Choi, 1999;Khaspekov et al., 1998). Neurons in hippocampal slice cultures from rats less than 10 days old are susceptible to ischemic injury, yet, EI for up to 30–45 min is needed to kill neurons (Xu et al., 2002;Saez-Valero et al., 2003;Weber et al., 2002;Zhong et al., 2003). This far exceeds the duration of ischemia that is neurotoxic *in vivo*. For preconditioning studies in slice cultures from rats less than 10 days old, 15 min of EI protected against a 45-min EI treatment. Acutely prepared slices from adult rats have been used to study short-term changes following preconditioning, but their short *in vitro* life span precludes their use to study of long-term changes. Thus, for the study of ischemic preconditioning, the age of the neurons is as critical a parameter *in vitro* as it is *in vivo*.

Investigators considering this model should also be aware of its disadvantages. The removal and transverse slicing of the hippocampus remove all extrinsic and intrinsic neural connections (Hassen et al., 2004). This includes all cholinergic, serotinergic, adrenergic and dopaminergic innervation. Longitudinal pathways of the hippocampus are also absent. These pathways are largely GABAergic, resulting in an altered balance of excitation and inhibition in the cultures. The consequences of the loss of these pathways to ischemic injury

are largely unknown. The major source of variability in this procedure or in any procedure using hippocampal slice cultures is the quality and uniformity of the cultures. We have unsuccessfully tested a large number of procedures in an attempt to improve the organotypic characteristics of these cultures (data not shown). Ultimately, the key parameter is the rapid and gentle removal and slicing of the hippocampus to reduce both ischemic and traumatic injury. To obtain highly reproducible results, 75% of the slice cultures should display a similar retention of neurons as the culture as shown by Xiang et al (Xiang et al., 2000). A second source of variability is the horse serum in the slice culture media. Not all horse sera give similar results. We routinely screen multiple lots of horse serum to find which lot yields the cultures with the highest percentage of cultures with superior retention of hippocampal structure. A third disadvantage that this model shares with all *in vitro* systems is the ratio of cell number to the size of the extracellular space. The 400-_m thick hippocampal slice culture is maintained in 1 ml of media. During mock-EI or EI treatment, the culture is placed in 50 ml of aCSF. In both conditions, the extracellular space is vast as compared to hippocampal neurons *in vivo*. As a result, substances released by neurons may rapidly diffuse from the cultures. Due to these caveats, the data obtained with this model of ischemic preconditioning should be viewed only as a starting point for comparable studies *in vivo* (Hassen et al., 2004).

There are future areas of improvement in this *in vitro* ischemic preconditioning model. Preconditioning was induced using simple hypoxia–hypoglycemia apparatus constructed with inexpensive materials.

Alterations in the design of the apparatus including multiple chambers or the addition of a perfusion system may further increase the uniformity of the neuroprotection following preconditioning.

Neuroprotection is slow to develop in ischemic preconditioning

During ischemic preconditioning, neurons are protected at 24 hours, but not 2 hours following a preconditioning treatment (Figure 5). This suggests that cellular changes observed at 2 hours are not directly protecting neurons as compared to changes observed at 24 hours. At 24 hours, but not 2 hours, there is an increased surface expression of the α_1 and α_2 Na/K ATPase isoforms as well as an increase in surface Na/K ATPase activity (Figure 7). Na/K ATPase activity also is elevated following ischemic preconditioning *in vivo*, yet the mechanism and the time course underlying the change in pumping was not examined. In our study, surface expression of the α_1 and α_2 isoform of Na/K ATPase increases after IPC. Increased surface expression is believed to result in the elevated Na/K ATPase activity observed after IPC. These data suggest that the changes in Na/K ATPase occur at a time when neurons are protected. PKMζ also increases following IPC. The increase in PKMζ did not occur at two hours after IPC but did occur at 24 hours. The time course of the increases in Na/K ATPase and PKMζ activity and expression is consistent with a role in neuroprotection.

Na/K ATPase and neuroprotection

This thesis did not directly address how increased Na/K ATPase activity is neuroprotective, but a protective role for increased pumping fits well with the current knowledge of the physiological changes that induce ischemic injury. During ischemia, there is a rapid loss of ion gradients. Na/K ATPase is critical in the maintenance and restoration of these ion gradients. Experimental ischemia in slice cultures induces a large loss of ATP (Table 1). During this period of low ATP, it is unlikely the Na/K ATPase can function. Therefore, prior to the loss of ATP, increased Na/K ATPase activity may protect neurons by delaying the loss of ion gradients. Alternatively, once ATP is again synthesized, increased Na/K ATPase activity could more rapidly restore membrane ion gradients. These two possibilities are not mutually exclusive resulting in a net effect of making the neuron more resistant to ischemic injury.

Increased surface expression of Na/K ATPase may also be neuroprotective independent of its role as an ion transporter. Recent studies have suggested that the Na/K ATPase also promotes second messenger activation. Low doses of ouabain protected against glutamate excitotoxicity *in vivo* or apoptosis in cerebellar granule cells (Golden and Martin, 2006). In addition to its inhibitory action on ion transport by the Na/K ATPase, ouabain also promotes the assembly of intracellular signaling enzymes on the cytoplasmic surface of the pump. Second messenger activation by these enzymes has been

shown to protect against excitotoxic or apoptotic neuronal loss. More Na/K ATPase is expressed on the cell surface after IPC. Increased receptor number may enhance the signaling of Na/K ATPase.by endogenous ouabain (Xie and Xie, 2005). This pathway may result in an additional mechanism of neuroprotection after IPC.

In our model, we showed that surface expression of Na/K ATPase increased without a change Na/K ATPase protein (Figure 7). These results suggest that the cell responds to the short term ischemia by modifying the pre-existing Na/K ATPase. New protein synthesis may also be important for neuroprotection following IPC. Unlike Na/K ATPase, PKMζ levels increased following IPC (Figure 9). PKMζ is believed to be a constitutively active kinase. The increase in PKMζ protein levels following IPC likely results in increased constitutive kinase activity. This results in increased surface expression of the α_1 isoform of Na/K ATPase. Therefore, the neuroprotective role of PKMζ may be to increase the surface expression of the α_1 Na/K ATPase. Further studies are needed to determine whether the mechanism seen in slice cultures is responsible for increased *in vivo* Na/K ATPase activity.

Isoform-specific alterations in the Na/K ATPase

This thesis examined three different Na/K ATPase isoforms that are expressed in slice cultures. These isoforms do not act uniformly; rather there are

clear isoform-specific changes. Isoform-specific changes suggest that these isoforms have different roles during ischemia. One important isoform-specific effect is PKM ζ regulation of α_1 isoform. Increased surface expression of α_1 isoform correlates with the higher level of PKMζ. In addition occlusion experiments strongly suggest that PKMζ regulates the activity of pumps containing α_1 (Figure 14). While α_1 expression is ubiquitous, PKMζ expression is restricted to neurons, suggesting that the PKMζ increase in α_1 isoform increased basal pump activity in neurons.

PKMζ may regulate α_1 directly or by phosphorylating other substrates which, in turn, regulate α_1. Conventional PKCs directly phosphorylate the α_1 subunit of Na/K ATPase at Ser-16. This residue is absent in α_2 and α_3 isoforms (Beguin et al., 1994). There is no evidence that aPKCs phosphorylate this site. This result suggests that α_1 isoform is the major isoform to underlie the PKC regulation or there are alternative phosphorylation sites in α_2 and α_3 isoforms. PKC also regulates the Na/K ATPase activity through phosphorylation associated substrates. Most studies focused on the regulation of cPKCs and nPKCs. As a result, the mechanism of PKMζ regulation of the α_1 isoform remains an open area for research.

Na/K ATPase activity was inhibited by ζ-peptide. The concentration of ζ-peptide needed to inhibit Na/K ATPase activity was much higher than previous studies in acute hippocampal slices when PKMζ was inhibited completely

inhibited using 1uM ζ-peptide (Ling et al., 2002). A major difference between acute and cultured hippocampal slices is the presence of serum in slice culture media. Serum contains proteases which may degrade ζ-peptide. In fact, in other cell cultures systems using serum required ζ-peptide concentrations between 50uM to 100uM to inhibit PKCζ (Standaert et al., 1999); (Muscella et al., 2005). This may explain why much more ζ-peptide was needed to inhibit PKM ζ in slice cultures than in slices. Additional experiments using pSinRep5-HA-PKMζ provided further confirmed that ζ-peptide inhibited PKMζ in slice cultures (Figure 13).

Six hours after infection of slice cultures pSinRep5-HA-PKMζ, Na/K ATPase was significantly increased. Sindbis virus vectors have been previously shown to selectively transduce neurons in hippocampal slice cultures (Ehrengruber et al., 1999). This increased pumping was blocked with ζ-peptide treatment. Increased pumping, however, was not observed at later time points after infection. An increase in Na/K ATPase activity at six hours, but not at later time points may be due to a toxic effect of PKMζ expression driven by strong sindbis viral promoters. EGFP expression, however, was observed in slice cultures 24 and 72 hours after transduction suggesting that the viral vector itself was not toxic.

The finding of upregulated Na/K ATPase activity 6 hours, but not 24 or 72 hours following pSinRep5-HA-PKMζ infection suggests a cytotoxic effect following PKMζ expression. This is unlikely due to the viral vector itself since

pSinRep5-HA-EGFP showed no cytotoxic effect at 24 or 72 after infection. A previous study also showed no rapid cytoxicity following infection with sindbis viral vectors. Rather cytotoxicity is thought to arise from the biological consequences of PKMζ over expression.

PKMζ and ischemic preconditioning

PKMζ has been previously identified as an important mediator of the maintenance phase of LTP (Ling et al., 2002;Pastalkova et al., 2006). Following LTP, PKMζ levels moderately increase, and a similar increase in PKMζ levels was observed following ischemic preconditioning (Figure 9). PKMζ levels increase within 10 minutes following LTP, in contrast, the increase in PKMζ levels following ischemic preconditioning is slow (Figure 9). The slower kinetics of PKMζ accumulation may reflect the greater than 90% drop in ATP within 5 minutes of the preconditioning stimulus that only partially recovered 2 hours later. The increase of PKMζ accumulation following IPC may be slower than LTP since protein and RNA synthesis must be restored before there are changes in gene expression.

This study shows PKMζ plays an important role in increased Na/K ATPase activity. This suggests that Na/K ATPase activity may increase following LTP. Recent experiments suggest increased surface expression of the α_1 isoform of Na/K ATPase during LTP maintenance (Matt Kelly, D.T., Todd. C. Sacktor,

and Peter Bergold, unpublished results). At this time, PKMζ levels are increased to a similar extent as the increase in IPC. The significance of Na/K ATPase activity increase during the maintenance phase of LTP is not clear. One theory of LTP maintenance is increased surface expression of the AMPA type of glutamate receptors. Alternatively, the increased synaptic strength in potentiated neurons may necessitate expression of neuroprotective mechanisms.

The demonstration of similar changes in Na/K ATPase and PKMζ in LTP and preconditioning suggests additional shared mechanisms between synaptic plasticity and neuroprotection. Establishment and maintenance of LTP requires NMDA receptors and elevated intracellular calcium. Maintenance of LTP requires PKMζ. Antagonists of NMDA receptors block the LTP induction. The NMDA receptors have been found play a role in the preconditioning of cortical slice model (Semenov et al., 2002;Bond et al., 1999). Inhibitors of PKMζ prevent the maintenance but not induction (Collingridge, 2003;Ling et al., 2002).

Our work first suggests that PKMζ is also necessary to establish the preconditioning (Figure 17). However, despite their similarities, the energy metabolism in potentiated and preconditioned neurons is very different in the first few hours following induction. This difference may result in change in the kinetics of new protein synthesis. In LTP, increased PKMζ may be due to *de novo* translation of pre-existing RNA or increased transcription. Evidence supports that the release from the translational block mediated by the long 5'-UTR of the PKMζ

mRNA by rapamycin-sensitive pathways or an internal ribosomal entry site are potential mechanisms for the increase in PKMζ synthesis (Dyer et al., 2003). However, the increase of PKMζ takes much longer time in preconditioning model than in LTP, which suggests there is increased PKMζ mRNA.

Calcium activated transcription factors may be responsible for increased PKMζ transcription. Potential transcription factors involved in the new PKMζ synthesis in LTP include, NFATc4 (nuclear factor of activated T-cells family members) (Graef et al., 1999), CREB (cAMP-response-element-binding) (Deisseroth et al., 1996), DREAM (downstream regulatory element antagonistic modulator) (Carrion et al., 1999). All these transcription factors are activated by an increase in intracellular Ca to nucleus. They have important differences in their model of activation. NFATc4 is translocated to nucleus after dephosphorylation by calcineurin. This signal transduction mechanism is particularly attractive to mediate IPC since it does not require ATP and could operate in conditions of low ATP. Similarly, DREAM directly responds to elevated intracellular Ca. CREB activation requires phosphorylation by the Ca calmodulin dependent protein kinase. Other potential candidates include hypoxia inducible factor (HIF) (Ran et al., 2005). During normoxia, the HIF is modified by hydroxylation and acetylation which lead to degradation. In hypoxia or anoxia condition, the hydroxylation and acetylation are inhibited which promote the translocation of HIF to the nucleus and initiate gene transcription. However, evidence showed that hypoxic preconditioning attenuates the HIF induction

initiated by ischemia in neuron (Ruscher et al., 1998). Despite its potential activation by hypoxia, HIF may not be the key regulator of PKMζ synthesis.

Regulation of Na/K ATPase by PKMζ is indirect

The results in this thesis strongly suggest that PKMζ regulates the α_1 isoform of Na/K ATPase. Preliminary results in this thesis do not support PKMζ regulation of Na/K ATPase by direct phosphorylation. An alternative possibility is that PKMζ regulates the α_1 isoform by phosphorylation of other substrates. Evidence for indirect regulation of Na/K ATPase by protein kinases has been shown in both non-neuronal and neuronal systems. In renal epithelial cells, Na/K ATPase activity is regulated by constitutive endocytosis (Chibalin et al., 1998b). In the rat brain, Na/K ATPase was enriched in the clathrin coated vesicles. Clathrin coated vesicles are clearly involved in membrane protein recycling (Heuser and Reese, 1973). PKMζ may regulate trafficking of the Na/K ATPase by phosphorylation of a component of the clathrin coated vesicle. Recent evidence showed that dopamine induces a time-dependent phosphorylation of adaptor protein-2 micro-2 subunit. This effect is partially regulated by PKCζ (Chen et al., 2006). Adaptor protein -2 is responsible for the endocytosis by recruiting clathrin.

The glial α_2 isoform is likely neuroprotective following ischemic preconditioning

Surface expression of the α_2 Na/K ATPase isoform is increased following IPC (Figure 7). α_2 is expressed in astrocytes suggesting a role for glia in protecting neurons during ischemic preconditioning. There are two potential benefits provided by increased pump activity in astrocytes. Astrocytes are essential for maintenance of the concentration of extracellular K. Maintenance of physiological extracellular K prevents the membrane depolarization, and spreading depression. Astrocytes also inhibit the excitotoxic neurotransmitter release of glutamate. A second potential benefit is prevention of glutamate toxicity. Glutamate excitotoxicity is also prevented by clearance of glutamate form synaptic cleft into astrocytes. Excessive glutamate causes the Ca overload and damage in neurons. The excitotoxicity of glutamate is a key factor of ischemic brain injury. The α_2 Na/K ATPase isoform is localized in the perisynaptic regions of astrocytes where it co-localizes with the sodium-dependent glutamate transporters GLT and GLAST (Cholet et al., 2002). GLAST is the dominant glutamate transporter in most regions of the central nervous system (CNS), accounting for up to 95% of total glutamate uptake in the brain (Tanaka et al., 1997b). The Na/K ATPase maintains the sodium gradient that is needed for sodium-dependent glutamate uptake. During ischemia large amounts of glutamate released kill neurons by excitotoxicity. Although some of this glutamate release is contained in synaptic vesicles, reversal of glutamate

transporters following loss of the inward Na gradient results in much more glutamate efflux during ischemia than vesicle release. Increased α_2 activity may reduce glutamate efflux by limiting reversal of the Na gradient or by enhancing sodium-dependent glutamate uptake after the Na^+ gradient is restored. An inward Na gradient is also needed for proper functioning of the Na/H, Na/Ca transporters and the Na/K/Cl co-transporter. Inhibition of any of these transporters aggravates ischemic injury. Improved maintenance of the Na gradient following preconditioning may better allow these transporters to protect neurons.

Following IPC, surface expression of α_2 is increased. Increased surface expression is unlikely due to PKMζ since peptide did not alter α_2 surface expression. This study used DHO to examine if α_2 activity is needed fro neuroprotection, but DHO is not a specific pharmacological inhibitor of the α_2 isoform and no specific inhibitors exist. As a result, these data support a neuroprotective role for α_2, but we can not rule out a role for α_3 as well. The regulation of the increased surface expression of α_2 isoform is another area for future studies (Figure 19).

To conclude, the Na/K ATPase appears to have an essential neuroprotective role following IPC. These data underscore the importance of the restoration of ion gradients not only for neuronal signaling, but to maintain the overall health of the brain.

Figure 19. Model of how PKMζ and Na/K ATPase are regulated during ischemic preconditioning. Neurons express the α_1 and α_3 Na/K ATPase isoforms and PKMζ, astrocytes express the α_1 and α_2 isoforms of Na/K ATPase. PKMζ activity is needed for the surface expression of the α_1 Na/K ATPase isoform. Ischemic preconditioning increases surface expression of the α_1 and α_2 isoforms. It is unknown if PKMζ or ischemic preconditioning increases surface pump expression by promoting vesicular pump insertion or by inhibiting removal of surface pump. In neurons, PKMζ activity regulates surface expression of the α_1 Na/K ATPase isoform. In astrocytes, an unknown factor regulates α_2 surface expression.

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