BRAIN RESEARCH 1213 (2008) 127–139

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Research Report

Protein kinase M zeta regulation of Na/K ATPase: A persistent neuroprotective mechanism of ischemic preconditioning in hippocampal slice cultures

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ARTICLE INFO

Article history:
Accepted 20 March 2008
Available online 3 April 2008

Keywords:
Ischemic tolerance
Protein trafficking
Na/K ATPase isoform
Atypical PKC

ABSTRACT

In ischemic preconditioning, a sublethal ischemic insult protects neurons from subsequent ischemia. In organotypic hippocampal slice cultures a sublethal 5-minute hypoxia–hypoglycemia treatment prevented neuronal loss after a 10-minute experimental ischemic (EI) treatment of hypoxia–hypoglycemia. Whereas preconditioning protected against EI given 24 h later, it did not protect when EI was given 2 h later, suggesting a slow development of neuroprotection. This model identified two regulators of ischemic preconditioning: the atypical protein kinase M zeta (PKMζ), and the Na/K ATPase. Two hours following preconditioning, when there was no neuroprotection, Na/K ATPase activity was unchanged. In contrast, Na/K ATPase activity significantly increased 24 h after the preconditioning treatment. Elevated Na/K ATPase activity was accompanied by increased surface expression of the α1 and α2 isoforms of the Na/K ATPase. Similarly, active PKMζ levels were increased at 24 h, but not 2 h, after preconditioning. PKMζ overexpression by sindbis virus vectors also increased Na/K ATPase activity. To examine PKMζ regulation of Na/K ATPase, occlusion experiments were performed using marinobufagenin to inhibit α1, dihydroouabain to inhibit α2/3 and a ζ-pseudosubstrate peptide to inhibit PKMζ. These experiments showed that PKMζ regulated both the activity and surface expression of the α1 isoform of the Na/K ATPase. Marinobufagenin, dihydroouabain, and ζ-pseudosubstrate peptide were used to determine if PKMζ or the α1 and α2 Na/K ATPase isoforms protected neurons. All three compounds blocked neuroprotection following ischemic preconditioning. PKMζ levels were elevated 3 days after ischemic preconditioning. These data indicate key roles of PKMζ and Na/K ATPase in ischemic preconditioning.

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Abbreviations: PKMζ, protein kinase M zeta; PKC, protein kinase C; Na/K ATPase, sodium potassium ATPase; IPC, ischemic preconditioning; EI, experimental ischemia; PI, propidium iodide; ζ-peptide, zeta-inhibitory pseudosubstrate peptide; TPA, phorbol 12-myristate 13-acetate; MBG, marinobufagenin; DHO, dihydroouabain; BSS, Earles Balanced Salt solution; BCA assay, bicinchoninic assay

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doi:10.1016/j.brainres.2008.03.046

1. Introduction

In ischemic preconditioning (IPC), brief ischemia, which itself does not kill neurons, protects neurons from a subsequent lethal ischemia (Badaut et al., 2005). Neuroprotective therapeutics for ischemia as well as other neurodegenerative disorders could be developed if the mechanisms underlying IPC were better understood. Neuroprotection following IPC develops

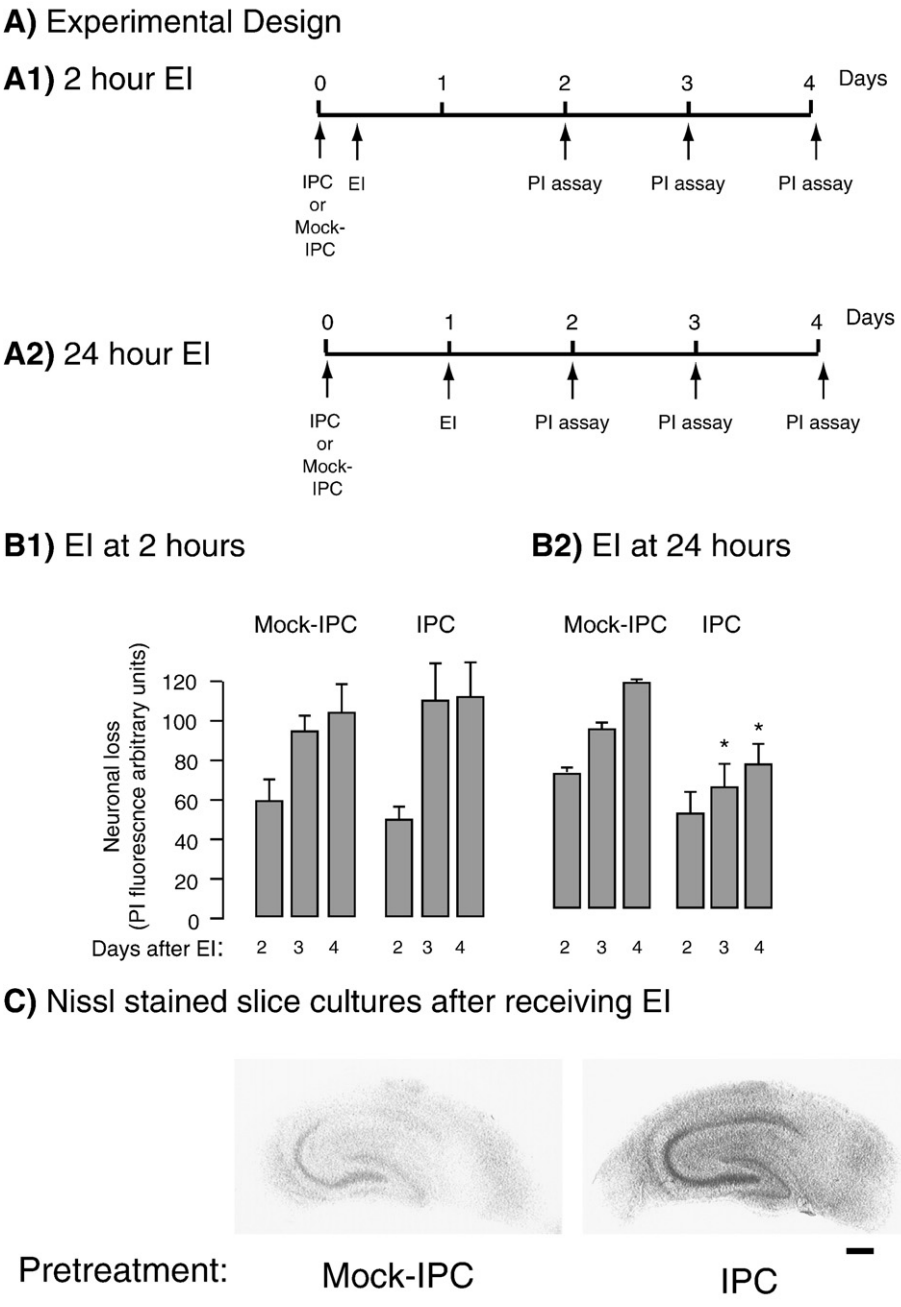


Fig. 1 – Neuroprotection develops slowly after ischemic preconditioning in slice cultures. Panel A, Schematic of the experimental design. On day 0, slice cultures received mock-IPC or IPC and were divided into two groups. One group received EI 2 h after mock-IPC or IPC (2 h EI, top). A second group received EI on day 1 (24 h EI, bottom). Neuronal loss in both groups was assayed using PI on days 2–4. Panel B, Summary of neuronal loss. Equivalent PI fluorescence was observed in the groups that received EI 2 h after IPC or mock-IPC (panel B1). In the group that received EI 24 h after IPC or mock-IPC, PI fluorescence was significantly less in the cultures receiving IPC (panel B2). (2 h, $n=6$ mock-IPC and IPC; 24 h, $n=6$ mock-IPC, $n=7$ IPC; ANOVA, $F=11.354$, $p<0.0001$; $^{*}p<0.01$ Student Neumann Keuls). These data suggest that neuroprotection developed between 2 and 24 h after IPC. Panel C, Nissl stained slice cultures. Slice cultures received either mock-IPC (left) or IPC (right). Twenty-four hours later, the cultures received EI. Seven days after EI the cultures were stained with cresyl violet. Cultures receiving IPC had better retention of the pyramidal cell layer, particularly in region CA1. Bar 500 μm.

over hours and persists for days. Long-term potentiation (LTP) also produces persistent changes in the brain. In anoxic LTP, preconditioning-like stimuli increased synaptic efficacy (Crepel et al., 2003; Hammond et al., 1994; Hsu and Huang, 1998, 1997) and IPC occludes LTP formation (Kawai et al., 1998). Tetanic stimulation produces LTP and protects against a subsequent ischemia (Youssef et al., 2001). Anoxic LTP and IPC require calcium influx through NMDA receptors suggesting similar mechanisms of induction (Raval et al., 2003). The atypical PKC, PKMζ, is a key mediator of the long-term maintenance of tetanic LTP (Ling et al., 2002; Sacktor et al., 1993). Therefore, we examined whether PKMζ is needed for neuroprotection following IPC.

PKMζ is a brain-specific, PKC isoform that lacks the autoinhibitory regulatory domain in PKCζ and other PKC isoforms (Hernandez et al., 2003). The lack of the autoinhibitory domain explains why PKMζ has constitutive kinase activity in the absence of second messengers needed to activate other PKC isoforms (Hernandez et al., 2003). Atypical PKCs also bind ATP with a 2–3 fold higher affinity than conventional or novel PKCs (Spitaler et al., 2000). This suggests retention of activity at the low ATP levels that occur during IPC (Hassen et al., 2004). Protein levels of atypical PKCs, including PKMζ were retained following ischemia while other PKC isoforms were downregulated (Libien et al., 2005). Therefore, substrates of PKMζ may remain phosphorylated during IPC. PKCζ positively regulates Na/K ATPase activity outside the brain (Bonizzi et al., 1999; Marsigliante et al., 2003). PKMζ is a potential regulator of brain Na/K ATPase since PKMζ and PKCζ share identical catalytic domains.

The Na/K ATPase maintains plasma membrane gradients of sodium and potassium (Kaplan, 2002). Preconditioning prevented loss of pumping following cardiac ischemia (Elmoselhi et al., 2003; Inserte, 2007; Nawada et al., 1997; Yorozuya et al., 2004). IPC also preserved brain Na/K ATPase activity following ischemia (de Souza Wyse et al., 2000). This study, however, did not test whether maintenance of Na/K ATPase activity protected neurons. The Na/K ATPase restores ionic gradients following action potentials as well as powering sodium-dependent membrane transporters. During ischemia, loss of Na^+, K^+, and Ca^{+2} gradients leads to membrane depolarization, cell swelling, glutamate release and cell death (Breder et al., 2000; Lipton, 1999; Sheldon and Church, 2004; Tanaka et al., 2002, 1997; Therien and Blostein, 2000; Martinez-Sanchez et al., 2004; Mergenthaler et al., 2004). The Na/K ATPase is critical for restoring these gradients (Lipton, 1999; Tanaka et al., 2002, 1997).

The Na/K ATPase has an α subunit containing its catalytic activity and the binding site for ouabain and related compounds and a β subunit that is needed for surface expression (Kaplan, 2002). Neurons express the α1 and α3 isoforms, and astrocytes express α1 and α2. In this study, we tested whether the Na/K ATPase was regulated by PKMζ and if this regulation was neuroprotective following IPC.

2. Results

2.1. *Neuroprotection develops slowly after preconditioning in hippocampal slice cultures*

Neuroprotection following IPC appeared rapidly within minutes or developed slowly within 1 day (Dirnagl et al., 2003; Kirino, 2002). We previously showed that a 5-minute IPC treatment protected against an experimental ischemia (EI) administered 24 h later (Hassen et al., 2004). To test whether IPC treatment protected 2 h after IPC, slice cultures received either IPC or mock-IP and EI was administered 2 or 24 h later (Fig. 1A). Neuronal loss was assayed using propidium iodide (PI). Equivalent amounts of PI staining were observed when EI was given 2 h following IPC or

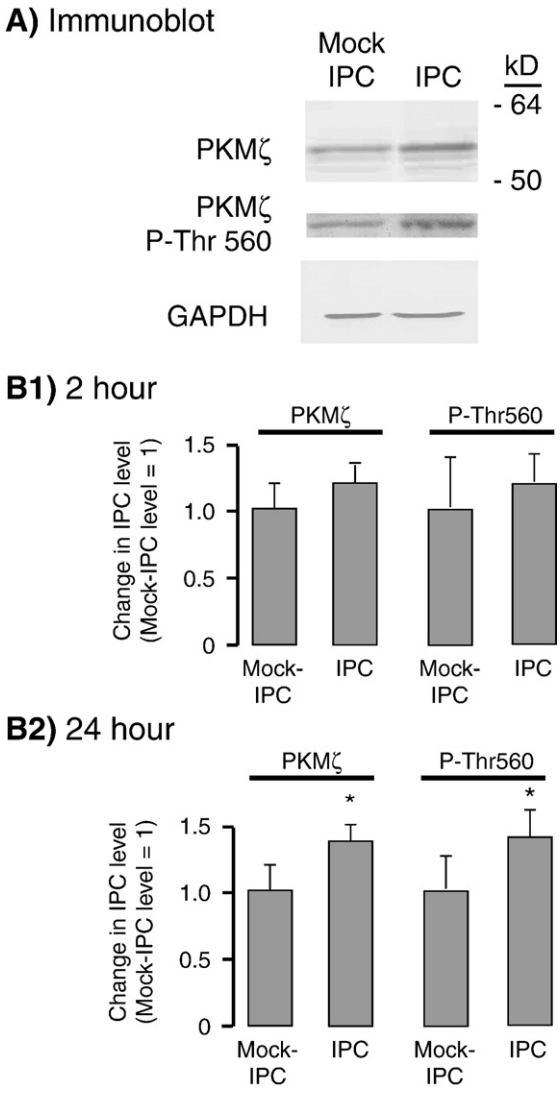


Fig. 2 – Ischemic preconditioning increases levels of active PKMζ. Panel A, Active PKMζ levels are increased 24 hours after IPC. Slice cultures received mock-IPC or IPC, maintained for 24 hours and immunoblotted with antisera against total PKMζ (PKMζ), phosphorylated PKMζ PKMζ P-Thr 560) or glyceraldehyde-3-dehydrogenase (GAPDH). Molecular weight markers are shown on the right. Panel B, Summary of immunoblot analysis of active PKMζ 2 or 24 h after IPC. Slice cultures received mock-IPC or IPC, maintained for 2 or 24 h and immunoblotted with antisera against PKMζ, phosphorylated PKMζ or GAPDH. At 24 h, but not at 2 h following IPC, levels of total PKMζ and phosphorylated PKMζ were significantly increased ($n=6$ for each group, $^*p<0.05$ Student's *t* test). Values are from densitometry scans with the level measured after mock-IPC normalized to 1.

mock-IPC treatment. In contrast, less PI fluorescence was assayed in the IPC group when EI was given 24 h later (Fig. 1B). Cultures receiving EI 24 h after IPC also had better preservation of hippocampal neuronal layers than cultures receiving mock-IPC (Fig. 1C). These data suggest that IPC protects neurons 24 h, but not 2 h following IPC.

2.2. *Preconditioning increases levels of active PKMζ*

PKMζ is a constitutively active kinase that can be assayed by the amount of Thr-560 autophosphorylation (Hernandez et al., 2003; Le Good et al., 1998; Standaert et al., 2001). We examined total levels of PKMζ and Thr-560 phosphorylation at 2 and 24 h following IPC (Fig. 2). Both PKMζ levels and Thr-560 phosphorylation significantly increased 24 h following IPC group suggesting that levels of active PKMζ increased following IPC at times when neurons were protected.

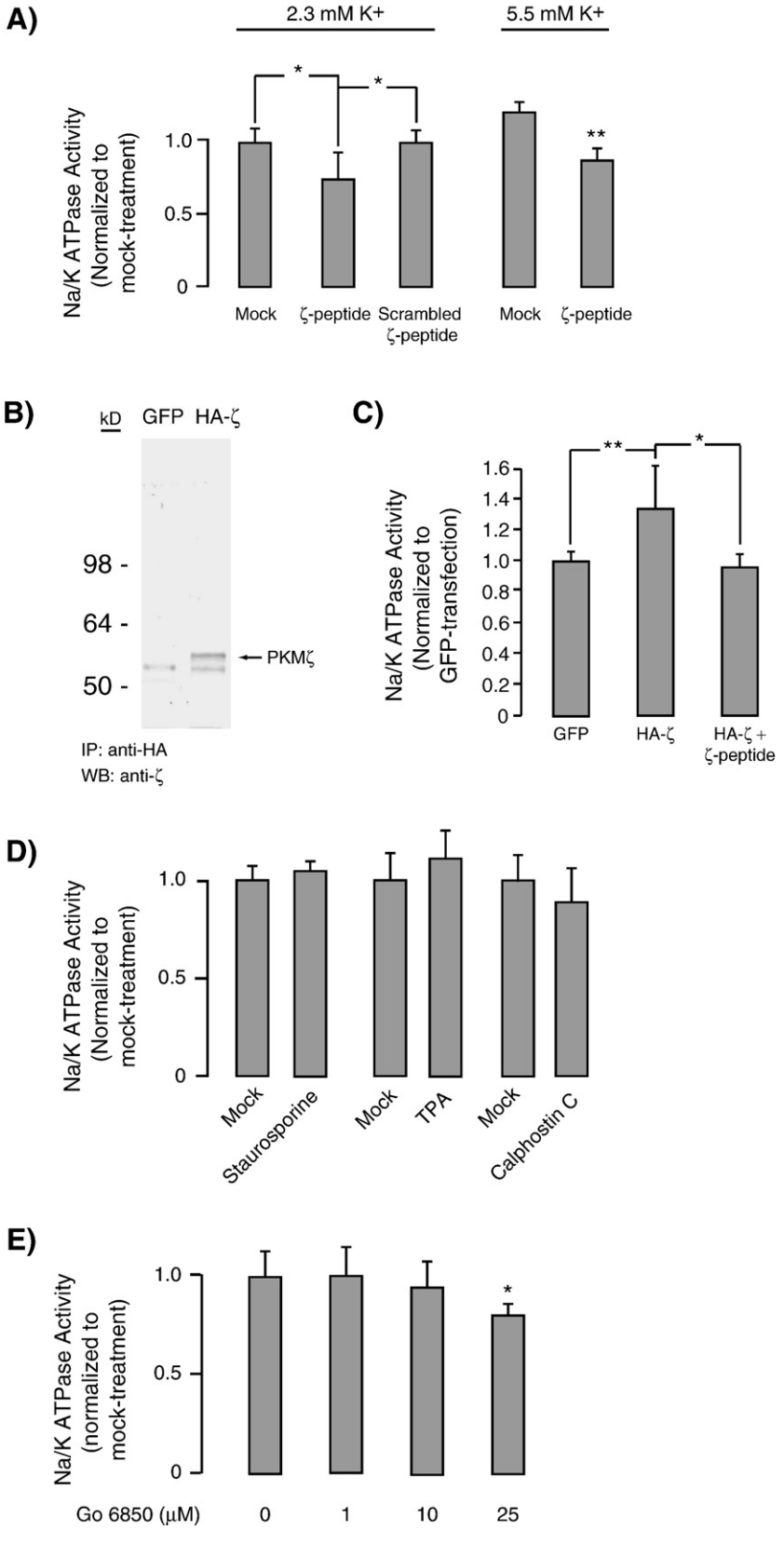
2.3. *PKMζ regulates Na/K ATPase activity*

PKCζ increases Na/K ATPase activity outside the nervous system (Bonizzi et al., 1999; Marsigliante et al., 2003). Unlike PKCζ, PKMζ is constitutively active suggesting it regulates basal Na/K ATPase activity (Hernandez et al., 2003). Slice cultures were mock-treated or treated with either myristoylated ζ-pseudosubstrate peptide (ζ-peptide) or a control scrambled peptide (Pastalkova et al., 2006). After 30 min of treatment, Na/K ATPase was assayed by ^{86}Rb uptake. ζ-Peptide treatment significantly decreased ^{86}Rb uptake while the scrambled peptide had no effect suggesting that PKMζ regulates basal Na/K ATPase activity (Fig. 3A). Elevated extracellular potassium stimulates Na/K ATPase activity (Kaplan, 2002). To test whether PKMζ regulated Na/K ATPase under conditions of elevated pumping, slice cultures were incubated in serum-free BSS containing 5.5 mM potassium. Na/K ATPase activity was measured following mock-treatment or treatment with ζ-peptide. Pumping in 5.5 mM potassium was also inhibited by ζ-peptide treatment (Fig. 3A). These data suggest that PKMζ regulates Na/K ATPase in conditions of stimulated pumping.

To further test whether PKMζ regulated Na/K ATPase activity, sindbis virus vectors were constructed that express a HA-PKMζ fusion protein, (SinRep5-HA-PKMζ) or enhanced green fluorescent protein (eGFP) (SinRep5-eGFP). Six hours after infection with sindbis viral vectors, protein extracts were prepared from the CA1 region of slice cultures, immunoprecipitated with anti-HA antibodies and immunoblotted with PKMζ antisera (Fig. 3B). SinRep5-HA-PKMζ infected cultures expressed a 55 kDa protein that reacted both the HA and ζ antibodies. This 55 kDa protein was absent in SinRep5-eGFP-infected cultures suggesting that SinRep5-HA-PKMζ directed expression of a 55 kDa HA-PKMζ protein. Six hours after SinRep5-HA-PKMζ or SinRep5-eGFP infection, the CA1 area was isolated and ^{86}Rb uptake measured (Fig. 3C). Cultures infected with SinRep5-HA-PKMζ had greater Na/K ATPase activity than cultures infected with SinRep5-eGFP. ζ-peptide (80 μM) blocked the increase in Na/K ATPase activity induced by SinRep5-HA-PKMζ suggesting that PKMζ stimulates Na/K ATPase activity. Na/K ATPase activity was not increased 24

Fig. 3 – PKMζ regulates Na/K ATPase activity. Panel A, ζ-peptide reduces Na/K ATPase activity in slice cultures. Slice cultures were either mock-treated (mock), or treated with myristoylated PKMζ-pseudosubstrate peptide (ζ-peptide, 80 μM) or a myristoylated peptide containing a scrambled ζ-peptide sequence (scrambled ζ-peptide). All groups were treated for 30 min. Pumping was assayed in 2.3 mM (left) or 5.5 mM (right) potassium. ^{86}Rb (1 μCi) or ^{86}Rb plus ouabain (2 mM) was added and the cultures incubated for 30 min. Ouabain-sensitive potassium uptake in the presence of 2.3 mM potassium in mock-treated cultures ($n=13$) is significantly higher than in ζ-peptide-treated ($n=6$) or scrambled peptide-treated ($n=6$) cultures (ANOVA, $F=8.3713$, $p<0.0001$, $^{*}p<0.01$, Student Neumann Keuls post-hoc test). In 5.5 mM potassium, ouabain-sensitive potassium uptake was significantly inhibited by ζ-peptide in the presence of 5.5 mM potassium (mock, $n=5$; ζ-peptide treatment, $n=8$; Student's t test, $^{**}p<0.001$). Panel B, Increased PKMζ expression following transduction into slice cultures. The CA1 pyramidal cell layer of slice cultures were transduced with either SinRep5-eGFP (GFP) or SinRep5-HA-PKMζ (HA-ζ). Six hours after infection, protein extracts were prepared from the CA1 region of the slice cultures and immunoprecipitated with anti-HA sera followed by immunoblot with antisera against the c-terminus of PKMζ (anti-ζ). An immunoreactive M_r 55 kDa PKMζ protein (arrow) was observed in extracts from the SinRep5-HA-PKMζ-transduced slice cultures that was absent in the SinRep5-eGFP-transduced cultures. These data suggest that SinRep5-HA-PKMζ directs expression of PKMζ protein 6 h after transduction. Panel C, Increased Na/K ATPase activity following infection with SinRep5-HA-PKMζ. The CA1 pyramidal cell region of slice cultures was transduced with either SinRep5-eGFP (GFP) or SinRep5-HA-PKMζ (HA-ζ). Six hours after transduction, Na/K ATPase activity was assayed by ^{86}Rb uptake. Na/K ATPase activity was significantly increased in SinRep5-HA-PKMζ-transduced cultures. To confirm that the increased pumping was due to the activity of PKMζ, some of the SinRep5-HA-PKMζ-transduced cultures were treated with ζ-peptide (80 μM) 30 min prior to the ^{86}Rb uptake assay. Na/K ATPase activity was significantly decreased by the ζ-peptide (80 μM) treatment. (ANOVA, $F=6.6626$, $p<0.01$; Student Neumann Keuls, GFP vs HA-ζ, $^{**}p<0.01$; HA-ζ vs HA-ζ plus ζ-peptide, $^{*}p<0.05$ $n=6$ for all groups). Panel D, Other protein kinases do not regulate basal Na/K ATPase activity. Slice cultures were pretreated for 30 min with staurosporine (100 nM), TPA (phorbol 12-myristate 13-acetate, 40 nM), or calphostin C (500 nM). Mock-treated cultures were treated with DMSO vehicle. ^{86}Rb uptake was assayed for 30 min and ouabain-dependent potassium uptake determined. No significant difference was observed between vehicle-treatment and treatment with staurosporine, TPA, or calphostin C (ANOVA, $F=0.3492$, $n=6$ for each group). Panel E, An atypical PKC regulates basal Na/K ATPase activity Slice cultures received Gö6850 (1 μM, 10 μM and 25 μM) or were mock-treated. Na/K ATPase assayed by ^{86}Rb uptake. Slice cultures treated with Gö6850 (1 μM, 10 μM) 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 (25 μM) ($n=6$ for each group; ANOVA, 5.2824, $p<0.01$; Student Neumann Keuls post-hoc test; $^{*}p<0.05$). The dose–response of slice culture Na/K ATPase activity to Gö6850 is consistent with regulation by an atypical PKC.

and 48 h following infection with SinRep5-HA-PKMζ. Immunofluorescence using anti-HA antibodies could not detect SinRep5-HA-PKMζ-infected cells in the CA1 pyramidal cell layer at either 6 h or at later times after infection (D.T. and P.J.B., data not shown). The inability to detect HA-PKMζ expression by immunofluorescence or by increased pump activity at time points later than 6 h may have resulted from a cytopathic effect of PKMζ overexpression. This cytopathic effect of SinRep5-HA-PKMζ is



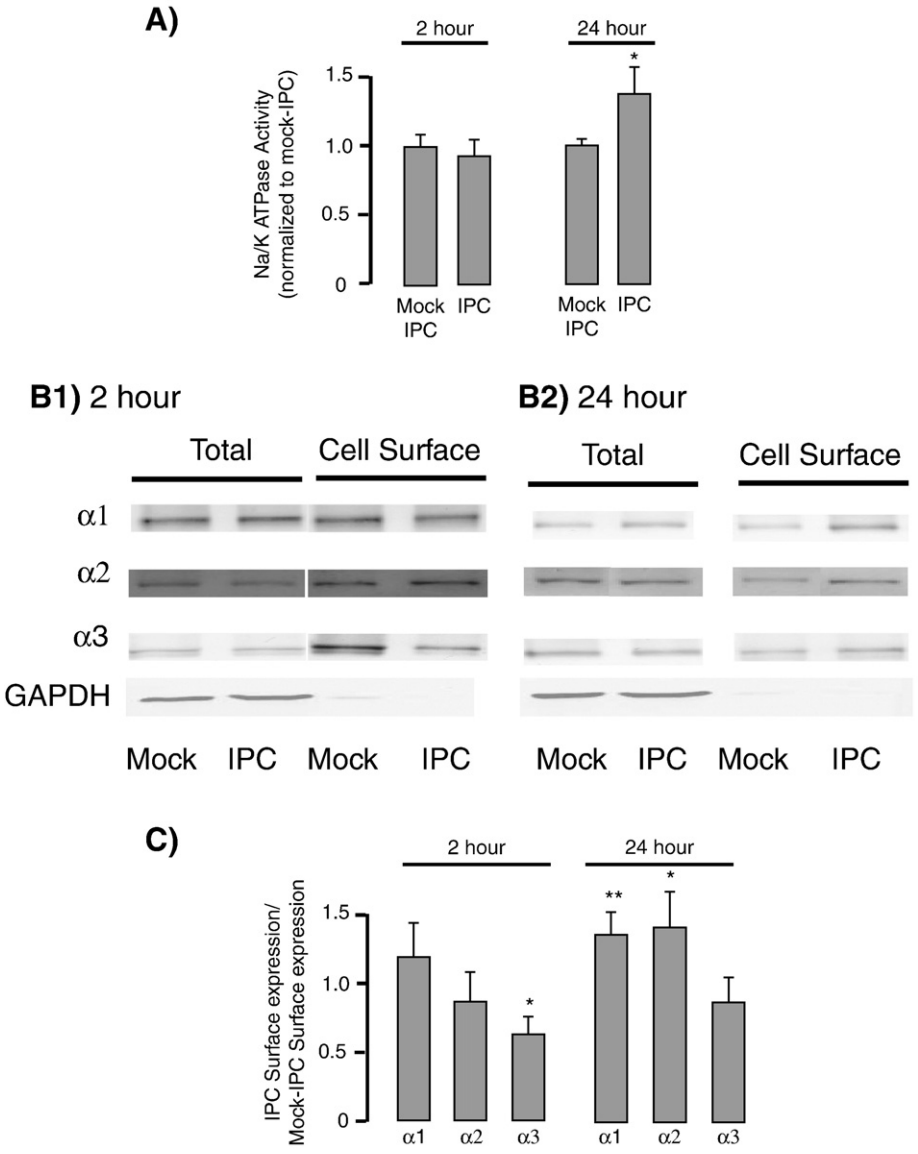


Fig. 4 – Ischemic preconditioning increases surface Na/K ATPase activity and expression. Panel A, Increased Na/K ATPase activity following IPC cell surface Na/K ATPase activity was assayed using ^{86}Rb uptake. Na/K ATPase activity in the IPC and mock-IPC groups did not significantly differ at 2 h. Pumping in IPC group significantly increased 24 h following IPC (ANOVA, $F=5.0812$, $p<0.01$; $^{*}p<0.05$, Student Newman Keuls; $n=6$, mock-IPC 2 h; $n=7$, IPC 2 h; $n=6$, mock-IPC 24 h; $n=5$, IPC 24 h). Panel B, IPC increased surface expression of Na/K ATPase isoforms following IPC. Slice cultures received either IPC or mock-IPC. Two or 24 h following treatment, biotinylated surface proteins were isolated and analyzed by immunoblot using antisera specific for individual Na/K ATPase α isoforms. Values are expressed as the ratio of surface expression in cultures receiving IPC as compared to cultures receiving mock-IPC. Panel B1, Two hours after IPC. Panel B2, Twenty-four hours after IPC. Panel C, Summary of the changes in surface Na/K ATPase isoforms following ischemic preconditioning. At 2 h, α3 isoform surface expression was significantly lower. At 24 h α1 and α2 isoform surface expression were significantly elevated ($n=6$ for all isoforms; α1, $^{**}p<0.005$, α2, $^{*}p<0.05$; Student's t test). Values are the mean of ratio of the expression of mock-IPC and IPC for each isoform ± SEM.

unlikely due to the sindbis virus vector since eGFP expression was readily observed time points later than 6 h post-infection of SinRep5-eGFP (D.T. and P.J.B., data not shown).

Other protein kinases regulate Na/K ATPase activity (Lopina, 2001). A panel of kinase inhibitors was used to examine if kinases other than PKMζ regulate basal Na/K ATPase activity (Fig. 3D). Staurosporine (100 nM) inhibits most major protein kinases expressed in brain, but has no effect on PKMζ activity (Tamaoki, 1991; Yanagihara et al., 1991; Ling et al., 2002). Basal Na/K ATPase activity was unaffected by staurosporine (100 nM) (Fig. 3D). TPA (phorbol 12-myristate 13-acetate, 400 nM) is a potent activator of conventional and novel PKCs and had no effect on basal Na/K ATPase (Battaini, 2001). Calphostin C irreversibly inhibits all PKC isoforms that contain a regulatory domain yet had no effect on basal Na/K ATPase activity (Fig. 3D). At 25 μM, Gö6850 inhibits all three classes of PKCs; at 1 μM, Gö6850 does not inhibit atypical PKCs. Gö6850 (1 μM) had no affect on basal Na/K ATPase activity, yet Gö6850 (25 μM) significantly reduced pumping (Fig. 3E).

Stimulation of pumping following PKMζ overexpression and inhibition of pumping by Gö6850 (25 μM) and ζ-peptide strongly suggest that PKMζ regulates Na/K ATPase activity in slice cultures.

2.4. *Ischemic preconditioning elevates Na/K ATPase activity and increases surface expression of the α1 and α2 isoforms*

The increase of PKMζ levels at 24 h following IPC suggests that Na/K ATPase activity will also be elevated. ^{86}Rb uptake was assayed 2 and 24 h after mock-IPC or IPC treatment (Fig. 4A). Two hours after IPC, no increase was assayed in Na/K ATPase while at 24 h, ATPase activity was increased.

Elevated surface Na/K ATPase activity may result from an enhanced pumping or an increase in the number of surface pumps. Three Na/K ATPase isoforms (α1, α2 and α3) are expressed in hippocampus and in slice cultures (McGrail et al., 1991) (Fig. 4B). Surface expression of Na/K ATPase isoforms was assayed by selective biotinylation and isolation of surface

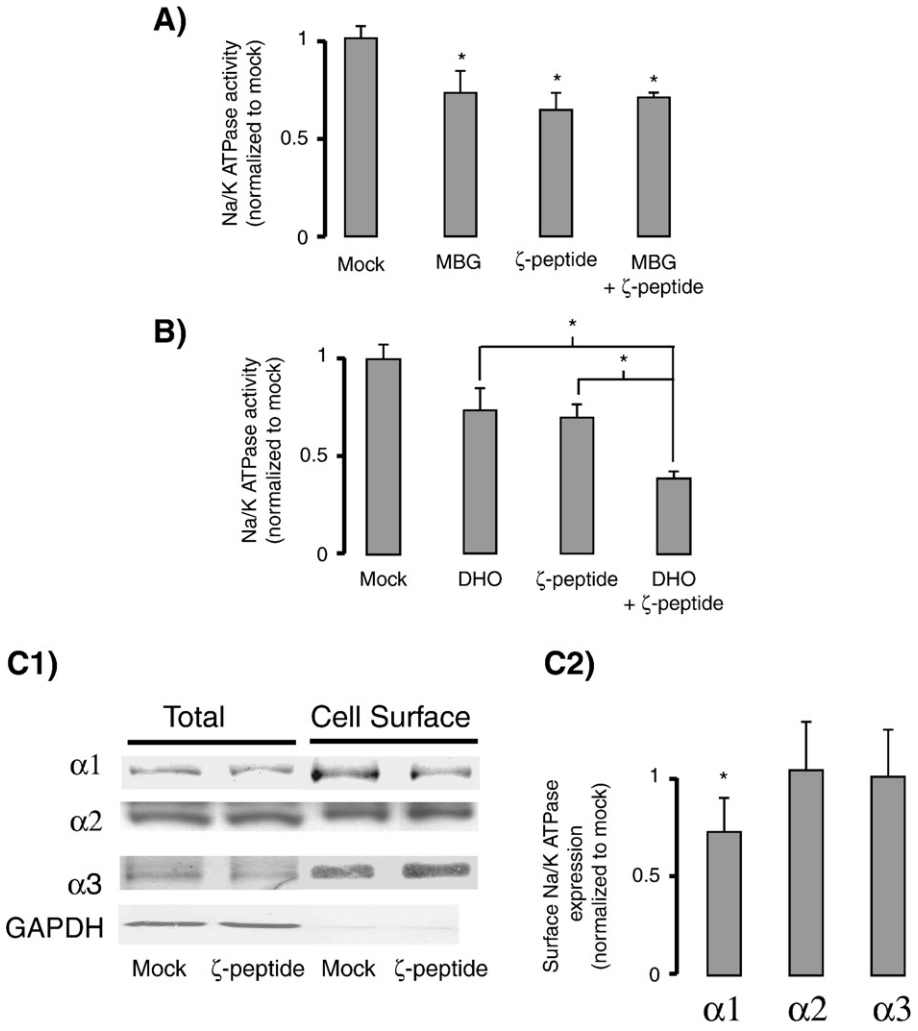


Fig. 5 – PKMζ regulates the Na/K ATPase α1 isoform. Panel A, Occlusion of ζ-peptide and marinobufagenin (MBG). Slice cultures received either mock-treatment ($n=7$), or were treated with MBG (2.1 nM, $n=4$), ζ-peptide (80 μM, $n=4$), or the combination of MBG and ζ-peptide ($n=6$). After a 30-minute treatment, Na/K ATPase in all groups were assayed using ^{86}Rb. Treatment with MBG, ζ-peptide, or MBG plus ζ-peptide significantly inhibited Na/K ATPase activity when compared with mock-treatment (ANOVA, $F=5.215$, $p<0.01$; $^{*}p<0.01$, Student Neumann Keuls). In contrast, inhibition by the combined treatment of ζ-peptide and MBG did not significantly differ from inhibition by MBG or ζ-peptide alone. Panel B, No occlusion between ζ-peptide and dihydroouabain (DHO). Slice cultures received either mock-treatment ($n=6$), or were treated with DHO (20 μM, $n=6$), ζ-peptide (80 μM, $n=5$) or the combination of DHO and ζ-peptide ($n=5$). Treatment with DHO or ζ-peptide significantly inhibited Na/K ATPase activity (ANOVA, $F=20.507$, $p<0.001$; $^{*}p<0.01$ Student Neumann Keuls). The inhibition of Na/K ATPase by the combined treatment of DHO and ζ-peptide was significantly greater than by DHO or ζ-peptide alone ($^{*}p<0.01$, Student Neumann Keuls). These data suggest no occlusion between ζ-peptide and DHO. Panel C, PKMζ regulates the surface distribution of the Na/K ATPase α1 isoform. Slice cultures were either mock-treated or treated with ζ-peptide (80 μm) for 2 h. Surface proteins were biotinylated, isolated on avidin beads and immunoblotted with Na/K ATPase isoform-specific antisera. Data was normalized to mock-treatment. Surface expression of the α1 isoform was lowered by ζ-peptide, despite the absence of change in its total expression (α1, $n=5$; α2, $n=4$; α3, $n=5$; Student's t test, $^{*}p<0.01$).

proteins followed by immunoblot with isoform-specific antisera. Total expression and surface expression of the α1, α2 or α3 isoforms were assayed 2 and 24 h after mock-IPC or IPC. At 2 h, surface expression of the α1 and α2 isoforms was unchanged and α3 was lower (Fig. 4B1). At 24 h, surface expression of the α1 and α2 was significantly increased with no change in α3 expression (Fig. 4B2). Increased surface α1 and α2 expression likely underlie the enhanced Na/K ATPase activity 24 h after IPC treatment.

2.5. *PKMζ regulates the α1 isoform of the Na/K ATPase*

Regulation of Na/K ATPase by PKMζ was further tested using Na/K ATPase inhibitors specific for the α1 isoform or for the α2 and α3 isoforms. Marinobufagenin (MBG) specifically inhibits α1 (α_1, IC_{50}=2.1 nM; α_3, IC_{50}=50 nM) (Fedorova and Bagrov, 1997). Slice cultures were either mock-treated or treated with differing concentrations of MBG (1–5 nM) and surface Na/K ATPase activity assayed by ^{86}Rb uptake assay. MBG (2.1 nM) inhibited 27.6±6.0% of Na/K ATPase activity suggesting selective inhibition of the α1 isoform (Fig. 5A). To test whether PKMζ also regulated α1, slice cultures were treated with ζ-peptide (80 μM) or a combination of MBG and ζ-peptide. Na/K ATPase inhibition by the combination of ζ-peptide and MBG did not significantly differ from the individual treatments suggesting that they both act on the α1 isoform of Na/K ATPase (Fig. 5A).

The low-affinity ouabain analog, dihydroouabain (20 μM) can specifically inhibit the α2 and α3 isoforms of Na/K ATPase (Reich et al., 2004; Vaillend et al., 2002). If PKMζ acts on α1, and dihydroouabain acts on α2/3, the inhibitory effect of dihydroouabain and ζ-peptide on Na/K ATPase activity will be additive. Slice cultures were either mock-treated or treated with dihydroouabain (20 μM), ζ-peptide (80 μM) or the combination of dihydroouabain and ζ-peptide. Na/K ATPase activity was assayed by ^{86}Rb uptake (Fig. 5B). Pumping was inhibited by 27.2±5.9% dihydroouabain and 30.5±6.0% by ζ-peptide (Fig. 3A). Inhibition by the combined treatment (57.7%) was very similar to the sum of the inhibition (60.9%) of the two compounds applied individually suggesting that PKMζ does not regulate the α2 or α3 isoforms of Na/K ATPase.

Regulation of Na/K ATPase surface expression by PKMζ was also examined. After mock-treatment or treatment with ζ-peptide (80 μM), biotinylated slice culture surface proteins were isolated and analyzed using Na/K ATPase isoform-specific antisera (Fig. 5C). ζ-peptide treatment lowered α1 surface expression without altering surface expression of the α2 and α3 isoforms (Figs. 5C, D). These data suggest PKMζ regulates Na/K ATPase activity through surface expression of α1.

Following IPC, levels of activated PKMζ, Na/K ATPase activity and the surface expression of α1 and α2 are increased (Figs. 2, 3 and 4). We therefore tested the role of PKMζ activity in the regulation of Na/K ATPase activity following IPC (Fig. 6). Slice cultures received mock-IPC or IPC. Twenty-four hours later, they were either mock-treated, or treated with ζ-peptide (80 μM) or MBG (2.1 nM). Na/K ATPase activity was assayed using ^{86}Rb uptake. In cultures receiving mock-IPC, MBG and ζ-peptide produced a similar inhibition of Na/K ATPase suggesting that PKMζ regulates α1 activity (Fig. 6). In cultures receiving IPC, the increase in Na/K ATPase activity

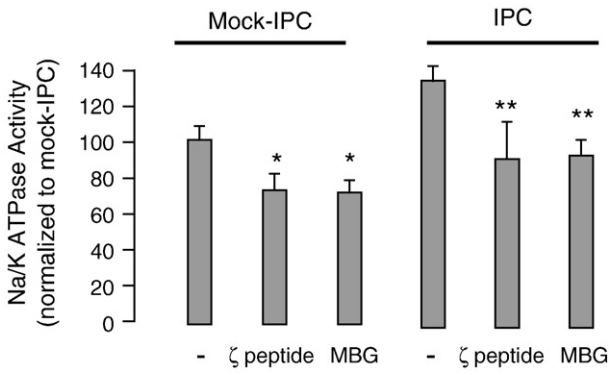


Fig. 6 – PKMζ regulates the α1 isoform of Na/K ATPase following IPC. Slice cultures received either mock-IPC or IPC. After 24 h, the IPC or mock-IPC groups were subdivided into three groups that received either mock-treatment or treatment with ζ-peptide (80 μM) or marinobufagenin (2.1 nM, MBG). After 30 min, the Na/K ATPase activity was measured using ^{86}Rb uptake. IPC induced a significant increase in Na/K ATPase activity. In cultures receiving either mock-IPC or IPC, MBG and ζ-peptide significantly decreased Na/K ATPase activity when compared to culture receiving mock-treatment (ANOVA, $F=10.856$, $p<0.0001$; Student Newman Keuls $^{*}p<0.05$, $^{**}p<0.01$).

was inhibited by ζ-peptide or MBG. The amount of Na/K ATPase activity remaining in cultures treated with ζ-peptide or MBG did not significantly differ from Na/K ATPase activity in mock-treated cultures. These data suggest that most of the increase in basal Na/K ATPase activity following IPC results from PKMζ regulation of α1.

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

IPC increased levels of active PKMζ (Fig. 2), Na/K ATPase activity (Fig. 3) and surface expression of α1 and α2 isoforms (Fig. 5). We tested whether these increases in PKMζ or Na/K ATPase activity mediated neuroprotection (Fig. 7). Slice cultures received either mock-IPC or IPC and returned to the incubator for 24 h. The IPC group was further subdivided into 4 groups that were either mock-treated, or treated with MBG (1 nM), dihydroouabain (20 μM), or ζ-peptide (80 μM). After 60 min of treatment, all groups received EI. Neuronal loss was assayed at 1 and 3 days following EI by PI assay (Fig. 7A). PI fluorescence in the group receiving IPC plus MBG (1 μM) or IPC plus dihydroouabain was significantly increased as compared the mock-IPC group suggesting a protective role for α1 or α2/α3 (Figs. 7B, C). The group receiving IPC plus ζ-peptide also displayed increased PI fluorescence suggesting a role for PKMζ in neuroprotection following IPC (Figs. 7B, C). Dihydroouabain (20 μM) inhibits both the α2 and α3 isoforms of Na/K ATPase. Despite the inhibition of both α2 and α3, α2 is believed to be the neuroprotective isoform since 1 day after IPC, surface expression of α2, but not α3 is elevated (Fig. 4C). An alternative, less likely, explanation for these findings is that ζ-peptide, MBG and dihydroouabain enhance ischemic injury in the absence of preconditioning.

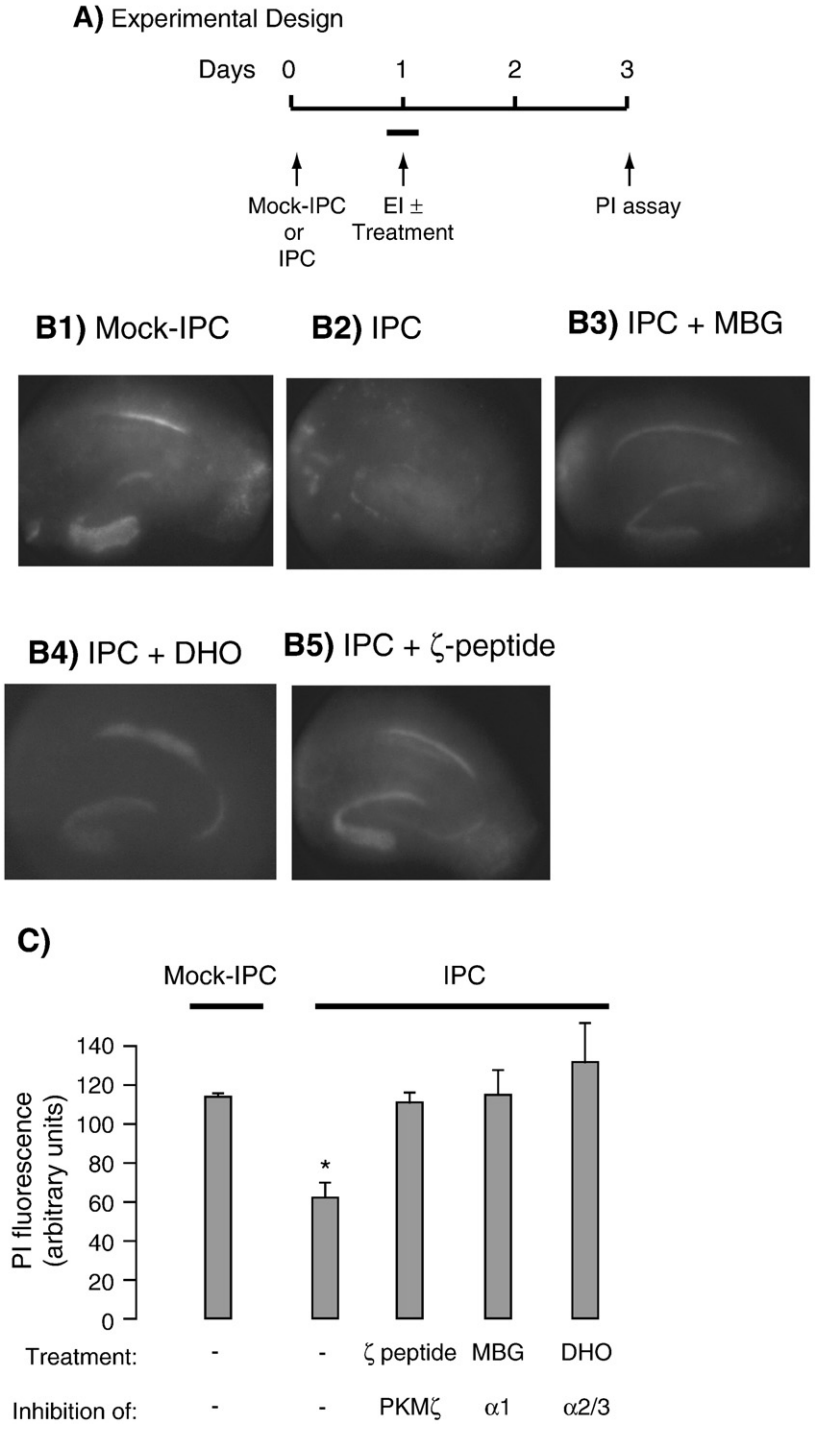


Fig. 7 – PKMζ and Na/K ATPase protect neurons following ischemic preconditioning. Panel A, Experimental design. Slice cultures received either mock-IPC or IPC and were returned to the incubator for 1 day. Sixty minutes before EI treatment, slice cultures were either mock-treated, or treated with ζ-peptide, marinobufagenin (MBG, 2.1 nM) or dihydroouabain (DHO, 20 μM). Three days later, PI was used to assay cell loss. Panel B, Representative images of PI fluorescence in slice cultures. Slice cultures received mock-IPC (panel B1), IPC (panel B2), IPC plus marinobufagenin (IPC+MBG) (panel B3), IPC plus dihydroouabain (IPC+DHO) (panel B4) or IPC or ζ-peptide (panel B5). Panel C, Summary of neuroprotection. 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 ($n=4–5$ for all groups; ANOVA, $F=7.932$, $p<0.0005$; $^{*}p<0.01$, Student Neumann Keuls).

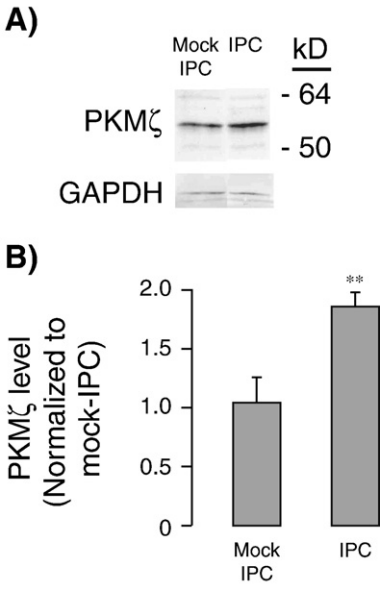


Fig. 8 – IPC induces a persistent elevation of PKMζ levels. Slice cultures received either mock-IPC or IPC and maintained in the incubator for 3 days. Protein extracts were prepared and immunoblotted using antisera against PKMζ (PKMζ) or GAPDH (GAPDH). Panel B, Summary of these data. The amount of the 55 kDa PKMζ or the 35 kDa GAPDH protein was measured and the PKMζ protein levels significantly increased following IPC as compared to mock-IPC (Student's *t* test $^{**}p<0.005$, $n=5$ for both groups).

2.7. *A persistent increase in PKMζ following IPC*

IPC lowered neuronal loss assayed 3 days following EI (Fig. 1). We tested whether PKMζ levels, that were increased 1 day following EI, remained elevated 3 days following EI. Slice cultures received either mock-IPC or IPC. Three days later PKMζ levels were assayed by immunoblot. PKMζ levels were increased suggesting that PKMζ was persistently increased following IPC (Fig. 8).

3. Discussion

This study indicates that neuroprotection slowly develops following IPC in slice cultures (Fig. 1). This neuroprotection results from PKMζ regulation of the Na/K ATPase. The evidence supporting this conclusion are: (1) IPC increases levels of activated PKMζ (Fig. 2). (2) PKMζ regulates basal Na/K ATPase activity since PKMζ inhibition decreases Na/K ATPase activity while increased PKMζ expression increases pumping (Fig. 3). (3) Na/K ATPase activity and surface expression of α1 and α2 isoforms increase 24 h after a preconditioning stimulus (Fig. 4). (4) PKMζ regulates the activity and surface expression of the α1 isoform of the Na/K ATPase (Fig. 5). (5) Most of the increase in basal Na/K ATPase activity following IPC results from a PKMζ-mediated increase in the α1 isoform. (6) The changes in PKMζ and Na/K ATPase occur at the time window when IPC protects neurons (Figs. 2 and 4). (7) PKMζ levels remain elevated 3 days after IPC. (8) The activity of α1 and PKMζ are likely needed for neuroprotection following IPC (Fig. 7). (9) Activity of the α2 and/or the α3 isoform is also likely needed for neuroprotection (Figs. 7 and 8).

Active PKMζ levels are increased 1 day following a preconditioning treatment that remained elevated for two additional days (Figs. 2 and 8). These data suggest that levels of active PKMζ are elevated throughout the time window for neuroprotection. PKMζ activity regulates Na/K ATPase activity as well as surface expression of α1 (Fig. 4). The α1 isoform is expressed throughout the brain while PKMζ is expressed only in neurons (Hernandez et al., 2003; McGrail et al., 1991). These data suggest that PKMζ only upregulates neuronal α1 during preconditioning.

This study suggests that PKMζ activity is neuroprotective. Earlier studies have suggested a necessary role for PKCζ activity in NMDA-dependent death of PC12 cells and embryonic cortical neurons (Crisanti et al., 2005; Koponen et al., 2003). Unlike PC12 cells or dissociated embryonic neurons, slice cultures retain the tissue architecture, cell density and synaptic connections of the hippocampus (Xiang et al., 2000). The brief episode of oxygen-glucose deprivation used in this study is similar to the stimuli preconditions the brain *in vivo*. The studies showing a neurotoxic role for PKCζ required a large and prolonged NMDA exposure (Crisanti et al., 2005; Koponen et al., 2003). Increased PKMζ expression is hypothesized to protect neurons. Unfortunately, increased PKMζ expression following infection with SinRep5-HA-PKMζ could not be used to address whether PKMζ protects neurons due to a cytopathic effect of PKMζ overexpression.

IPC also increased Na/K ATPase activity. The Na/K ATPase α2 isoform is localized in the perisynaptic regions of astrocytes where it co-localizes with the sodium-dependent glutamate transporters GLAST and GLT-1 suggesting that IPC increases glutamate uptake (Cholet et al., 2002). The Na/K ATPase is also needed for the Na^+/H^+ and Na^+/Ca^{+2} transporters as well as the $Na^+/K^+/Cl^-$ co-transporter (Beck et al., 2003; O'Donnell et al., 2004; Sun and Murali, 1999). Inhibition of any of these transporters aggravates ischemic injury. Improved maintenance of the Na^+ gradient following IPC may better allow these transporters to protect neurons.

PKMζ has been previously identified as an important mediator of the maintenance phase of LTP (Ling et al., 2002; Pastalkova et al., 2006; Sacktor et al., 1993). PKMζ levels increase to a similar degree following IPC (Fig. 2) and LTP (Sacktor et al., 1993). PKMζ levels increase within 10 min following LTP and between 2 and 24 h following IPC occurs (Sacktor et al., 1993) (Fig. 3). Establishment and maintenance of LTP and preconditioning requires NMDA receptors, elevated intracellular calcium and PKMζ. This study also showed an important role for increased Na/K ATPase activity during IPC. PKMζ may also regulate Na/K ATPase activity during LTP. Recent experiments have suggested increased surface expression of the α1 isoform of Na/K ATPase during LTP maintenance (Mathew Kelly, D.T., T.C.S., and P.B, unpublished results). The demonstration of similar changes in Na/K ATPase and PKMζ in LTP and IPC suggests additional shared mechanisms between synaptic plasticity and neuroprotection.

4. Experimental procedures

4.1. *Preparation of slice cultures and ischemic preconditioning*

Slice cultures were prepared by the method of Hassen et al. (2004). Adequate measures were taken to minimize pain or discomfort in

rats during the preparation of slice cultures. All cultures were maintained at 32 °C in a 5% CO_2 atmosphere for at least 2 weeks prior to all experiments. One day before an experiment, the cultures were shifted to a 37 °C incubator in a 5% CO_2 atmosphere. IPC was performed as described by Hassen et al. (2004). For IPC, slice cultures were submerged in Earles Balanced Salt solution (BSS) without glucose for 5 min and bubbled vigorously with 95% N_2, and 5% CO_2. A mock-IPC group were submerged in BSS with 5 mM glucose for 5 min and bubbled vigorously with 20% O_2, 75% N_2, and 5% CO_2. Twenty-four hours later, both groups received a neurotoxic experimental ischemia (EI) treatment of 10 min of BSS without glucose and bubbled vigorously with 95% N_2, and 5% CO_2.

Propidium iodide assay, a standard method for assaying neuronal loss in slice cultures, was performed as described by Hassen et al. (2004). Briefly, PI intensity was assayed in the pyramidal cell layer of the slice culture CA1 region. Changes in PI fluorescence were also observed in the granule cell layer of the slice culture dentate gyrus. The PI fluorescence in the dentate gyrus was not observed consistently in the experiments in this study therefore assay of neuronal loss was restricted to the CA1 pyramidal cell layer. Other studies using slice cultures isolated from post-natal day 7–10 day old animals have normalized increased PI fluorescence to PI fluorescence obtained in which 100% of the neurons were killed. Presumably, this normalization is needed when the number of neurons in the cultures is highly variable. In this study, the variability in the neuron number between cultures is sufficiently small to obtain the statistically significant difference between the control and experimental groups without an additional normalization step.

4.2. *Preparation and titration of sindbis virus vectors and infection of hippocampal slice cultures*

A PKMζ cDNA was cloned into pSinRep5 using a PCR based strategy. Briefly, an insert corresponding to amino acids 184–592 of PKCζ was obtained using a plasmid containing the mouse cDNA as a template. A forward primer (5′-AAATCTAGAACATGGCATACCCATACGACGTCCCAGACTACGCTATGGATTCTGTCATGCCTTCC-3′) was synthesized with a restriction site for Xba I followed by the sequence of influenza hemagglutinin. A reverse primer was constructed (5′-AATTCTAGATCACACGGACTCCTCAGC-3′) with a second restriction site for Xba I. pSinRep5-HA-PKMζ was constructed by inserting the PCR amplified product into the pCRT7/NT TOPO TA vector (Invitrogen, Carlsbad CA) according to the manufacturer's instructions, digested with Xba I and subcloned into the pSinRep5 Xba I site. RNA was prepared from pSin5PKMζ and electroporated into baby hamster kidney (BHK) cells with RNA prepared from DH-BB(tRNA-TE12). RNA for electroporation was also prepared from pSinRep5GFP. pSinRep5GFP and DH(tRNA-TE12) were gifts from Pavel Osten (Northwestern University, IL). The supernatant of the transfected BHK cells was isolated. pSinRep5GFP and pSin5PKMζ viral stocks were titrated on primary neuronal cultures. Primary hippocampal neurons were prepared from embryonic days 18–19 Sprague–Dawley rats. Hippocampal cells were disassociated by trituration and 6×10^4 cells were plated on poly-D-lysine treated 18 mm coverslips in neurobasal media supplemented with B27, penicillin, streptomycin, and L-glutamine. (InVitrogen, Carlsbad, CA) on 9.6 cm^2 6-well plates (BD Falcon). For viral titration or immunoblot analysis, cultures 21 days *in vitro* were infected with of virus stock diluted to a final volume of 500 μl with Neurobasal–B27 medium. The pSinRep5GFP transfected cells were visualized using fluorescein filters. pSinRep5PKMζ transfected cells were fixed with paraformaldehyde (4% w/v) and immunostained with anti-HA serum (1:500, Sigma, St. Louis, MO). The titer of the sindbis viral vectors used in this study varied from 1.2×10^6–2.3×10^6 infectious units/ml.

One hundred nanoliters of either SinRep5-HA-PKMζ or SinRep5-eGFP were injected 8–12 times into the CA1 pyramidal cell layer of slice cultures and returned to the incubator for 6 h before assay by immunoprecipitation and immunoblot or by ^{86}Rb uptake.

4.3. *^{86}Rubidium uptake assay of Na/K ATPase activity*

^{86}Rb uptake was assayed with modification of the method of Peng et al. (1996). ^{86}RbCl (1 μCi, >1 μCi/μg, PerkinElmer, Boston, MA) was added to the slice cultures in BSS containing 2.3 mM KCl (2.3 mM K BSS) and incubated for 30 min at 37 °C. The reaction was stopped with ice-chilled 2.3 mM K BSS and extracellular ^{86}RbCl removed with 3 washes of 2.3 mM K BSS. The slices were homogenized with 1 ml 0.1 N NaOH. A 200 μl aliquot of the homogenized sample was added to 10 ml of scintillation fluid (Ecolume, ICN Biomedicals, Irvine, CA) and counted on a Beckman LS6000IC scintillation counter (Beckman Instruments, Fullerton, CA). Protein content was measured by using a BCA assay (Pierce Chemical, Rockford, IL). ζ-peptide or a peptide containing a scrambled ζ-peptide sequence (Biosource, Camarillo, CA) were prepared as 10 mM solutions in water and stored at −20 °C. Immediately prior to use it was diluted to a final concentration of 80 μM in BSS. Aggregates of ζ-peptide or scrambled ζ-peptide were not observed in the slice culture medium during the 30-minute incubations.

4.4. *Biotinylation assay of surface expression of Na/K ATPase isoforms*

Selective biotinylation of surface proteins was modified from the method of Martin and Henley (2004). This method selectively biotinylates surface proteins throughout the thickness of the slice (Thomas-Crusells et al., 2003). Briefly, slice cultures were biotinylated in phosphate-buffered saline (PBS) containing 1.0 mg/ml EZ-Link sulfossuccinimido-biotin (sulfo-NHS-S-S-biotin; Pierce Chemical, Rockford, IL) for 40 min at 4 °C on a shaker. The free unreacted sulfo-NHS-S-S-biotin sample was quenched with PBS containing 0.1 M glycine at 4 °C followed by 2 washes with PBS. Slice cultures were treated with lysis buffer (Tris–HCl 10 mM, pH 7.5, EDTA, 10 mM, Triton X-100 1% (v/v), SDS 0.1% (w/v) and a mammalian protease inhibitor cocktail 1% (v/v) (Sigma)). The cultures were lysed by sonication and the supernatant was collected after centrifuge at 13,000 rpm for 20 min. The protein concentration from the each sample was determined using the bicinchoninic assay (BCA assay, Pierce Chemical, Rockford, IL). The sample was then divided into two aliquots containing equal amount of protein as determined from the BCA assay. One aliquot was used to assay total slice culture protein and the second aliquot was used to analyze biotinylated surface proteins. Biotinylated proteins were incubated with streptavidin-agarose beads (Immunopure streptavidin; Sigma) overnight at 4 °C. Following extensive washes with lysis buffer, proteins were eluted from the streptavidin beads by

boiling in reducing Laemmli sample buffer. The aliquots containing total protein or biotinylated surface proteins were resolved by SDS-PAGE and immunoblotted with 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 by Dr. T. Pressely, Texas Tech Univ. (Pressley, 1992)), α_3 (1:1000, Affinity Bioreagents, Golden, CO), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000, Sigma). Only the samples containing total slice culture protein had GAPDH immunoreactivity since GAPDH is not a cell surface protein. The analysis of GAPDH immunoreactivity, however, did ensure that similar amounts of protein were loaded into each gel lane. NA/K ATPase and GAPDH immunoreactive bands were scanned and quantified using Image J 1.30 software (NIH). The amount of immunoreactivity in the Na/K ATPase α subunits or GAPDH following IPC was normalized to immunoreactivity from cultures receiving mock-IPC treatment.

4.4. *Immunoprecipitation and immunoblot analysis*

Twelve slice cultures were infected with either pSinRep5-eGFP or pSinRep5-HA-PKMζ. After 6 h in a 32 °C incubator, the CA1 area was isolated from the infected cultures and homogenized in 50 μl of 50 mM Tris, pH 7.5, 5 mM DTT, and 1% (w/v) SDS. The protein concentration of each extract was determined using the BCA assay. Protein (200 μg in 500 μl) from both extracts was treated with protein G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h in working buffer (working buffer: 50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, aprotinin (17 kallikrein U/ml), 0.1 mM leupeptin) at 4 °C. The beads were pelleted by centrifugation for 20 s at 5000 g. HA monoclonal antibody (Covance HA11, 1 mg/ml, 3.5 μl) was added to the supernatant and rotated for 1 h at 4 °C. Protein G agarose beads (450 μl) was added into supernatant and rotated at 4 °C overnight. The beads were collected by spinning at 5000 g for 20 s at 4 °C. The beads were washed 3 times in working buffer. The immunoprecipitate was eluted by treatment with reducing Laemmli sample buffer (40 μl) for 10 min at 95 °C. One half of the eluate was used for immunoblot analysis.

Immunoblots were performed as described in Sacktor et al. (1993). The PKMζ antisera have been previously characterized (Sacktor et al., 1993). The p410 and p560 antisera used in this study previously recognized PKMζ in brain protein extracts but did not recognize PKMζ when the same extracts were treated with calf alkaline phosphatase (J.F.C and T.C.S., personal communication).

Acknowledgments

This work was supported by a Grant-in-Aid from the American Heart Association (to P.J.B.). This paper is dedicated to the memory of Dr. James H. Schwartz.

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