εPKC Is Required for the Induction of Tolerance by Ischemic and NMDA-Mediated Preconditioning in the Organotypic Hippocampal Slice

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Glutamate receptors and calcium have been implicated as triggering factors in the induction of tolerance by ischemic preconditioning (IPC) in the brain. However, little is known about the signal transduction pathway that ensues after the IPC induction pathway. The main goals of the present study were to determine whether NMDA induces preconditioning via a calcium pathway and promotes translocation of the protein kinase C ϵ (εPKC) isozyme and whether this PKC isozyme is key in the IPC signal transduction pathway. We corroborate here that IPC and a sublethal dose of NMDA were neuroprotective, whereas blockade of NMDA receptors during IPC diminished IPC-induced neuroprotection. Calcium chelation blocked the protection afforded by both NMDA and ischemic preconditioning significantly, suggesting a significant role of calcium. Pharmacological preconditioning with the nonselective PKC isozyme activator phorbol myristate acetate could not emulate IPC, but blockade of PKC activation with chelerythrine during IPC blocked its neuroprotection. These results suggested that there might be a dual involvement of PKC isozymes during IPC. This was corroborated when neuroprotection was blocked when we inhibited εPKC during IPC and NMDA preconditioning, and IPC neuroprotection was emulated with the activator of εPKC. The possible correlation between NMDA, Ca^{2+} , and εPKC was found when we emulated IPC with the diacylglycerol analog oleoylacetyl glycerol, suggesting an indirect pathway by which Ca^{2+} could activate the calcium-insensitive εPKC isozyme. These results demonstrated that the εPKC isozyme played a key role in both IPC- and NMDA-induced tolerance.

Key words: metabolism; *in vitro* cultures; glutamate receptors; anoxia; tolerance; signal transduction

Introduction

Ischemic preconditioning is an intrinsic adaptive condition that results in tolerance in different organs when they are subjected to mild ischemic insults before a “lethal” ischemic insult. The exact mechanism underlying ischemic preconditioning (IPC)-induced tolerance remains unclear, although a number of possible induction pathways have been investigated, including, among others, neuroactive cytokines (Nawashiro et al., 1996; Ohtsuki et al., 1996), activation of glutamate receptors (Best et al., 1996; Pringle et al., 1996), adenosine receptors, the ATP-sensitive potassium channel ($\text{K}^{+}_{\text{ATP}}$) (Heurteaux et al., 1995; Perez-Pinzon et al., 1996, 1999; Riepe et al., 1997; Blondeau et al., 2000), nitric oxide (Caggiano and Kraig, 1998; Centeno et al., 1999; Gonzalez-Zulueta et al., 2000), oxidative stress (Ohtsuki et al., 1992), hypothermia (Nishio et al., 2000), and hyperthermia (Chopp et al., 1989; Rordorff et al., 1991).

It now appears that almost any sublethal stress may render cells tolerant against lethal stress. For example, it has been established that overstimulation of glutamate receptors promotes neuronal cell death (Rothman and Olney, 1986; Albers et al., 1992; Choi, 1995; Pellegrini-Giampietro et al., 1999), whereas blocking NMDA receptors during ischemia appears to be neuro-

protective (Simon et al., 1984; Goldberg et al., 1987; Ozyurt et al., 1988; Steinberg et al., 1988; Swan and Meldrum, 1990; Rao et al., 2000; Bruno et al., 2001; Liniger et al., 2001; Nishizawa, 2001). However, MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate], an NMDA antagonist, administered during sublethal ischemia blocked the development of ischemic tolerance in gerbils (Kato et al., 1992a). *In vitro* studies also supported the role of NMDA receptors during IPC but not kainate or AMPA receptors (Bond et al., 1999; Grabb and Choi, 1999).

Subsequent increases of cytosolic calcium result from NMDA receptor activation during IPC, and this Ca^{2+} increase may promote a signal transduction cascade. It has been suggested that a putative neuroprotective pathway may involve a calcium-induced activation of PKC, because PKC translocation and phosphorylation of several membrane proteins are mediated by NMDA receptors through calcium influx (Vaccarino et al., 1991). Strong evidence exists of the involvement of PKC in the induction of IPC tolerance in the heart (Downey et al., 1994). In brain, however, different preconditioning models have shown contradictory results (Perez-Pinzon and Born, 1999; Tauskela et al., 1999; Reshef et al., 2000).

We reported recently that sublethal *in vitro* ischemia in organotypic hippocampal slice cultures protects against neuronal cell death produced by lethal *in vitro* ischemia (Xu et al., 2002). The present study, using the organotypic slice cultures, investigates three issues concerning the mechanism of IPC: (1) whether the NMDA receptors are involved in the triggering phase of IPC via

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calcium, (2) whether the PKC isozymes are involved in induction of neuroprotection, and (3) whether ϵ PKC is involved in the signaling pathway of IPC neuroprotection as shown in the heart (Souroujon and Mochly-Rosen, 1998).

Materials and Methods

Preparation of cultures

All of the protocols were approved by the University of Miami Animal Care and Use Committee. Organotypic slice cultures of the hippocampus were made according to the methods described by Bergold and Casaccia-Bonnel (1997). Sprague Dawley neonatal rats (9–11 d old) were anesthetized by intraperitoneal injections of ketamine (1.0 mg/pup). The pups were decapitated, and the hippocampi were dissected out and sliced transversely (400 μ m) on a McIlwain tissue chopper. Slices were placed in Gey's balanced salt solution (Invitrogen, San Diego, CA) supplemented with 6.5 mg/ml glucose (Sigma, St. Louis, MO) for 1 hr at 4°C. They were then transferred onto 30-mm-diameter membrane inserts (Millicell-CM; Millipore, Bedford, MA). Each insert had two slices obtained from two different pups. The inserts were placed into six-well culture trays with 1 ml of slice culture medium per well. The slice culture medium consisted of 50% minimum essential medium (Invitrogen), 25% HBSS (Invitrogen), and 25% heat-inactivated horse serum (Invitrogen) supplemented with 6.5 mg/ml glucose and glutamine (1 mM). The cultures were maintained at 36°C in an incubator (CF autoflow; NuAire, Plymouth, MN) with an atmosphere of 100% humidity and 5% CO₂. The slice culture medium was changed twice per week. Slices were kept in culture for 14–15 d before experiments.

Oxygen–glucose deprivation

We defined the ischemia and preconditioning protocols in a previous study (Xu et al., 2002). The organotypic cultures have been used to study mechanisms underlying neuronal death induced by hypoxia–aglycemia (Pringle et al., 1997a) and excitotoxins (Sakaguchi et al., 1997). To model ischemic events, organotypic cultures were exposed to oxygen–glucose deprivation (OGD) using an anaerobic chamber. Cimarosti et al. (2001) and Laake et al. (1999) suggested the suitability of this model for the study of ischemic lesions and neuroprotective drugs. They observed that the lesions induced by OGD were similar to those shown by animals submitted to transient cerebral ischemia. We corroborated recently that, like global cerebral ischemia, OGD promotes selective cell death in the CA1 subregion of the hippocampus (Xu et al., 2002). The slices were washed three times with glucose-free HBSS, pH 7.4, containing the following (in mM): 1.26 CaCl₂ · 2 H₂O, 5.37 KCl, 0.44 KH₂PO₄, 0.49 MgCl₂, 0.41 MgSO₄ · 7 H₂O, 136.9 NaCl, 4.17 NaHCO₃, 0.34 Na₂HPO₄ · 7 H₂O, and 15 sucrose (all from Sigma). The slices were then placed into an airtight chamber, and 95% N₂–5% CO₂ gas, preheated to 36°C, was blown through the chamber for 5 min (4 l/min) to achieve anoxic conditions. After 5 min, the chamber was sealed and placed in the incubator, in which the temperature was maintained at 36°C, for 10 min (for a total of 15 min for preconditioning) or 35 min (for a total of 40 min for ischemic insult). After OGD, the slices were placed back into the incubator in normal culture medium.

Assessment of cell death by image analysis using propidium iodide

Propidium iodide (PI) (Sigma) was used for identifying dead cells. Before experimental treatment (OGD or preconditioning), slices were incubated in culture medium supplemented with 2 μ g/ml PI for 1 hr and then removed and replaced by regular media. The slices were studied using an inverted fluorescence microscope (Olympus IX 50; Olympus Optical, Tokyo, Japan). Fluorescence digital pictures were taken using a SPOT charge-coupled device (CCD) camera (Diagnostic Instruments, Sterling Heights, MI) and SPOT advanced software. The bright-field (0.254 sec exposure time) and PI (1.902 sec exposure time, red filter) images of cultured slices were taken before the experiment, followed by preconditioning treatment. After preconditioning, the slices were reperused for 48 hr followed by 40 min of “test” ischemia. The PI fluorescence was taken 24 hr after test ischemic insult, and then NMDA was added onto

the slices (100 μ M) (Sigma) for 1 hr. The last image was taken 24 hr after NMDA treatment. The bright-field image was used to align the slice in the same position during subsequent measurements. The intensity of PI fluorescence in the CA1 subfield of the hippocampal slices was used as an index of cell death.

For quantification purposes, a set of fluorescence images taken for one slice was opened in the following sequence: (1) 24 hr after NMDA treatment, (2) 24 hr after test ischemia, and (3) obtained at the onset of the experiment. They were then stacked using Scion Image software (Windows version) (Scion, Frederick, MD). The region of interest (ROI) was selected from the final image depicting total neuronal cell death, which remained constant for a particular slice. The ROI was subsequently superimposed on the fluorescence images taken 24 hr after test ischemia and at the beginning of the experiment. Relative cell death was calculated from each ROI as follows: relative percentage cell death = $(F_{\text{exp}} - F_{\text{min}}) / (F_{\text{max}} - F_{\text{min}}) \times 100$, where F_{exp} is the fluorescence of the test condition, F_{max} is maximum fluorescence (100 μ M NMDA treatment for 1 hr), and F_{min} is background fluorescence (before preconditioning or OGD). In all groups, experiments (except Western blot analysis) were terminated by superfusing slices with an overdose of NMDA 24 hr after the end of the experiments to determine total number of cells using the PI technique (F_{max} above).

Cell fractionation and Western blot analysis

To determine whether the ϵ PKC isozyme was translocated after IPC in organotypic slices, we used Western blot analysis. This method is adapted from one described previously (Mackay and Mochly-Rosen, 2001). Hippocampal organotypic slices were frozen in liquid nitrogen at different time intervals and stored at –80°C until the analysis. At the time of Western blot analysis, the hippocampal organotypic slice cultures were separated from the supporting membrane. For one sample, ~32 slices were pooled together. The separated pooled slices were washed twice with cold PBS. Slices were pelleted at 1000 $\times$ g, and PBS was removed. The pellet was resuspended in 400 μ l of cell lysis buffer (4 mM ATP, 100 mM KCl, 10 mM imidazole, 2 mM EGTA, 1 mM MgCl₂, 20% glycerol, 0.05% Triton X-100, 17 μ g/ml PMSF, 20 μ g/ml soybean trypsin inhibitor, 25 μ g/ml leupeptin, and 25 μ g/ml aprotinin). The suspended slices were homogenized using an all-glass homogenizer. The homogenate was then centrifuged at 4°C at 1000 $\times$ g for 10 min. The supernatant (soluble fraction) was carefully taken off and recentrifuged at 16,000 $\times$ g for 15 min to exclude any contaminating pellet material. The initial pellet was resuspended in 250 μ l of cell lysis buffer containing 1% Triton X-100 and was extracted on ice for 60 min. Samples were centrifuged at 16,000 $\times$ g for 15 min. The supernatant is the particulate fraction. Both fractions were analyzed for protein content by the Bradford assay, and 40 μ g of protein from each fraction was separated by 12% SDS-PAGE. One soluble and one particulate fraction of each group were run on the same gel and analyzed at the same time. Each group consisted of three samples. Protein was transferred to Immobilon-P (Millipore) membrane and incubated with the primary antibody anti- ϵ PKC (Calbiochem, La Jolla, CA) (1:500). In the case of phorbol myristate acetate (PMA), the translocation of two additional isozymes of PKC (i.e., δ PKC and γ PKC) was also determined by blotting the membrane with primary antibody anti- δ PKC (1:1000) and anti- γ PKC (1:500) (Calbiochem). Immunoreactivity was detected using enhanced chemiluminescence (ECL Western blotting detection kit; Amersham Biosciences, Little Chalfont, UK). Autoradiographic images were digitized at eight-bit precision by means of a CCD-based camera (8–12 bits) (Xillix Technologies, Vancouver, British Columbia, Canada) equipped with a 55 mm Micro-Nikkor lens (Nikon, Tokyo, Japan). The camera was interfaced to an advanced image-analysis system (MCID model M2; Imaging Research, St. Catharines, Ontario, Canada). The digitized immunoblots were subjected to densitometric analysis using MCID software.

Experimental design

The organotypic slices were divided into five major groups (Fig. 1), as follows.

Figure 1. Description of different experimental groups. *Group-I*, Sham; *Group-II*, ischemia with any other treatment; *Group-III*, preconditioning experiments; *Group-IV*, pharmacological preconditioning (PPC) with NMDA, PMA, ψ eRACK, and OAG; *Group-V*, experimental design of pharmacological blockade preconditioning (PC) using MK-801, BAPTA AM, BAPTA, chelerythrine chloride, and ϵ V1-2.

Group I: sham

These slices were incubated for 15 min with HBSS supplied with equimolar concentrations of glucose instead of sucrose (sham OGD). Subsequently, slices were transferred back to regular media. After 48 hr, the slices were exposed for 40 min of sham OGD.

Group II: ischemia

Slices were exposed to sham IPC (group I), and, 48 hr later, test ischemia (40 min of OGD) was induced.

Group III: IPC

Slices were exposed to IPC (15 min of OGD) 48 hr before test ischemia.

Group IV: pharmacological emulation of IPC

NMDA treatment. NMDA at 1 μ M (Sigma) was administered onto slices for 15, 30, and 60 min. Subsequently, the drug was washed out, and, after 48 hr, test ischemia was induced.

PMA-induced translocation of PKCs. PMA at 1 μ M (Sigma) was administered onto slices for 30 min. Subsequently, the drug was washed out, and, after 48 hr, test ischemia was induced.

Pharmacological preconditioning using ψ eRACK (a ϵ PKC agonist). In this group, ψ eRACK (receptor for activated C kinase) was administered onto slices for 15 min using different concentrations (0.02, 0.2, 0.6, and 1 μ M). The drug was washed out, and, after 48 hr, test ischemia was induced.

Pharmacological preconditioning with oleoylacetate glycerol. Oleoylacetate glycerol (OAG) (10 nM; Sigma), an analog of diacylglycerol (DAG), was administered onto slices for 15 min. Subsequently, the drug was washed out, and, after 48 hr, test ischemia was induced.

Group V: pharmacological blockade of IPC

Blocking NMDA receptors with MK-801 maleate. Slices were bathed with MK-801 (10 μ M; Sigma) either during IPC alone or for 30 min before and during IPC (to ensure that the NMDA receptor was blocked during IPC). The drug was washed out at the end of IPC, and, after 48 hr, test ischemia was induced.

Treatment with calcium chelators. Slices were divided into three subgroups: (1) slices were bathed with the cytoplasmic calcium chelator BAPTA AM (100 μ M; Sigma) for 45 min and during IPC; (2) slices were treated in a similar manner as in 1 but using the extracellular BAPTA (10 μ M; Sigma) instead of the cytoplasmic calcium chelator; and (3) slices were bathed with both intracellular and cytoplasmic calcium chelators BAPTA AM for 45 min and during IPC. Subsequently, the drug was washed out, and, after 48 hr, test ischemia was induced.

Calcium chelators and NMDA preconditioning. In this group, slices were bathed with both BAPTA AM (100 μ M) and BAPTA (10 μ M) for 45 min. Subsequently, slices were bathed with 1 μ M NMDA in the presence of both calcium chelators for 1 hr. The drugs were washed out, and, after 48 hr, test ischemia was induced.

Blocking of PKC activation by chelerythrine chloride. Slices were bathed

Figure 2. Typical images of hippocampal slice cultures. *A, C*, Bright-field images of ischemic and IPC groups, respectively. *B, D*, PI fluorescence images showing neuronal death in the ischemic and IPC groups taken 24 hr after the test ischemic insult. Scale bars, 2.25 mm.

with chelerythrine (10 μ M) during IPC. The drug was washed out at the end of IPC, and, after 48 hr, test ischemia was induced.

Blocking of ϵ PKC isozyme activation with ϵ V1-2. Slices were bathed with ϵ V1-2 (0.02, 0.2, 0.6, and 1 μ M) during IPC. The drug was washed out at the end of IPC, and, after 48 hr, test ischemia was induced.

NMDA preconditioning along with blockade of ϵ PKC. The slices were preconditioned for 60 min with NMDA (1 μ M) along with ϵ V1-2 (0.02 μ M). The drug was washed out at the end of NMDA preconditioning, and, after 48 hr, test ischemia was induced.

OAG preconditioning along with blockade of ϵ PKC. The slices were bathed for 15 min with OAG (10 nM) in the presence of ϵ V1-2 (0.02 μ M).

To provide better controls in these experiments, all six-well plates used contained at least one well for the sham, IPC, and ischemic groups. In addition, for statistical purposes, each insert had two slices obtained from two different pups. Thus, $n = 1$ (one slice) represents a different animal.

Peptide preparation and delivery

The ϵ V1-2 [ϵ PKC inhibitor, amino acids 14–21 (EAVSLKPT)] (Gray et al., 1997) and ψ eRACK [ϵ PKC activator, amino acids 85–92 (HDAPIGYD)] (Dorn et al., 1999) peptides were synthesized at Stanford's Protein and Nucleic Acid facility and conjugated to Tat [carrier peptide, amino acids 47–57 (YGRKKRRQRRR)] (Schwarze et al., 1999) via a cysteine–cysteine bond at their N termini, as described previously (Chen et al., 2001).

In the past, peptides have been delivered initially into the cells by transient permeabilization of the cells or by microinjection. However, an alternative to this method was used recently in which the peptides are conjugated via disulfide bonds to short cell-permeable peptides (Souroujon and Mochly-Rosen, 1998). Once the peptide crosses the cell membrane, the disulfide bond breaks, the peptide cargo is trapped in the cell, and the peptide carrier can be washed out. These studies already demonstrated that the peptides have highly selective effects; they inhibit or induce translocation of their corresponding isozymes but not that of others (Gray et al., 1997; Dorn et al., 1999). In addition, at concentrations up to ~ 10 μ M, the peptides or the carrier have no toxic effects on the cell. Finally, peptides are introduced to all of the cells at a final concentration that does not exceed 10% of that applied (Souroujon and Mochly-Rosen, 1998).

Statistical analysis

The results are expressed as mean $\pm$ SD. Statistical significance was determined with an ANOVA test, followed by a Bonferroni's *post hoc* test.

Results

In the present study, we confirm our previous findings in which IPC conferred protection against test ischemia in hippocampal

Figure 3. PI fluorescence values measured 24 hr after test ischemia in seven experimental groups: (1) sham, (2) ischemia, (3) IPC, (4) NMDA-induced preconditioning, (5) IPC with MK-801, (6) calcium chelators with NMDA preconditioning, and (7) calcium chelators with IPC. Significance compared with sham ($^Ap < 0.01$; $^ap < 0.001$), ischemia ($^bp < 0.01$; $^bp < 0.001$), or IPC ($^cp < 0.01$; $^cp < 0.001$).

organotypic slices (Fig. 2). The hippocampal organotypic slices that were preconditioned by 15 min of sublethal ischemia 48 hr before 40 min of test ischemia showed $34.13 \pm 19.25\%$ ($n = 20$) of PI fluorescence in the CA1 region of the hippocampus compared with sham ($5.45 \pm 2.85\%$; $n = 8$; $p < 0.01$). In contrast, 40 min of test ischemia led to $59.64 \pm 20.82\%$ ($n = 24$) of PI fluorescence (Fig. 3). Thus, IPC significantly lowered cell death by $\sim 42\%$ ($p < 0.01$).

One of the suggested mechanisms by which IPC promotes ischemic tolerance in other animal models of cerebral ischemia is by activation of the NMDA receptor. We tested this hypothesis in the organotypic cultures by bathing slices with NMDA ($1 \mu\text{M}$) alone for 15 min at 48 hr before the test ischemic insult, which resulted in $42.67 \pm 17.05\%$ ($n = 16$) of PI fluorescence. This level of PI fluorescence was significantly lower than in test ischemia ($p < 0.05$). Increasing the time of NMDA exposure to 30 or 60 min further reduced percentage PI fluorescence by $\sim 34\%$ ($n = 8$) and 69% ($n = 5$), respectively, compared with test ischemia ($p < 0.001$).

To further characterize the role of the NMDA receptor during IPC, the NMDA receptor was blocked (MK-801, $10 \mu\text{M}$) during the preconditioning insult. This treatment significantly eliminated IPC-induced neuroprotection. The PI fluorescence values were $87.25 \pm 12.43\%$ ($n = 7$) and $59.64 \pm 20.82\%$ for MK-801-treated and ischemic groups, respectively. Similar results were obtained when the NMDA receptor was blocked before and during the IPC insult (Fig. 3). In control experiments, when MK-801 was administered to slices without any other treatment, percentage PI fluorescence was $55.43 \pm 13.65\%$ ($n = 5$) of PI fluorescence, similar to that of ischemia.

A suggested pathway by which NMDA promotes ischemic tolerance is by influx of Ca^{2+} into the cytoplasm. To determine whether influx of calcium plays a role in preconditioning-induced neuroprotection in the organotypic slice, we removed both extracellular and cytoplasmic calcium using calcium chelators. Both extracellular and intracellular calcium chelators (BAPTA and BAPTA-AM) administered before and during IPC blocked the protection afforded by IPC ($p < 0.001$) (Fig. 3). Furthermore, chelation of either extracellular or cytoplasmic calcium alone before and during IPC followed by OGD 48 hr later resulted in $62.09 \pm 13.65\%$ ($n = 8$) or $75.01 \pm 10.43\%$ ($n = 8$) of PI fluorescence values, respectively, which were significantly higher than in the IPC group ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 3). Chelation of either extracellular or cytoplasmic calcium alone followed by OGD 48 hr later resulted in $54.84 \pm 5.96\%$ ($n = 4$) and $60.68 \pm 7.10\%$ ($n = 4$) of PI fluorescence, respectively.

Figure 4. PI fluorescence values measured 24 hr after test ischemia in five experimental groups: (1) sham, (2) ischemia, (3) IPC, (4) PMA, and (5) chelerythrine (CHL) plus IPC. Significance compared with sham ($^Ap < 0.01$; $^ap < 0.001$), ischemia ($^bp < 0.01$; $^bp < 0.001$), or IPC ($^cp < 0.01$; $^cp < 0.001$).

In control experiments, extracellular or cytoplasmic calcium chelation without any OGD insult resulted in $3.24 \pm 1.13\%$ ($n = 5$) and $4.86 \pm 0.39\%$ ($n = 5$) of PI fluorescence, respectively. No significant increases in PI fluorescence were observed with these treatments compared with sham values.

To examine whether NMDA-induced preconditioning also occurred via calcium influx, we chelated calcium during NMDA preconditioning. The ischemic tolerance afforded by NMDA pretreatment was abolished by bathing hippocampal slices with calcium chelators before and during NMDA-induced preconditioning ($p < 0.001$) (Fig. 3). The PI fluorescence values of calcium-chelated NMDA-preconditioned and ischemic groups were $72.14 \pm 9.71\%$ ($n = 11$) and $59.64 \pm 20.85\%$, respectively (Fig. 3).

An increase in cytosolic calcium by itself can directly and indirectly activate PKC isozymes (Downey et al., 1994). Thus, we examined whether hippocampal organotypic slices could be preconditioned by PMA ($1 \mu\text{M}$), a nonselective PKC isozyme translocator. The percentage cell death (PI fluorescence) in the CA1 region of the hippocampus was $87.34 \pm 15.43\%$ ($n = 10$), $67.66 \pm 30.77\%$ ($n = 7$), $28.90 \pm 17.35\%$ ($n = 10$), and $5.45 \pm 2.85\%$ for the PMA-treated, ischemia, IPC, and sham groups, respectively. The ischemia and PMA-treated groups were significantly higher than both the sham and IPC groups ($p < 0.001$) (Fig. 4), demonstrating that this nonselective PKC translocation did not emulate IPC. Next, PKC activity was blocked with chelerythrine (nonselective inhibitor of PKC) ($10 \mu\text{M}$) during the preconditioning insult. IPC-induced neuroprotection was partially reduced by this treatment (Fig. 4). The PI fluorescence values were $49.03 \pm 11.08\%$ ($n = 14$) and $67.66 \pm 30.77\%$ for the chelerythrine-treated and ischemia groups, respectively (Fig. 4).

The PMA and chelerythrine results were in contradiction to each other. A plausible explanation was that different PKC isozymes played different roles during IPC. Thus, we explored the possible role of ϵ PKC isozyme that is key for ischemic tolerance in the heart (Souroujon and Mochly-Rosen, 1998). Slices were bathed with the specific blocker of ϵ PKC (ϵ V1-2 peptide at different concentrations, i.e., 0.02, 0.2, 0.6, and $1 \mu\text{M}$) during IPC. This treatment significantly blocked IPC-induced neuronal protection at all of the concentrations used ($p < 0.01$). The PI fluo-

Figure 5. PI fluorescence values measured 24 hr after test ischemia in five experimental groups: (1) sham, (2) ischemia, (3) IPC, (4) ϵ V1-2 (0.02 μ M) treatment during IPC, and (5) preconditioning with ϵ PKC activator peptide $\psi\epsilon$ RACK (0.02 μ M). Significance compared with sham (^a $p < 0.01$; ^a $p < 0.001$), ischemia (^b $p < 0.01$; ^b $p < 0.001$), or IPC (^c $p < 0.01$; ^c $p < 0.001$).

rescence was $64.85 \pm 15.46\%$ ($n = 8$) with ϵ V1-2 peptide (0.02 μ M) treatment compared with IPC ($28.90 \pm 17.35\%$; $n = 10$) (Fig. 5).

To further corroborate the role of ϵ PKC, we emulated IPC with the $\psi\epsilon$ RACK peptide (agonist of ϵ PKC) for 15 min at different concentrations (i.e., 0.02, 0.2, 0.6, and 1 μ M). All of these treatments resulted in significant neuroprotection compared with the ischemia group ($p < 0.001$). In control experiments, the Tat carrier peptide administered for 15 min at 48 hr before OGD at different concentrations did not promote any significant protection compared with the ischemia group. The PI fluorescence values of Tat peptide treatment at 0.02, 0.2, 0.6, and 1 μ M concentrations were $61.44 \pm 11.15\%$ ($n = 5$), $63.24 \pm 8.38\%$ ($n = 4$), $65.48 \pm 5.24\%$ ($n = 4$), and $60.84 \pm 7.76\%$ ($n = 6$), respectively.

To determine whether ϵ PKC was also involved during NMDA-induced preconditioning, the ϵ PKC inhibitor (ϵ V1-2 peptide, 0.02 μ M) was administered during the NMDA preconditioning treatment. This treatment resulted in significant inhibition of protection ($p < 0.001$) compared with IPC and NMDA preconditioning. The PI fluorescence values of NMDA preconditioning along with the ϵ V1-2 peptide group and IPC- and NMDA-preconditioned groups were $82.09 \pm 7.91\%$ ($n = 7$), $28.90 \pm 17.35\%$ ($n = 9$), and $21.44 \pm 10.97\%$ ($n = 6$), respectively (Fig. 6).

Activation of PKC is associated with translocation from the soluble to the particulate fraction of cells (Kraft and Anderson, 1983; Dorn et al., 1999). Therefore, to confirm that IPC translocates ϵ PKC isozyme in hippocampal organotypic slices, the effect of IPC on subcellular localization of ϵ PKC isozyme was determined by cell fractionation and Western blot analysis. The subcellular fractionation method was used to separate proteins into a soluble and a particulate fraction. ϵ PKC isozyme translocated in response to PMA (1 mM, 30 min) from the soluble to the particulate fraction (Fig. 7C). Relative to the control group, ϵ PKC decreased in the soluble fraction by 43%, with a corresponding increase in the particulate fraction. We also confirmed that incubation with PMA (1 mM, 30 min) translocated other PKC isozymes, such as δ PKC and γ PKC isozymes. We found that the δ PKC and γ PKC isozymes also translocated from the soluble to the particulate fraction. δ PKC and γ PKC isozymes decreased in the soluble fraction by 25 and 35%, respectively, with a corresponding increase in the particulate fraction (Fig. 7C). The ϵ PKC isozyme translocated from the soluble to the particulate fraction by 7, 28 ($p < 0.05$), 24, and 8% after 15, 30, 60, and 180 min of

Figure 6. PI fluorescence values measured 24 hr after test ischemia in seven experimental groups: (1) sham, (2) ischemia, (3) IPC, (4) NMDA (1 μ M) preconditioning for 60 min, (5) NMDA preconditioning with ϵ V1-2 (0.02 μ M), (6) OAG treatment for 15 min, and (7) OAG with ϵ V1-2. Significance compared with sham (^a $p < 0.01$; ^a $p < 0.001$), ischemia (^b $p < 0.01$; ^b $p < 0.001$), IPC (^c $p < 0.01$; ^c $p < 0.001$), or NMDA (^d $p < 0.01$).

Figure 7. A, Immunoblot of ϵ PKC isozyme in control baseline (untreated) and 15 min, 30 min, 1 hr, and 3 hr after IPC. Organotypic slices underwent subcellular fractionation and Western blot analysis, probed with antibodies detecting the ϵ PKC isozyme. The soluble and particulate fractions are shown. One experiment representative of three is shown. B, Immunoblots as represented in A were subjected to densitometric analysis, and percentage translocation from soluble to particulate fraction was determined. Results are expressed as percentage of total ϵ PKC in soluble fraction for each sample, which was then expressed as percentage of control. For a decrease in soluble fraction, there is a corresponding increase in particulate (data not shown). Results are expressed as mean $\pm$ SD of three experiments. ^a $p < 0.05$ versus control. C, Immunoblots blotted with anti- ϵ PKC, anti- δ PKC, and anti- γ PKC of soluble and particulate fraction of organotypic slices harvested 30 min after exposure to PMA were subjected to densitometric analysis, and percentage translocation from soluble to particulate fraction was determined. Results are expressed as percentage of total ϵ PKC, δ PKC, and γ PKC in soluble fraction after PMA treatment, which was then expressed as percentage of control. For a decrease in soluble fraction, there is a corresponding increase in particulate (data not shown). Results are expressed as mean $\pm$ SD of three experiments.

reperfusion after IPC, respectively, with a corresponding increase in the particulate fraction. The ϵ PKC isozyme concentration remained the same in the soluble and particulate fractions after 60 min of reperfusion when IPC was performed in presence of chelerythrine. Administration of the specific ϵ PKC antagonist ϵ V1-2 during IPC inhibited translocation of ϵ PKC (Fig. 7).

ϵ PKC belongs to the Ca^{2+} -independent type of PKC isozymes, and NMDA-induced tolerance was blocked by the analog of ϵ PKC; thus, we tested the hypothesis that NMDA plus ϵ PKC was indirectly linked by DAG, which is produced by Ca^{2+} -stimulated phospholipase C activation (Nishizuka, 1992). Slices were preconditioned with the DAG analog OAG. OAG pretreatment provided protection from ischemia similar to that afforded by IPC. PI fluorescence values for OAG and IPC were $12.25 \pm 5.77\%$ ($n = 5$) and $28.90 \pm 17.35\%$ ($n = 9$), respectively. The ϵ PKC inhibitor ϵ V1-2 blocked protection afforded by OAG. The PI fluorescence was $67.99 \pm 22.11\%$ ($n = 5$) when OAG and ϵ V1-2 were administered together 48 hr before test ischemia.

Discussion

In this study, we demonstrated that IPC 48 hr before the test ischemic insult induced neuroprotection in the CA1 region of the hippocampal organotypic slices. We also showed that preconditioning by activation of sublethal NMDA receptor was highly neuroprotective, whereas antagonizing the NMDA receptor by MK-801 blocked the protection. We further demonstrated that calcium chelation prevented both IPC- and NMDA-mediated neuroprotection, suggesting that increased cytosolic calcium plays a key role in the induction of IPC and NMDA neuroprotection. Pharmacological preconditioning with the PKC activator PMA could not emulate IPC neuroprotection, whereas chelerythrine blocked the preconditioning effect, suggesting that different PKC isozymes play different roles. This was corroborated by our findings that we could emulate IPC neuroprotection with the agonist of ϵ PKC (ψ ϵ RACK) and block it with its antagonist ϵ V1-2. This result demonstrates that the preconditioning-induced neuroprotective effect of the ψ ϵ RACK is caused by selective activation of ϵ PKC. In addition, we found that pharmacological preconditioning using the DAG analog OAG also promoted neuroprotection, suggesting a link between the calcium pathways with the Ca^{2+} -independent PKC isozyme (ϵ PKC) (Nishizuka, 1992).

A number of studies have shown that glutamate and glutamate receptors play a central role in acute neurodegeneration after cerebral ischemia (Rothman and Olney, 1986; Albers et al., 1992; Choi, 1995; Pellegrini-Giampietro et al., 1999). However, mild (nonlethal) depolarization of brain cells before ischemia, such as that produced by spreading cortical depression, promoted protection against such insults *in vivo* and *in vitro* (Kobayashi et al., 1995; Matsushima et al., 1996; Kawahara et al., 1997; Plumier et al., 1997). These studies suggested the possible role of glutamate receptors in the induction phase of IPC. This hypothesis was supported by studies *in vivo* in which inhibition of NMDA receptors with MK-801 during IPC resulted in blockade of ischemic tolerance by IPC in gerbils (Kato et al., 1992b) and *in vitro* (Pringle et al., 1997b). Gonzalez-Zulueta et al. (2000) showed that, during OGD preconditioning in neuronal cell cultures, the signaling cascade was initiated by activation of the NMDA receptors, calcium influx, and production of nitric oxide in an RAS–extracellular signal-regulated protein kinase (ERK)-dependent manner, leading to the development of neuronal tolerance to ischemia.

The results from the present study support the hypothesis that implicates NMDA receptors mediating IPC. A previous study by Pringle et al. (1997b) in organotypic slice culture demonstrated that administration with $1 \mu\text{M}$ NMDA for 3 hr significantly reduced the neuronal damage produced by either 45 or 60 min of ischemia. In contrast to that study, our data show robust neuroprotection when slices were exposed to NMDA for only 1 hr.

However, the time of ischemia in the present study was only 40 min. The role of the NMDA receptor in inducing tolerance is also supported by our findings that blockade of the NMDA receptor before and during IPC significantly ameliorates IPC- and NMDA-induced neuroprotection. Because the longest duration of NMDA exposure (60 min) was more efficacious than that observed with 15 and 30 min of NMDA preconditioning, we can surmise that continuous stimulation of the NMDA receptor was necessary to build a critical level of intracellular calcium to promote tolerance.

The hypothesis that calcium plays a key role in the induction of tolerance by IPC- and NMDA-induced preconditioning is supported by our findings that removal of cytoplasmic and extracellular calcium before and during IPC blocks neuroprotection. Furthermore, the extracellular and intracellular calcium chelation was also efficacious in blocking the induction of tolerance by NMDA preconditioning. We conjecture that a critical level of calcium is necessary to stimulate calcium-dependent enzymes, including PKC (Huang et al., 1999; Katsura et al., 1999) and nitric oxide synthase (Gonzalez-Zulueta et al., 2000; Nandagopal et al., 2001), that will lead to a signal transduction pathway that could ultimately play a role in neuroprotection.

Recently, the role of PKC during IPC in brain was investigated. Tauskela et al. (1999) found that neuroprotection by IPC was maintained despite pharmacological inhibition of PKC, suggesting that PKC does not play a role during IPC. In contrast, Reshef et al. (2000) showed that the preconditioning-induced neuroprotection initiated by adenosine receptors was mediated through activation of PKC in primary rat neuronal cultures. Because results from these studies contradicted each other, we surmised that differences between these studies might be a result of the different roles of PKC isozymes. In both heart (Brooks and Hearse, 1996) and brain (Savolainen et al., 1995; Sieber et al., 1998; Tan et al., 1998; Koponen et al., 2000), the role of PKC is controversial, probably because of the use of non-isozyme-specific tools. We also found contradictory data using the general PKC activator PMA and non-isozyme-specific inhibitor of PKC chelerythrine in organotypic slice culture. In the present study, we also show that the PMA translocated δ PKC and γ PKC along with translocation of ϵ PKC. The lack of protection with PMA (emulating IPC) and the inhibition of IPC produced by chelerythrine may be because of activation of more than one PKC isozyme that may have different roles in neurons. It has been reported that δ PKC and ϵ PKC isozymes have opposite effects to ischemia in the heart, in which δ PKC promoted pathology, whereas ϵ PKC was protective (Chen et al., 2001). It is likely that, in brain as well, different PKC isozymes have opposite effects. For example, activation of δ PKC exacerbates damage during ischemia in three different models: isolated myocytes, intact heart *ex vivo*, and intact heart *in vivo* (Chen et al., 2001). Furthermore, overexpression of a catalytic fragment of δ PKC in PC12 cell leads to apoptosis (Bharti et al., 1998; Anantharam et al., 2002). γ PKC is a brain-specific PKC isozyme, and its translocation has been correlated with ischemic cell death, whereas its downregulation by ischemic preconditioning resulted in neuroprotection, thus suggesting that this isozyme may play a negative role after cerebral ischemia (Shamlou and Wieloch, 1999; Katsura et al., 2001). Thus, it is possible that there might be a dual involvement of PKC isozymes after PMA superfusion.

We demonstrate in the present study that, like in cardiac preconditioning, ϵ PKC is a key player in the induction of IPC neuroprotection. To the best of our knowledge, these results are the

first evidence that a specific PKC isozyme plays a key role in the induction of tolerance by IPC in the brain.

Because NMDA-induced preconditioning neuroprotection was also blocked by the ϵ PKC blocker ϵ V1-2, activation of this isozyme by calcium had to occur indirectly. In fact, a transient increase in $[Ca^{2+}]_i$ activates phospholipase C-induced release of 1,4,5-inositol triphosphate and DAG, which can stimulate Ca^{2+} -independent isozymes of PKC, such as γ PKC and ϵ PKC (Nishizuka, 1992). In the heart, transient increase in calcium activates diacylglycerol (Wang and Ashraf, 1999), which subsequently activates both classic (α , β I, β II, and γ) and novel (δ , ϵ , η , and θ) PKC isozymes (Guang et al., 1999). Our data demonstrate that we could emulate preconditioning neuroprotection with the DAG analog OAG and that this protection could be blocked by addition of the ϵ PKC antagonist, indicating that ϵ PKC is activated via DAG.

The signal transduction pathway that ensues after ϵ PKC activation remains undefined. Other candidates in the signal transduction pathway after IPC include the RAS–ERK pathway (Gonzalez-Zulueta et al., 2000). It remains to be determined whether ϵ PKC and the RAS–ERK pathway are linked together or are two different pathways that promote tolerance against cerebral ischemia. Finally, the similarity in the signal transduction pathways in the heart and brain is a significant finding, because it is possible that a novel therapeutic approach could emerge that would protect both organs from global ischemic conditions.

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