Role and mechanism of PKC in ischemic preconditioning of pig skeletal muscle against infarction

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The phenomenon of ischemic preconditioning (IPC) of myocardium against infarction was first recognized by Murry et al. (35). Despite intensive investigation by numerous laboratories to elucidate the cellular mechanism of IPC against myocardial infarction, the mediator, signal-transduction pathway, and effector mechanism responsible for the IPC phenomenon remain unresolved, and they seem to vary with species of laboratory animals. Specifically, there is evidence to indicate that adenosine (A1), mediated by A1 receptors, initiates the protective effect of IPC in the rabbit, dog, and pig (14, 28, 53, 56, 58), but not the rat (24). Subsequently, Auchampach and Gross (3) demonstrated a central role for ATP-sensitive K+ (KATP) channels in IPC against myocardial infarction in the dog. There is also evidence in the dog to indicate a link between A1 receptors and KATP channels in myocardial IPC (3, 14). So far, the evidence supporting an important role for KATP channels is unequivocal in the dog and pig (3,11,14,34,48), but equivocal in small laboratory animals such as the rat (13,30,45) and rabbit (54,55). On the other hand, Downey and coworkers (29,32,61) demonstrated in the rabbit that inhibition of protein kinase C (PKC) with staurosporin, polymyxin B (Poly B), or chelerythrine (Chel) (29,32) and disruption of cytoskeletal microtubules with colchicine (32) during sustained ischemia abolished the cardioprotective effect of IPC against infarction, whereas activation of PKC by preischemic treatment with 4b-phorbol 12-myristate 13-acetate (PMA) or 1-oleoyl-2-acetyl glycerol (OAG) mimicked the protective effect of IPC (61). These observations were taken to indicate that PKC membrane translocation and activation are essential during sustained ischemia to realize the protective effect of IPC (9). There is evidence from several laboratories to indicate that the anti-infarction effect of IPC is also dependent on PKC activation (45). Furthermore, PKC activation is necessary for the development of myocardial IPC in the rabbit (46). More recently, Liu et al. (43) observed that brief periods of ischemia and reperfusion in rabbit hearts caused cytosol-to-particulate translocation of PKCε and PKCγ isozymes. Further study from this group revealed that PKCε is the specific isoform responsible for the development of myocardial IPC in the rabbit (46).
(27) observed that the protective effect from IPC or the PKC activator OAG in rabbit cardiomyocytes was abolished by a highly selective PKCε peptide inhibitor, but not by peptide inhibitors selective for PKCβ, PKCδ, or PKCγ. These findings strongly support the PKC hypothesis and further suggest that the PKCε isoform is responsible for the cardioprotection of IPC, at least in rabbit cardiomyocytes.

Our particular interest is in IPC of skeletal muscle against infarction. So far, we have demonstrated in vivo that A1 receptors and KATP channels are involved in IPC of noncontracting pig latissimus dorsi (LD) muscle against infarction (38, 40). The objective of the present project is twofold. We planned to use the same pig LD muscle model to investigate 1) the role and mechanism of PKC in the anti-infarction effect of IPC and 2) the possibility of a link between PKC and KATP channels in the infarct-limiting mechanism of IPC.

**MATERIALS AND METHODS**

**Experimental Surgery**

The experimental protocols for use of pigs in this project were approved by the Animal Care Committee of The Hospital for Sick Children and were in compliance with the guidelines of the Canadian Council of Animal Care.

**Anesthesia.** Castrated Yorkshire pigs (19.0 ± 1.4 kg) were used. Pigs were anesthetized during surgery, IPC, and sustained muscle ischemia and biopsy. Surgical anesthesia was induced with intramuscular ketamine (25 mg/kg) and intravenous pentobarbital sodium (20–25 mg/kg) and maintained by intravenous infusion of isotonic saline (2 ml/min) containing pentobarbital sodium (1 mg · kg⁻¹ · min⁻¹). The pig was intubated and mechanically ventilated (tidal volume 15 ml/kg) with O₂ and N₂O (1:1 volume). The pig was kept warm by intravenous pentobarbital sodium (20–25 mg/kg) and maintained muscle ischemia and biopsy. Anesthesia was withdrawn. The pig was allowed to wake up and was returned to the animal holding room with controlled light (0700–1900) and temperature (22°C). The pig was killed 24 h later with an overdose of pentobarbital sodium, and the LD muscle flaps were immediately excised and cut transversely into 12 cm segments for assessment of muscle infarction, using the nitroblue tetrazolium dye staining technique previously described for this muscle flap model (31–33).

**Acute ischemic preconditioning.** A complete IPC procedure for pig LD muscle flaps consisted of three cycles of 10 min of ischemia and 10 min of reperfusion (42).

**Drug Delivery**

Saline (20 ml) or saline containing dissolved drugs was infused into the LD muscle flap through a 23-gauge catheter that was placed in a small side branch of the axillary artery that supplied blood to the thoracodorsal artery of the LD muscle flap. The brachial artery and the subscapular trunk distal to the thoracodorsal artery were occluded temporarily with 2–0 silk suture during drug infusion that lasted for 10 min. Ten minutes of equilibration were allowed after drug infusion before the muscle flaps were subjected to 4 h of sustained global ischemia.

**Assessment of Muscle Blood Flow in LD Muscle Flaps**

Co-labeled microspheres (New England Nuclear, Boston, MA) of 15.5 ± 0.1-μm diameter were used (~150,000 microspheres/kg) for measurement of muscle blood flow using the reference blood sampling technique previously described for pig LD muscle flaps (42). Mean arterial blood pressure was monitored continuously, and the pig was killed with an overdose of pentobarbital sodium at the end of the experiment.

**Muscle Biopsies**

Timed biopsies (0.5 × 0.5 cm) for myeloperoxidase (MPO) or PKC assays were sequentially taken 1 cm from the dorsal edge of the LD muscle flap in a cephalad direction, starting 8 cm from the vascular pedicle. Each biopsy was immediately rinsed with cold (4°C) isotonic saline, frozen in liquid nitrogen, and stored at −85°C. It has previously been documented that muscle infarction as well as the protective effect of IPC occurred consistently in this biopsied region of the LD muscle flap, and it is unlikely that harvesting of each biopsy would have significant effect on the blood supply to the remaining muscle flap (42).

**MPO Assay**

Muscle samples (~300 mg) were homogenized in 3 ml of ice-cold 0.9% sodium chloride solution and centrifuged at 20,000 g for 15 min. The supernatant was decanted, and the pellet was suspended in 50 mM potassium phosphate buffer (4°C) containing 0.5% hexadecyl trimethyl ammonium bromide at pH 6.0. This supernatant was homogenized and then sonicated for 10 min at 4°C. The supernatant was assayed for MPO activity using a spectrophotometric technique (38, 40). One unit of enzyme activity was defined as the amount of MPO that produced an absorbance change of 1.0 optical density · min⁻¹ · g wet wt⁻¹ at 37°C.
Detection of PKC Isoforms in Skeletal Muscle

Muscle cell fractionation. All muscle biopsies were processed within 24 h of harvesting to minimize the possibility of subcellular realocalization of PKC during storage. Cytosol and a sarcolemma- and sarcoplasmic reticulum (sarcolemma-SR)-enriched membrane fraction were prepared by a modified method of Deems et al. (8). Crude particulate (total membrane) fractions were prepared by the method of Ping et al. (43). All steps were performed on ice or at 4°C.

For preparation of the sarcolemma-SR-enriched membrane fraction, the muscle biopsies were individually minced with scissors for 3 min in fractionation buffer (10 ml/g), mechanically homogenized for 8 s (Pro 250, ProScientific, Monroe, CT), and centrifuged at 1,500 g for 10 min. The supernatant was then centrifuged at 9,000 g for 10 min. The fractionation buffer consisted of 20 mM Tris·HCl (pH 7.5), 1.2 mM EGTA, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupeptin, and 0.1 mg/ml aprotinin. The final pellet, which contained nuclear and mitochondrial membranes, was discarded and the supernatant was centrifuged at 190,000 g for 1 h. The resulting supernatant represented the cytosol fraction. A soluble membrane fraction was prepared by incubating the pellet in fractionation buffer containing 1% Triton X-100 (detergent) supplemented to 5 mM EGTA and 2 mM EDTA for 45 min, then centrifuging at 100,000 g for 30 min. The resulting supernatant was a solubilized, relatively enriched fraction of sarcolemmal and sarcoplasmic reticulum proteins. To confirm successful fractionation, equal amounts of membrane and cytosol fractions were subjected to immunoblot analysis using a monoclonal antibody against Ca2+-ATPase (a sarcoplasmic reticulum-associated protein).

To prepare a crude membrane fraction of the total cellular protein, frozen muscle biopsies were individually powdered in a prechilled mortar and pestle. Glass-glass homogenization of the powdered tissue was performed in sample buffer containing 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 50 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 0.3% β-mercaptoethanol. The homogenate was centrifuged at 45,000 g for 30 min without a preceding low-speed fractionation step. The resulting supernatant represented the cytosol fraction of total cellular proteins, whereas the pellet represented the crude (total) membrane fraction. Both cytosol and crude membrane fractions were stored at −85°C before analysis.

Protein Assay

The method of Bradford was used to assay the protein concentrations of the cytosol and crude particulate fraction for each tissue sample (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA). Due to the high Triton X-100 concentration of the membrane solubilization buffer, the method of Lowry was used to assay the sarcolemmal-rich membrane fractions (Bio-Rad Detergent Compatible Protein Assay, Bio-Rad Laboratories).

Western Blot Analysis

Equal amounts of samples (30 μg of protein) were subjected to denaturing 8% Tris-glycine gel electrophoresis in Laemmli buffer, then electrophoretically transferred, using a semi-dry technique, to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) at constant voltage (25 V) for 100 min at room temperature. Nonspecific binding sites on the membrane were blocked by a 1-h incubation at room temperature in blocking solution (5% wt/vol non-fat dry milk in phosphate-buffered saline, pH 6.5 with 0.1% Tween 20). Immunoblots were performed on the membrane-bound proteins at room temperature with a 1-h incubation in a 1:1,000 dilution of primary PKC isofrom-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution, followed by washing in blocking solution and a 1-h incubation in a 1:7,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody (BioRad Laboratories, Richmond, CA) in blocking solution. After a final wash in blocking solution, signal detection was performed using enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) on Kodak X-OMAT AR Scientific Imaging Film (Eastman Kodak, Rochester, NY) and was quantified using scanning laser densitometry (The Discovery Series Analysis Software for One-Dimensional Gel Analysis, Protein Databases, New York, NY). Equal loading technique was confirmed by visualization of total protein on loaded gels using Coomassie brilliant blue G-250 (CBB), and equal transfer was confirmed by staining of the PVDF membrane-bound proteins either with Ponceau S (3-hydroxy-4-[2-sulfo-4-(sulfo-phenylazophenylazo)phenylazo]-2,7-naphthalene disulfonic acid in 30% trichloroacetic acid and 30% sulfosalicylic acid before immunoblotting or with CBB after immunoblotting. These nonspecific total protein stains demonstrated no observable difference in overall amount of protein between the various lanes. Isoform specificity of the antibodies was confirmed by loss of signal on incubation with isoform-specific control peptide and by the characteristic banding pattern and migration distance of each isoform (37).

Biochemicals

All chemical reagents and drugs were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise stated. Sodium 5-hydroxydecanoate (5-HD) was purchased from Research Biochemicals (Natick, MA). Anti-Ca2+-ATPase monoclonal antibody was a generous gift from Dr. D. MacLennon, Banting Institute, University of Toronto.

Experimental Protocols

Protocol 1: To investigate the effect of PKC inhibitors (Chel and Poly B) on the anti-infarction effect of IPC and adenosine. Pigs with bilateral LD muscle flaps were assigned to eight groups: 1) ischemic control, 2) IPC, 3) adenosine (0.5 mg/muscle), 4) Chel (0.6 mg/muscle) starting immediately after IPC, 5) Poly B (1.0 mg/muscle) starting immediately after IPC, 6) Chel starting at 10 min after adenosine infusion, 7) Chel alone, and 8) Poly B alone. Drugs were delivered to each muscle flap by 10 min of local intra-arterial infusion followed by 10 min of equilibration. All muscle flaps were subjected to 4 h of global ischemia and 24 h of reperfusion. We have previously observed that infarction did not occur in this 8 × 12-cm muscle flap model if the muscle was not subjected to ischemic insult (42), therefore a sham (nonischemic) group was not planned for this protocol.

Protocol 2: To investigate the anti-infarction effect of PKC activators (PMA and OAG) in the absence and presence of the KATP channel blocker 5-HD. Pigs with bilateral LD muscle flaps were assigned to nine groups: 1) ischemic control, 2) IPC, 3) PMA (0.05 μg/muscle) before 4 h of ischemia, 4) OAG (0.1 mg/muscle) before 4 h of ischemia, 5) OAG at the onset of reperfusion, 6) intravenous 5-HD (27 mg/kg) before IPC, 7) intravenous 5-HD before PMA, 8) intravenous 5-HD before OAG, and 9) intravenous 5-HD alone. Drugs, except 5-HD, were given by 10 min of local intra-arterial infusion. All

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PKC AND K_{ATP} CHANNELS IN ISCHEMIC PRECONDITIONING

muscles were subjected to 4 h of global ischemia, and the pigs were killed after 24 h of reperfusion for assessment of muscle infarction.

Protocol 3: To investigate the effect of IPC, adenosine, Chel, and OAG on muscle blood flow. Pigs with bilateral LD muscle flaps were assigned to five groups: 1) Control, 2) IPC, 3) Adenosine (0.5 mg/muscle), 4) Chel (0.6 mg/muscle), and 5) OAG (0.1 mg/muscle). At the end of IPC or 10 min of local intra-arterial drug infusion, blood flow in LD muscle flaps was measured using the radioactive microsphere technique. The mean arterial blood pressure was monitored continuously during infusion and blood flow study.

Protocol 4: To investigate the effect of IPC on cytosol-to-membrane translocation of PKC isoforms in pig LD muscles. Effect of IPC on the Subcellular Distribution of PKC Isoforms in Cytosol and Sarcolemma-SR Membrane. Muscle biopsies harvested from preconditioned LD muscle flaps (n = 4 pigs) were fractionated into cytosol and sarcolemma-SR-enriched membrane components and were screened for PKC isoforms of conventional (cPKCs α, β1, βII, and γ), novel (nPKCs δ, ε, η, and θ), and atypical (aPKCs i, j, and λ) types by Western blotting using commercially available antibodies. Muscle biopsies were taken at three time points: immediately before the first brief ischemia period of IPC (baseline control value) and at 10 and 20 min after the end of the third and final period of ischemia of IPC. We did not harvest biopsies at the end of the first or second cycle of IPC, because we demonstrated previously that a minimum of three cycles of 10-min ischemia and reperfusion were required to precondition pig skeletal muscle against infarction (42). Muscle flaps used in this experiment to study IPC-induced PKC translocation were not exposed to any sustained period of ischemia after the IPC stimulus. PKC band density was reported as a percentage of the band density of the baseline biopsy. A value > 100% would indicate an IPC-induced increase in the PKC isoform, whereas a value < 100% would indicate an IPC-induced decrease. We chose to use the pre-IPC baseline as individual control instead of using a parallel non-IPC control group, because it has been reported that the baseline PKC band density in skeletal muscle varies greatly between animals and individual control rather than individual comparison (42). The mean arterial blood pressure and muscle blood flow in LD muscle flaps were not significantly different among the control and treatment groups. Specifically, the necessary phorbol ester binding motif.

The time course for IPC-induced PKC cytosol-to-crude membrane translocation. Muscle biopsies harvested from preconditioned LD muscle flaps (n = 4 pigs) were fractionated into cytosol and crude total membrane components. The biopsies were harvested at three time points: immediately before the first brief ischemia period of IPC (baseline value) and at 10 min and 20 min after the end of the third and final brief ischemia period of IPC. Muscle flaps used in this experiment were not exposed to any sustained period of ischemia after completion of the IPC stimulus. The crude membrane and cytosol fractions were analyzed for PKC isoforms using Western blotting. Only those isoforms that had demonstrated an IPC-induced decrease in cytosol band density were analyzed. The purpose was to confirm that the decrease in isoform present in the cytosol after IPC was associated with a corresponding increase in the crude membrane fraction, consistent with IPC-induced PKC translocation.

Protocol 5: To investigate the effect of IPC and the PKC activator OAG on neutrophilic MPO activity in muscle flaps during sustained ischemia and reperfusion. Pigs with bilateral LD muscle flaps were assigned to ischemic control, IPC, preischemic OAG, and postischemic OAG treatment groups. Each muscle flap in the control and preischemic OAG group received 10 min of local intra-arterial infusion of saline containing 0 and 0.1 mg of OAG, respectively. Muscle flaps in the postischemic OAG group received the same dose of OAG infusion at the onset of reperfusion. Time-matched muscle biopsies were taken from these four groups of muscle flaps immediately before sustained ischemia, at the end of 4 h of ischemia, and at 1.5 and 16 h of reperfusion. All biopsies were processed and assayed for MPO activity.

Statistical Analysis

All values are expressed as means ± SD. The number of observations and specific statistical analysis are mentioned in the legends of Tables 1 and 2 and Figs. 1–6. One-way ANOVA was used for detection of treatment effect, and t-test with Bonferroni correction was used for multiple comparison of means. Statistical significance was set at P < 0.05 for all tests.

RESULTS

Effect of PKC Inhibitors (Chel and Poly B) on the Anti-Infarction Effect of IPC and Adenosine

IPC and preischemic adenosine treatment decreased (P < 0.05) muscle infarction by 40 and 64%, respectively, compared with ischemic controls in pig LD muscle flaps subjected to 4 h of global ischemia and 24 h of reperfusion. This infarct protective effect of IPC and adenosine was blocked in muscle flaps pretreated with a PKC inhibitor, Chel or Poly B, but Chel or Poly B alone did not affect the muscle infarct size compared with ischemic controls (Fig. 1).

Anti-Infarction Effect of PKC Activators (PMA and OAG) in the Absence or Presence of the K_{ATP} Channel Blocker 5-HD

IPC and preischemic treatment with PMA or OAG reduced (P < 0.05) muscle infarction by 44, 55, and 68%, respectively, compared with ischemic controls in LD muscle flaps subjected to 4 h of ischemia and 24 h of reperfusion (Fig. 2). When given at the onset of reperfusion, OAG did not reduce muscle infarction. The infarct-limiting effect of IPC, OAG, and PMA was completely blocked by pretreatment with 5-HD, a K_{ATP} channel blocker, but 5-HD alone produced no effect on infarct size compared with ischemic controls (Fig. 2).

Effect of IPC, Adenosine, OAG, and Chel on Muscle Blood Flow

The mean arterial blood pressure and muscle blood flow in LD muscle flaps were not significantly different among the control and treatment groups. Specifically,
arterial infusion over 10 min. 5-hydroxydecanoate (5-HD; 27 mg/muscle) were given by local intra-
muscle), phorbol myristate acetate (PMA; 0.05
mg/muscle), chelerythrine (Chel; 0.6 mg/muscle), and polymyxin B (Poly B; 1.0 mg/muscle) were given by local intra-
the rest of the treatment groups. 1-Oleoyl-2-acetyl glycerol (OAG; 0.1
muscle) were given by local intra-

**Treatment Groups**

Fig. 1. Effect of protein kinase C (PKC) inhibitors on the anti-
infarction effect of ischemic preconditioning (IPC) and adenosine. Values are means ± SD; n = 4 or 5 pigs. There were bilateral latissimus dorsi (LD) muscle flaps in each pig, with a total of 8–10 muscle flaps in each group. *Similar and significantly (P < 0.05) different from the control and the rest of the treatment groups. Adenosine (0.5 mg/muscle), chelerythrine (Chel; 0.6 mg/muscle), and polymyxin B (Poly B; 1.0 mg/muscle) were given by local intra-
arterial infusion over 10 min.

The mean arterial blood pressure of the control, IPC, adenosine, OAG, and Chel groups of pigs was 109 ± 6,
115 ± 10, 107 ± 9, 114 ± 2, and 112 ± 6 mmHg (n = 4 pigs), respectively, and the corresponding muscle blood flow in LD muscle flaps was 4.5 ± 0.9, 5.5 ± 1.5, 4.7 ± 1.8, 5.4 ± 2.8, and 4.8 ± 1.3 ml·min⁻¹·100 g⁻¹

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115 ± 10, 107 ± 9, 114 ± 2, and 112 ± 6 mmHg (n = 4 pigs), respectively, and the corresponding muscle blood flow in LD muscle flaps was 4.5 ± 0.9, 5.5 ± 1.5, 4.7 ± 1.8, 5.4 ± 2.8, and 4.8 ± 1.3 ml·min⁻¹·100 g⁻¹

**Effect of IPC on Membrane PKC Translocation in LD Muscle**

Using Western blot analysis, eight PKC isoforms (α, β₁, β₂, δ, ε, θ, ζ, ϵ) were detected in the cytosol fraction of the muscle homogenate (Table 1), and only six isoforms (α, β₂, δ, ε, θ, ζ) were detected in the sarcolemma-SR-enriched fraction of preconditioned LD muscles before IPC (baseline, 100%) and at 10 and 20 min after the last cycle of ischemia in IPC (Table 2). The apparent molecular masses of all bands ranged from 64 to 90 kDa. Of the isoforms present in the cytosol fraction, nPKCe alone demonstrated a progressive decrease

![Graph](image_url)

**Table 1. Change in cytosol fraction immunoblot band density in muscle after IPC with no prolonged ischemia**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
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<tbody>
<tr>
<td>cPKCa</td>
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<td>97 ± 11</td>
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<tr>
<td>cPKCbI</td>
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<td>cPKCbII</td>
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<tr>
<td>cPKCy</td>
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<tr>
<td>nPKCa</td>
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<td>99 ± 14</td>
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<td>35 ± 10*</td>
<td>21 ± 11*</td>
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<tr>
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<tr>
<td>nPKCc</td>
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<tr>
<td>nPKCa</td>
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</table>

Values (means ± SD) are expressed as percent of baseline (pre-ischemic preconditioning [Pre-IPC]) band density (n = 4 pigs). UD, undetectable. Of the 7 isoforms detected in the cytosol fraction, only novel (n) protein kinase C (PKC) isoform (nPKCe) demonstrated a significant (*P < 0.05) IPC-associated decrease in band density assessed at 10, 20, and 30 min after IPC, compared with pre-IPC baseline. cPKC, conventional PKC.

(n = 4 pigs). There were two muscle flaps in each pig, with a total of eight muscle flaps in each group.

**Table 2. Change in sarcolemma- and sarcoplasmic reticulum-enriched membrane fraction immunoblot band density in muscle after IPC with no prolonged ischemia**

<table>
<thead>
<tr>
<th>Isoform</th>
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<tr>
<td>cPKCa</td>
<td>95 ± 11</td>
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<td>nPKCa</td>
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Values (means ± SD) are expressed as percent of baseline (Pre-IPC) band density (n = 4 pigs). Of the 6 isoforms detected in the sarcolemma- and sarcoplasmic reticulum-enriched fraction, none demonstrated a significant IPC-associated change in band density compared with pre-IPC baseline.
in band density relative to the baseline biopsy (Table 1). Representative blots are shown in Fig. 3. The band density of nPKCe dropped to 55±13 and 35±10% of the pre-IPC baseline density (100%) at 10 and 20 min after the third cycle of IPC, respectively. A third biopsy was harvested in this group at 30 min after the third IPC cycle, and a further decrease (P < 0.05) in nPKCe was observed at 21±11% of baseline. In comparison, there were no significant changes in band densities of the PKC isoforms, including nPKCe, in the sarcosome-SR-enriched membrane fraction at 10 and 20 min after IPC compared with the pre-IPC baseline biopsy (Table 2). Representative blots for nPKCe are shown in Fig. 4.

Because nPKCe had demonstrated an IPC-induced decrease in band density of the cytosol fraction without a corresponding increase in the sarcosome-SR-enriched membrane fraction, a crude (total) membrane fraction of the muscle homogenate was prepared and probed for this isoform. The decrease in nPKCe band density relative to the baseline biopsy observed in the cytosol fraction at 10 and 20 min after the final cycle of IPC was now observed to be associated with a corresponding progressive increase in the crude (total) membrane fraction (Table 3). Representative immunoblots are shown in Fig. 5.

**Effect of IPC and the PKC Activator OAG on Muscle Neutrophilic MPO Activities During Ischemia and Reperfusion**

Preischemic muscle MPO activities were similar among the control, IPC, and pre- and postischemic OAG-treated muscle flaps (Fig. 6). The muscle MPO activity did not change significantly in these four groups of muscle flaps at the end of 4 h of global ischemia. There was a significant (P < 0.05) increase in muscle MPO activity in the control and pre- and postischemic OAG-treated muscle flaps at 1.5 and 16 h of reperfusion. It is important to note that at 1.5 h of reperfusion, MPO activities in the IPC and preischemic OAG-treated muscle flaps were significantly lower (P < 0.05) than the time-matched control and postischemic OAG groups. Between 1.5 and 16 h of reperfusion, the MPO activity of the postischemic OAG-treated muscle flaps continued to increase significantly (P < 0.05), reaching a level similar to that of the control at 16 h of reperfusion. However, the MPO activities in the IPC and preischemic OAG-treated muscle flaps remained 97 and 71%, respectively, lower than controls at 16 h of reperfusion.

<table>
<thead>
<tr>
<th>Time After IPC</th>
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<th>Cytosol</th>
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<tbody>
<tr>
<td>10 min</td>
<td>156±15</td>
<td>61±9</td>
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<tr>
<td>20 min</td>
<td>187±16</td>
<td>29±11</td>
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</table>

Values (means ± SD) are expressed as percent of baseline (Pre-IPC) band density (n = 4 pigs). The significant (P < 0.05) decrease in cytosol nPKCe band density from baseline density (100%) at 10 and 20 min after IPC was associated with corresponding significant (P < 0.05) increase in nPKCe band density above the baseline (100%) in the crude (total) membrane fraction.

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**Table 3. Change in crude membrane and cytosol fraction of nPKCe band density in muscle after IPC with no prolonged ischemia**

<table>
<thead>
<tr>
<th>Time After IPC</th>
<th>Membrane</th>
<th>Cytosol</th>
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Values (means ± SD) are expressed as percent of baseline (Pre-IPC) band density (n = 4 pigs). The significant (P < 0.05) decrease in cytosol nPKCe band density from baseline density (100%) at 10 and 20 min after IPC was associated with corresponding significant (P < 0.05) increase in nPKCe band density above the baseline (100%) in the crude (total) membrane fraction.

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**Fig. 3.** Representative Western blots demonstrating cytosol and sarcosome-sarcoplasmic reticulum-enriched membrane band density of PKC isoforms immediately before IPC (B) and at 10 and 20 min after completion of the final ischemia cycle of IPC. Note that of the isoforms present in the cytosol fraction, novel PKC isoform (nPKCe) alone demonstrated a progressive decrease in band density relative to the baseline (B). However, there was no corresponding increase in membrane nPKCe band density in the sarcosome and sarcoplasmic reticulum-enriched membrane. cPKC and aPKC, conventional and atypical PKC isoforms, respectively.

**Fig. 4.** Representative Western blots demonstrating the progressive decrease in cytosol nPKCe band density from baseline density after IPC without a corresponding increase in the sarcosome-sarcoplasmic reticulum-enriched membrane nPKCe band density. Biopsies were harvested before IPC (B, baseline density) and at 10, 20, and 30 min after completion of the last ischemic cycle of ischemia.

**Fig. 5.** Representative Western blots demonstrating the progressive decrease in cytosol nPKCe band density after IPC with a corresponding increase in crude (total) membrane nPKCe band density. Biopsies were harvested before IPC (B, baseline density) and at 10 and 20 min after completion of the last ischemic cycle of IPC.
groups. The quantity of DMSO required for our studies did not affect muscle blood flow in the pig skeletal muscle. Values are means ± SD; n = 4 pigs. There were bilateral LD muscle flaps in each pig, with a total of 8 muscle flaps in each group. *At each time point, significantly different (P < 0.05) from the time-matched control and other treatment groups. (P < 0.05) than the time-matched control at the end of 16 h of reperfusion (Fig. 6).

DISCUSSION

We previously demonstrated in vivo that IPC against infarction can be induced in noncontracting pig skeletal muscle (42) and that the adenosine A
receptor is the initiator of this phenomenon (38). Here, we demonstrate for the first time that 1) PKC most likely plays a central role in the postreceptor signal transduction pathway of IPC against infarction in noncontracting pig skeletal muscle; 2) among the seven PKC isoforms detectable in pig skeletal muscle, nPKCε alone appears to translocate from the cytosol within 10 min after the final ischemic cycle of IPC; 3) nPKCε translocates to a membrane component of the skeletal muscle cell but does not appear to translocate to the sarcosomal membrane or sarcoplasmic reticulum compartment; 4) the KATP channel, downstream to PKC, appears to be involved in the IPC signal-transduction pathway; and 5) the anti-infarction effect of IPC and the PKC activator OAG is associated with a lower neutrophilic MPO activity in postischemic muscle during 16 h of reperfusion compared with the time-matched ischemic control. These observations provide for the first time in vivo evidence that PKC is most likely involved in the IPC of skeletal muscle against infarction. We previously demonstrated in the same pig LD muscle flap model that the KATP opener lemakalim mimicked the anti-infarction effect of IPC, and this infarct protective effect of lemakalim was antagonized by a KATP channel blocker, glibenclamide (Glib) or 5-HD (41). In the present study, we observed that the anti-infarction effect of PKC activators, OAG and PMA, was blocked by the KATP channel antagonist 5-HD (Fig. 2). 5-HD alone was not proischemic and had no effect on muscle blood flow or systemic blood pressure. These observations are taken together to indicate that the KATP channel may be involved downstream of PKC in the signal-transduction pathway of IPC in pig skeletal muscle. There is in vitro cellular evidence from other laboratories to support a link between these two components in a signal-transduction pathway in IPC. Specifically, it was demonstrated that KATP channels could be activated by PKC in ventricular myocytes of the rabbit and human (18, 19). It was also observed that the protective effect of PKC activation against contractile dysfunction in ischemic superperfused human right atrial trabeculae was blocked by the KATP channel antagonist Glib (49).

Recently, much attention has focused on the potential role of sarcoemmal KATP (sarKATP) and mitochondrial KATP (mitoKATP) channels in myocardial IPC. It was observed that the myocardial anti-infarction effect of the KATP channel opener bimakalim and BMS-18048 were not associated with a significant shortening of action potential in the dog (60) and guinea pig (12), respectively. These observations on the anti-infarction effect of KATP opener independent of sarcoemmal action potential is consistent with the observation that the phenomenon of IPC can be induced in quiescent human cardiomyocytes (20). Recent studies provide further evidence to indicate that mitoKATP rather than sarKATP channels are involved in IPC. Specifically, diazoxide, a selective mitoKATP channel opener in cardiomyocytes is cardioprotective (10, 31), and the mitoKATP channel antagonist 5-HD (19, 47) blocked the cardioprotective effect of diazoxide (10, 31) and IPC (4, 16, 49). Last, but not least, activation of PKC activators or inhibitors were only given for 10 min before 4 h of sustained ischemia with no collateral blood flow and with muscle infarction assessed after 24 h of reperfusion. We observed that the anti-infarction effect of IPC in pig LD muscle flaps could be mimicked by adenosine, OAG, or PMA and blocked by Chel or Poly B. Neither Chel nor Poly B was proischemic when administered alone (Figs. 1 and 2).
PKC potentiated opening of mitoK\textsubscript{ATP} channels (45). There is also evidence to indicate that mitoK\textsubscript{ATP} rather than sarK\textsubscript{ATP} channels may be involved in IPC of skeletal muscle. We previously demonstrated that the phenomenon of IPC could be induced in noncontracting pig LD muscle flaps, and the K\textsubscript{ATP} channel inhibitors Glib and 5-HD blocked the infarct protective effect of IPC in pig LD muscle flaps (40). The infarct protective effect of K\textsubscript{ATP} channel openers lemakalim and diazoxide was also blocked by 5-HD in the pig LD muscle (39, 40). However, using whole cell patch clamp technique, other investigators observed that the K\textsubscript{ATP} channel openers levcromakalim and pinacidil caused a concentration-dependent (10–400 \textmu M) increase in conductance in rat skeletal muscle cells, but diazoxide was inactive up to the concentration of 300 \textmu M. The levcromakalim-induced current was blocked by classical K\textsubscript{ATP} channel inhibitors such as glibencamide, tolbutamide, and glipizide, but 5-HD was completely inactive (5). Therefore, neither diazoxide nor 5-HD had any effect on the sarK\textsubscript{ATP} channels. Taken together, the in vivo and in vitro observations indicated that K\textsubscript{ATP} channels other than sarK\textsubscript{ATP} channels are involved in the anti-infarction effect of IPC in skeletal muscle.

**Evidence of IPC-induced PKC Membrane Translocation**

Studies using PKC activators and inhibitors to probe the putative role of PKC in IPC have thus far supported a central role for PKC in IPC of intact heart in the rat (25, 33, 52), rabbit (29, 32, 61), and dog (23) and also in the pig in the presence of a tyrosine kinase inhibitor (57). Here, we have also demonstrated an important role of PKC in IPC of skeletal muscle. The pharmacological studies discussed thus far could be criticized for their lack of proven specificity of the PKC activators and inhibitors and direct proof of involvement of PKC (7). Several recent studies have helped to confirm an important role of PKC in IPC of myocardium at the molecular level. Specifically, Ping et al. (43) demonstrated that out of 11 PKC isoforms that were detectable in rabbit myocardium, IPC caused selective cytosol to particulate translocation of PKC\textsubscript{\gamma} and PKC\textsubscript{\varepsilon}. They also demonstrated that measurements of total cytosol and particulate PKC activities were not sufficiently sensitive to detect the translocation of PKC in myocardial IPC, because IPC only activates one or two PKC isoforms. This may explain why other investigators failed to observe changes in myocardial cytosolic and particulate PKC activities in IPC of rabbit heart (50). Further study from this laboratory demonstrated that PKC\textsubscript{\varepsilon} is the specific isoform responsible for the development of myocardial IPC in the rabbit heart (46). More recently, Liu et al. (27) demonstrated that the protective effect of IPC or the PKC activator OAG in rabbit cardiomyocytes was abolished by a highly selective PKC\textsubscript{\varepsilon} peptide inhibitor, but not by peptide inhibitors specific for PKC\textsubscript{\beta}, PKC\textsubscript{\delta}, or PKC\textsubscript{\gamma}. These recent studies provided further support for PKC as a critical part of signal-transduction pathway in IPC and for PKC\textsubscript{\varepsilon} as the specific PKC isoyme involved in IPC of the myocardium. However, not all studies to illustrate a role for PKC are consistent, and it is not known if this is due to a difference between species. For example, PKC translocation was not observed during brief episodes of IPC in the canine heart (44). Two studies in rat myocardium reported an IPC-induced translocation of PKCs \delta and \varepsilon in one (22) and PKCs \alpha, \varepsilon, and \iota in the other (2).

In our in vivo study with pig LD muscle, we observed that the seven PKC isoforms (\alpha, \beta\textsubscript{I}, \beta\textsubscript{II}, \delta, \varepsilon, \zeta, \iota) detected in the cytosol fraction of preconditioned pig LD muscle flaps by Western blot analysis, the band density of nPKC\textsubscript{\varepsilon} alone decreased progressively after the final ischemia cycle of IPC (Table 1 and Fig. 3). However, there was no corresponding increase in nPKC\textsubscript{\varepsilon} band density in the sarcolemma-SR-enriched membrane fraction prepared with the method of Deems et al. (8) (Table 2 and Fig. 4). This fractionation technique is known to discard the majority of the nuclear and mitochondrial membranes in the low-speed centrifugation step. When the muscle biopsies were fractionated according to the method of Ping et al. (43), which preserved all cell membrane proteins in the particulate fraction, an IPC-induced increase in nPKC\textsubscript{\varepsilon} band density was observed in this crude membrane fraction, corresponding to the decrease in the cytosol fraction (Table 3 and Fig. 5). These observations would indicate that within 10 min after the final ischemia cycle of IPC, nPKC\textsubscript{\varepsilon} translocated from the cytosol to a membrane compartment of the skeletal muscle cell but that this membrane destination did not appear to be the sarcolemma or sarcoplasmic reticulum.

It could be argued that failure to detect a corresponding increase in PKC\textsubscript{\varepsilon} in the sarcolemma-SR-enriched membrane fraction could be related to a lack of sensitivity. This was unlikely the case for two reasons. First, at the baseline level, the nPKC\textsubscript{\varepsilon} band density in the cytosol was about three times higher than that in the sarcolemma-SR-enriched membrane, measured in the same blot with equal loading. At 20 min after IPC, 65% of the nPKC\textsubscript{\varepsilon} was translocated from the cytosol (Table 1). This large amount of nPKC\textsubscript{\varepsilon} translocation would have been detected as an increase in the sarcolemma-SR-enriched membrane blot if that was the site of translocation. Second, when the membrane fraction included the nuclear and mitochondrial membranes, an IPC-induced increase in nPKC\textsubscript{\varepsilon} band density was seen in the crude membrane fraction.

Therefore, the failure to detect translocation of nPKC\textsubscript{\varepsilon} from cytosol to sarcolemma-SR-enriched membrane was not the result of sensitivity, but the lack of nuclear and mitochondrial membranes that were discarded in the low-speed centrifugation step. In the future, it is important to investigate directly nPKC\textsubscript{\varepsilon} translocation to the nuclear and mitochondrial membranes and to investigate how this signal-transduction pathway contributes to the mechanism of IPC.
Cause of Neutrophil Inhibition Associated with IPC and OAG Treatment in Skeletal Muscle

We observed that IPC, or preischemic OAG treatment, in pig LD muscle flaps were associated with a lower muscle neutrophilic activity after up to 16 h of reperfusion after 4 h of global ischemia, compared with the time-matched control (Fig. 6). The mechanism for this lowering of neutrophil activity is not known at the present time. It has been speculated in dog skeletal muscle that IPC can activate KATP channels, which in turn may attenuate the formation and/or release of chemotactic stimuli from the muscle parenchyma, or limit the regulation of adhesion molecules on the endothelium or neutrophil, thereby decreasing neutrophil accumulation (21). More recently, observations made with intravital microscopy in murine cremaster muscles indicated that IPC may enhance muscle adenosine formation during ischemia and reperfusion, and that this elevated level of adenosine in turn inhibits neutrophil adhesion (1). There is evidence in the present study that IPC and OAG treatment may have caused opening of KATP channels, because the anti-infarction effect of IPC and OAG was blocked by 5-HD, a KATP channel antagonist (Fig. 2). However, activation of KATP channels or adenosine release cannot explain the neutrophil inhibition effect of OAG treatment. Specifically, we have previously observed that the anti-infarction effect of IPC or preischemic lemakalim (a KATP channel opener) treatment was not associated with an increase in muscle content of adenosine during sustained ischemia or reperfusion (39, 40). Other investigators have also observed that the anti-infarction effect of IPC and KATP channel activation in dog myocardium did not cause any increase in venous adenosine concentration during 60 min of regional ischemia and subsequent reperfusion (26). In addition, we have demonstrated that lemakalim or adenosine treatment given at the onset of reperfusion did not reduce neutrophil accumulation or infarction in ischemic pig LD muscles (38, 40). It is most likely that reduction of ischemic injury by IPC or OAG treatment decreased cellular inflammation, thus reducing neutrophil activation and accumulation at reperfusion, as seen in the present experiment.

In summary, we have demonstrated in the pig LD muscle that the anti-infarction effect of IPC could be mimicked by PKC activators (PMA, OAG) and blocked by PKC inhibitors (Chel, Poly B). The anti-infarction effect of IPC and PKC activators was also blocked by the KATP channel antagonist 5-HD, and this observation was in line with our previous finding that the KATP channel opener lemakalim mimicked the anti-infarction effect of IPC. IPC and preischemic OAG treatment were associated with reduced muscle MPO activity after up to 16 h of reperfusion after 4 h of global ischemia. In addition, Western blot analysis indicated progressive cytosol to membrane nPKCe translocation within 10 min of the final ischemia period of IPC. The specific membrane destination of the nPKCe did not appear to be the sarcolemma or the sarcoplasmic reticulum. Our present and previous observations led us to conclude that PKC most likely play a central role in the anti-infarction effect of IPC in pig skeletal muscle, probably through a PKC-KATP channel-linked pathway. However, the location of PKC and KATP channels associated with IPC remains conjectural.

Limitations in the Present Studies

The commercial antibodies used in the Western blot analysis were raised in rabbits, and we assumed that these antibodies were valid for pig tissue. On the basis of densitograms available in the literature, the various PKC isoforms appear to be highly conserved across most species, with the exception of cPKCo, which has a 20% divergence in humans from rats and rabbits (28). However, only the α-, β-, δ-, ε-, and θ-PKC isoforms have been reported in the membrane fraction of rat skeletal muscle (47), and because we also did not detect cPKCy, nPKCη, and nPKCλ, we assumed that these PKC isoforms are not present in pig skeletal muscle.

We did not use a “housekeeping” protein as a loading control in the present study. Instead, measures were taken to ensure even gel loading and transfer in our immunoblotting technique and this was discussed in the text. In addition, because only one (nPKCe) out of the six PKC isoforms examined in the translocation study actually demonstrated significant simultaneous changes in band density in both the cytosol and membrane fractions, we have no reason to suspect that the cytosol-to-membrane nPKCe translocation was due to uneven loading. It should also be pointed out that the demonstration of IPC-induced nPKCe membrane translocation in the present study implied but did not demonstrate nPKCe activation.

Perspectives

Unpredictable perioperative and postoperative complications in vascular and musculoskeletal reconstructive surgery can sometimes cause prolonged and/or repeated ischemic insult to skeletal muscles, resulting in irreversible muscle damage. Muscle necrosis can cause morbidity and life-threatening systemic complications in severe cases. Understanding the mechanism of IPC in skeletal muscle will most likely lead to the identification of novel pharmacological agents to be used as prophylactic drugs for augmentation of muscle ischemic tolerance in musculoskeletal and vascular reconstructive surgery (41).

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REFERENCES

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