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Reactive Oxygen Species Precede the ϵ Isoform of Protein Kinase C in the Anesthetic Preconditioning Signaling Cascade

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Background: **Protein kinase C (PKC) and reactive oxygen species (ROS) are known to have a role in anesthetic preconditioning (APC). Cardiac preconditioning by triggers other than volatile anesthetics, such as opioids or brief ischemia, is known to be isoform selective, but the isoform required for APC is not known. The authors aimed to identify the PKC isoform that is involved in APC and to elucidate the relative positions of PKC activation and ROS formation in the APC signaling cascade.**

Methods: **Isolated guinea pig hearts were subjected to 30 min of ischemia and 120 min of reperfusion. Before ischemia, hearts were either untreated or treated with sevoflurane (APC) in the absence or presence of the nonspecific PKC inhibitor chelerythrine, the PKC-δ inhibitor PP101, or the PKC-ϵ inhibitor PP149. Spectrofluorometry and the fluorescent probes dihydroethidium were used to measure intracellular ROS, and effluent dityrosine as used to measure extracellular ROS release.**

Results: **Previous sevoflurane exposure protected the heart against ischemia–reperfusion injury, as previously described. Chelerythrine or PP149 abolished protection, but PP101 did not. ROS formation was observed during sevoflurane exposure and was not altered by any of the PKC inhibitors.**

Conclusions: **APC is mediated by PKC-ϵ but not by PKC-δ. Furthermore, PKC activation probably occurs downstream of ROS generation in the APC signaling cascade.**

ANESTHETIC preconditioning (APC) is the phenomenon whereby previous exposure of the heart to a volatile anesthetic leads to a state of increased resistance to the injurious effects of ischemia–reperfusion. This has been demonstrated for each of the volatile agents and has been demonstrated in all species studied, including humans.[1–4]

Certain components of the cell-signaling cascade that lead to the preconditioned phenotype have been identified. These include reactive oxygen species (ROS)[5–8] and protein kinase C (PKC).[1] PKC is suggested to be a "final common pathway" during preconditioning by various stimuli.[9] It is activated by brief ischemia and by pharmacologic agents known to induce preconditioning, including volatile anesthetic agents.[10] Inhibition of PKC has been shown to prevent APC in hearts from rabbits[1] and dogs.[11]

PKC, however, consists of a family of isoenzymes, each with specific cellular and subcellular localizations, indicative of isoenzyme-specific functions.[12] Recently, specific PKC isoform inhibitors have become available. Use of these new agents has demonstrated that different isoforms have specific biologic functions during conditions of cardiac stress. The isoforms implicated in preconditioning pathways are PKC-δ and PKC-ϵ. PKC-ϵ is required for preconditioning by brief ischemia[13] and for preconditioning by κ-opioid receptor stimulation.[14] In contrast, PKC-δ is required for preconditioning by μ-opioid receptor stimulation.[15] Paradoxically, activation of PKC-δ by some agents, such as ethanol,[16] may exacerbate rather than prevent ischemic injury. Although PKC is known to be involved in APC, there is no information about isoform-specific effects. The purposes of this study were to identify the roles of PKC-δ and PKC-ϵ in APC and to clarify the relative sequence of PKC activation and ROS formation in the APC signaling cascade.

Materials and Methods

Langendorff Heart Preparation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health publication 85-23, revised 1996) and was approved by the Institutional Animal Use and Care Committee. The preparation has been described in detail previously.[3,5,8] Guinea pig hearts (n = 102) were perfused at 37°C at a constant pressure of 55 mmHg with an oxygenated Krebs-Ringer's solution of the following composition (in mM): Na^+ 138, K^+ 4.5, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134, HCO_3^- 14.5, $H_2PO_4^-$ 1.2, glucose 11.5, pyruvate 2, mannitol 16, probenecid 0.1, EDTA 0.05, and 5 U/l insulin.

Left ventricular pressure (LVP) was measured isovolumetrically with a transducer connected by stiff saline-filled tubing to a latex balloon placed in the left ventricle through an incision in the left atrium. Systolic and diastolic LVPs were measured, and developed (systolic − diastolic) LVP was calculated. Coronary inflow was measured by an ultrasonic flowmeter (T106X; Transonic, Ithaca, NY). Atrial and ventricular bipolar leads were used to measure spontaneous heart rate. Coronary inflow (a) and coronary venous (v) Na^+, K^+, Ca^{2+}, Po_2, pH, and Pco_2 were measured off-line with an intermit-

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tently self-calibrating analyzer (ABL 505; Radiometer, Copenhagen, Denmark). Coronary sinus P_{O_2} tension (P_{VO_2}) was also measured continuously on-line with a Clark electrode (model 203B; Instech, Plymouth Meeting, PA). Myocardial O_2 consumption ($M\dot{V}O_2$) was calculated as coronary inflow/heart weight (g) × (P_{aO_2} − P_{VO_2}) × 24 ml O_2/ml at 760 mmHg.

Global ischemia was achieved by clamping the aortic inflow line. If ventricular fibrillation (VF) occurred on reperfusion and did not convert within 30 s, a bolus of lidocaine (250 μg) was given. At the end of 120 min, reperfusion hearts were removed, cut into six transverse sections, and stained with 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M KH_2PO_4 buffer (pH 7.4, 38°C) for 10 min as described previously. Infarct size was expressed as a percentage of total heart weight.[5,8]

Specific Isoforms of PKC

To determine which isoform is required for APC-induced cardioprotection, we used recently developed isoform-selective inhibitors of PKC-δ and PKC-ϵ. Each PKC isoform translocates to a unique subcellular location after activation, responding to a specific anchoring protein, collectively termed RACK (receptor for activated C-kinase).[17] The isoform-selective modulators of PKC function that have been developed are peptides that inhibit translocation. These were synthesized at the Stanford Protein and Nucleic Acid Facility.[17] A recent review summarized the rationale that led to the identification of these peptides.[18]

Measurement of ROS in Coronary Effluent Using Dityrosine Fluorescence

We used the method of Yasmin *et al.*[19] in isolated hearts to estimate production of ROS. Briefly, the heart is infused throughout the experiment with L-tyrosine in Krebs-Ringer's solution. L-Tyrosine reacts with peroxynitrite to form the fluorescent product dityrosine, which is released in coronary effluent and measured off-line by spectrofluorimetry.[20] The sensitivity, linearity, and stability of this reaction have been described and tested in detail previously.[5,19,21] Formation of dityrosine was analyzed in the incubation solution by measuring fluorescence spectra, with excitation wavelength (λ_{ex}) of 320 nm and emission wavelength (λ_{em}) of 410 nm, at room temperature with a spectrophotofluorometer (model LS 50B; Perkin Elmer, Beaconsfield, Buckinghamshire, UK). Collected effluent samples were kept at 3°C until measured for dityrosine concentration within 15 min at 25°C.

Protocol

Hearts were randomized into eight L-tyrosine–treated groups subjected to ischemia (n = 8 hearts for each group) and one control group not subjected to ischemia (n = 6, data not shown). This group is referred to as the nonischemic control. Each experiment lasted 200 min, beginning 30 min after equilibration (fig. 1).

One group was subjected to 30 min of global ischemia, *i.e.*, index ischemia, and 120 min of reperfusion without pretreatment (ISC group). In four groups, hearts were exposed to two 2-min periods of perfusion with sevoflurane delivered by vaporizer, *i.e.*, preconditioning stimuli, separated by 5 min of perfusion without sevoflurane, ending 20 min before 30 min of index ischemia and 120 min of reperfusion. Sevoflurane was detected by gas chromatography (GC-8AIF; Shimadzu Corp., Kyoto, Japan) as described previously.[3] Aortic inflow concentration was 0.35 ± 0.02 mM (2.48 ± 0.20 vol%). Sevoflurane was not detectable in the effluent at the end of the 20-min washout period before global ischemia.

One group did not receive additional treatment during sevoflurane exposure (APC group). Three groups were treated with PKC inhibitors during sevoflurane exposure. The PKC inhibitors used were chelerythrine (CHE, 50 μM), the PKC-δ isoform inhibitor PP101 (1 μM), and the PKC-ϵ isoform inhibitor PP149 (1 μM). To determine the relative roles of PKC-δ and PKC-ϵ isoforms in APC, these peptides were perfused into the intact guinea pig heart for 5 min before, during, and for 5 min after sevoflurane exposure and followed by a 15-min washout period with Krebs-Ringer's solution before the onset of index ischemia (APC+CHE, APC+PP101, and APC+PP149 groups). Three additional groups of hearts were pretreated with the PKC inhibitors alone to rule out direct effects of these drugs (CHE, PP101, and PP149 groups).

Coronary flow responses to bolus adenosine (0.2 ml of 200 mM stock), 100 μM nitroprusside, and 10 nM bradykinin were tested at the end of reperfusion (at 200 min). For dityrosine concentration measurements, coronary effluent samples (2.7 ml) were collected at baseline (15 min), 1 min after each exposure to sevoflurane, 1 min before global ischemia, each minute during the first 5 min of reperfusion, and at 10 min of reperfusion.

Measurement of Intracellular ROS Using Dihydroethidium

To clarify the relative sequence of PKC activation and ROS formation in the APC signaling cascade, additional experiments were performed for each experimental group using the fluorescent probe dihydroethidium to measure ROS formation during sevoflurane exposure. Using this technique, we reported recently that the fluorescent signal increases during exposure to sevoflurane and decreases during infusion of ROS scavengers.[8]

Details of this technique to measure ROS in intact hearts have been described previously.[8] Briefly, the distal end of a trifurcated fiberoptic cable (optical surface area, 3.85 mm^2) was placed against the left ventricular free wall through a hole in the tissue bath. Netting was applied around the heart for optimal contact without

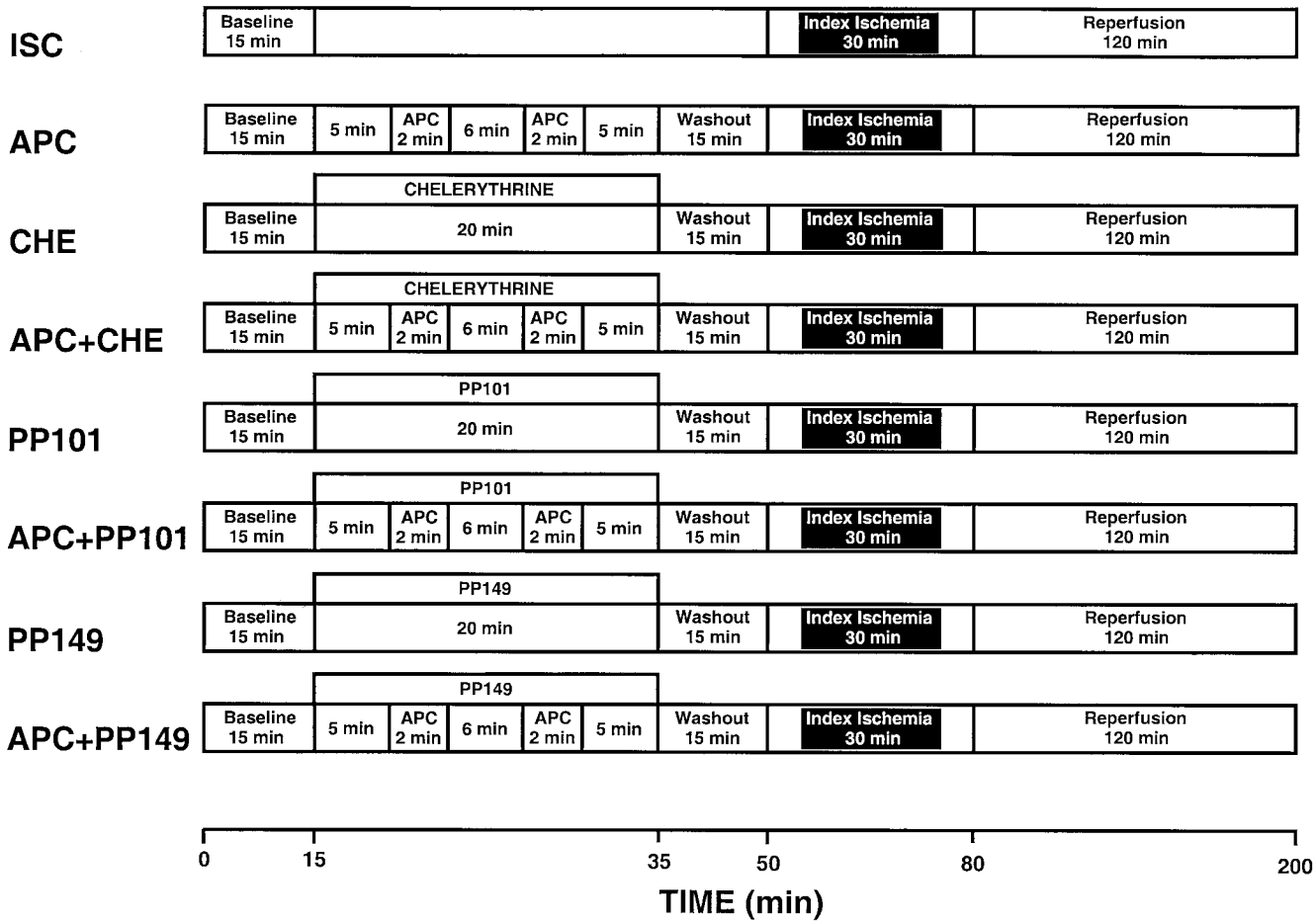

Fig. 1. Schema for protocols used in eight randomized groups of guinea pig hearts. APC = anesthetic preconditioning; APC+CHE = APC and chelerythrine; APC+PP101 = APC and protein kinase C–δ isoform inhibitor; APC+PP149 = APC and protein kinase C–ϵ isoform inhibitor; CHE = chelerythrine alone; ISC = ischemic control; PP101 = PP101 alone; PP149 = PP149 alone.

impeding relaxation. The fiberoptic cable was connected to a modified spectrophotofluorometer (SLM Aminco-Bowman II; Spectronic Instruments, Urbana, IL). Dihydroethidium is converted to fluorescent ethidium (ETH) in the presence of strong oxidants, particularly superoxide. Hearts were loaded with 10 μM dihydroethidium for 25 min, followed by a 15-min washout with Krebs-Ringer's solution. Fluorescent emissions (λ_{em}) at 590 nm (bandwidth, 4 nm) were amplified by a photomultiplier tube (700 V) and recorded after excitation (λ_{ex}) with a 150-W xenon arc lamp filtered through a 540-nm monochromator (bandwidth, 4 nm). The excitation and emission wavelengths penetrate, with decreasing intensity, through the whole 4 mm of the guinea pig left ventricular wall. In 32 hearts (n = 4 hearts for each group), the effect of 2 min of exposure to sevoflurane on ETH fluorescence was evaluated in the absence (APC) and presence (APC+CHE) of chelerythrine, the PKC-δ isoform inhibitor PP101 (APC+PP101), and the PKC-ϵ isoform inhibitor PP149 (APC+PP149). These drugs were given from 5 min before, during, and until 5 min after sevoflurane exposure. These drugs were also given alone without sevoflurane (CHE, PP101, and PP149 groups). Measured ETH fluorescence was averaged for a 100-ms sampling time. A new recording was made every 6 s.

Statistical Analysis

All data were expressed as mean ± SEM. Within-group data (time effect) for a given variable were compared with a baseline control period (at 15 min) by the Duncan comparison of means test whenever univariate ANOVA for repeated measures showed significant differences (Super ANOVA 1.11® software for Macintosh® from Abacus Concepts, Inc, Berkeley, CA). Among-group data (treatment effect) at specific time points (at 15, 23, 30, 81, 110, 140, and 200 min) were analyzed by multivariate analysis for repeated measures. Infarct size was analyzed similarly. The incidence of VF *versus* sinus rhythm was determined by chi-square analysis, and differences in VF duration were determined by unpaired *t* tests. Differences among means were considered statistically significant when $P < 0.05$.

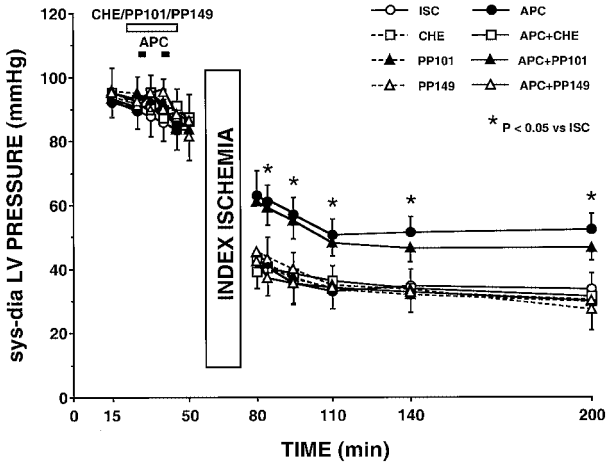

Fig. 2. Time course for developed systolic − diastolic (sys − dia) left ventricular pressure before, during, and after index ischemia. APC = anesthetic preconditioning; APC+CHE = APC and chelerythrine; APC+PP101 = APC and protein kinase C–δ isoform inhibitor; APC+PP149 = APC and protein kinase C–ϵ isoform inhibitor; CHE = chelerythrine alone; ISC = ischemic control; PP101 = PP101 alone; PP149 = PP149 alone. Developed left ventricular pressure was least reduced in the APC and APC+PP101 groups on reperfusion, with no differences among the remaining groups. Values are mean ± SEM; n = 8 for each group. *$P < 0.05$ *versus* ISC.

Results

Baseline Measurements

There were no differences in baseline values (at 15 min) for all cardiac variables among all groups. For the nonischemic group, there were no significant changes over time (15–200 min) for any variable (data not shown).

Mechanical Effects

Systolic − diastolic (developed) LVP was not different among groups before index ischemia (fig. 2), but it was reduced in all groups after ischemia compared with the nonischemic control (data not shown). During reperfusion, developed LVP was significantly greater in the APC and APC+PP101 groups than in the ISC group. The presence of the nonspecific PKC inhibitor chelerythrine (APC+CHE group) or the PKC-ϵ isoform–specific inhibitor PP149 (APC+PP149 group) during the APC period abolished this protective effect of preconditioning on developed LVP. The PKC-δ isoform–specific inhibitor PP101 (APC+PP101) did not abolish preconditioning. When these agents were given alone, no direct effects were observed. End-diastolic LVP (table 1) was lower in the APC and APC+PP101 groups than in the other groups at 200 min (end of reperfusion), indicating that the beneficial effect of previous sevoflurane exposure on developed LVP was a result of attenuated diastolic contracture.

Metabolic Effects

Table 1 shows that myocardial oxygen consumption ($M\dot{V}o_2$) dropped below baseline in each group after index ischemia but was significantly higher in the APC and APC+PP101 groups than in the ISC group. The presence of chelerythrine (APC+CHE group) or the PKC-ϵ isoform–specific inhibitor PP149 (APC+PP149 group), but not the PKC-δ isoform–specific inhibitor PP101 (APC+PP101 group), given before, during, and after the APC period, abolished the protective effect of preconditioning on $M\dot{V}o_2$. The cardiac efficiency index, $(mmHg \cdot beats^{-1})/(100\ nl\ O_2 \cdot g^{-1})$, calculated from the above data and shown in table 1, mirrors the above observations.

Table 2 shows that coronary flow was higher throughout reperfusion in the APC and APC+PP101 groups than in any other group. Moreover, the postischemic reactive-flow increase during the initial 1- to 2-min reperfusion period was apparent only in the APC and APC+PP101 groups. Coronary flow responses (table 2) to adenosine, nitroprusside, and bradykinin, after 120 min of reperfusion, were significantly higher in the APC and APC+PP101 groups than all other groups.

Table 1. End-diastolic Left Ventricular Pressure (mmHg), $M\dot{V}o_2$ ($\mu l \cdot min^{-1} \cdot g^{-1}$), EI ($mmHg \cdot beats^{-1}$)/($100\ nl\ O_2 \cdot g^{-1}$), VF, and MI Size (% Total Heart Weight) and after Index Ischemia in ISC, APC, CHE, APC + CHE, PP101, APC + PP101, PP149, and APC + PP149 Groups

	EDLVP, 200 min	$M\dot{V}o_2$, 200 min	EI, 200 min	VF, %	MI, %
ISC	16 ± 3	49 ± 3	11.6 ± 4	99	47 ± 3
APC	3 ± 3*	62 ± 4*	17.8 ± 3*	48*	26 ± 2*
CHE	17 ± 4	46 ± 4	11.5 ± 4	96	42 ± 3
APC + CHE	16 ± 4	43 ± 4	12.3 ± 5	93	44 ± 3
PP101	18 ± 4	45 ± 3	12.5 ± 2	98	46 ± 3
APC + PP101	5 ± 4*	60 ± 4*	16.8 ± 4*	52*	30 ± 4*
PP149	17 ± 4	50 ± 4	12.6 ± 2	95	44 ± 4
APC + PP149	18 ± 3	47 ± 3	12.0 ± 3	96	42 ± 3

Values are mean ± SEM; n = 8 for each group.

* $P < 0.05$ *vs.* ISC.

APC = anesthetic preconditioning; APC + CHE = APC and chelerythrine; APC + PP101 = APC and protein kinase C-δ isoform inhibitor; APC + PP149 = APC and protein kinase C-ϵ isoform inhibitor; CHE = chelerythrine alone; EDLVP = end-diastolic left ventricular pressure; EI = efficiency index; ISC = ischemic control; MI = myocardial infarct; $M\dot{V}o_2$ = myocardial oxygen consumption; PP101 = PP101 alone; PP149 = PP149 alone.

Table 2. Coronary Flow ($ml \cdot min^{-1} \cdot g^{-1}$) before and after Index Ischemia and Responses to ADE, NP, and BK, Respectively, in ISC, APC, CHE, APC + CHE, PP101, APC + PP101, PP149, and APC + PP149 Groups

	Baseline, 15 min	APC Periods, 30 min	Washout, 50 min	RP 1 min, 81 min	RP 60 min, 140 min	RP 120 min, 200 min	ADE	NP	BK
ISC	7.9 ± 0.3	7.8 ± 0.3	7.3 ± 0.3	4.2 ± 0.3	3.9 ± 0.4	3.9 ± 0.4	6.7 ± 0.4	5.2 ± 0.3	5.6 ± 0.3
APC	7.8 ± 0.3	7.7 ± 0.4	7.3 ± 0.4	6.8 ± 0.4*	5.4 ± 0.4*	5.6 ± 0.4*	8.9 ± 0.4*	7.7 ± 0.3*	7.5 ± 0.4*
CHE	7.8 ± 0.2	7.8 ± 0.4	7.2 ± 0.3	4.4 ± 0.5	4.3 ± 0.4	4.1 ± 0.4	6.6 ± 0.3	5.5 ± 0.3	5.6 ± 0.3
APC + CHE	7.7 ± 0.4	7.7 ± 0.4	6.9 ± 0.4	4.3 ± 0.5	4.4 ± 0.4	4.1 ± 0.5	6.7 ± 0.3	5.4 ± 0.3	5.5 ± 0.3
PP101	7.8 ± 0.4	7.7 ± 0.4	7.1 ± 0.3	4.0 ± 0.5	3.9 ± 0.4	3.8 ± 0.4	6.5 ± 0.3	5.3 ± 0.4	5.4 ± 0.4
APC + PP101	7.7 ± 0.4	7.8 ± 0.4	6.9 ± 0.4	6.5 ± 0.3*	5.3 ± 0.4*	5.5 ± 0.3*	8.7 ± 0.4*	7.5 ± 0.4*	7.3 ± 0.3*
PP149	7.8 ± 0.3	7.7 ± 0.4	7.0 ± 0.4	4.1 ± 0.5	3.9 ± 0.5	3.4 ± 0.4	6.3 ± 0.4	5.2 ± 0.3	5.6 ± 0.4
APC + PP149	7.7 ± 0.3	7.7 ± 0.2	7.2 ± 0.3	4.2 ± 0.3	4.0 ± 0.3	3.7 ± 0.4	6.4 ± 0.3	5.4 ± 0.4	5.3 ± 0.3

Values are mean ± SEM; n = 8 for each group.

*$P < 0.05$ *vs.* ISC.

ADE = adenosine; APC = anesthetic preconditioning; APC + CHE = APC and chelerythrine; APC + PP101 = APC and protein kinase C-δ isoform inhibitor; APC + PP149 = APC and protein kinase C-ϵ isoform inhibitor; BK = bradykinin; CHE = chelerythrine alone; ISC = ischemic control; NP = nitroprusside; PP101 = PP101 alone; PP149 = PP149 alone; RP = reperfusion period.

Electrical Effects

For all groups before index ischemia (at 50 min) and after reperfusion (at 200 min), there were no differences in heart rate (249 ± 4 and 251 ± 3 beats/min, respectively) or atrioventricular conduction time (77 ± 4 and 74 ± 3 ms, respectively); these values were averaged for all groups. The only dysrhythmia observed on reperfusion was VF, which occurred in all ischemic groups. The incidence of VF for each group, including repeat VF, is shown in table 1. When VF occurred, its onset was within 1 min of reperfusion, except in the APC and APC+PP101 groups, in which the onset was much later, at 5.7 ± 0.3 and 5.6 ± 0.3 min, respectively ($P < 0.05$).

Infarct Size

The inhibition of the functional and metabolic cardioprotective effects of APC by the nonspecific PKC inhibitor chelerythrine (APC+CHE group) or the PKC-ϵ isoform–specific inhibitor PP149 (APC+PP149 group) was accompanied by a significant increase in infarct size (table 1). Myocardial infarct size was significantly smaller in the APC and APC+PP101 groups. There were no differences in infarct size among the ISC, APC+CHE, and APC+PP149 groups.

ROS Release in Coronary Effluent

On early reperfusion, dityrosine fluorescence was observed in all ischemic groups but was decreased markedly in the APC and APC+PP101 groups (fig. 3). The presence of either the nonspecific PKC inhibitor chelerythrine or the PKC-ϵ isoform–specific inhibitor PP149 during the preconditioning stimuli caused the dityrosine fluorescence on reperfusion to be increased to control levels. The changes in dityrosine fluorescence were not attributable to changes in coronary flow. Relative fluorescence normalized for coronary flow remained significantly lower in the APC and APC +PP101 groups than the other groups during the first 3 min of reperfusion.

Intracellular ROS Measured with Dihydroethidium during Sevoflurane Exposure

In additional experiments using hearts loaded with dihydroethidium, ETH fluorescence, a marker of intracellular ROS formation, increased significantly during sevoflurane exposure (fig. 4). This change was independent of coronary flow, because it occurred similarly when flow was fixed at 8 $ml \cdot min^{-1} \cdot g^{-1}$. Bracketing sevoflurane administration with the PKC inhibitors chelerythrine, PP101, or PP149 had no effect on ETH fluorescence during sevoflurane exposure. No direct effect on ETH fluorescence was observed when these drugs were given alone.

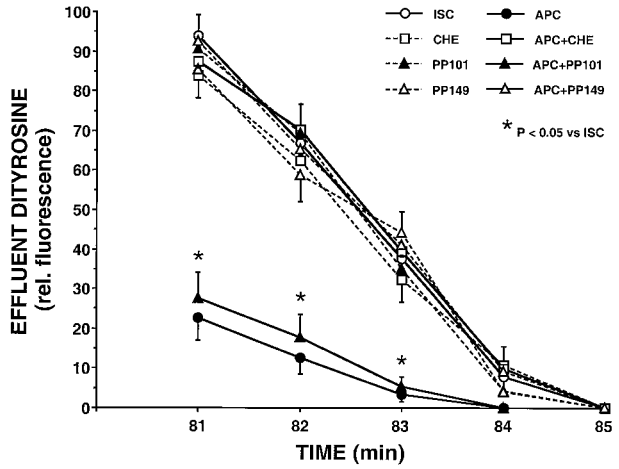

Fig. 3. Release of fluorescent dityrosine (in arbitrary units) into the coronary effluent on initial reperfusion after index ischemia. APC = anesthetic preconditioning; APC+CHE = APC and chelerythrine; APC+PP101 = APC and protein kinase C–δ isoform inhibitor; APC+PP149 = APC and protein kinase C–ϵ isoform inhibitor; CHE = chelerythrine alone; ISC = ischemic control; PP101 = PP101 alone; PP149 = PP149 alone. Values are expressed as the change in relative (*rel.*) units of fluorescence from the baseline value measured in effluent before treatments. Dityrosine concentration increased least in the APC and APC+PP101 groups on reperfusion after index ischemia. Values are mean ± SEM; n = 8 for each group. *$P < 0.05$ *versus* ISC.

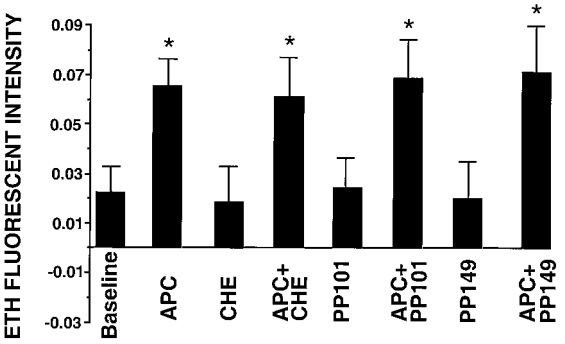

Fig. 4. Ethidium (ETH) fluorescent intensity in arbitrary units, measured in real time at the left ventricular free wall by spectrofluorometry. Hearts are loaded with dihydroethidium, which is converted to fluorescent ETH by intracellular reactive oxygen species, particularly superoxide. APC = anesthetic preconditioning; APC+CHE = APC and chelerythrine; APC+PP101 = APC and protein kinase C–δ isoform inhibitor; APC+PP149 = APC and protein kinase C–ϵ isoform inhibitor; CHE = chelerythrine alone; PP101 = PP101 alone; PP149 = PP149 alone. Averaged signal intensity during 5 min of perfusion with Krebs-Ringer's solution (baseline) and during exposure to sevoflurane alone (APC) or with protein kinase C inhibitors (APC+CHE, APC+PP101, APC+PP149) and during Krebs-Ringer's solution perfusion with protein kinase C inhibitors in the absence of sevoflurane (CHE, PP101, PP149). Values are mean ± SEM; n = 4 for each group. *$P < 0.05$ *versus* baseline.

Discussion

We have identified the PKC isoform specifically involved in the cell-signaling pathway responsible for APC. The isoform-nonspecific inhibitor chelerythrine abolished anesthetic-induced preconditioning, as did inhibition of the PKC-ϵ isoform by PP149. In contrast, inhibition of the PKC-δ isoform by PP101 did not abolish APC. Volatile anesthetics have recently been shown to generate ROS in the heart to trigger APC.[5–8] In this study, we found that PKC inhibition did not prevent ROS formation during sevoflurane exposure. We therefore propose a signaling pathway in which ROS, generated in response to volatile anesthetics, lead to activation of PKC-ϵ. This isoform may then modulate mitochondrial K_{ATP} channel opening, leading to a state of resistance to the effects of ischemia–reperfusion injury.

That PKC is a mediator of ischemic preconditioning (IPC) was first reported by Ytrehus *et al.*[22] in 1994. They demonstrated that the PKC inhibitors staurosporine and polymyxin B abolished the cardioprotective effects of IPC in rabbits. These findings were replicated by others; for example, Goto *et al.*[23] reported that in intact rats, inhibition of PKC partially or completely abolished the effects of IPC, depending on the strength of the preconditioning stimulus. Pharmacologic preconditioning by opioids,[15] ethanol,[16] and adenosine[24,25] have also been reported to require PKC activation. Cope *et al.*[1] first demonstrated that nonspecific PKC inhibition with chelerythrine inhibited isoflurane-induced preconditioning in the rabbit heart. Toller *et al.*[11] subsequently found similar results using another nonspecific PKC antagonist, bisindolemaleimide, in intact dogs. Volatile anesthetics have previously been shown to stimulate PKC translocation from the cytosolic to the particulate compartment and to increase its activity,[26] possibly by interacting with the regulatory domain of the enzyme.[11] Thus, PKC activation seems to be a component of a common pathway that leads to preconditioning in response to ischemia or pharmacologic stimuli, including volatile anesthetics.

The PKC isoform family is a large group of serine/threonine protein kinases that are distinguished by variable regulatory domains and cofactors. These kinases display diverse tissue and species distribution.[12] The major subfamilies have been defined, and they include the conventional (α, β, γ) and novel (δ, ϵ, η, θ) subfamilies. Volatile anesthetic agents may have opposing effects depending on which isoforms are activated. For example, in pulmonary arteries, isoflurane has been reported to increase or decrease vascular tone when acting through the novel PKC or conventional PKC subfamily, respectively.[27] PKC involvement in the cardioprotective pathway is known to be isoform specific. The δ and ϵ isoforms have been particularly implicated, because translocation of PKC-δ and PKC-ϵ has been detected in ischemic preconditioned hearts.[13] Both are members of the Ca^{2+}-independent, diacylglycerol-activated novel PKC subfamily.[28,29] There is evidence that it is the PKC-ϵ isoform that is specifically required for the triggering of IPC, because IPC does not occur in PKC-ϵ knock-out mice,[30] and inhibition of PKC-δ does not block IPC.[31] κ-Opioid– but not μ-opioid–induced preconditioning is similarly dependent on PKC-ϵ,[14] whereas μ-opioid–induced preconditioning, in contrast, requires PKC-δ.[15] Ethanol-induced preconditioning requires PKC-ϵ, whereas PKC-δ activation by ethanol is reported to exacerbate ischemic cardiac injury.[16]

To the best of our knowledge, no previous study has sought to identify the specific PKC isoform required for APC. We found no direct effect of PKC inhibition alone, either by the nonspecific PKC inhibitor chelerythrine or by either of the isoform-specific inhibitors. When given with the sevoflurane pulses, however, PKC-ϵ isoform–specific inhibition prevented cardioprotection. This was manifested in the postischemic period by worsened mechanical and metabolic function, increased free radical release, and increased infarct size compared with the preconditioned hearts. When a PKC-δ isoform–specific inhibitor was administered during the sevoflurane pulses, cardioprotection was intact. Therefore, PKC-δ seems not to alter cardiac resistance to ischemia–reperfusion, in contrast to its deleterious role reported after ethanol exposure.[16]

We[5,8] and others[6] have previously shown that ROS are components of the APC signaling pathway, because the administration of scavengers with the preconditioning pulses abolished protection. Recent reports have directly demonstrated generation of ROS during exposure

to volatile anesthetics.[7,8] In the present study, we found that ROS generation during sevoflurane exposure was not prevented by PKC inhibition. This is supportive of a postulated pathway in which volatile anesthetics cause generation of ROS, most likely at complex I of the mitochondrial electron transport chain,[32] and in turn, the ROS then activate PKC-ϵ. We can then speculate that PKC-ϵ modulates the sensitivity of the mitochondrial K_{ATP} channel. Work in cardiomyocytes supports this proposed pathway. For instance, Zhang *et al.*[33] found that ROS selectively activated the ϵ isoform of PKC, and Liu *et al.*[34] found that PKC activation sensitizes the K_{ATP} channel. Specific PKC consensus sites have been identified on the sarcolemmal form of the K_{ATP} channel,[24] indicating a molecular basis for phosphorylation and activation of this channel by the enzyme. The mitochondrial K_{ATP} channel has yet to be cloned, but our pharmacologic studies and those of other investigators[35] strongly support a direct interaction of PKC with this form of the channel.

Recent findings strongly suggest that volatile anesthetic–induced PKC translocation and activation is indeed necessary to open K_{ATP} channels and produce myocardial protection. For example, chelerythrine abolished sevoflurane-induced increases in mitochondrial K_{ATP} channel activity in rat ventricular myocytes and prevented protection from ischemic damage.[36] Patch clamp experiments demonstrated that isoflurane does not directly facilitate K_{ATP} channel opening in excised membrane patches but enhances K_{ATP} channel current in a whole cell configuration concomitant with PKC stimulation.[37] PKC may also protect the heart by attenuating myocardial calcium loading through L-type calcium channels.[38]

Limitations of this model include difficulty in making conclusions across species and the possibility that there may be other enzyme intermediates in this pathway; for example, other intracellular kinases may be activated in series or in parallel with PKC. In addition, PKC is known to stimulate tyrosine-activated[39] and mitogen-activated[40] protein kinases. These enzymes have also been implicated in IPC.[41,42] Although we did not test other isoforms of PKC, available evidence points to PKC-ϵ and PKC-δ as the isoforms involved in cardiac preconditioning pathways. In addition, we did not directly measure PKC isoform translocation after anesthetic exposure; therefore, our conclusions are based solely on the effects of pharmacologic inhibition. Finally, isolated guinea pig hearts have limitations with respect to the choice of external solutions and perfusion substrate, and they may have unphysiologically low workloads.

In summary, we have demonstrated that PKC-ϵ but not PKC-δ is involved in preconditioning in response to sevoflurane exposure and that its effect is downstream from volatile anesthetic–induced ROS generation. This elucidates another key step in the signaling pathway by which volatile anesthetics induce a state of resistance to ischemia–reperfusion injury in the heart.

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