Anesthesiology
2000; 92:1400–7

Isoflurane Preconditions Myocardium Against Infarction via *Activation of Inhibitory Guanine Nucleotide Binding Proteins*

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Background: **Isoflurane-induced myocardial protection during ischemia is mediated by adenosine triphosphate–regulated potassium (K_{ATP}) channels; however, the intracellular signal transduction cascade responsible for this process has been incompletely evaluated. The authors tested the hypothesis that isoflurane reduces myocardial infarct size through a G_i protein–mediated process.**

Methods: **Forty-eight hours after pretreatment with vehicle (0.9% saline) or the G_i protein inhibitor pertussis toxin (10 μg/kg intravenously), barbiturate-anesthetized dogs (n = 43) were instrumented for measurement of aortic and left ventricular pressures and maximum rate of increase of left ventricular pressure. All dogs were subjected to a 60-min left anterior descending coronary artery occlusion followed by 3-h reperfusion. In four separate groups, vehicle- or pertussis toxin–pretreated dogs were studied with or without administration of 1 minimum alveolar concentration isoflurane. In two additional groups, dogs received the direct K_{ATP} channel agonist nicorandil (100 μg/kg bolus and 10 $\mu g \cdot kg^{-1} \cdot min^{-1}$ intravenous infusion) in the presence or absence of pertussis toxin pretreatment. Myocardial perfusion and infarct size were measured with radioactive microspheres and triphenyltetrazolium staining, respectively.**

Results: **Isoflurane significantly ($P < 0.05$) decreased infarct size to 7 ± 2% of the area at risk compared with control experiments (26 ± 2%). Pertussis toxin pretreatment alone had no effects on myocardial infarct size (31 ± 4%) but blocked the beneficial effects of isoflurane (21 ± 3%). Nicorandil decreased infarct size (11 ± 2%), but, in contrast to isoflurane, this effect was independent of pertussis toxin pretreatment (11 ± 1%).**

Conclusion: **Isoflurane reduces myocardial infarct size by a G_i protein–mediated mechanism *in vivo*. (Key words: Myocardial ischemia; pertussis toxin.)**

A GROWING body of evidence indicates that volatile anesthetics reduce reversible[1,2] and irreversible[3–7] myocardial ischemic injury *in vivo*, a process termed anesthetic-induced preconditioning (APC). Activation of adenosine triphosphate–regulated potassium (K_{ATP}) channels plays a central role in these protective effects.[1] The mechanism by which volatile anesthetics activate K_{ATP} channels is incompletely understood. Recent findings demonstrate that volatile anesthetic–mediated protection is attenuated by administration of adenosine subtype 1 (A_1)-receptor antagonists[1] and protein kinase C (PKC) inhibitors,[2,3] suggesting that volatile agents may activate K_{ATP} channels by a similar signal transduction pathway as demonstrated during ischemic preconditioning. Inhibitory guanine (G_i) nucleotide-binding proteins have previously been shown to couple A_1 receptors to K_{ATP} channels,[8] and blockade of G_i proteins with pertussis toxin (PTX) abolishes the cardioprotective effects of ischemic preconditioning.[9] Thus, we tested the hypothesis that antagonism of G_i proteins with PTX also attenuates reductions in myocardial infarct size pro-

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Received from the Departments of Anesthesiology, Pharmacology and Toxicology, and Medicine (Division of Cardiovascular Diseases), the Medical College of Wisconsin and the Clement J. Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin. Submitted for publication October 11, 1999. Accepted for publication January 10, 2000. Supported in part by a Max Kade Research Fellowship from the Austrian Science Foundation (to Dr. Toller) Vienna, Austria; grants no. HL 03690 (to Dr. Kersten), AA 12331 (to Dr. Pagel), and HL 54280 (to Dr. Warltier) and Anesthesiology Research Training Grant No. GM 08377, from the United States Public Health Service, Bethesda, Maryland (to Dr. Warltier).

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duced by isoflurane but not by the direct K_{ATP} channel opener nicorandil.[10]

Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. All procedures were in conformity with the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society[11] and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*.[12]

Surgical Preparation

The experimental methods have been previously described in detail.[13] Briefly, mongrel dogs (weight = 23 ± 1 kg; mean ± SEM) were anesthetized with sodium barbital (200 mg/kg) and sodium pentobarbital (15 mg/kg) and ventilated with an air/oxygen mixture (fraction of inspired oxygen = 0.25) after tracheal intubation. Tidal volume and respiratory rate were adjusted to maintain arterial blood gas tensions within a physiologic range. A double pressure transducer–tipped catheter was inserted into the aorta and left ventricle (LV) *via* the left carotid artery to measure aortic and LV pressures, respectively. The maximum rate of increase of LV pressure ($+dP/dt_{max}$) was obtained by electronic differentiation of the LV pressure waveform. The femoral artery and vein were cannulated for the withdrawal of reference blood flow samples and fluid administration, respectively. A thoracotomy was performed at the left fifth intercostal space. A heparin-filled catheter was inserted into the left atrial appendage for administration of radioactive microspheres. A 1.0-cm segment of the left anterior descending (LAD) coronary artery was dissected immediately distal to the first diagonal branch, and a silk ligature was placed around this vessel for production of coronary artery occlusion and reperfusion. Regional myocardial perfusion was measured in the ischemic (LAD) and normal (left circumflex coronary artery) zones using radioactive microspheres. Myocardial infarct size was determined with triphenyltetrazolium chloride staining at the completion of each experiment as previously described.[4] End-tidal concentrations of isoflurane were measured at the tip of the endotracheal tube by an infrared anesthetic gas analyzer. The canine minimum alveolar concentration value of isoflurane used in the present investigation was 1.28%.[14] Hemodynamic data were continuously monitored throughout the experiment, recorded on a polygraph, and digitized using a computer interfaced with an analog-to-digital converter.

Experimental Protocol

The experimental design is illustrated in figure 1. Forty-eight hours before each dog was subjected to a 60-min LAD occlusion followed by 3 h of reperfusion, they were randomly assigned to receive an intravenous bolus of vehicle (0.9% saline) or PTX (10 μg/kg; Sigma Chemical, St. Louis, MO). In four separate experimental groups, dogs pretreated with vehicle or PTX were studied in the presence or absence of administration of 1.0 minimum alveolar concentration isoflurane (end-tidal concentration) that was discontinued immediately before the 60-min LAD occlusion. These experiments tested the hypothesis that isoflurane-mediated myocardial protection involves activation of G_i proteins. In two additional groups of experiments, vehicle- or PTX-pretreated dogs received intravenous nicorandil (100 μg/kg bolus and 10 $\mu g \cdot kg^{-1} \cdot min^{-1}$ infusion) initiated 15 min before LAD occlusion and discontinued at the onset of reperfusion. This dose of nicorandil has been previously shown to reduce myocardial infarct size in the absence of systemic hemodynamic effects in dogs.[15] These experiments tested the hypothesis that direct activation of K_{ATP} channels protects ischemic myocardium independent of G_i proteins. At the completion of each experiment, dogs received intravenous injections of acetylcholine (4 and 10 μg/kg), and mean arterial and LV pressure responses were recorded. The latter experiments verified the efficacy of PTX-induced G_i-protein inhibition, as previously described.[9,16]

Statistical Analysis

Statistical analysis of data within and between groups was performed using multiple analysis of variance for repeated measures with *post hoc* analysis by the Student *t* test with Bonferroni's correction for multiplicity. Changes within and between groups were considered statistically significant at $P < 0.05$. All data are expressed as mean ± SEM.

Results

Forty-three dogs were instrumented to obtain 38 successful experiments. Two dogs were excluded because

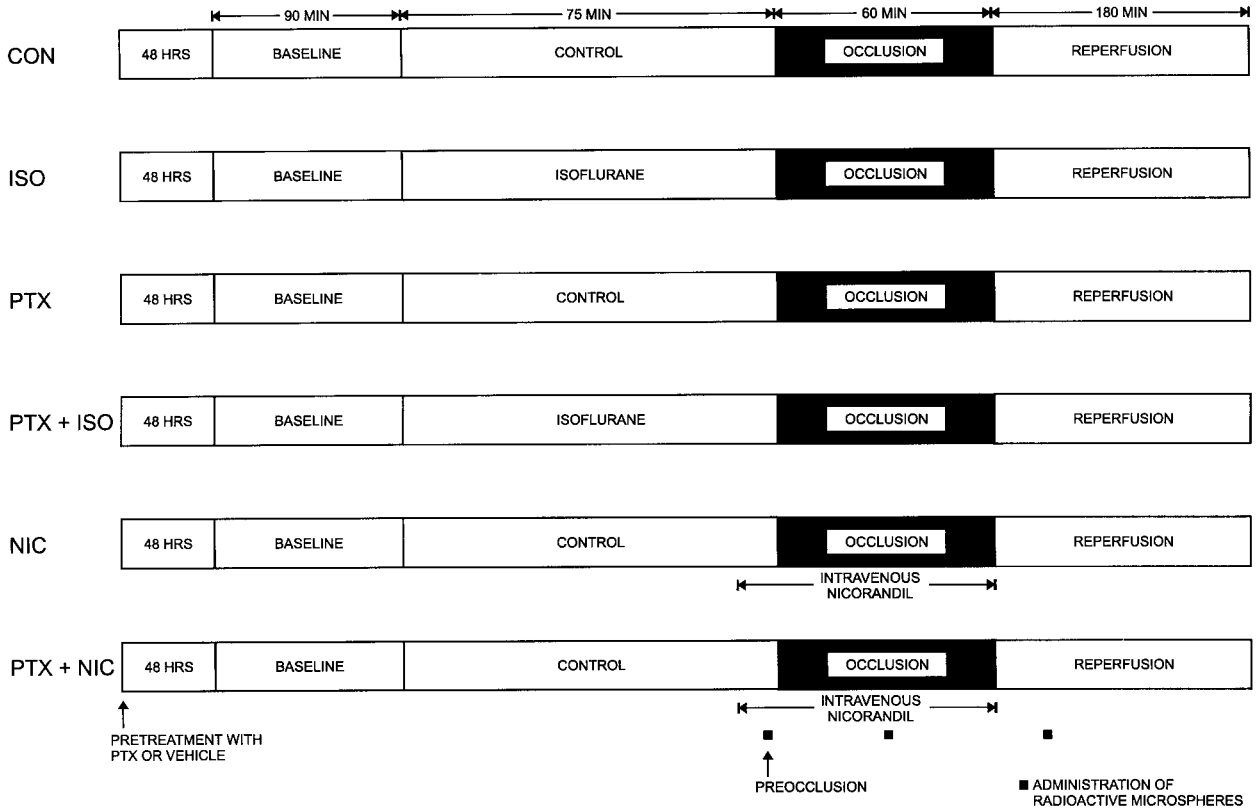

Fig. 1. Schematic illustration of the experimental protocol used in the present investigation (see text). CON = control; ISO = isoflurane; PTX = pertussis toxin; NIC = nicorandil.

of intractable ventricular fibrillation during LAD occlusion or reperfusion (one control; one PTX plus nicorandil). Two dogs were excluded because transmural coronary collateral blood flow exceeded 0.2 $ml \cdot min^{-1} \cdot g^{-1}$ (one control; one PTX plus isoflurane). One dog (control) was excluded because of the presence of heartworms.

Systemic Hemodynamics

Pertussis toxin pretreatment significantly ($P < 0.05$) reduced mean arterial and LV systolic pressures at baseline (table 1). No other differences in baseline systemic hemodynamics were observed between experimental groups. Isoflurane decreased heart rate, mean arterial and LV systolic pressures, rate–pressure product, and LV $+dP/dt_{max}$. Isoflurane produced similar hemodynamic effects in the presence and absence of PTX pretreatment. Nicorandil caused minimal cardiovascular effects. LAD occlusion increased LV end-diastolic pressure in all experimental groups. Hemodynamics were similar between groups during LAD occlusion and reperfusion.

Regional Myocardial Perfusion

Transmural myocardial blood flow in the ischemic (LAD) and normal (left circumflex coronary artery) regions is summarized in table 2. There were no intergroup differences in myocardial blood flow before, during, or after LAD occlusion.

Myocardial Infarct Size

The area at risk was similar between groups (control, 40 ± 3%; isoflurane alone, 38 ± 2%; PTX alone, 42 ± 2%; PTX and isoflurane, 42 ± 3%; nicorandil, 45 ± 1%; PTX and nicorandil, 40 ± 2% of the LV). Isoflurane significantly ($P < 0.05$) reduced myocardial infarct size to 7 ± 2% of the area at risk (fig. 2) compared with control experiments (26 ± 2%). PTX pretreatment abolished the protective effects of isoflurane (21 ± 3%) but had no effect on infarct size when administered alone (31 ± 4%). Nicorandil decreased infarct size independent of PTX (11 ± 1% and 11 ± 2% in the presence and absence of PTX pretreatment, respectively; fig. 2).

Table 1. Systemic Hemodynamics

				Reperfusion		
	Baseline	Preocclusion	30 min CAO	1 h	2 h	3 h
HR (beats/min)						
CON	129 ± 9	129 ± 9	123 ± 7	117 ± 8	117 ± 9	118 ± 10
ISO	134 ± 9	105 ± 6*	114 ± 8*	114 ± 6*	110 ± 6*	111 ± 7*
PTX	155 ± 3	155 ± 3†	149 ± 5	140 ± 11	136 ± 10	134 ± 10
PTX + ISO	147 ± 4	122 ± 5*‡	129 ± 7	133 ± 10	136 ± 9	133 ± 11
NIC	126 ± 5	124 ± 5	127 ± 4	122 ± 7	122 ± 7	119 ± 7
PTX + NIC	142 ± 4	140 ± 6	135 ± 6	121 ± 4*	119 ± 5*	118 ± 5*
MAP (mmHg)						
CON	94 ± 4	94 ± 4	82 ± 6	93 ± 7	97 ± 7	97 ± 7
ISO	112 ± 5	76 ± 3*	94 ± 4*	103 ± 3	107 ± 5	108 ± 4
PTX	87 ± 4	87 ± 4	88 ± 6	95 ± 7	92 ± 6	96 ± 4
PTX + ISO	86 ± 3§	68 ± 5*†	86 ± 4	91 ± 5	97 ± 2	93 ± 3
NIC	100 ± 6	91 ± 5	71 ± 4*	88 ± 5	96 ± 4	97 ± 5
PTX + NIC	92 ± 4	89 ± 4	82 ± 5	90 ± 5	93 ± 8	89 ± 7
RPP (beats/min · $mmHg^{-1} \cdot 10^{-3}$)						
CON	13.6 ± 1.3	13.6 ± 1.3	11.1 ± 1.2*	11.8 ± 1.4	12.5 ± 1.6	12.6 ± 1.6
ISO	16.4 ± 1.0	9.2 ± 0.8*	12.0 ± 1.3*	12.9 ± 1.1*	12.9 ± 1.4*	13.2 ± 1.4*
PTX	15.8 ± 0.7	15.8 ± 0.7	14.7 ± 1.3	15.0 ± 2.1	13.9 ± 1.9	14.5 ± 1.7
PTX + ISO	15.2 ± 0.8	10.2 ± 1.0*‡	13.0 ± 1.3	14.0 ± 1.6	14.9 ± 1.3	14.2 ± 1.5
NIC	13.8 ± 1.4	12.6 ± 1.3	9.8 ± 0.8*	11.6 ± 0.9	12.5 ± 1.0	12.4 ± 1.1
PTX + NIC	14.7 ± 0.9	14.0 ± 0.9	12.0 ± 1.1*	11.7 ± 1.0*	11.9 ± 1.5*	11.4 ± 1.4*
LVSP (mmHg)						
CON	103 ± 4	103 ± 4	87 ± 7	97 ± 8	102 ± 9	102 ± 9
ISO	120 ± 5	85 ± 3*	101 ± 5*	110 ± 4	114 ± 6	116 ± 5
PTX	99 ± 3§	99 ± 3	95 ± 5	103 ± 6	100 ± 5	105 ± 4
PTX + ISO	99 ± 3§	81 ± 4*†	98 ± 4	108 ± 8	110 ± 4	108 ± 3
NIC	105 ± 5	97 ± 5	72 ± 3*§	90 ± 3*	102 ± 4	101 ± 6
PTX + NIC	104 ± 4	99 ± 5	90 ± 7	95 ± 6	98 ± 9	95 ± 9
LVEDP (mmHg)						
CON	6 ± 1	6 ± 1	13 ± 2*	14 ± 2*	14 ± 2*	13 ± 2
ISO	8 ± 2	9 ± 1	13 ± 1*	11 ± 1	12 ± 1	11 ± 1
PTX	8 ± 1	8 ± 1	16 ± 2*	15 ± 4	14 ± 2	15 ± 2
PTX + ISO	6 ± 1	7 ± 1	15 ± 2*	11 ± 2	11 ± 2	12 ± 3
NIC	9 ± 2	8 ± 2	13 ± 2*	12 ± 2	13 ± 2	13 ± 2
PTX + NIC	4 ± 1	4 ± 1	13 ± 2*	10 ± 3	6 ± 1	7 ± 1
$+dP/dt_{max}$ (mmHg/s)						
CON	1920 ± 220	1920 ± 220	1420 ± 200*	1410 ± 120*	1400 ± 130*	1360 ± 140*
ISO	1950 ± 190	1120 ± 80*†	1520 ± 110*	1300 ± 120*	1350 ± 160*	1390 ± 150*
PTX	2340 ± 250	2340 ± 250	1760 ± 200	1540 ± 150*	1530 ± 120*	1470 ± 120*
PTX + ISO	2010 ± 260	1140 ± 100*‡	1580 ± 160	1490 ± 140*	1540 ± 160	1450 ± 160*
NIC	1550 ± 150	1580 ± 130	1150 ± 90*	1350 ± 100	1350 ± 90	1260 ± 80
PTX + NIC	2370 ± 230	2450 ± 210‖	1810 ± 230*	1430 ± 100*	1410 ± 160*	1430 ± 100*

Data are mean ± SEM.

* Significantly ($P < 0.05$) different from baseline.

† Significantly ($P < 0.05$) different from the corresponding value in dogs receiving saline (CON).

‡ Significantly ($P < 0.05$) different from the corresponding value in dogs pretreated with pertussis toxin alone (PTX).

§ Significantly ($P < 0.05$) different from the corresponding value in dogs receiving isoflurane alone (ISO).

‖ Significantly ($P < 0.05$) different from the corresponding value in dogs receiving nicorandil alone (NIC).

CAO = coronary artery occlusion; HR = heart rate; MAP = mean aortic blood pressure; RPP = rate-pressure product; LVSP and LVEDP = left ventricular systolic and end-diastolic pressures, respectively; $+dP/dt_{max}$ = maximal rate of increase of left ventricular pressure; CON = control; ISO = isoflurane; PTX = pertussis toxin; NIC = nicorandil.

Table 2. Transmural Myocardial Blood Flow in the Ischemic and Normal Region (ml · min^{-1} · g^{-1})

	Preocclusion	Coronary Artery Occlusion	Reperfusion
Ischemic region			
CON	0.95 ± 0.11	0.06 ± 0.01*	1.70 ± 0.25*
ISO	0.72 ± 0.08	0.07 ± 0.02*	1.10 ± 0.16
PTX	1.20 ± 0.18	0.09 ± 0.02*	1.64 ± 0.29
PTX + ISO	0.82 ± 0.12	0.11 ± 0.02*	1.51 ± 0.15*
NIC	0.87 ± 0.10	0.08 ± 0.01*	1.42 ± 0.22*
PTX + NIC	1.30 ± 0.27	0.10 ± 0.01*	1.23 ± 0.13
Normal region			
CON	1.35 ± 0.30	1.21 ± 0.12	1.37 ± 0.19
ISO	0.96 ± 0.11	0.96 ± 0.11	1.09 ± 0.13
PTX	1.58 ± 0.19	1.41 ± 0.23	1.15 ± 0.24
PTX + ISO	1.02 ± 0.14	1.11 ± 0.08	1.19 ± 0.15
NIC	1.14 ± 0.16	0.94 ± 0.05	0.90 ± 0.06
PTX + NIC	1.63 ± 0.25	1.34 ± 0.22	0.93 ± 0.09*

Data are mean ± SEM.

* Significantly ($P < 0.05$) different from preocclusion.

CON = control; ISO = isoflurane; PTX = pertussis toxin; NIC = nicorandil.

Acetylcholine-induced Hypotension

Mean arterial pressure responses to acetylcholine are depicted in figures 3 and 4. Acetylcholine (4 and 10 μg/kg) decreased mean arterial pressure in control experiments (65 ± 5% and 62 ± 6% of baseline values, respectively). Pretreatment with PTX significantly attenuated these effects.

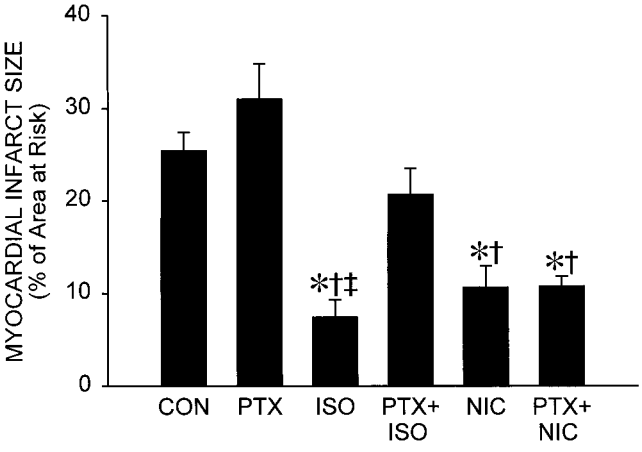

Fig. 2. Histograms depicting myocardial infarct size as a percentage of area at risk in dogs pretreated with vehicle (CON) or pertussis toxin (PTX) in the presence or absence of either 1.0 minimum alveolar concentration isoflurane (ISO) or nicorandil (NIC). *Significantly ($P < 0.05$) different from CON; †significantly ($P < 0.05$) different from PTX alone; ‡significantly ($P < 0.05$) different from PTX plus ISO.

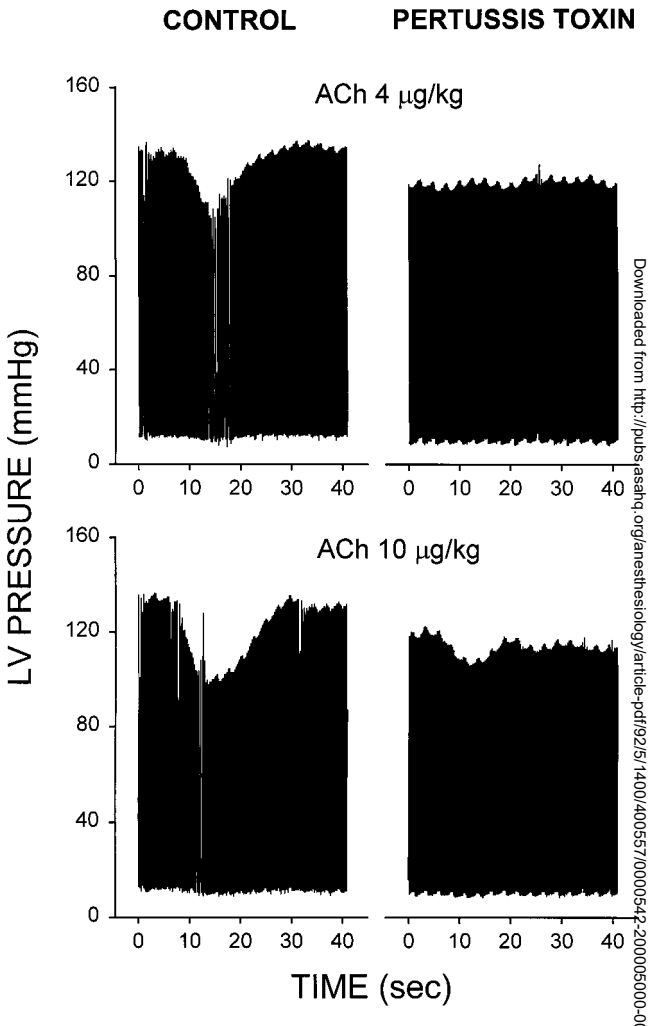

Fig. 3. Alterations in left ventricular (LV) pressure tracings recorded during administration of intravenous acetylcholine in representative dogs pretreated with vehicle (control, *left*) and pertussis toxin (*right*).

Discussion

Experimental evidence indicates that volatile anesthetics exert protective actions during ischemia and reperfusion by activating K_{ATP} channels.[4–7] Opening of K_{ATP} channels has also been shown to play a pivotal role in mediating the protective effects of ischemic preconditioning (IPC).[17] These findings suggest that the intracellular signal transduction pathways responsible for both APC and IPC may be similar. The mechanism by which APC or IPC activates the K_{ATP} channel is incompletely understood. It has been proposed that IPC causes activation of adenosine A_1 receptors, which are coupled to G_i proteins. Activation of PKC[18,19] may subsequently

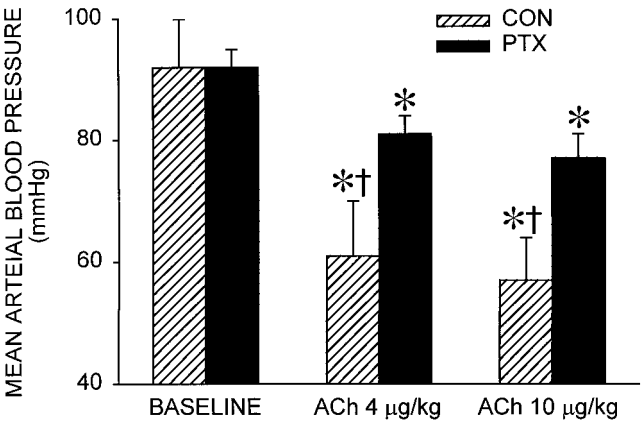

Fig. 4. Histograms depicting the acetylcholine-induced decreases in mean arterial pressure in dogs pretreated with vehicle (CON) or pertussis toxin (PTX). *Significantly ($P < 0.05$) different from baseline values; †significantly ($P < 0.05$) different from PTX-pretreated dogs.

phosphorylate and enhance K_{ATP} channel opening by decreasing its sensitivity to inhibition by ATP.[20] The beneficial effects of IPC are abolished by pharmacologic blockade of A_1 receptors,[17] G_i proteins,[9,21,22] and PKC.[23] Blockade of both A_1 receptors and PKC also attenuates the protective effects of isoflurane to enhance recovery of stunned myocardium[1,2] and blocks the protective effects of isoflurane[3,5] and halothane[3] during experimental myocardial infarction. These findings suggest that G_i proteins may also be involved in signal transduction during APC.

The present results indicate that G_i proteins are an essential element of isoflurane-induced K_{ATP} channel activation. G_i-protein blockade with PTX alone did not alter myocardial infarct size but completely abolished the protective effects of isoflurane independent of the alterations of systemic hemodynamics produced by this volatile agent. In contrast, PTX pretreatment did not block the protective effects of the direct K_{ATP}-channel agonist nicorandil. These findings indicate that K_{ATP} channels remain functionally intact during inhibition of G_i proteins, and direct stimulation of these channels by nicorandil, at a site presumably independent of the ATP inhibition site, is capable of producing a cardioprotective effect. Thus, the present findings support the contention that volatile anesthetics may activate K_{ATP} channels through second messengers.

The direct effects of volatile anesthetics on the K_{ATP} channel *in vitro* remain unclear. Using patch-clamp techniques in rabbit ventricular myocytes, Han *et al.*[24] demonstrated that isoflurane directly inhibits K_{ATP}-channel activity but paradoxically increases the probability of K_{ATP}-channel opening. The latter action probably occurred through an effect of isoflurane to decrease channel sensitivity to inhibition by ATP. Adenosine has also been shown to enhance K_{ATP}-channel opening by altering channel sensitivity to ATP, and this effect is mediated by activation of A_1 receptors and G_i proteins.[25,26] Recent evidence indicates that isoflurane does not potentiate K_{ATP}-channel activity in the presence of adenosine in a cell-free environment[27] but increases K_{ATP}-channel current in whole ventricular myocytes. These data suggest that cellular mechanisms underlying anesthetic-induced activation of K_{ATP} channels require the presence of an intracellular second messenger system. The present results support the latter hypothesis because blockade of G_i proteins abolished the protective effects of isoflurane but not the actions of the direct K_{ATP}-channel agonist nicorandil, whose actions are thought to occur at a site distinct from the ATP regulatory site.[10,26]

Pertussis toxin was used in the present investigation to block G_i proteins, and the mechanism and duration of action of this toxin have been previously characterized. Endoh *et al.*[28] demonstrated in isolated rat atria that intravenous administration of PTX blocked the G_i protein–mediated negative chronotropic and inotropic effects of the muscarinic cholinergic agonist carbachol. These effects were time-dependent and most pronounced 48 h after administration. Accordingly, several *in vitro*[22,29,30] and *in vivo*[9,16,21,31] studies have used PTX to block G_i proteins, and the dose used in the present investigation has been shown to be effective in the dog.[16] The efficacy of PTX to block G_i proteins was confirmed in the present investigation using an acetylcholine challenge, as previously described.[9,16] Intravenous acetylcholine causes pronounced decreases in arterial pressure mediated through activation of G_i proteins, and pretreatment with PTX modulates this response. We observed marked attenuation of acetylcholine-induced decreases in arterial pressure in PTX-pretreated dogs, indicating that G_i proteins were effectively blocked in the present investigation. PTX pretreatment is well tolerated in a variety of animal species, but decreases in baseline arterial pressure are often observed.[9,31,32] In the present investigation, PTX pretreatment also caused slight decreases in baseline mean arterial and LV systolic pressures. However, it is unlikely that these small hemodynamic changes were responsible for the failure of isoflurane to reduce myocardial infarct size in PTX-pretreated dogs.

The present findings must be interpreted within the constraints of several other potential limitations. Isoflurane-induced decreases in heart rate, mean arterial pressure, and myocardial contractility may have caused favorable alterations in myocardial oxygen supply–demand relations and contributed to a reduction in infarct size. However, blockade of G_i proteins with PTX completely abolished the protective effect of isoflurane without affecting the hemodynamic actions of this anesthetic agent. Nevertheless, coronary venous oxygen tension was not measured and myocardial oxygen consumption was not directly quantified in the present investigation. Interpretation of the present findings should also be qualified because only a single end-tidal concentration of isoflurane was used. Higher inspired concentrations of isoflurane may have produced reductions of myocardial infarct size *via* effects on K_{ATP} channels despite pretreatment with PTX. Experiments with nicorandil were completed as positive controls to demonstrate that PTX does not prevent direct K_{ATP}-channel activation and reductions of myocardial infarct size. Nicorandil possesses nitrate-like characteristics that could contribute to cardioprotection independent of K_{ATP} channels. However, Mizumura *et al.*[33] demonstrated that the infarct size–reducing effect of nicorandil is specifically mediated by activation of K_{ATP} channels *in vivo* and is not blocked by nitric oxide inhibition with methylene blue. Nicorandil has also been shown to activate mitochondrial K_{ATP} channels,[34] and these channels have been suggested to be critical mediators of ischemic preconditioning.[35] The subcellular location (sarcolemmal *vs.* mitochondrial) of K_{ATP} channels modulated by isoflurane is unknown, but preliminary results with desflurane suggest that mitochondrial K_{ATP} channels are also involved in anesthetic-mediated myocardial protection.[7] Whether PTX-induced G_i-protein inhibition differentially alters sarcolemmal *versus* mitochondrial K_{ATP} channel–linked cardioprotective mechanisms is unknown.

In summary, the present results indicate that G_i proteins play a critical role in isoflurane-mediated reductions of experimental myocardial infarct size in dogs and support the contention that ischemic preconditioning and volatile anesthetics activate similar signal transduction pathways.

The authors thank Drs. Werner List and Helfried Metzler (Department of Anesthesiology and Intensive Care Medicine, University of Graz, Austria) for their gracious support, and David Schwabe for technical assistance.

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