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Differential Effects of Anesthetics on Mitochondrial K_{ATP} Channel Activity and Cardiomyocyte Protection

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Background: **Mitochondrial adenosine triphosphate–sensitive potassium (mitoK$_{ATP}$) channels play a pivotal role in mediating cardiac preconditioning. The effects of intravenous anesthetics on this protective channel have not been investigated so far, but would be of importance with respect to experimental as well as clinical medicine.**

Methods: **Live cell microscopy was used to visualize and measure autofluorescence of flavoproteins, a direct reporter of mitoK$_{ATP}$ channel activity, in response to the direct and highly selective mitoK$_{ATP}$ channel opener diazoxide, or to diazoxide following exposure to various anesthetics commonly used in experimental and clinical medicine. A cellular model of ischemia with subsequent hypoosmolar trypan blue staining served to substantiate the effects of the anesthetics on mitoK$_{ATP}$ channels with respect to myocyte viability.**

Results: **Diazoxide-induced mitoK$_{ATP}$ channel opening was significantly inhibited by the anesthetics *R*-ketamine, and the barbiturates thiopental and pentobarbital. Conversely, urethane, 2,2,2-trichloroethanol (main metabolite of α-chloralose and chloral hydrate), and the opioid fentanyl potentiated the channel-opening effect of diazoxide, which was abrogated by coadministration of chelerythrine, a specific protein kinase C inhibitor. *S*-ketamine, propofol, xylazine, midazolam, and etomidate did not affect mitoK$_{ATP}$ channel activity. The significance of these modulatory effects of the anesthetics on mitoK$_{ATP}$ channel activity was substantiated in a cellular model of simulated ischemia, where diazoxide-induced cell protection was mitigated by *R*-ketamine and the barbiturates, while urethane, 2,2,2-trichloroethanol, and fentanyl potentiated myocyte protection.**

Conclusions: **These results suggest distinctive actions of individual anesthetics on mitoK$_{ATP}$ channels and provide evidence that the choice of background anesthesia may play a role in cardiac protection in both experimental and clinical medicine.**

SINGLE or multiple brief episodes of sublethal cardiac ischemia produce a marked protection against subsequent prolonged ischemia. This phenomenon, which is termed ischemic preconditioning, is one of the most powerful means of attaining myocardial protection (30–90% reduction in infarct size).[1] The protection is typically present immediately after preconditioning but vanishes after 2–3 h (classic or early preconditioning). An additional sustained protection occurs 12–24 h after the initial preconditioning stimulus, lasting for up to 72 h (second window of protection or late preconditioning). Although it was initially hypothesized that the surface or sarcolemmal adenosine triphosphate–sensitive potassium (sarcK$_{ATP}$) channel mediates this protection, overwhelming evidence now supports the concept that mitochondrial K$_{ATP}$ (mitoK$_{ATP}$) channels, and not sarcK$_{ATP}$ channels, play the pivotal role in early and late preconditioning.[2,3] Accordingly, the mitoK$_{ATP}$ channel may serve as the putative end effector in the multiple and complex signaling cascades toward the preconditioned state of the myocardium, whereby its opening leads to optimization of the mitochondrial energy production,[4] decreased mitochondrial Ca^{2+} overload,[5] and increased gene expression of myocyte-inherent cytoprotective proteins.[3]

Preconditioning can also be elicited and amplified by pharmacological means. Our previous study clearly showed that the volatile anesthetics isoflurane and sevoflurane mimic cardiac preconditioning by priming the activation of the mitoK$_{ATP}$ channel in adult rat ventricular myocytes.[6] The study also demonstrated that this protection is specifically mediated by multiple protein kinase C (PKC)-coupled signaling pathways. Many anesthetics have profound effects on mitochondrial membranes at concentrations as low as those known to produce general anesthesia and can destabilize lipid–protein interactions or induce conformational changes in proteins. It therefore appears likely that other anesthetics than volatile anesthetics may also affect mitoK$_{ATP}$ channel activity and thereby modulate myocyte protection. The aim of the present study was to investigate whether the finding of the modulatory effect by volatile anesthetics on the mitoK$_{ATP}$ channel activity also pertains to other commonly employed intravenous anesthetics.

Materials and Methods

This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Zurich.

Preparation of Isolated Cardiac Myocytes

Ca^{2+}-tolerant adult rat ventricular myocytes were isolated from hearts of male Sprague-Dawley rats (300–350 g)

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by standard enzymatic technique, as previously described.[7] The animals were heparinized (500 U intraperitoneally) and 30 min later decapitated. To avoid putative effects on K_{ATP} channel activity,[8,9] no anesthetics were administered. The isolated myocytes were resuspended in serum-free defined culture medium consisting of DMEM with 2 mg/ml bovine serum albumin, 2 mM L-carnitine, 5 mM creatine, and 5 mM taurine. No antibiotics were added to culture medium to avoid any putative effect on K_{ATP} channels. Myocytes were cultured for 3 h before experiments to allow reestablishment of normal electrolyte gradients. Purity of cardiomyocyte cultures was determined by counting the percentage of myosin positive–staining cells using immunofluorescence with a myosin heavy chain specific antibody, MF-20.[7] Ninety-nine percent of cells stained positive.

Time-Lapse Analysis of Flavoprotein-induced Autofluorescence

Isolated myocytes were cultured at a density of 100–150 cells/mm^2 on 20-mm round glass coverslips precoated with laminin (1 μg/cm^2; Sigma, St. Louis, MO) placed in 35-mm plastic culture dishes. The fluorescence of flavoproteins (succinate dehydrogenase, glygerol-3-phosphate dehydrogenase, acyl-CoA dehydrogenase) served to determine mitochondrial redox state, which directly reflects mitoK_{ATP} channel activity.[10,11] After 3 h, the dishes were washed with phosphate-buffered saline to remove unattached cells. Experiments were performed over the next 6 h. Similar treatment protocols and concentrations of reagents were described previously.[6,12,13] Briefly, myocytes on glass coverslips were placed in a customized perfusion chamber with a volume of 0.5 ml, covered with a 25-mm glass coverslip, which was sealed with vacuum grease (Fisher, Pittsburgh, PA). The chamber was perfused at room temperature (~25°C) with a buffer solution at a flow rate of 0.5 ml/min containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM HEPES (pH 7.4). Myocytes were exposed in series to the following solutions: plain buffer solution (baseline), buffer with 100 μM diazoxide (Biomol, Plymouth Meeting, PA) followed by a washout with plain buffer, buffer with the anesthetic alone (for some experiments in the presence or absence of 2 μM chelerythrine [Biomol]) followed by buffer with the anesthetic and 100 μM diazoxide, and finally buffer with 100 μM 2,4-dinitrophenol (Sigma). The effects of the following anesthetics on autofluorescence were determined: urethane (ethyl carbamate) at 10 mM, thiopental at 100 μM, pentobarbital at 100 μM (all purchased from Sigma), *R*-ketamine at 10 μM and *S*-ketamine at 10 and 100 μM (Pfizer AG, Zurich, Switzerland), propofol (as chemical reagent dissolved in dimethyl sulfoxide [DMSO]; Sigma) at 10 and 200 μM, xylazine at 10 and 100 μM (Bayer, Provet AG, Lyssach, Switzerland), midazolam at 0.1 and 1 μM (Roche, Basel, Switzerland), etomidate (dissolved in propylene glycol; Sigma) at 100 and 200 μM (Janssen-Cilag, Baar, Switzerland), fentanyl (Janssen-Cilag) at 100 nM, and 2,2,2-trichloroethanol (Sigma) at 1 mM, respectively. Separate experiments evaluated the effects of 100 μM 5-hydroxydecanoate (Sigma) and 50 μM HMR-1098 (a gift from Aventis AG, Frankfurt am Main, Germany) on diazoxide-induced flavoprotein oxidation. Control experiments were done for propylene glycol. Propylene glycol was prepared as a 35% vol/vol solution similar to the concentration in the clinical formulation of etomidate, and the same volume of that solution was added to the preparations. Propofol and diazoxide were dissolved in buffer containing DMSO 0.1%. Importantly, propylene glycol and DMSO alone at the concentrations used in the experiments ($<$ 1% for propylene glycol and 0.1% for DMSO) had no effect on autofluorescence of myocytes (percent flavoprotein oxidation: $<$ 1% propylene glycol 15 $\pm$ 3%; 0.1% DMSO 16 $\pm$ 3%; baseline 15 $\pm$ 4%; $P > 0.98$). Also, separate experiments showed that chelerythrine alone or followed by diazoxide did not affect autofluorescence of flavoproteins.[6] For each experimental group, myocytes of 8 different rat hearts were used (n = 8). An upright microscope (Axioplan2; Zeiss, Jena, Germany) equipped with a xenon arc lamp and the appropriate filter set (excitation at 480 nm and emission at 530 nm) was used to monitor the flavoprotein-induced autofluorescence of myocytes. Images were captured using a cooled CCD camera (ORCA-100, 12 bit digital output; Hamamatsu Photonics, Herrsching, Germany) controlled by the image acquisition software (Openlab; Improvision, Lexington, MA). Every 15 s, fluorescence intensity was recorded by exposing myocytes for 125 ms using a computer-controlled high-speed shutter (Orbit; Improvision). Four sequential images were averaged. Calibration of flavoprotein fluorescence was achieved by setting fluorescence obtained after 2,4-dinitrophenol exposure to 100% (fully oxidized flavoproteins). All measurements of flavoprotein oxidation were expressed as percentage of 2,4-dinitrophenol–induced maximal autofluorescence. In each experiment, the fluorescence of 5–10 myocytes was monitored with a 20$\times$ objective lens (LD Achroplane, NA = 0.4; Zeiss). A binary mask was separately drawn for each myocyte using Openlab software (Improvision) to exclude artifacts and background noise. The time-lapse of flavoprotein fluorescence of multiple individual myocytes could be selectively and simultaneously tracked. An artificial color scale was used to visualize the relative intensity of mitochondrial flavoprotein oxidation states. Myocytes with an increased initial autofluorescence greater than 30% of the peak 2,4-dinitrophenol–induced autofluorescence were excluded from analysis and considered as damaged cells.[6]

Simulated Ischemia of Cardiomyocytes

Myocytes were suspended in the incubation buffer containing 119 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 11 mM glucose, 24.9 mM creatine, and 58.5 mM taurine and supplemented with 1% basal medium Eagle amino acids (GIBCO, Paisley, Scotland) and 1% minimum essential medium nonessential amino acids (Sigma) (at pH 7.4). The various anesthetics were administered to myocytes at increasing concentrations 15 min before initiation of 60 min of ischemia in the presence or absence of 100 μM diazoxide. Depending on the treatment group, 2 μM chelerythrine, 100 μM 5-hydroxydecanoate, or 50 μM HMR-1098 was administered 5 min prior to the administration of the anesthetics. Diazoxide and propofol were dissolved in DMSO containing buffer solution (final concentration 0.1%), and etomidate was dissolved in propylene glycol (final concentration $< 1\%$). Because DMSO and propylene glycol were used as solvents for these drugs, a series of experiments was performed in which DMSO and propylene glycol alone were administered to myocytes. DMSO and propylene glycol alone, at the concentration used in the experiments, had no effect on survival of myocytes. After the various treatment modalities, 1 ml of the myocyte suspension was pipetted from the bottom of the 10-ml tube into a 1.5-ml microcentrifuge tube and centrifuged for 20 s at 15*g*. The supernatant was discarded, and 0.25 ml of mineral oil (Sigma) was layered onto the myocyte pellet to inhibit gaseous diffusion of oxygen.[13,14] Following 60 min of incubation in a standard incubator at 37°C, 15 μl of myocytes was sampled through the mineral oil layer and mixed with 150 μl of a hypotonic trypan blue staining solution containing 11.9 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 2.7 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.5% glutaraldehyde, and 0.5% trypan blue.[15,16] For each experimental group, cells of 8 different rat hearts (n = 8) were used, and experiments were performed in duplicate. Myocyte viability was assessed by counting the number of myocytes staining clearly after 3 min of exposure to the hypoosmolar trypan blue staining solution. Five randomly chosen fields (1 mm^2) were counted at 10 $\times$ 10 magnification with a phase-contrast microscope in duplicate and expressed as the total percentage of total viable myocytes before ischemia. The percentage of myocytes viable at the beginning of the experiments was 90 $\pm$ 3% (n = 30). The small percentage of nonviable myocytes was due to the enzymatic isolation procedure.

Statistical Analysis

Data are expressed as mean $\pm$ SD. Analysis of variance with *post hoc* Scheffé test for multiple comparisons was performed to determine statistical significance of multiple treatments. $P < 0.05$ was considered to be significant (StatView Version 4.5; Abacus Concepts, Berkeley, CA).

Results

Urethane, 2,2,2-Trichloroethanol, and Fentanyl Potentiate Diazoxide-induced Mitochondrial K_{ATP} Channel Opening, which is Dependent on Protein Kinase C Activation

Mitochondrial K_{ATP} channel activity is directly reflected by flavoprotein redox state. Diazoxide (a highly specific opener of the mitoK$_{ATP}$ channel) administration alone produced a significant increase in oxidation of flavoproteins, which was clearly reversible (fig. 1). The diazoxide peak was abolished by 5-hydroxydecanoate but unaffected by HMR-1098. Exposure of myocytes to urethane, 2,2,2-trichloroethanol, and fentanyl as long as 30 min did not alter flavoprotein oxidation as compared to baseline values. Conversely, diazoxide administration to myocytes preexposed to urethane, 2,2,2-trichloroethanol, or fentanyl significantly enhanced diazoxide-induced increases in flavoprotein-mediated autofluorescence (figs. 1A–C). Also, diazoxide-induced fluorescence signals were significantly accelerated by urethane, 2,2,2-trichloroethanol, and fentanyl (latency to peak mitoK$_{ATP}$ channel activity: diazoxide, 11 $\pm$ 3 min; urethane + diazoxide, 5 $\pm$ 2 min; 2,2,2-trichloroethanol + diazoxide, 5 $\pm$ 1 min; fentanyl + diazoxide, 5 $\pm$ 2 min; $P <$ 0.001 diazoxide *vs.* urethane + diazoxide, 2,2,2-trichloroethanol + diazoxide, or fentanyl + diazoxide). Coadministration of chelerythrine, a specific PKC inhibitor, to urethane, 2,2,2-trichloroethanol, or fentanyl abrogated the potentiating effect of these anesthetics on mitoK$_{ATP}$ channel activity (figs. 1B and C). Chelerythrine did not affect baseline autofluorescence of the flavoproteins or modify the subsequent diazoxide-induced fluorescence peak at the low concentration of 2 μM used in this study.[6] Autofluorescence with chelerythrine alone was 18 $\pm$ 3% as compared to baseline of 16 $\pm$ 2%. Taken together, these observations suggest that urethane, 2,2,2-trichloroethanol, and fentanyl can significantly enhance opening of the mitoK$_{ATP}$ channel.

R-*ketamine and Barbiturates (Thiopental, Pentobarbital) Mitigate Diazoxide-induced Mitochondrial K_{ATP} Channel Opening*

To test whether the intravenous anesthetics *R*-ketamine, *S*-ketamine, thiopental, pentobarbital, xylazine, etomidate, propofol, and midazolam affect mitoK$_{ATP}$ channel activity, myocytes were exposed to the anesthetics alone, to diazoxide, or to diazoxide in the presence of the anesthetics, and the flavoprotein oxidation of myocytes was continuously recorded. Exposure of the anesthetics alone as long as 30 min did not alter flavoprotein oxidation in myocytes as compared with baseline values. Diazoxide administration alone produced a significant increase in oxidation of flavoproteins (fig. 2). In contrast, exposure of myocytes to *R*-ketamine, thiopental, and pentobarbital abolished diazoxide-induced

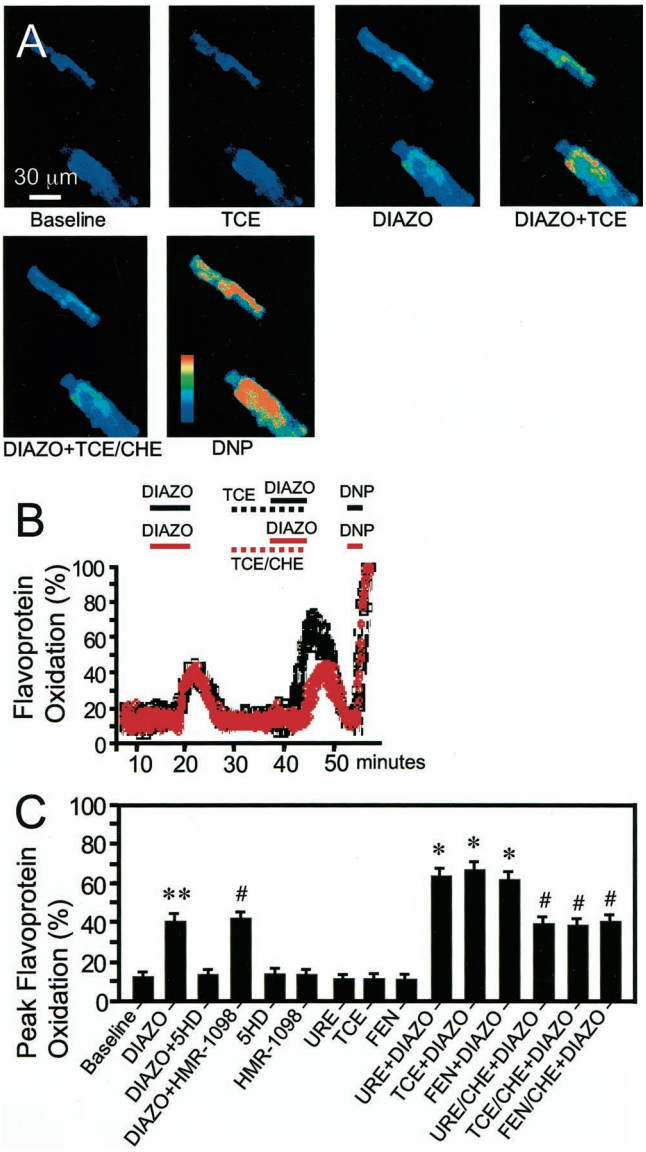

Fig. 1. Effects of the anesthetics urethane (URE), 2,2,2-trichloroethanol (TCE), and fentanyl (FEN) on diazoxide (DIAZO)-induced flavoprotein oxidation. (*A*) Baseline, 1 mM TCE, 100 μM DIAZO, 100 μM DIAZO + 1 mM TCE, 100 μM DIAZO + 1 mM TCE–2 μM chelerythrine (CHE), at 100 μM 2,4-dinitrophenol. (*B*) Time lapse of fluorescence intensity expressed as percentage of 2,4-dinitrophenol–induced fluorescence in TCE-treated myocytes. TCE enhances DIAZO-induced fluorescence (black symbols), and this enhancement is inhibited by coadministration of CHE (red symbols). The black and red symbols represent eight different experiments each. (*C*) Mean percentages of peak flavoprotein fluorescence in response to the various treatment modalities. *P < 0.001 URE + DIAZO, TCE + DIAZO, FEN + DIAZO *versus* DIAZO. #P not significant *versus* DIAZO. **P < 0.0001 DIAZO *versus* baseline. Data are mean ± SD.

increases in flavoprotein-mediated autofluorescence, indicating a significant inhibitory effect of these anesthetics on mitoK$_{ATP}$ channel activity (fig. 2A–C). *S*-ketamine (10 and 100 μM), xylazine (10 and 100 μM), etomidate (10 and 100 μM), propofol (10 and 200 μM), and midazolam (0.1 and 1 μM) did not affect diazoxide-induced mitoK$_{ATP}$ channel opening. In all cases (n = 8 for each concentration), the mean peak flavoprotein oxidation ranged from 41 to 43% as compared to 41% for diazoxide alone (P > 0.98). Notably, the concentrations used cover the full range previously reported for experimental as well as clinical conditions.[17–20] The results of these experiments clearly show that the intravenous anesthetics *R*-ketamine and the barbiturates thiopental and pentobarbital exert pronounced inhibitory effects on the mitoK$_{ATP}$ channel.

Modulatory Effects of Anesthetics on Cytoprotection in a Simulated Model of Ischemia

To test whether opening of mitoK$_{ATP}$ channels exerts cytoprotection in a cellular model of ischemia, myocytes were exposed to diazoxide, diazoxide + 5-hydroxyde-

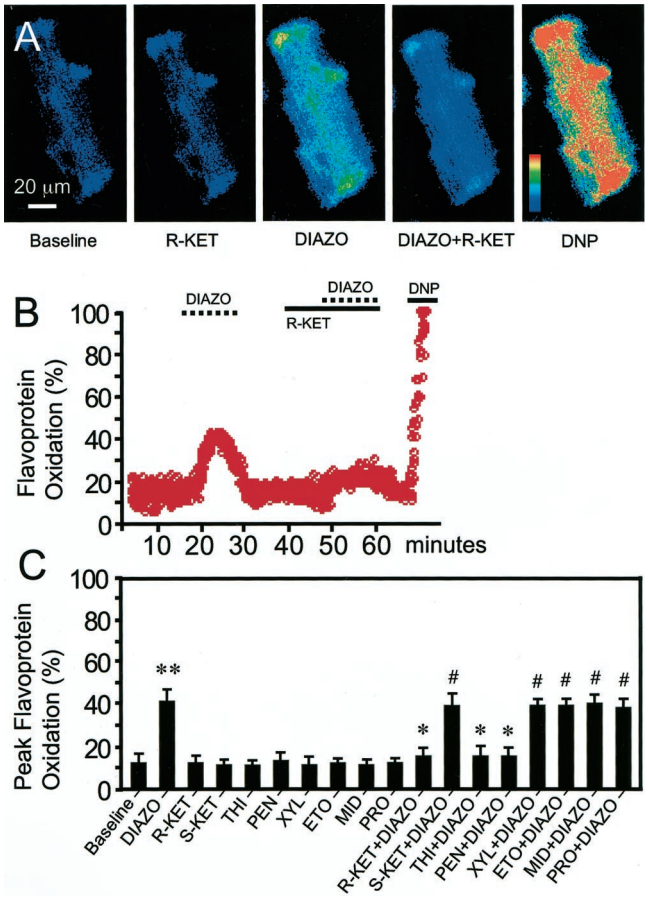

Fig. 2. Effects of the anesthetics *R*-ketamine (R-KET), *S*-ketamine (S-KET), pentobarbital (PEN), thiopental (THI), xylazine (XYL), etomidate (ETO), midazolam (MID), and propofol (PRO) on diazoxide (DIAZO)-induced flavoprotein oxidation. Pseudocolor scale: red indicates fully oxidized cells and black indicates fully reduced cells. (*A*) Baseline, 10 μM R-KET alone, 100 μM DIAZO, 100 μM DIAZO + 10 μM R-KET, 100 μM 2,4-dinitrophenol. (*B*) Time lapse of fluorescence intensity expressed as percentage of 2,4-dinitrophenol-induced fluorescence in R-KET–treated myocytes. The red symbols represent eight different experiments. Coadministration of R-KET abolishes the DIAZO-induced peak in fluorescence. (*C*) Mean percentages of peak flavoprotein fluorescence in response to the various anesthetics. *P not significant *versus* baseline. #P not significant *versus* DIAZO. **P < 0.0001 DIAZO *versus* baseline. Data are mean ± SD.

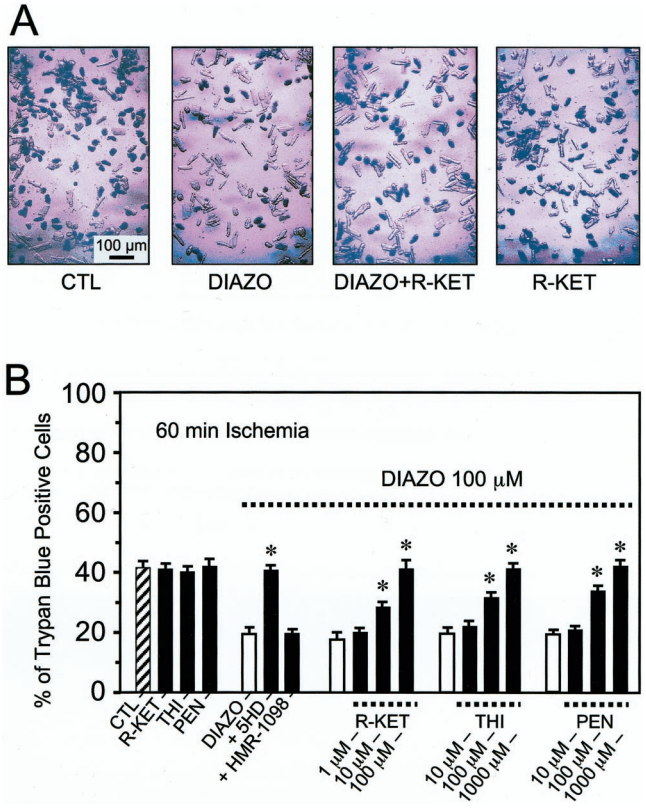

Fig. 3. Effects of *R*-ketamine (R-KET), thiopental (THI), and pentobarbital (PEN) on diazoxide (DIAZO)-induced ischemic cell protection. Myocytes were exposed to 60 min of ischemia and stained with hypoosmolar trypan blue. (*A*) R-KET at 10 μM was used in the experiments shown at the top. (*B*) R-KET (100 μM), THI (1000 μM), and PEN (1,000 μM) alone did not affect the number of surviving cells. Administration of R-KET, THI, and PEN dose-dependently inhibited the protective effects of DIAZO. Open bar indicates DIAZO alone. CTL = control; 5HD = 5-hydroxydecanoate, a specific mitoK$_{ATP}$ channel blocker; HMR-1098 = a specific sarcK$_{ATP}$ channel blocker. Data are mean ± SD.

canoate, and diazoxide + HMR-1098 and subsequently exposed to 60 min of ischemia. While 5-hydroxydecanoate, a specific blocker of the mitoK$_{ATP}$ channel, abolished the protective effect of diazoxide, HMR-1098, a specific blocker of the sarcK$_{ATP}$ channel, did not alter cell survival (control: 41 ± 3%, diazoxide: 19 ± 3%, diazoxide + 5-hydroxydecanoate: 40 ± 2%, diazoxide + HMR-1098: 20 ± 2%, *P* not significant for diazoxide + HMR-1098 *vs.* diazoxide and diazoxide + 5-hydroxydecanoate *vs.* control) (fig. 3). This observation clearly indicates that diazoxide-induced myocyte protection exclusively depends on mitoK$_{ATP}$ channel opening in the experimental model used. To further evaluate the effects of the various intravenous anesthetics on cell viability, and to test whether the observed differential modulatory effects of these anesthetics on mitoK$_{ATP}$ channel activity would result in alterations in cell survival, myocytes were exposed to anesthetics in the presence and absence of 100 μM diazoxide and subsequently exposed to 60 min of ischemia. Myocytes, which could not exclude trypan blue staining under hypoosmolar conditions after ischemia, were considered irreversibly damaged. *R*-ketamine, thiopental, and pentobarbital dose-dependently mitigated the protective effect of diazoxide (fig. 3). Importantly, significant inhibitory effects were detectable for *R*-ketamine at 10 μM (27 ± 2%) and for thiopental (31 ± 3%) and pentobarbital (34 ± 2%) at 100 μM ($P < 0.001$ *vs.* diazoxide [19 ± 3%]). Conversely, urethane, 2,2,2-trichloroethanol, and fentanyl potentiated diazoxide-induced protection at increasing concentrations, with urethane, 2,2,2-trichloroethanol, and fentanyl being most protective at 100 mM (11 ± 2%), 10 mM (10 ± 3%), and 1 μM (9 ± 2%), respectively (fig. 4A). At these concentrations, urethane, 2,2,2-trichloroethanol, and fentanyl alone provided similar protection as did diazox-

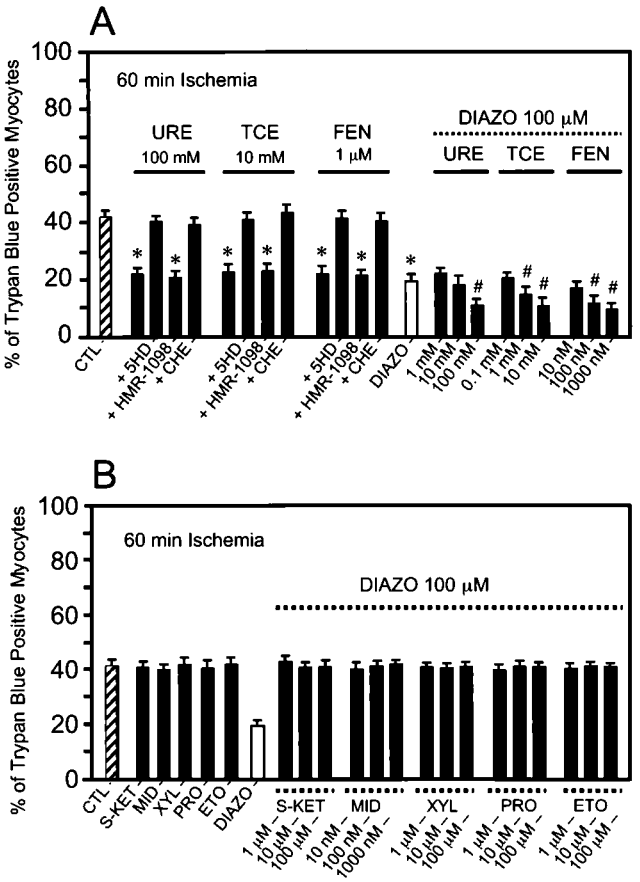

Fig. 4. Effects of various anesthetics on diazoxide (DIAZO)-induced ischemic cell protection. After 60 min of ischemia, the number of myocytes stained with hypoosmolar trypan blue was counted. Data are mean ± SD. (*A*) Urethane (URE), 2,2,2-trichloroethanol (TCE), and fentanyl (FEN) alone exerted significant protection similar to DIAZO at 100 μM (open bar). This protection was abolished by coadministration of 5-hydroxydecanoate (5HD) at 100 μM or chelerythrine (CHE) at 2 μM. Administration of URE, TCE, and FEN dose-dependently enhanced the protective effects of DIAZO. *$P < 0.0001$ *versus* control (CTL). #$P < 0.001$ *versus* DIAZO. CTL = control; 5HD = 5-hydroxydecanoate, a specific mitoK$_{ATP}$ channel blocker; HMR-1098 = a specific sarcK$_{ATP}$ channel blocker. (*B*) Effects were observed neither alone nor in combination with DIAZO for *S*-ketamine (S-KET), midazolam (MID), xylazine (XYL), propofol (PRO), and etomidate (ETO). Drugs alone were tested at the highest given concentration.

ide at 100 μM. *S*-ketamine, midazolam, xylazine, propofol, and etomidate did not affect cell viability at various concentrations (fig. 4B).

Urethane-, 2,2,2-Trichloroethanol–, and Fentanyl-Enhanced Myocyte Survival is Mediated by Activation of Protein Kinase C

To test whether urethane-, 2,2,2-trichloroethanol–, and fentanyl-induced myocyte protection would be dependent on PKC activation, chelerythrine was coadministered to myocytes in the presence of the anesthetics. Chelerythrine clearly abolished the protective effect of urethane, 2,2,2-trichloroethanol, and fentanyl, which indicates a pivotal role of PKC activity in mediating the protective effect of these intravenous anesthetics (100 mM urethane: 22 ± 2%, 10 mM 2,2,2-trichloroethanol: 23 ± 2%, 1 μM fentanyl: 21 ± 3%, 100 mM urethane + 2 μM chelerythrine: 38 ± 3%, 10 mM 2,2,2-trichloroethanol + 2 μM chelerythrine: 43 ± 2%, 1 μM fentanyl + 2 μM chelerythrine: 40 ± 3%, control: 41 ± 3%, *P* not significant for urethane + chelerythrine, 2,2,2-trichloroethanol + chelerythrine, or fentanyl + chelerythrine *vs.* control) (fig. 4).

Discussion

In this study, we showed that acute exposure of isolated adult rat ventricular myocytes to commonly used intravenous anesthetics induces differential effects on ischemic cardiomyocyte survival by modifying the preconditioning mimicking effects of diazoxide, a highly specific mitoK$_{ATP}$ channel activator. Although none of the investigated anesthetics affected baseline mitoK$_{ATP}$ channel activity, diazoxide-induced opening of the mitoK$_{ATP}$ channel was significantly enhanced by urethane, 2,2,2-trichloroethanol, and fentanyl, whereas *R*-ketamine, thiopental, and pentobarbital diminished this effect (table 1). The changes in diazoxide-induced mitoK$_{ATP}$ channel activity were detectable at anesthetic concentrations that are known to occur *in vivo*[17–20] and directly translated into significant changes in viability of diazoxide-treated myocytes exposed to 60 min of ischemia. Furthermore, we demonstrated that the protection afforded by acute exposure to urethane, 2,2,2-trichloroethanol, and fentanyl is attributable to activation of PKC (inhibited by chelerythrine), a key enzyme in the signal transduction of the preconditioned state. Similar to volatile anesthetics,[6] urethane, 2,2,2-trichloroethanol, and fentanyl do not directly open the mitoK$_{ATP}$ channel but increase its open probability (priming) at the initiation of ischemia. In addition, a number of anesthetics, including *S*-ketamine, etomidate, propofol, xylazine, and midazolam, did not affect mitoK$_{ATP}$ channel activity and modify myocyte protection.

The contributions of mitochondria to cellular physiology have reemerged into the spotlight of science.[21] Mitochondria play a pivotal role in oxygen sensing and in Ca^{2+} signaling, and are a rheostat in determining myocyte survival *versus* apoptotic and necrotic cell death. Evidence that mitoK$_{ATP}$ channels are involved in cardioprotection was first reported by Garlid *et al.*[22] These researchers observed that selective activation of mitoK$_{ATP}$ channels by diazoxide produced massive infarct size reduction following ischemia in a perfused rat heart model. Using specific blockers for the mitoK$_{ATP}$ channel and sarcK$_{ATP}$ channel, numerous subsequent studies identified the mitoK$_{ATP}$ channel as a key element in mediating the protection afforded by ischemic and pharmacological preconditioning in the myocardium.[23,24] Administration of diazoxide to myocytes mimics preconditioning by generation of a prooxidant environment through specific opening of mitoK$_{ATP}$ channels.[25] Since urethane, 2,2,2-trichloroethanol, and fentanyl do not alter baseline channel activity, but enhance its activity in a PKC-dependent manner, it is reasonable to postulate that these anesthetics prime the mitoK$_{ATP}$ channels, resulting in an easier opening of the channel at the initiation of ischemia. Sato *et al.* reported similar effects on mitoK$_{ATP}$ channels by adenosine.[13] Conversely, the inhibitory effects of *R*-ketamine, thiopental, and pentobarbital may be caused by interactions of these anesthetics with the mitoK$_{ATP}$ channel itself, or its vicinity, or by their influence on key components of cellular signaling. However, evidence for a direct interaction of these anesthetics with the channel protein has not yet been reported.

Two studies evaluated the effects of racemic ketamine[9] and thiamylal[8] on sarcK$_{ATP}$ channel activity and revealed a dose-dependent inhibition of this channel. Recently, fluorescence microscopy facilitated the study of mitochondria in their native habitat and the collection of data

Table 1. Summary of Effects of Anesthetics on Diazoxide-induced MitoK$_{ATP}$ Channel Activity in Isolated Adult Rat Cardiomyocytes

Anesthetic	Effect
Urethane	Potentiation
Trichloroethanol	Potentiation
Fentanyl	Potentiation
Isoflurane*	Potentiation
Sevoflurane*	Potentiation
Thiopental	Inhibition
Pentobarbital	Inhibition
R-Ketamine	Inhibition
Etomidate	No effect
Propofol	No effect
S-Ketamine	No effect
Midazolam	No effect
Xylazine	No effect

Anesthetics are grouped according to their enhancing effect (increase in protection), their inhibitory effect (decrease in protection), or no effect on the channel activity as measured by intrinsic flavoprotein fluorescence in the presence of 100 μM diazoxide (for details see Methods section).

* Taken from Zaugg *et al.*[6]

about their specific properties.[26] Using myocyte-inherent flavoprotein-induced fluorescence (autofluorescence) in live cell imaging microscopy, Marbán and his group showed that the redox state of these endogenous fluorophores directly reflects mitoK$_{ATP}$ channel activity, and that opening of this channel is closely associated with significant protection against ischemia.[10–13] By the same methodological approach, we recently demonstrated that volatile anesthetics mediate their protection in cardiomyocytes by selectively priming mitoK$_{ATP}$ channels through multiple triggering PKC-coupled signaling pathways.[6] The present study now extends these findings by demonstrating modulatory effects of intravenous anesthetics on this important cardioprotective channel (table 1). In accordance with our results, propofol and pentobarbital alone did not affect baseline flavoprotein autofluorescence in guinea pig cardiomyocytes.[27] Interestingly, these two anesthetics inhibited the isoflurane-induced flavoprotein oxidation.

How does opening of the mitoK$_{ATP}$ channel elicit its protection in cardiomyocytes? Two main concepts, which do not exclude, but rather complement each other, are currently under intensive investigation. According to Marbán and his group, opening of mitoK$_{ATP}$ channel leads to a depolarization of the inner mitochondrial membrane.[5] Although this change is modest, it has a significant impact on mitochondrial Ca^{2+} load due to the nonlinear dependence of Ca^{2+} flux on the membrane potential. It is hypothesized that depolarization of the inner mitochondrial membrane attenuates mitochondrial Ca^{2+} accumulation by lowering the driving force for Ca^{2+} uptake. The decreased mitochondrial Ca^{2+} overload during ischemia may prevent opening of the mitochondrial permeability transition pores and guarantee optimal conditions for ATP production.[28] Garlid *et al.* proposed that opening of the mitoK$_{ATP}$ channels decreases the ischemia-induced swelling of the mitochondrial intermembrane space, which would preserve functional coupling between adenine nucleotide translocase and creatine kinase.[4,29] This, in turn, secures the transport of newly synthesized ATP from the site of production by the ATP-synthase at the inner mitochondrial membrane to the cytosol. Thus, both mechanisms contribute to an uninterrupted supply of high-energy phosphate substrates from the mitochondria to the sites of energy consumption.

The modifying effects of anesthetics on infarct size were previously reported. Increased myocardial infarct size was observed in a barbiturate-anesthetized dog model when compared with conscious dogs.[30] Furthermore, rabbits exposed to regional ischemia exhibit half as large infarct size under halothane, enflurane, and isoflurane anesthesia as compared with pentobarbital, ketamine–xylazine, or propofol anesthesia.[31] This is different from observations in a dog model, where halothane was associated with the largest infarct size.[32] Similar to their modifying effect on infarct size, anesthetics further modify the magnitude of infarct limitation by ischemic preconditioning. Accordingly, infarct limitation by ischemic preconditioning is abolished by glibenclamide during ketamine–xylazine anesthesia, but not during pentobarbital anesthesia in a rabbit model.[33] In another study, the effects of pentobarbital, isoflurane, and ketamine–xylazine were evaluated in a rabbit model of regional ischemia.[34] Although infarct size was not different among nonpreconditioned hearts, the magnitude of infarct limitation by preconditioning varied significantly with the anesthetic employed. Taken together, these studies clearly stress the concept that anesthetics profoundly modify the consequences of myocardial ischemia. Using a more basic cellular approach, the results of the present study now support the concept that mitoK$_{ATP}$ channels significantly participate in modifying infarct size in response to anesthetics.

Racemic ketamine was found to block sarcK$_{ATP}$ channels in isolated cardiomyocytes at concentrations that may be clinically relevant.[9] Also, racemic ketamine, but not the stereoisomer *S*-ketamine, was found to block early and late preconditioning in rabbit hearts.[35,36] Our results now extend the stereoselective effect of ketamine on mitoK$_{ATP}$ channels, with the *R*-isomer having pronounced inhibitory effects and the *S*-isomer having no influence. Since stereoselectivity is consistent with mechanisms related to a specific binding site, it is likely that *R*-ketamine directly blocks the channel. However, ketamine also inhibits nitric oxide synthase,[37] and nitric oxide is a potent opener of mitoK$_{ATP}$ channels. Similarly, the barbiturate thiamylal, which closely resembles thiopental in its chemical structure, inhibits sarcK$_{ATP}$ channels in myocytes.[8] Our results again extend this effect now to mitoK$_{ATP}$ channels for two commonly used barbiturates. This finding is in line with the observation that barbiturates are known to competitively antagonize the adenosine A1 receptors, a pivotal signaling pathway in cardiac preconditioning.[38]

Recently, the cardioprotective role of ethanol in ischemia–reperfusion was demonstrated.[39] Acute exposure to ethanol mimics preconditioning by activation of PKC, and the protection occurs at "physiologic" levels. Our results now clearly show that 2,2,2-trichloroethanol, the halogenated analog of ethanol and main metabolite of chloral hydrate and α-chloralose, enhances mitoK$_{ATP}$ channel activity also by activation of PKC. Notably, these two anesthetics are still used extensively in physiologic and pharmacological experiments. Our finding that fentanyl provides significant protection by enhancing mitoK$_{ATP}$ channels activity is in clear accordance to previous studies, which related this effect to δ_1-opioid receptor stimulation and subsequent activation of PKC.[40] Finally, urethane, a carcinogenic anesthetic used in the laboratory, also enhanced preconditioning in a PKC-dependent manner. Conversely, we did not find an

effect of xylazine on mitoK$_{ATP}$ channels. However, since preconditioning may be induced by norepinephrine in ischemic myocardium *via* stimulation of α_1- and β-adrenergic receptors,[41,42] xylazine may limit preconditioning *in vivo*. Also, xylazine is known to promote hyperglycemia, which impairs mitoK$_{ATP}$ channel activity.[43] Propofol, etomidate, and midazolam did not have any effect on mitoK$_{ATP}$ channels or ischemic myocyte survival.

Although we recognize that mechanistic information on preconditioning in rat myocytes may not be transferable to other species, particularly to humans, and that isolated myocyte models have limitations with respect to the choice of external solutions, substrate selection, and unphysiologically low workload, the results of the present study have two important implications. First, many investigators used ketamine, thiopental, pentobarbital, urethane, or α-chloralose as background anesthetic in their animal preparations to elucidate the phenomenon of preconditioning. However, an analysis of the effects of anesthetics on preconditioning has not yet been performed, and the role of the anesthetic chosen appears to be critical in the interpretation of the experimental results. The experimental dilemma becomes particularly concerning in studies where a combination of up to four different anesthetics was used in a single experimental protocol.[36] Investigations into the precise mechanisms of preconditioning therefore need to address more carefully the effects of the anesthetic "black box" on experimental results in the future. Second, the observation that patients may receive anesthetics with inhibitory effects on mitoK$_{ATP}$ channels raises the concern that they might be at increased risk should myocardial ischemia occur, since preconditioning protection afforded by preceding ischemic events may be blocked. This may be specifically relevant for patients with coronary artery disease in the perioperative period, where ischemia frequently occurs. Although it was not shown so far that the choice of anesthetics may play a pivotal role with respect to outcome in even high-risk cardiac patients,[44] inhibition of the endogenous cardioprotection by preconditioning represents a potential hazard. Of note, sulfonylurea hypoglycemic agents prevent ischemic preconditioning in human myocardium and are thought to be responsible for the reported increased cardiovascular mortality in patients treated with these agents.[45] Conversely, administration of anesthetics known to elicit or amplify preconditioning such as opioids and volatile anesthetics,[46] in combination with specific openers of the mitoK$_{ATP}$ channel,[47] may limit the detrimental effects of perioperative myocardial ischemia.

In summary, commonly used anesthetics modulate the magnitude of cardioprotection elicited by cardiac preconditioning through enhancing (increase in protection) or inhibiting (decrease in protection) mitoK$_{ATP}$ channel activity. Therefore, the interpretation of results from experiments elucidating the complex phenomenon of preconditioning should acknowledge the effects of background anesthesia. Also, careful evaluation of the anesthetic agents and perioperative medication employed in the clinical care of patients with coronary artery disease is necessary.

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