

Oxygen Tension Modulates Inhibition of L-type Calcium Currents by Isoflurane in Human Atrial Cardiomyocytes

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Background: Myocardial L-type Ca^{2+} currents ($I_{\text{Ca,L}}$) are inhibited by isoflurane in the presence of a partial pressure of oxygen (Po_2) of 150 mmHg. In guinea pig cardiomyocytes, $I_{\text{Ca,L}}$ are inhibited by reduced oxygen tensions. The authors therefore analyzed the effects of Po_2 on $I_{\text{Ca,L}}$ in human cardiomyocytes and the effects of isoflurane at reduced Po_2 .

Methods: Atrial cardiomyocytes were prepared from specimens of patients undergoing open-heart surgery and superfused with either a high or a low Po_2 (150 or 12 ± 1 mmHg) while $I_{\text{Ca,L}}$ were measured with the whole cell patch clamp technique.

Results: Basal $I_{\text{Ca,L}}$ were not changed by the Po_2 (range, 9–150 mmHg) at 21° or 36°C . The reducing agent 1,4-dithiothreitol (DTT) left $I_{\text{Ca,L}}$ unaffected, and the oxidizing agent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) irreversibly inhibited $I_{\text{Ca,L}}$. The Po_2 significantly affected the inhibition of $I_{\text{Ca,L}}$ by isoflurane (1 minimum alveolar concentration) that decreased $I_{\text{Ca,L}}$ by $17 \pm 2.0\%$ at the high Po_2 but only by $5.8 \pm 2.9\%$ ($P = 0.037$) at the low Po_2 . The inhibition of $I_{\text{Ca,L}}$ by isoflurane was also significantly diminished ($P = 0.018$) by a low Po_2 when isoflurane effects at both Po_2 conditions were compared in the same cell.

Conclusions: In contrast to the situation in guinea pigs, basal $I_{\text{Ca,L}}$ in human atrial cardiomyocytes was not sensitive to acute Po_2 changes over a wide range. This might be explained by a lack of oxygen-sensitive splice variants of L-type calcium channel subunits. The Po_2 , however, has a decisive role for the effects of isoflurane on $I_{\text{Ca,L}}$.

MYOCARDIAL contractility is essentially determined by the amount of Ca^{2+} entering the sarcoplasm during electrical systole and the amount of Ca^{2+} released from intracellular calcium stores. For both processes, L-type Ca^{2+} channels are decisive because they provide the main voltage-dependent Ca^{2+} entry pathway in myocardial cells and because Ca^{2+} entering through these channels triggers Ca^{2+} -induced Ca^{2+} release.^{1,2} Moreover, the sympathetic regulation of contractile force is, to a major part, mediated by phosphorylation of L-type Ca^{2+} channels, enabling enhanced Ca^{2+} influx.^{3,4} Therefore, any interference with these channels is expected to exert immediate effects on myocardial contractility. Sev-

eral inhalational anesthetics such as halothane, isoflurane, and sevoflurane (but not the noble gas xenon) have been demonstrated to reduce L-type Ca^{2+} currents ($I_{\text{Ca,L}}$) in cardiomyocytes.^{5–8} These findings have been used to explain in part the negative inotropic effects of the anesthetics.

A further role of L-type Ca^{2+} channels has recently become apparent because they exhibited profoundly reduced currents in hypoxic conditions. This was first shown on recombinant L-type channels after expression of the pore-forming α_{1C} subunits of human cardiac tissue in human embryonic kidney 293 cells.⁹ Later, $I_{\text{Ca,L}}$ in guinea pig cardiomyocytes was demonstrated to be decreased by approximately 22% when the partial pressure of oxygen (Po_2) was decreased from 150 mmHg to 17 mmHg.¹⁰ In the same line, $I_{\text{Ca,L}}$ was also inhibited by the thiol-specific reducing agent 1,4-dithiothreitol (DTT), and this effect was antagonized by the thiol-specific oxidizing agent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB).^{10,11} The results on recombinant channels⁹ were interpreted as evidence for a structural change of the channels by oxygen tension. Therefore, L-type channels may act as oxygen sensors mediating shortening of action potentials and reduction of contractile force in response to hypoxia.^{12,13} This mechanism may be understood as cardioprotective, especially in regional hypoxia, because it enables energy saving in the presence of short oxygen supply. Notably, these properties of the channels allow a direct response to hypoxia, not necessitating more complex pathways such as the activation of adenosine 5'-triphosphate-dependent K^+ channels that reduces action potential duration when the adenosine 5'-triphosphate/adenosine 5'-diphosphate ratio decreases intracellularly.^{14–16}

The anesthetic gas isoflurane has been shown to exert moderate inhibition of $I_{\text{Ca,L}}$ in voltage clamp experiments on isolated cardiomyocytes, prepared from either animal or human heart tissue.^{5,17,18} It should be kept in mind that these experiments were all performed at standard experimental conditions that use oxygen tensions in the range of a Po_2 of 150 mmHg. The effects of isoflurane have been attributed to an interaction with channel proteins.¹⁸ In light of the evidence for an interaction of the channels with oxygen, we considered it necessary to reexamine the effects of isoflurane on myocardial $I_{\text{Ca,L}}$ in the presence of lower oxygen tensions that better represent physiologic and pathophysiologic conditions. The aim of the current study, therefore, was twofold. First, we wished to quantify the modulation of $I_{\text{Ca,L}}$ by oxygen in human cardiomyocytes. This is be-

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Received from RWTH Aachen, Aachen, Germany. Submitted for publication December 23, 2005. Accepted for publication January 4, 2007. Supported by grant Hu 951/1-2 by the Deutsche Forschungsgemeinschaft, Bonn, Germany (to Dr. Hüneke), and faculty support to the Institute of Physiology, University Hospital, Rheinisch-Westfälische Technische Hochschule, Aachen, Germany.

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cause previous experiments have been performed on cardiomyocytes from guinea pigs, whereas studies on human α_{1C} subunits revealed that only one of three splice variants conferred oxygen sensitivity to recombinant L-type channels.^{10,19} Although all three variants are reportedly expressed in human hearts,²⁰ their functional contribution is not known. Second, we intended to analyze L-type currents in the presence of isoflurane and reduced oxygen tensions. We report that, surprisingly, no effect of the P_{O_2} on basal $I_{Ca,L}$ can be demonstrated over a range from 9 to 150 mmHg in human atrial cardiomyocytes. However, a lower P_{O_2} dramatically reduced or even abrogated the inhibition of $I_{Ca,L}$ by isoflurane that proved to be quite inert on $I_{Ca,L}$ at a P_{O_2} of approximately 12 mmHg.

Materials and Methods

Isolation of Single Atrial Myocytes

Right atrial appendages were obtained as surgical specimens from patients undergoing heart surgery. The patients ($n = 30$, 23 men, 7 women) had a mean age of 67 yr (range, 45–85 yr), and all underwent a coronary artery bypass operation ($n = 23$) or aortic valve replacement ($n = 7$). All patients were in sinus rhythm and had no evidence of right heart failure. Their obligatory medication included β blockers and nitrates. The examinations were performed in accordance with the principles outlined in the declaration of Helsinki and approved by the local institutional review board (Ethik Kommission, Rheinisch-Westfälische Technische Hochschule Aachen, Germany). All patients gave written informed consent before surgery.

Human atrial cardiomyocytes were prepared according to Hatem *et al.*,²¹ as modified and described in detail by Skasa *et al.*²² Briefly, the myocardial specimens were carefully cut into chunks and washed in Ca^{2+} -free buffer twice for approximately 5 min. Afterward, the tissue was incubated in Ca^{2+} -free buffer that contained protease XXIV (Sigma, Deisenhofen, Germany) and collagenase V (Sigma). Incubation was finished as soon as microscopic examination revealed intact single cardiomyocytes. After centrifugation, cardiomyocytes were resuspended in a buffer containing the following: 120 mM NaCl, 5.4 mM KCl, 5 mM $MgSO_4$, 5 mM sodium pyruvate, 20 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Because visible cell damage to these cells would occur if a physiologic Ca^{2+} concentration was immediately restituted, the $CaCl_2$ concentration was gradually increased to 1.8 mM immediately before measurements of $I_{Ca,L}$. Only well-striated, bleb-free, rod-shaped myocytes were used for the studies, performed within 4 h after isolation.

Patch Clamp Experiments

Cardiomyocytes were allowed to adhere to a glass coverslip that was transferred into a perfusion chamber.

The cells were continuously superfused as described previously.⁵ $I_{Ca,L}$ were recorded with the whole cell patch clamp technique using an amplifier (EPC-9; HEKA, Lambrecht, Germany) and a personal computer equipped with Pulse 8.5 software (HEKA) for data acquisition and analysis. The patch pipettes, pulled from borosilicate glass and fire polished, had a tip resistance of 3–5 M Ω when filled with pipette solution. A holding potential of -60 mV was chosen to minimize sodium currents. $I_{Ca,L}$ were evoked by a series of depolarization pulses to potentials ranging from -50 to $+60$ mV. Ca^{2+} currents are given here as peak current amplitude of the respective depolarizing step. To save time, pulses only to $+10$ mV were used to evaluate the effects of low P_{O_2} and anesthetics in some experiments.

Preparation of Solutions

The bath solution contained 136 mM tetraethylammonium chloride, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with tetraethylammonium hydroxide. The pipette solution contained 140 mM CsCl, 2 mM $MgCl_2$, 0.3 mM adenosine 5'-triphosphate, 0.3 mM guanosine 5'-triphosphate, 10 mM EGTA, and 10 mM HEPES, pH-adjusted to 7.2 with CsOH.

Bath solutions were bubbled with either 100% nitrogen or with air in a gastight glass flask equipped with a frit and a membranous septum. Gas could leave the flask through a valve. The P_{O_2} values were measured with a calibrated oxygen-sensitive probe (Unisense, Aarhus, Denmark; tip diameter, 500 μ m). Gassing with nitrogen for approximately 30 min resulted in a decrease of the P_{O_2} to approximately 1 mmHg. Bath solutions containing isoflurane were bubbled with an appropriate gas mixture (isoflurane plus air or nitrogen). An anesthetic gas analyzer (Vamos; Dräger, Lübeck, Germany) was used to continuously monitor the concentration of isoflurane in the gas phase. A fraction (10–20 ml) of gassed solutions was taken through the septum of the reservoir into gastight syringes (1010 TLL; Hamilton, Bonaduz, Switzerland) and immediately used for experiments. The bath chamber (volume approximately 300 μ l) was continuously perfused through high-performance liquid chromatography-grade steel capillaries at a rate of 2–5 ml/min. The surface of the bath was covered with a thin layer of paraffin oil (DAB10; Wasserfuhr, Bonn, Germany) saturated with nitrogen to minimize diffusional exchange of oxygen and isoflurane. The isoflurane concentration in the reservoir as well as in the bath chamber was verified by headspace gas chromatography-mass spectrometry-selected ion monitoring (GC/MS/SIM, SSQ 7000; Finnegan, Bremen, Germany) as described previously.⁵

To examine the effects of a lowered P_{O_2} on $I_{Ca,L}$, the myocytes were exposed to a hypoxic solution in the bath chamber by rapid solution exchange, followed by continuous perfusion of the chamber with the same

hypoxic solution. The PO₂ was continuously recorded with an oxygen-sensitive probe (Unisense) placed into the chamber close to the examined cell. A final PO₂ of 12 ± 1 mmHg was achieved in the bath chamber. This condition could be held stable throughout the measurements of I_{Ca,L} in one myocyte (approximately 5–7 min).

The thiol-specific oxidizing agent DTNB (Sigma) was dissolved in ethanol (100%) to yield a stock solution with 2 mM that was stored at 4°C. Bath solutions contained 200 μM DTNB; in all experiments using DTNB, ethanol was added to control solutions resulting in the same final concentration (1% vol/vol) as the DTNB solutions. The thiol-specific reducing agent DTT (Sigma) was dissolved in water.

Temperature Control

Most experiments were carried out at room temperature (21°C). Some experiments were performed at 36°C. In this case, the temperature of the solution in the double-barreled stainless steel tubes and the bath chamber was kept constant with a feedback-controlled Peltier device (Strothmann, Aachen, Germany). The temperature was continuously monitored with a thermocouple probe mounted in the chamber wall.

Data Analysis And Statistics

Current amplitudes mark the peak current during one depolarizing pulse. Current densities were calculated by dividing current amplitudes by the whole cell capacitance amounting to 85 ± 7 pF in 59 cells. Values are expressed as mean ± SEM. Statistical comparisons were performed with SPSS version 12.0 software (SPSS Inc., Chicago, IL). Differences in I_{Ca,L} were tested for statistical significance using the Wilcoxon nonparametric test for paired data. Unpaired data were tested using the nonparametric Mann-Whitney U test. A probability of error of *P* < 0.05 was considered significant.

Results

To study effects of the oxygen tension on I_{Ca,L} in cardiomyocytes, we continuously measured the PO₂ in the immediate neighborhood of a patch clamped cell. When the superfusate was changed from a solution with a PO₂ of 150 mmHg to a solution gassed with nitrogen, a decrease in PO₂ resulted that fully developed over approximately 1 min. Then a stable plateau of 12 ± 1.0 mmHg (range, 9–15 mmHg) was reached. Lower PO₂ values could not be obtained. When the superfusate was switched back to the original solution with the high PO₂, PO₂ values were restored within 20 s. I_{Ca,L} was measured in intervals of approximately 20 s as peak current values after depolarizing steps from a holding potential of –60 mV to +10 mV. Unexpectedly, I_{Ca,L} did not exhibit any noticeable changes when the PO₂ was changed from the

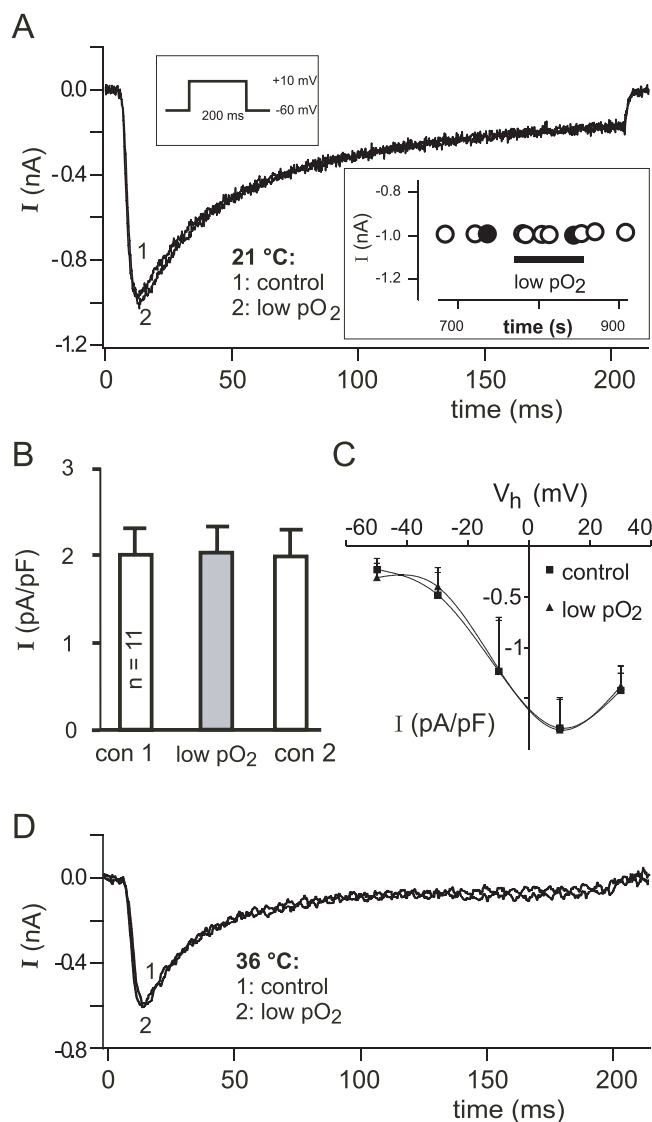


Fig. 1. Lack of effects of low oxygen tension on L-type Ca²⁺ currents (I_{Ca,L}) in human atrial cardiomyocytes. (A) Original traces from one cell in which I_{Ca,L} was first measured in a solution with a partial pressure of oxygen (PO₂) of 150 mmHg (control, trace 1) and then in a solution with a PO₂ of 9 mmHg (low PO₂, trace 2). The trace obtained after restitution of control conditions is not shown because it was almost identical with trace 1. The bath temperature was 21°C. The inset on top shows the voltage protocol. The other inset shows the time course of I_{Ca,L} during the experiment; at time 0, the whole cell configuration was obtained. Closed circles represent the value derived from the two traces shown. (B) Summary of the experiments (n = 11) on the effects of low PO₂ on the current density of I_{Ca,L} with the protocol as in A (bath temperature 21°C). (C) Current-voltage (I-V) relation of I_{Ca,L} in those experiments (n = 4) in which test pulses to several depolarizing potentials were tested, in the presence of high or low PO₂ conditions. (D) An experiment in which I_{Ca,L} was measured at 36°C, in control and hypoxic conditions (PO₂ 13 mmHg).

control conditions (150 mmHg) to 12 mmHg (fig. 1A) and back to 150 mmHg (not shown). In the mean, I_{Ca,L} was 2.00 ± 0.30 pA/pF (n = 11) in control conditions, 2.03 ± 0.31 during low PO₂, and 1.97 ± 0.31 after restoration of the control PO₂ (fig. 1B). I_{Ca,L} at other

membrane potentials ranging from -30 to $+30$ mV were not significantly altered by changes in the P_{O_2} , either (fig. 1C). Moreover, no modifications of $I_{Ca,L}$ were observed during the transition phases of the P_{O_2} . Likewise, the kinetics of the currents during each depolarizing step were virtually identical in each P_{O_2} condition (fig. 1A).

Although the range over which the P_{O_2} was varied in our experiments was well within the range for which significant changes of $I_{Ca,L}$ have previously been reported,^{9,10} we reasoned that the P_{O_2} might not be sufficiently low to affect $I_{Ca,L}$ in human cardiomyocytes. Because oxygen contamination, probably due to diffusion, led to a failure of our attempts to reduce the P_{O_2} further by oxygen-free superfusion of the cells, we tried to remove oxygen enzymatically^{23,24} by adding 100–300 mU/ml Oxyrase (Oxyrase Inc., Mansfield, OH) to the superfusate. Indeed, very low P_{O_2} values could be obtained with Oxyrase (even below 1 mmHg). $I_{Ca,L}$ was completely suppressed by these solutions. However, this suppression was irreversible. Moreover, the effects of Oxyrase could not be attributed to P_{O_2} reductions because exposure to Oxyrase evoked the same irreversible inhibition of $I_{Ca,L}$ in the presence of a P_{O_2} of 150 mmHg (data not shown).

The experiments reported so far were performed at room temperature. We also tested whether a potential sensitivity of $I_{Ca,L}$ to decreased oxygen tensions might reveal in physiologic temperatures (36°C). The problem with experiments at this temperature is that a significant run-down of $I_{Ca,L}$ occurs in the majority of experiments, in contrast to those at room temperature when $I_{Ca,L}$ was usually stable over 10–15 min. Typically, run-down at 36°C reduces $I_{Ca,L}$ by as much as 50% over 8 min, making the unequivocal detection of small inhibitions on top of the run-down impossible. However, in none of the experiments in which a low P_{O_2} was applied to cells at 36°C ($n = 7$) was any decrease of $I_{Ca,L}$ observed that was not explainable by run-down. Moreover, the restoration of a P_{O_2} of 150 mm did not result in a deceleration of time-dependent run-down of $I_{Ca,L}$. In two experiments, run-down was slower than usual (fig. 1D); in these cells, $I_{Ca,L}$ was identical in a P_{O_2} of 150 and of 14 or 12 mmHg. Therefore, no evidence was obtained that a low P_{O_2} suppressed $I_{Ca,L}$ at 36°C .

Next, we studied the interaction between oxygen and isoflurane with respect to their effects on L-type calcium channels. Isoflurane was added to superfusates at a final concentration of 1.2% (vol/vol), resulting in 0.38 mm and corresponding to a minimum alveolar concentration of 1 at 21°C . Previous experiments at a P_{O_2} of 150 mmHg had shown that isoflurane evokes a rapid, stable, and quickly reversible inhibition of $I_{Ca,L}$.^{5,17,18} In the current experiments, cells were kept at 150 mmHg P_{O_2} (control conditions) and then exposed either to isoflurane at a low P_{O_2} (9–15 mmHg) or to isoflurane at a control P_{O_2} (150

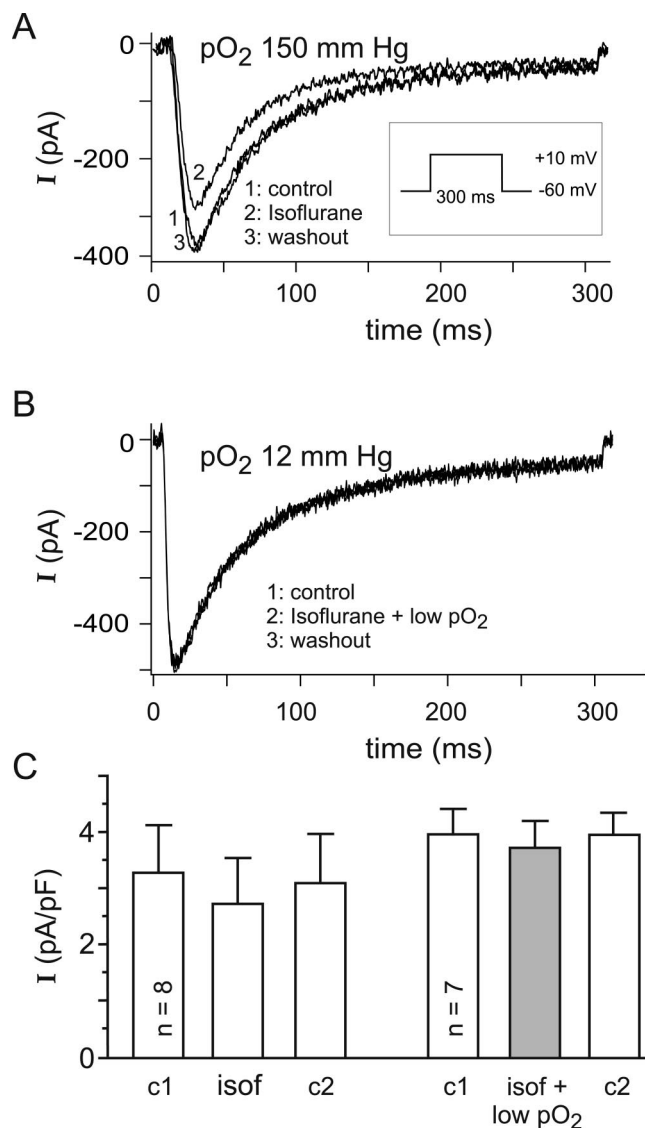


Fig. 2. Effects of the partial pressure of oxygen (P_{O_2}) on the inhibition of L-type Ca^{2+} currents induced by the anesthetic gas isoflurane. (A) Traces from one experiment in which the cell was kept at a P_{O_2} of 150 mmHg. After obtaining a control trace without the addition of isoflurane, isoflurane (at a minimum alveolar concentration of 1) was applied, followed by restitution of control conditions. (B) Traces from one experiment in which isoflurane was applied during a P_{O_2} of 12 mmHg. The control traces before and after the addition of isoflurane were obtained at a P_{O_2} of 150 mmHg. (C) Summary of the experiments with the protocol of A and B. Note that all controls were performed at a P_{O_2} of 150 mmHg. The inhibition by isoflurane was stronger ($P = 0.037$) when the gas was applied at a P_{O_2} of 150 mmHg ($n = 8$) than at a P_{O_2} of 12–15 mmHg ($n = 7$).

mmHg). Afterward, the original control conditions were restored. The results (fig. 2) show a weaker inhibition of $I_{Ca,L}$ by isoflurane at the lower than the control P_{O_2} . Isoflurane reduced $I_{Ca,L}$ by $17 \pm 2.0\%$ at the control P_{O_2} but only by $5.8 \pm 2.9\%$ at the low P_{O_2} . When the decreases in $I_{Ca,L}$ induced by isoflurane at a P_{O_2} of 150 mmHg ($n = 8$) were compared with those at 9–15 mmHg ($n = 7$), a slightly significant difference was revealed ($P = 0.037$, Mann-Whitney U test). To demon-

strate more conclusively that isoflurane-mediated inhibitions were dependent on the oxygen tension, we designed a protocol in which the effect of two PO₂ values on isoflurane-induced current inhibitions was studied in each cell, allowing the application of a paired test for statistical significance. Isoflurane was applied at a PO₂ of 9–15 mmHg to cells kept previously at 150 mmHg PO₂. Then, with isoflurane still present in the superfusate, the PO₂ was increased to 150 mmHg. Finally, isoflurane was removed while the PO₂ was kept at 150 mmHg (fig. 3). In these experiments, there was a significant stronger inhibition of I_{Ca,L} by isoflurane at 150 than at 12 mmHg PO₂ ($P = 0.018$, Wilcoxon rank test, $n = 7$). The data furthermore show that hardly any inhibition of I_{Ca,L} was evoked by isoflurane at the low PO₂. Washout of isoflurane consistently led to a full recovery of I_{Ca,L}.

The results so far are consistent with the view that not so much isoflurane but rather the high PO₂ (because 150 mmHg represents an oxygen tension considerably exceeding the physiologic values) is the decisive factor that accounts for the reduction of I_{Ca,L} when isoflurane is applied at a PO₂ of 150 mmHg. Because the PO₂ might affect membrane proteins such as calcium channels through oxidation and reduction of sulfhydryl groups, we tested the thiol-specific oxidant DTNB in comparison with the thiol-specific reducing agent DTT. The reducing agent DTT (2 mM) did not affect I_{Ca,L} at basic conditions (fig. 4A) or in the presence of isoflurane (fig. 4B). Specifically, DTT did not change I_{Ca,L} when the agent was added to a standard bath (3.41 ± 1.38 vs. 3.46 ± 1.36 pA/pF, $n = 6$). Furthermore, when a control value of I_{Ca,L} was determined in a standard bath and then the known inhibition by isoflurane was provoked, the further addition of DTT (in the continuous presence of isoflurane) left I_{Ca,L} at the same level as in the presence of isoflurane alone (2.80 ± 0.88 vs. 2.86 ± 0.94 pA/pF, $n = 5$). In contrast to DTT, the oxidizing agent DTNB (200 μ M) had profound effects on I_{Ca,L} (figs. 4C and D). Addition of the agent to a standard bath evoked an immediate reduction of I_{Ca,L} by $11 \pm 2\%$ ($n = 10$). However, this effect was irreversible; after extended washout, I_{Ca,L} remained on the diminished level for several minutes, demonstrating that inhibitions of I_{Ca,L} by DTNB cannot be attributed to run-down of I_{Ca,L} (fig. 4C). Therefore, I_{Ca,L} in human cardiomyocytes are more sensitive to oxidation and high oxygen tensions than to reduction and low oxygen tensions.

Discussion

In this study, we have examined how oxygen tension affects I_{Ca,L} in human atrial cardiomyocytes at basal conditions as well as in the presence of the anesthetic gas isoflurane, previously shown to inhibit these currents at high PO₂ values.⁵ As main findings, a PO₂ between 9 and

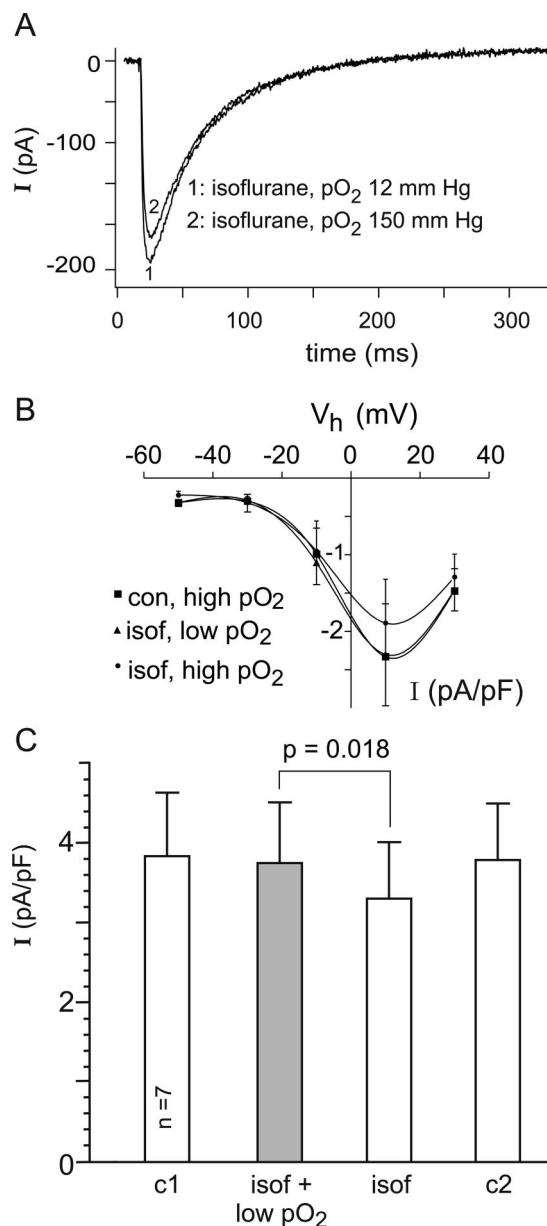


Fig. 3. Effects of the partial pressure of oxygen (PO₂) on the inhibition of L-type Ca²⁺ currents induced by isoflurane. In contrast to the experiments in figure 2, two different PO₂ conditions were examined in each cell. (A) Isoflurane (at minimum alveolar concentration of 1) was applied first along with a PO₂ of 12 mmHg and then with a PO₂ of 150 mmHg. Two control traces (without the addition of isoflurane at a PO₂ of 150 mmHg) were obtained before trace 1 and after trace 2 but are not shown because they were almost identical with trace 2. (B) Current-voltage (IV) relation of L-type Ca²⁺ currents ($n = 4$) under the conditions used in the experiment of A. (C) Summary of the experiments ($n = 7$) with the protocol of A. The current densities in the presence of isoflurane were significantly ($P = 0.018$) smaller at the control than at the decreased PO₂.

150 mmHg did not affect I_{Ca,L} in the absence of isoflurane, in discrepancy to previous findings on guinea pig cardiomyocytes. However, the PO₂ exerted significant effects on the inhibition of I_{Ca,L} by isoflurane. Only at high PO₂ levels were inhibitory effects of the anesthetic gas observed. Inhibition of I_{Ca,L} was also evoked by the

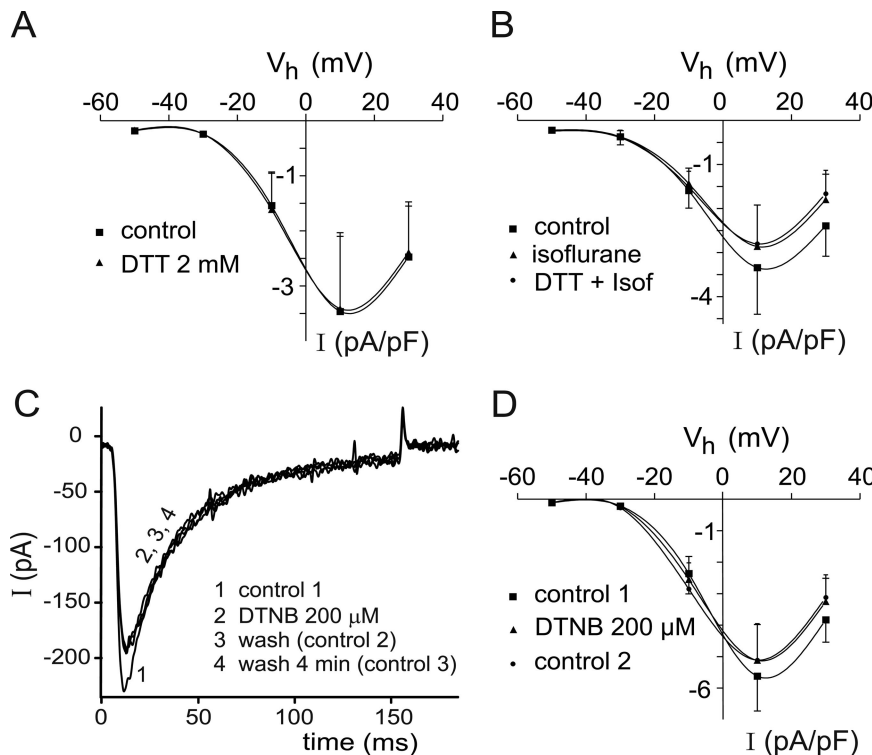


Fig. 4. Effects of the thiol-specific reducing agent 1,4-dithiothreitol (DTT) and of the thiol-specific oxidant 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) on L-type Ca^{2+} currents ($I_{\text{Ca,L}}$). (A) Effects of DTT (2 mM, $n = 6$) on $I_{\text{Ca,L}}$ as measured at various holding potentials (V_h). (B) Effects of DTT (2 mM, $n = 5$) in the presence of isoflurane. Control values were obtained before addition of isoflurane (1 minimum alveolar concentration). (C) Effects of DTNB (original traces from one experiment). After obtaining a control value for $I_{\text{Ca,L}}$ (trace 1), DTNB (200 μM) was added and trace 2 was recorded. Traces 3 and 4 were recorded immediately after washout of DTNB and 4 min later, during which the cells were continuously superfused with control solution. (D) Current-voltage relation of $I_{\text{Ca,L}}$ before, during, and after application of DTNB (200 μM , $n = 10$).

oxidizing agent DTNB, whereas the reducing agent DTT did not modify $I_{\text{Ca,L}}$.

Our results indicate that $I_{\text{Ca,L}}$ in the human heart are inhibited by oxidizing agents and by high Po_2 levels (in the presence of isoflurane) rather than by reducing agents and low Po_2 . What could the reasons be for the apparent discrepancy of the current results, when compared with those obtained in guinea pig cardiomyocytes? First, one could assume that the temperature is relevant. The author of the study in guinea pig cells¹⁰ chose a temperature of 37°C, and such experimental conditions are more difficult to establish than room temperature. From our experience, experiments at physiologic temperatures have a high failure rate due to rapid run-down of $I_{\text{Ca,L}}$.²⁵ The situation could not be improved by use of the perforated patch technique because this requires more time without producing more stable conditions in our hands. Despite the problems with run-down, we considered it essential to test the hypothesis that Po_2 effects on $I_{\text{Ca,L}}$ would become apparent at physiologic temperatures. Not any indication was found for an inhibition of $I_{\text{Ca,L}}$ by decreased Po_2 values, but run-down of $I_{\text{Ca,L}}$ prevented the definite exclusion of a Po_2 -induced inhibition in most experiments. However, we have two experiments in which only moderate run-down of $I_{\text{Ca,L}}$ occurred. Even in these experiments, we observed no reduction of $I_{\text{Ca,L}}$ by a low Po_2 , indicating that human cardiomyocytes are not sensitive to Po_2 in the way of guinea pig cardiomyocytes.¹⁰

The second reason for the discrepancy could be the existence of species-dependent (human *vs.* guinea pig)

and cell-dependent (atria *vs.* ventricles) differences in the oxygen sensitivity of cardiomyocytes. In this context, it is noteworthy that no strict correlation between the extent of Po_2 reductions and the effects on $I_{\text{Ca,L}}$ was reported in guinea pig cardiomyocytes.¹⁰ On the other hand, such a correlation was clearly established in human embryonic kidney 293 cells expressing α_{1C} subunits.⁹ Moreover, no saturation of the Po_2 -induced $I_{\text{Ca,L}}$ suppression was evident in the latter cell model, even at the lowest attainable Po_2 values. Therefore, the oxygen-sensing capability of a cell may apply to different ranges of Po_2 variations. Unfortunately, we could not achieve hypoxic conditions with Po_2 values considerably lower than 12 mmHg. Our attempts with Oxyrase did not allow us to attribute the observed marked alterations of $I_{\text{Ca,L}}$ to hypoxia but rather to some oxygen-independent effects of the enzyme. However, it should be stressed that our experiments tested a range of Po_2 values that produced dramatic changes of $I_{\text{Ca,L}}$ in both guinea pig cardiomyocytes and human embryonic kidney 293 cells expressing human α_{1C} subunits, again pointing to distinctly different Po_2 sensitivity in human cardiomyocytes than in these other models.

Finally, the results that have been reported from studies on different isoforms of Ca^{2+} channel α_{1C} subunits deserve consideration. Only one of three isoforms, or splice variants, of the proteins conferred oxygen sensitivity to $I_{\text{Ca,L}}$ in human embryonic kidney 293 cells.¹⁹ Although all three isoforms are likely to exist in the human heart,²⁰ a quantitative or functional analysis has not yet been performed. Such an analysis should also

consider regional differences, in particular between atria and ventricles. Currently, a quantitative determination of the expression pattern and of $I_{Ca,L}$ could not be performed simultaneously and in identical cells. If there were a preponderance of oxygen-insensitive over oxygen-sensitive Ca^{2+} channel proteins in our cells, the experimental results would be explained.

The molecular basis of how the two gases oxygen and isoflurane modulate ion currents has not been fully elucidated. An interaction of isoflurane with channel proteins is likely and may be a predominant factor in many conditions.¹⁸ Similarly, oxygen may interact with L-type Ca^{2+} channels directly, as proposed on the basis of the experiment of heterologously expressed channels²⁶ and of results obtained with thiol-specific reducing and oxidizing agents.²⁷ It has been concluded that the open probability of L-type Ca^{2+} and other channels is critically dependent on the oxidative state of various sulfhydryl groups which are differently amenable to redox-modulating compounds.^{26,27} However, the literature on effects of thiol-reducing and oxidizing agents is full of inconsistencies, as summarized in a recent review.²⁸ For example, in guinea pigs where $I_{Ca,L}$ is sensitive to decreased PO_2 values, the reducing agent DTT mimicked the effects of low PO_2 , whereas the oxidizing agent DTNB partly reversed the inhibition by low oxygen tensions.¹⁰ Mostly in line with these results, DTT attenuated and DTNB stimulated basal $I_{Ca,L}$ in ferret cardiomyocytes.¹¹ In definite contrast, the sulfhydryl oxidant 2,2'-dithiopyridine was inhibitory on heterologously expressed α_{1C} subunits of smooth muscle from rabbits, whereas DTT had only negligible effects on basal $I_{Ca,L}$.²⁷ The oxidizing agent p-chloromercuribenzenesulfonic acid led to an irreversible inhibition of calcium currents,²⁶ as did DTNB in our hands. To explain the discrepancies between various studies, again general species differences may be considered as well as differences in the oxygen sensitivity of various channel splice variants. Moreover, even in guinea pigs, the effects of oxidizing agents are not unequivocal because DTNB alone evoked some effects inconsistent with a pure antagonism of low PO_2 ,¹⁰ and another oxidizing agent, p-hydroxy-mercuric-phenylsulfonic acid, reduced rather than enhanced $I_{Ca,L}$, and these effects were at best partly reversed by DTT.²⁹ To our best knowledge, the current study is the first one that analyzes effects of DTNB and DTT in human cardiomyocytes; as in the case of the oxygen sensitivity, a distinct divergence to the situation in guinea pigs is evident. However, our results are fully in line with those of Fearon *et al.*,²⁶ who heterologously expressed one human α_{1C} subunit and demonstrated lack of effects of a reducing agent and irreversible inhibition by an oxidizing agent.

From a clinical point of view, it is an interesting question how the cardiac actions of isoflurane are modulated by clinically relevant PO_2 values. In the past, there has

been much debate regarding which PO_2 around a cell represents physiologic conditions and which hypoxia.³⁰⁻³⁴ A PO_2 of 150 mmHg is likely to exceed the physiologic range,³³ although such a PO_2 is standard in most experiments in isolated cells. Therefore, it could be argued that isoflurane inhibitions of $I_{Ca,L}$ at a PO_2 of 150 mmHg represent a situation where the anesthetic gas acts in the presence of another gas, *i.e.*, oxygen, in unphysiologic and potentially toxic concentrations. In this line, isoflurane may be viewed as an agent unmasking inhibitory effects of oxygen on $I_{Ca,L}$, whereas isoflurane-dependent inhibitions of $I_{Ca,L}$ are dramatically reduced when the PO_2 is decreased to probably more physiologic values. It remains to be clarified how sulfhydryl groups in L-type channel proteins govern the interaction of isoflurane and oxygen.

In conclusion, the current experiments reveal a striking functional difference of $I_{Ca,L}$ in human atrial cardiomyocytes, in comparison with cells from guinea pig ventricles, because $I_{Ca,L}$ was not modulated by PO_2 in human cells in the way demonstrated for guinea pig cells. Our results might be explained by a predominant expression of oxygen-insensitive α_{1C} subunits. Regional differences of the expression pattern of α_{1C} subunits may be present in the human heart, but species differences seem to be more relevant. Furthermore, the study demonstrates a clear dependence of the isoflurane-induced inhibition of $I_{Ca,L}$ on PO_2 . An inhibition occurs mostly at standard experimental PO_2 conditions, but these are likely to represent hyperoxia not normally present in the microenvironment of living cells.^{33,34} At lower oxygen tensions, isoflurane becomes almost inert on $I_{Ca,L}$ in human cardiomyocytes from atrial appendages.

The authors thank Manfred Möller, Ph.D. (Assistant Professor, Department of Hygiene and Environment Medicine, University Hospital, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany), for analysis of gas concentrations with gas chromatography. The authors acknowledge the excellent cooperation of the staff of the Department of Thoracic and Cardiovascular Surgery, University Hospital, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany.

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