Halothane and isoflurane preferentially depress a slowly inactivating component of Ca^{2+} channel current in guinea-pig myocytes

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1. The effects of the inhalational anaesthetics halothane and isoflurane on the high-voltage-activated Ca^{2+} channels were determined in isolated guinea-pig ventricular myocytes using the patch-clamp technique.

2. Recording solutions were equilibrated with inhalational anaesthetic vapour delivered from a calibrated vaporizer set at clinically relevant ranges of partial pressure. Anaesthetic concentrations in solution were determined using gas chromatography.

3. Halothane (0·9 mM in solution) and isoflurane (0·8 mM in solution) decreased peak whole-cell Ca^{2+} current (I_{Ca}) by ~40 and ~20%, respectively, while increasing the apparent rate of inactivation.

4. The sum of fast and slow exponential decay functions was required to fit the inactivation phase of I_{Ca}. The anaesthetics preferentially affected the slow component of inactivation while also increasing the rate of slow inactivation. The physiological significance of these effects was addressed by examining I_{Ca} evoked by a ventricular action potential waveform.

5. Measurement of the current carried by Ba^{2+} through Ca^{2+} channels (I_{Ba}) permitted the isolation of the slow component of inactivation. Halothane and isoflurane diminished peak I_{Ba} at 0 mV by ~45 and ~20%, respectively, with similar changes in rate and magnitude of the slowly inactivating component as with I_{Ca}.

6. Cell-attached patch-clamp measurements of Ca^{2+} channel activity revealed that halothane did not alter single-channel conductance. Instead, the anaesthetic reduced channel open probability to the same extent as observed during the whole-cell recording, an effect partially due to an increase in null sweeps. In patches with a single channel present, the open-time distribution, fitted by a single exponential, showed a decrease in mean open time. The closed-time distribution, fitted by the sum of slow and fast exponential components, revealed an anaesthetic-induced increase in the duration of the slow component with no effect on the fast component. Results are presented in terms of a channel-gating model, and model predictions are examined with a computer simulation.

It is well known that inhalational anaesthetics depress myocardial contractility. Halothane, and to a lesser extent isoflurane (Stevens, Cromwell, Halsey, Eger, Shakespeare & Balman, 1971), induce a negative inotropic effect in the myocardium, in which several cellular mechanisms are implicated. Anaesthetics diminish the release of Ca^{2+} to the contractile elements through depletion of sarcoplasmic reticulum (SR) Ca^{2+} (Wheeler, Rice, Hansford & Lakatta, 1988) by reduced uptake (Wilde, Knight, Sheth & Williams, 1991; Miao, Frazer & Lynch, 1994), and enhanced release from the ryanodine receptor (Connelly & Coronado, 1994; Lynch & Frazer, 1994); the latter effect is specific to halothane. Electrophysiological studies based on single microelectrode techniques (Lynch, Vogel & Sperelakis, 1981; Lynch, 1986; Terrar & Victory, 1988*a*,*b*) and the whole-cell patch-clamp method (Bosnjak, Supan & Rusch, 1991; Eskinder, Rusch, Supan, Kampine & Bosnjak, 1991) have shown that the volatile anaesthetics also inhibit sarcolemmal Ca^{2+} channels. Slow action potential estimates of Ca^{2+} channel function from guinea-pig papillary muscle revealed that halothane produced a more pronounced blockade of the voltage-gated Ca^{2+} current (I_{Ca}) than isoflurane (Lynch *et al.* 1981; Lynch, 1986). In addition to the more potent effect of halothane over isoflurane, the anaesthetics enhanced the apparent rate of inactivation of I_{Ca} of guinea-pig ventricle (Terrar & Victory, 1988*a*,*b*) and vascular smooth muscle (Wilde, 1994). Indeed, a faster rate of I_{Ca} inactivation might be expected, at least transiently,

with application of halothane since the initial decay phase appears to be influenced by the sub-sarcolemmal Ca^{2+} concentration ($[Ca^{2+}]_i$) (Neely, Olcese, Wei, Birnbaumer & Stefani, 1994), and modulation of the Ca^{2+}-induced Ca^{2+} release from the SR can affect I_{Ca} inactivation (Imredy & Yue, 1994). Whole-cell voltage-clamp studies of isolated canine ventricular myocytes (Bosnjak *et al.* 1991) and cardiac Purkinje cells (Eskinder *et al.* 1991) suggested that each anaesthetic produced a similar inhibition of I_{Ca} at approximately equi-anaesthetic concentrations. Furthermore, changes in the decay kinetics of I_{Ba}, i.e. the current carried by Ba^{2+} ions through Ca^{2+} channels, were not apparent with application of halothane or isoflurane (Bosnjak *et al.* 1991).

The purpose of the present study was to describe in detail the effects of inhalational anaesthetics on Ca^{2+} channels from isolated guinea-pig ventricular myocytes. Inhalational anaesthetics were delivered to the recording solutions using calibrated vaporizers, and concentrations in solution were derived using gas chromatography. A preliminary account of this work has been presented at the American Society of Anesthesiology (Pancrazio & Lynch, 1993).

METHODS

Cardiac myocyte isolation

English Shorthair guinea-pigs (~300 g) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}) via I.P. injection following the guidelines of the University of Virginia Animal Research Committee. The heart was quickly excised and perfused using a Langendorff perfusion system to isolate ventricular myocytes as described earlier (Park, Pancrazio & Lynch, 1994). Isolated cells were stored in a 95% air–5% CO_2 incubator at 37 °C and used only on the day of isolation. Rod-shaped cells with apparent striations and which remained quiescent in solution with 2 mM $CaCl_2$ were studied at room temperature (20–23 °C) in an open perfusing chamber on an inverted microscope.

Solutions

Prior to establishing the whole-cell configuration, the external recording solution contained (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes; pH adjusted to 7·4 with 1 N NaOH. The patch pipette solution contained (mM): 120 CsCl, 20 TEA-Cl, 1 $CaCl_2$, 11 EGTA–CsOH, 10 Hepes and 5 Mg-ATP; pH adjusted to 7·3 with 1 N HCl. Once the whole-cell configuration was achieved, the recording solution was exchanged to (mM): 125 CsCl, 20 TEA-Cl, 2 $CaCl_2$ or $BaCl_2$, 1 $MgCl_2$ and 10 Hepes; pH adjusted to 7·4 with 1 N CsOH. Pilot experiments clearly demonstrated that the inclusion of 5 mM Mg-ATP in the patch pipette solution greatly minimized Ca^{2+} channel rundown or washout. The use of Na^+-free recording solutions eliminated any confounding Na^+–Ca^{2+}-exchange current, and furthermore, addition of tetrodotoxin (2 mg ml^{-1}) eliminated a residual component of inward current through Na^+ channels.

For single-channel experiments, myocytes were bathed in a solution containing (mM): 120 potassium aspartate, 20 KCl, 2 EGTA and 10 Hepes; pH adjusted to 7·4 with 1 M KOH. The effect of this solution was to zero the resting membrane potential. The patch pipette contained (mM): 120 $BaCl_2$, 10 Hepes and 1 EGTA; pH adjusted to 7·4 with 1 M CsOH. To prolong Ca^{2+} channel openings in several experiments, the patch pipette solution contained 5 μM Bay K 8644 (Calbiochem), made from a 1 mM stock solution dissolved in DMSO.

Inhalational anaesthetics were equilibrated in the recording solution at room temperature by bubbling for at least 20 min with filtered air passed through a calibrated vaporizer. The equi-anaesthetic levels, which were arbitrarily chosen for emphasis, were multiples of the anaesthetic ED_{50} or minimum alveolar (gas) concentration (MAC): 0·75 and 1·25% atm for halothane and isoflurane, respectively. These concentrations, which were produced by the vaporizer, were 90–100% of the anticipated value calculated from the gas : aqueous partition coefficient (Firestone, Miller & Miller, 1986) at 20 °C, based on gas chromatography sampling directly from the recording chamber. For example, 1·5% halothane and 2·5% isoflurane yielded 0·9 and 0·8 mM in solution, respectively. Control solutions were bubbled with filtered air only. Control, anaesthetic treatment and recovery were performed on each cell tested. On rare occasions where peak I_{Ca} failed to recover to at least 80% of the control amplitude, the data were excluded from analysis. Complete solution exchange was accomplished within 20 s and measurements were made approximately 90 s after solution application. Alterations in I_{Ca} or I_{Ba} after anaesthetic treatment persisted for several minutes regardless of whether or not the perfusion was constant. For single-channel measurements, cell-attached membrane patches equilibrated in the anaesthetic-containing solution for a minimum of 4 min and data were collected during constant perfusion.

Patch-clamp recording, data acquisition and analysis

Standard patch-clamp methods were employed as described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). A period of 4–6 min was allowed after initiating the whole-cell recording configuration to establish a stable baseline. Voltage-clamp measurements were performed using the Axopatch 200A (Axon Instruments) patch-clamp amplifier. Patch electrodes were prepared from borosilicate glass (model KIMAX-51; American Scientific, Charlotte, NC, USA) and had typical resistances of less than 1 MΩ when filled with internal solution. Following the fabrication of pipettes with a two-stage micropipette puller (Narishige), pipette tips were heat polished with a microforge (Narishige). For single-channel experiments, pipette tips were coated with Sylgard 184 (Dow Corning) prior to heat polishing.

Unless otherwise noted, myocytes were voltage clamped at −80 mV and the commonly employed P/n approach ($n = -4$) was used to estimate leakage and capacitive current. Also, whole-cell currents were filtered at 2 kHz with a four-pole Bessel low-pass filter and digitized at 10 kHz. Current records were analysed off-line using a custom-made program capable of preparing current–voltage (I–V) relations and non-linear curve fitting (Pancrazio, 1993) or with the Clampfit routine from pCLAMP 6 (Axon Instruments). For steady-state activation and inactivation estimates, data were fitted to the Boltzmann function:

$$f(V) = \frac{1}{1 + \exp((V_n - V)/k_n)}, \quad (1)$$

where V_n is the voltage where the Boltzmann function ($f(V)$) has a value of 0·5 and k_n is the slope factor.

For a set of experiments, a cardiac action potential (AP) waveform was used as the voltage-clamp command potential. Data were acquired using a system described earlier (Pancrazio, Johnson & Lynch, 1994) which permits leak and capacitive current compensation.

Unitary Ca^{2+} channel currents were measured in the cell-attached patch-clamp configuration, filtered at 1 kHz with a four-pole low-pass Bessel filter and digitized at 10 kHz. Voltage pulses, 200 ms in duration in groups of sixty-four episodes, were applied at a rate of 0·5 Hz with a total of 192–256 sweeps collected for each experimental condition. Leak and capacitive current artifacts were subtracted by fitting a null sweep, i.e. a record with no observable openings, with the sum of three exponential decay functions and subtracting the fitted result from each data trace. Data were analysed off-line with a custom-made program designed to estimate open-channel amplitude, kinetics and the open probability (P_O). For voltage-dependent channel openings, which are non-stationary, P_O is a function of time; however, for convenience, P_O is reported as the average over the duration of the applied voltage step. For patches containing only one or two channels, the fraction of null sweeps was determined. For kinetic analysis, event detection was achieved using a half-amplitude threshold. No correction was made for events which may have been missed due to their brevity of opening or closing and data filtering. For patches containing a single Ca^{2+} channel, the open and closed times were separated into bins and fitted to an exponential function, $f(t)$:

$$f(t) = \sum_{i=1}^{3} A_i \exp(-t/\tau_i), \quad (2)$$

where i is an index of 1, 2 or 3. To verify that the single openings observed in a membrane patch were due to a single channel, the probability (P_1) that a data record from a patch with N channels will exhibit only single openings was calculated. According to Colquhoun & Hawkes (1983):

$$P_1 \approx \exp\left(-T\left(\frac{N-1}{N}\right)\frac{m_O}{m_C^2}\right), \quad (3)$$

where m_O is the mean open time, m_C is the mean closed time, and T is the total observation time. For non-stationary channel behaviour, m_C may be biased against long duration closures which may not be detected since each closure must be bounded by two openings. Thus, using the relationship $P_O = m_O/(m_O + m_C)$, P_1 was written without dependence on m_C:

$$P_1 \approx \exp\left(-T\left(\frac{N-1}{N}\right)\frac{P_O^2}{(1-P_O)^2 m_O}\right). \quad (4)$$

Statistics

Where appropriate, data are presented as mean percentages of control ± S.E.M. and the number of cells tested by n. Statistical significance of a drug effect was determined using Student's t test with $P < 0{\cdot}05$ considered as significant.

RESULTS

Whole-cell Ca^{2+} current is dominated by L-type Ca^{2+} channels at depolarized potentials

I_{Ca} was evoked by step depolarizations from −70 to +80 mV from a holding potential of −80 mV. Although a small, low-threshold, dihydropyridine-insensitive (T-type) Ca^{2+} current could be occasionally distinguished in response to small amplitude depolarizations to voltages ranging from −60 to −40 mV, ventricular myocyte I_{Ca} was dominated by high-threshold Ca^{2+} channels. Consistent with past work also using guinea-pig ventricular myocytes (Balke, Rose, Marban & Wier, 1992), voltage steps to potentials more depolarized than −20 mV triggered I_{Ca} almost exclusively carried by dihydropyridine-sensitive L-type Ca^{2+} channels. Preliminary studies verified that I_{Ca} evoked from a holding potential of −50 mV was suppressed by 1 μM nifedipine as expected for L-type Ca^{2+} channels (data not shown).

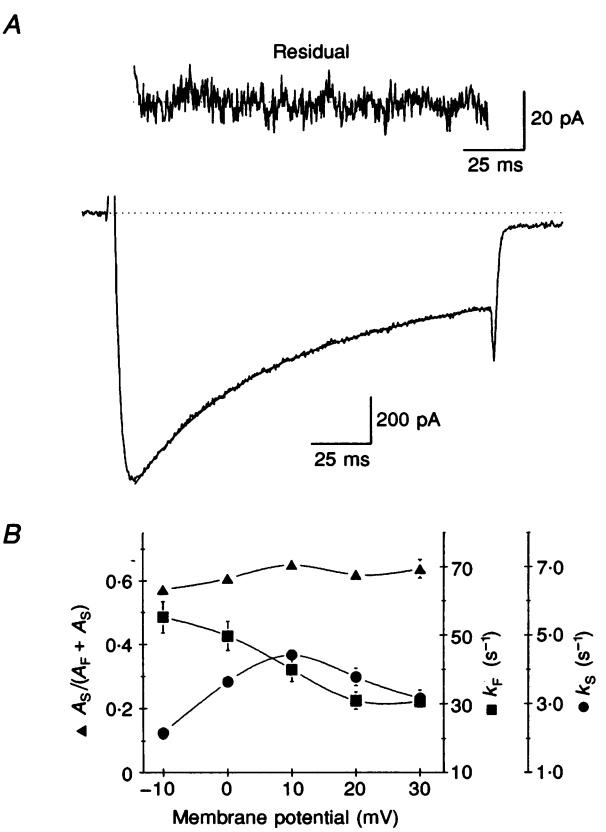

Figure 1. Two distinct components of I_{Ca} inactivation
Two exponential decay functions, each of the form $A\exp(-kt)$, were required to fit the inactivation phase of high-threshold I_{Ca}. *A*, I_{Ca} evoked by a step potential to +10 from −80 mV with a superimposed curve derived from eqn (5). In this example, $A_F = -486$ pA, $k_F = 25\ s^{-1}$, $A_S = -648$ pA and $k_S = 2{\cdot}73\ s^{-1}$. Upper trace is the residual current derived by subtracting the curve–fit result from the corresponding data points of I_{Ca}. Dotted line shows the zero transmembrane current level in this and following figures. *B*, summary of curve fitting parameters as a function of membrane potential. Data are given as means ± S.E.M. for $n = 29$ myocytes.

I_{Ca} exhibits slow and fast inactivating components

Fig. 1*A* shows I_{Ca} triggered by a voltage pulse to +10 mV from a holding potential of −80 mV. Peak I_{Ca} at +10 mV ranged from −0·26 to −2·11 nA. In every myocyte examined, the I_{Ca} inactivation phase was well described by the sum of two exponential decay functions:

$$I_{Ca}(t) = A_F \exp(-k_F t) + A_S \exp(-k_S t), \quad (5)$$

where A_F and A_S are the magnitudes and k_F and k_S are the rate constants of the fast and slow components, respectively. The suitability of the curve fitting function is clearly shown by the residual (or difference) waveform derived by subtracting the I_{Ca} record from the curve fitting result. The requirement for two exponential functions to describe the inactivation phase is consistent with past studies from isolated rat (Richard, Tiaho, Charnet, Nargeot & Nerbonne, 1990; Richard, Charnet & Nerbonne, 1993) and guinea-pig (Balke & Wier, 1991) ventricular myocytes. Richard *et al.* (1990, 1993) suggested that the fast and slow inactivation components reflect distinct, parallel gating paths of the same L-type Ca^{2+} channel (but see Imredy & Yue, 1994).

Detailed kinetic examination of I_{Ca} evoked by test pulses from −10 to +30 mV indicated that A_F and A_S were approximately equal over the voltage range (Fig. 1*B*), whereas k_F and k_S differed by an order of magnitude. For voltage-dependent inactivation, one would expect the rate constants to increase with more positive potentials. Instead, k_F decreased over this voltage range, whereas k_S increased between −10 and +10 mV then decreased between +10 and +30 mV (Fig. 1*B*). If Ca^{2+}-dependent inactivation is largely due to the $[Ca^{2+}]_i$ near the pore of the Ca^{2+} channel (Neely *et al.* 1994), the downward trend in k_F would be expected as the driving force for Ca^{2+} entry is decreased. A combination of voltage and Ca^{2+} dependence might explain the form of k_S *versus* membrane potential.

Anaesthetics preferentially depress the slowly inactivating current component of I_{Ca}

Application of the inhalational anaesthetics halothane (Fig. 2*A*) and isoflurane (Fig. 2*B*) reversibly decreased the peak I_{Ca} in a concentration-dependent manner (Fig. 2*C* and *D*, respectively) and also enhanced the apparent rate of inactivation. Neither the voltage for peak I_{Ca} (+10 mV), nor

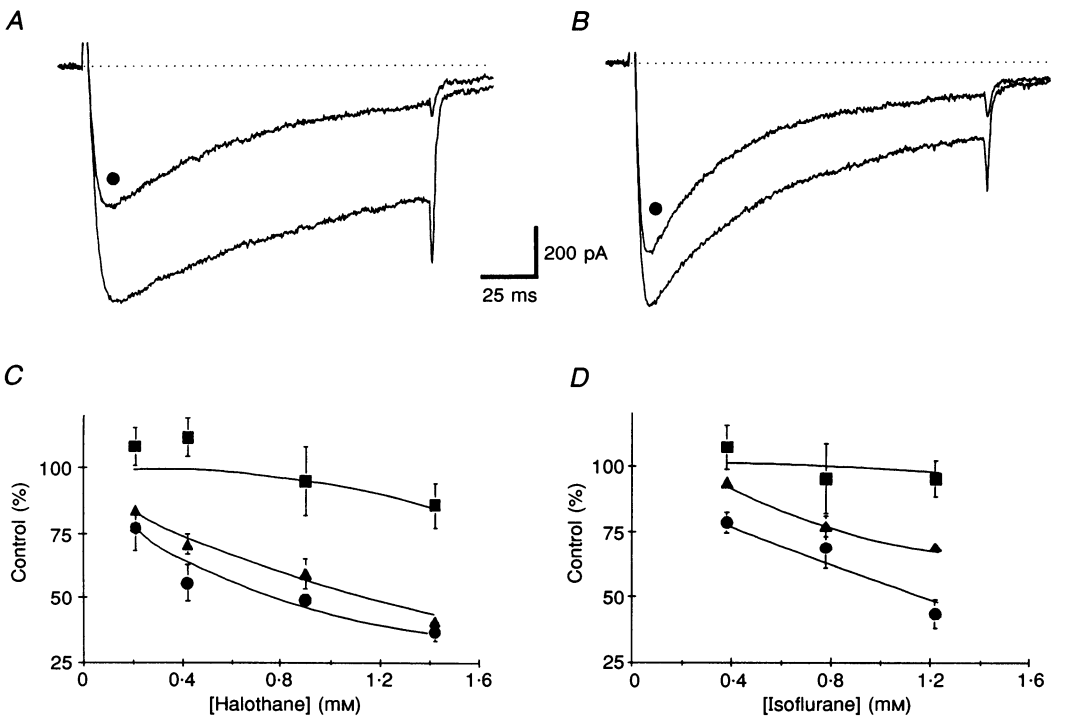

Figure 2. Effect of inhalational anaesthetics halothane and isoflurane on I_{Ca}

I_{Ca} waveforms shown were elicited by step potentials to +10 mV from a holding potential of −80 mV. The parameters A_F, A_S, k_F and k_S were derived from fitting the inactivation phase of I_{Ca} at +10 mV to eqn (5). *A*, control I_{Ca} and after administration of 0·9 mM halothane (●). *B*, control I_{Ca} and after administration of 0·8 mM isoflurane (●). Note that the two control traces appear to be different, however the inactivation kinetics, k_S and k_F, are virtually identical but the magnitude terms, A_S and A_F, differ. For the control trace in *A*, fitting the inactivation phase yielded $A_F = -248$ pA, $k_F = 29\ s^{-1}$, $A_S = -895$ pA and $k_S = 3{\cdot}62\ s^{-1}$. For the control record in *B*, $A_F = -627$ pA, $k_F = 28\ s^{-1}$, $A_S = -654$ pA and $k_S = 4{\cdot}38\ s^{-1}$. *C*, concentration dependence of the halothane-induced depression of both A_F (■) and A_S (●) compared with the reduction of peak I_{Ca} (▲) by halothane. *D*, concentration dependence of the isoflurane-induced depression of both A_F (■) and A_S (●) compared with the reduction of peak I_{Ca} (▲) by isoflurane.

the I_{Ca} reversal potential (+69 ± 1 mV; $n = 29$) was affected by the anaesthetics. Peak I_{Ca} fell by 41 ± 6% ($n = 5$) and 23 ± 4% ($n = 7$) in the presence of 0·9 mM halothane and 0·8 mM isoflurane, respectively. The anaesthetic effect was largely attributed to a preferential action on the magnitude and, to a lesser extent, the inactivation rate of the slowly inactivating current component. For I_{Ca} evoked by a voltage step to +10 mV, halothane (0·9 mM) and isoflurane (0·8 mM) depressed A_S by 50 ± 3% ($n = 5$) and 31 ± 8% ($n = 6$), respectively, while exerting no observable effect on A_F. The anaesthetics also enhanced k_S in a concentration-dependent manner with little observable action on k_F. For example, halothane at 0·9 and 1·4 mM significantly increased k_S at +10 mV from a control level of 3·43 ± 0·19 s^{-1} ($n = 5$) to 4·44 ± 0·22 s^{-1} ($n = 5$) and 6·30 ± 0·37 s^{-1} ($n = 6$), respectively. A similar effect at higher concentrations of isoflurane (> 0·8 mM) was observed.

If one was to calculate the total movement of Ca^{2+} ions by integrating I_{Ca} evoked by test potentials, one might expect both halothane and isoflurane to be profound myocardial depressants. To gain insight into how the anaesthetics may affect voltage-gated Ca^{2+} entry under physiological conditions, I_{Ca} was evoked using a cardiac AP waveform as the command voltage. The AP waveform rose from a resting potential of −86 mV to a peak voltage of +36 mV with a duration of 260 ms. As shown in Fig. 3, I_{Ca} triggered by the AP waveform exhibited a rapid inward transient during the AP upstroke followed by a slower plateau current during repolarization over potentials ranging from +24 to +8 mV. Halothane (0·9 mM) depressed the rapid inward transient and the plateau currents to the same extent, by 48 ± 2% ($n = 5$) and 52 ± 3%, respectively. In contrast, isoflurane (0·8 mM) diminished the plateau component to a greater extent than the rapid inward transient component, by 38 ± 2% ($n = 6$) *versus* 22 ± 2%, respectively. Upon complete repolarization, a small inward tail current was consistently observed. By introducing instantaneous voltage steps during this time span and performing ion substitution experiments, it was determined that the small inward tail current was carried by Cs^+ ions (data not shown), possibly through inwardly rectifying K^+ channels (Mitra & Morad, 1991). This small inward tail current was insensitive to both of the anaesthetics.

To determine whether or not a shift in the voltage dependence of inactivation can explain, at least in part, the anaesthetic effect, the steady-state inactivation profile of I_{Ca} was examined. Myocytes were held at a prepulse voltage ranging from −110 to +35 mV in 5 mV increments for 5 s and I_{Ca} was triggered by a test pulse to 0 mV. Richard *et al.* (1990, 1993) reported that the two components of I_{Ca} inactivation in rat myocardium exhibited a differential sensitivity to the holding potential; however, no difference was observed in the contribution of fast and slow

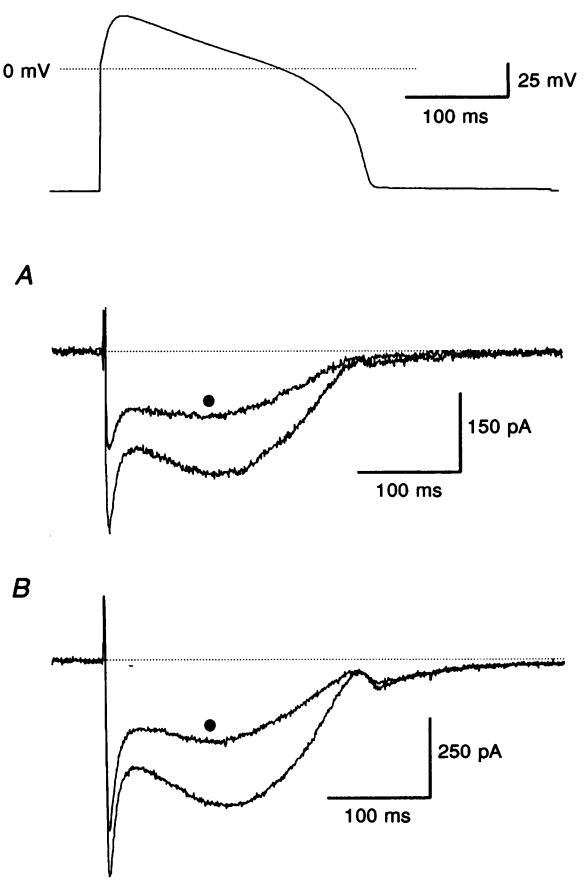

Figure 3. Inhalational anaesthetic action on I_{Ca} evoked by a cardiac action potential (AP) waveform

The rapid initial inward current component and the inward plateau current component were carried by Ca^{2+} ions, whereas the small inward tail current was carried by Cs^+ ions. I_{Ca} waveforms shown were elicited by step potentials to +10 mV from a holding potential of −80 mV. Current records were corrected for leak and capacitive artifacts by a modified $P/-4$ method. *A*, control I_{Ca} and after administration of 0·9 mM halothane (●). *B*, control I_{Ca} and after application of 0·8 mM isoflurane (●).

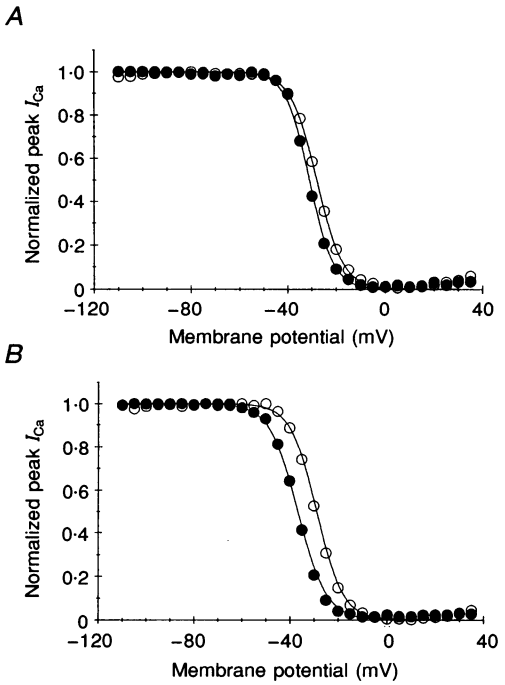

Figure 4. Effect of halothane and isoflurane on steady-state inactivation of I_{Ca}

Myocytes were held at a prepulse voltage ranging from −110 to +35 mV for 5 s prior to triggering a test pulse to 0 mV to measure peak I_{Ca}. Currents were not corrected for leak and capacitive artifact; instead leakage current was considered negligible at 0 mV. For each myocyte, the peak inward current evoked from each prepulse voltage was normalized to the peak I_{Ca} and fitted to the Boltzmann function with parameters describing the prepulse potential for half-steady-state inactivation (V_n) and the slope factor (k_n). *A*, steady-state inactivation from a typical cell before (control; ○) and after exposure to 1·4 mM halothane (●). V_n, −28 and −31 mV; k_n, −5·3 and −4·9 mV, for control and halothane exposure, respectively. *B*, steady-state inactivation from a typical cell before (control; ○) and after exposure to 1·2 mM isoflurane (●). V_n, −29 and −37 mV; k_n, −5·4 and −5·7 mV, for control and halothane exposure, respectively.

components as a function of prepulse potential in guinea-pig myocytes. For each cell, steady-state inactivation was quantified by normalizing the peak inward current from each prepulse potential to the maximum current. The resulting curves were well fitted by the Boltzmann function; under control conditions, V_n and k_n were −26·7 ± 0·9 mV ($n = 23$) and −5·7 ± 0·2 mV ($n = 23$), respectively (for example of a typical cell see Fig. 4*A*). Halothane (1·4 mM) produced a modest, reversible shift in V_n of 2·5 ± 0·3 mV ($n = 7$) towards more hyperpolarized potentials (Fig. 4*A*), with no significant effect on k_n. In contrast, isoflurane produced a more obvious shift in steady-state inactivation (Fig. 4*B*, typical cell); isoflurane (1·2 mM) reversibly shifted V_n by 7·7 ± 0·8 mV ($n = 5$) towards more hyperpolarized potentials. This effect of isoflurane was concentration dependent; V_n shifted by −4·3 ± 0·9 mV ($n = 7$) and −9·7 ± 0·6 mV ($n = 4$) in the presence of 0·8 and 1·6 mM isoflurane, respectively.

Since the main effect of the anaesthetics appeared to be a decrease in the amplitude of a component of I_{Ca} that exhibits slow inactivation, one possibility was that the voltage-dependent activation of this slowly inactivating component is shifted to more depolarized potentials. However, since I_{Ca} inactivation is incomplete for test

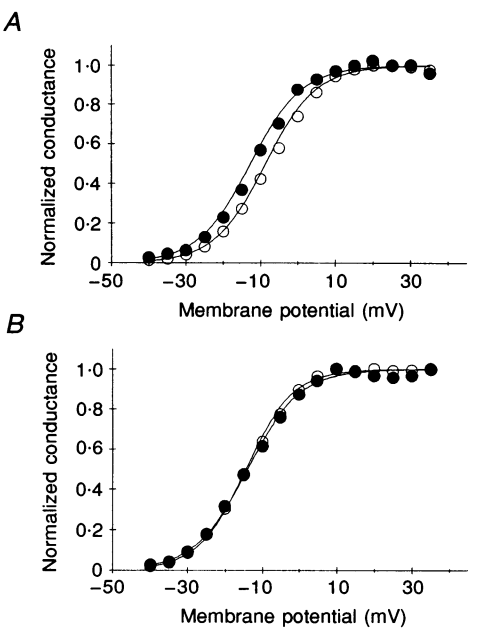

Figure 5. Anaesthetics have little or no effect on steady-state activation of I_{Ca}

Myocytes were voltage clamped to −50 mV to inactivate any T-type Ca^{2+} channels and voltage steps ranging from −40 to +35 mV were applied in 5 mV increments to measure peak I_{Ca}. Peak I_{Ca} was converted to conductance according to Ohm's law, normalized by the maximal Ca^{2+} conductance and fitted to the Boltzmann function. *A*, steady-state activation from a typical cell before (control; ○) and after exposure to 1·4 mM halothane (●). V_n, −9 and −13 mV; k_n, 6·9 and 7·0 mV, for control and halothane exposure, respectively. *B*, steady-state activation from a typical cell before (control; ○) and after exposure to 1·2 mM isoflurane (●). V_n, −14 and −14 mV; k_n, 6·4 and 7·1 mV, for control and halothane exposure, respectively.

potentials more negative than 0 mV, where activation is most sensitive to voltage, the fast and slowly inactivating components cannot be reliably separated over the voltage range of interest. Nevertheless, the effect of large concentrations of anaesthetics on net I_{Ca} activation was estimated by measuring peak inward current. I_{Ca} activation was assessed from a holding potential of −50 mV to eliminate any T-type I_{Ca} while providing a safety margin for the isoflurane-induced shift in steady-state inactivation. Cells were depolarized in 5 mV increments from −40 to +35 mV. The reversal potential was estimated graphically and current data were converted to conductance, $G(V)$, according to Ohm's law, normalized to maximum Ca^{2+} conductance, and plotted. The resulting curve was well fitted by the Boltzmann function where control V_n and k_n were −12·1 ± 1·4 ($n = 10$) and 6·5 ± 0·2 mV, respectively. Halothane (1·4 mM) caused a small hyperpolarizing shift in V_n by 3·8 ± 0·7 mV ($n = 5$) (Fig. 5*A*) while isoflurane (1·2 mM) produced no observable effect in V_n ($n = 5$) (Fig. 5*B*). Neither anaesthetic affected k_n. These data suggest that the anaesthetics exert little or no effect on the voltage dependence of I_{Ca} activation.

To examine the rate of recovery from inactivation, a standard two-pulse protocol (to 0 mV) was employed where the interpulse interval (to −80 mV) was progressively shortened from 580 to 100 ms. Each episode was separated by 6 s, which allowed complete recovery of I_{Ca}. Peak I_{Ca} during the test potential was then normalized to the peak I_{Ca} during the conditioning pulse. Facilitation of I_{Ca}, which has been described under similar recording conditions (Yuan & Bers, 1994), was observed in particular when comparing the first set of conditioning and test current records separated by an interpulse interval of 580 ms. Under control conditions, as well as in the presence of 1·4 mM halothane, a 4 ± 1 % ($n = 4$) increase was observed in peak I_{Ca} during the first episode, suggesting a lack of anaesthetic effect on the mechanism(s) underlying facilitation. No facilitation of I_{Ba} was observed with this protocol, consistent with a role for $[Ca^{2+}]_i$ in this process (Yuan & Bers, 1994). Examination of the normalized current

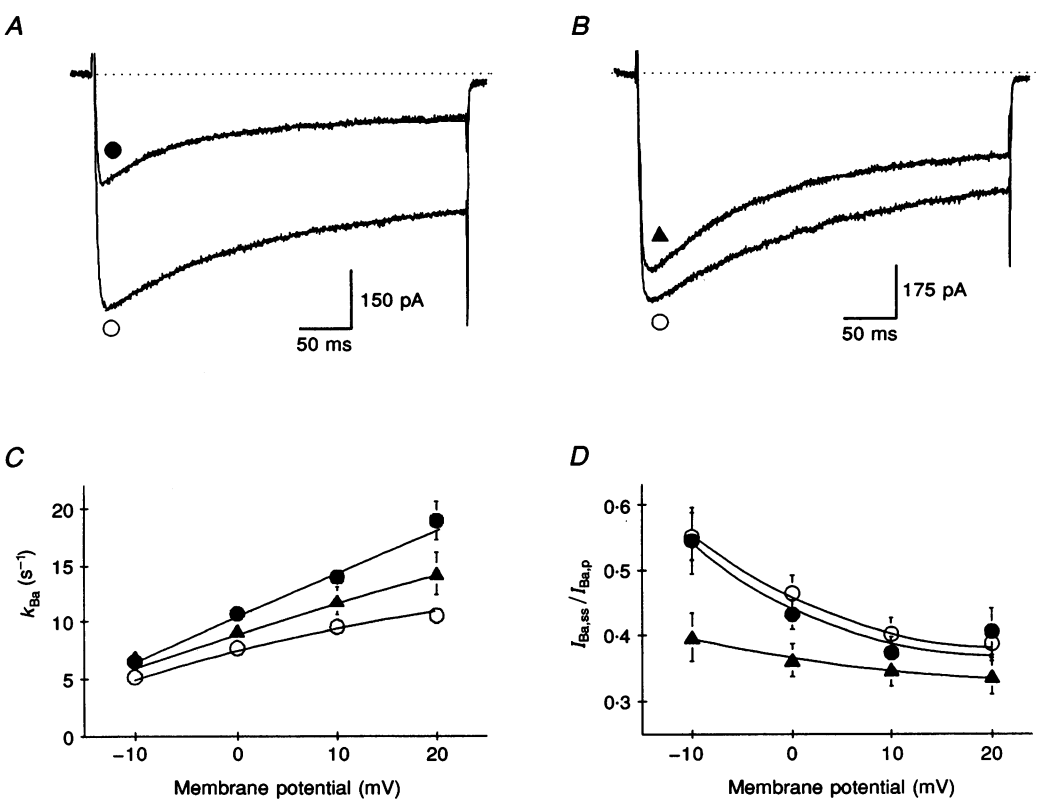

Figure 6. Effect of the anaesthetics on inactivation kinetics of I_{Ba}

Inward current was evoked by step potentials to 0 mV from a holding voltage of −80 mV. *A*, I_{Ba} under control and halothane (0·9 mM) conditions with a superimposed curve derived by fitting the inactivation phase of I_{Ba} to eqn (6), where $I_{Ba,ss}$ is the offset term and $I_{Ba,p}$ is the peak I_{Ba}. Control (○): $I_{Ba,p} = -610$ pA, $k_{Ba} = 6{\cdot}55\ s^{-1}$ and $I_{Ba,ss} = -331$ pA. Halothane (●): $I_{Ba,p} = -280$ pA, $k_{Ba} = 12{\cdot}68\ s^{-1}$, and $I_{Ba,ss} = -124$ pA. *B*, I_{Ba} under control and isoflurane (0·8 mM) conditions. Inactivation phases were fitted to eqn (6) with control (○): $I_{Ba,p} = -768$ pA, $k_{Ba} = 5{\cdot}38\ s^{-1}$ and $I_{Ba,ss} = -334$ pA, and isoflurane (▲): $I_{Ba,p} = -667$ pA, $k_{Ba} = 8{\cdot}29\ s^{-1}$, and $I_{Ba,ss} = -258$ pA. Summary of curve fitting parameters k_{Ba} (*C*) and the ratio of $I_{Ba,ss}$ to $I_{Ba,p}$ (*D*) as a function of membrane potential under control conditions. ○, control; ●, halothane; ▲, isoflurane. Data are given as means ± S.E.M. for $n = 5$–12 myocytes.

amplitudes for control and halothane-treated conditions indicated that the anaesthetic had little or no effect on the rate of recovery from I_{Ca} inactivation. Isoflurane (1·2 mM) was also without effect.

Elimination of rapid inactivation with Ba^{2+}: anaesthetic effects on I_{Ba}

In the presence of extracellular Ca^{2+}, both halothane and isoflurane primarily affected the rate of slow Ca^{2+} channel inactivation which has been largely attributed to a voltage-dependent mechanism (Kass & Sanguinetti, 1984; Lee, Marban & Tsien, 1985; Zong, Zhou & Tanabe, 1994). Therefore, the efficacy of the anaesthetics to alter I_{Ba}, which eliminates the Ca^{2+}-dependent inactivation pathway (Lee *et al.* 1985), was examined. If Ca^{2+}-dependent inactivation constitutes an anaesthetic-insensitive distinct pathway, one might expect I_{Ba} to be more sensitive to the inhalational agents than I_{Ca}. In fact, at the highest halothane concentration employed, sensitivity differences between I_{Ca} and I_{Ba} became statistically significant. In a paired study, where both I_{Ca} and I_{Ba} were measured in the same myocyte, halothane at ~1·8 mM decreased peak I_{Ca} by 57 ± 4% ($n = 4$) whereas I_{Ba} was diminished by 68 ± 4%; in comparison, I_{Ba} was 11 ± 5% more sensitive than I_{Ca}.

I_{Ba} activation was quantified by the time from the onset of depolarization required to reach one-half of the peak inward current ($t_{½}$). Overall, control $t_{½}$ at 0 mV was 6·1 ± 0·3 ms ($n = 17$) without a significant change after administration of either halothane (1·4 mM) or isoflurane (1·2 mM). For experiments assessing anaesthetic effects on I_{Ba} inactivation, the command pulse was extended in duration to 320 ms to assess adequately the slower decay phase. Under control conditions, the primary difference between I_{Ca} and I_{Ba} was the lack of fast inactivation ($k \approx 30$–$45\ s^{-1}$) of I_{Ba}. Inactivation of I_{Ba} was well fitted by a single-exponential decay function and an offset term as previously reported (Zong *et al.* 1994) under both control and anaesthetic-treated conditions (Fig. 6*A* and *B*):

$$I_{Ba}(t) = I_{Ba,ss} + (I_{Ba,p} - I_{Ba,ss}) \exp(-k_{Ba}t), \quad (6)$$

where $I_{Ba,ss}$ is the offset term, k_{Ba} is the rate of I_{Ba} inactivation and $I_{Ba,p}$ is the peak Ba^{2+} current. This quantitative description of the I_{Ba} inactivation phase is consistent with a simple two-state model describing an $O \rightleftharpoons I$ transition, where O and I are open and inactivated states, respectively. Unlike k_S derived from I_{Ca} inactivation curve fitting, k_{Ba} showed clear voltage dependence (Fig. 6*C*) as did the ratio of $I_{Ba,ss}$ to $I_{Ba,p}$ (Fig. 6*D*). Application of halothane (Fig. 6*A*) and isoflurane (Fig. 6*B*) depressed $I_{Ba,p}$ evoked by a step potential to 0 mV to the same degree as peak I_{Ca}. $I_{Ba,p}$ reversibly decreased by 46 ± 3% ($n = 9$) and 17 ± 6% ($n = 7$) with administration of 0·9 mM halothane and 0·8 mM isoflurane, respectively. Detailed examination of the I_{Ba} inactivation kinetics revealed that both anaesthetics enhanced k_{Ba} to the same extent. Under control conditions, k_{Ba} at 0 mV was 7·70 ± 0·50 s^{-1} ($n = 12$) increasing by 38 ± 9% ($n = 6$) and 30 ± 10% ($n = 6$) with halothane and isoflurane, respectively (Fig. 6*C*). Therefore, the anaesthetic-induced change in k_{Ba} appeared to be voltage independent since the same effect was observed for I_{Ba} evoked by pulses from −10 to +20 mV and +110 mV. Differences between the anaesthetics were apparent with respect to the ratios of the amplitude terms $I_{Ba,ss}$ and $I_{Ba,p}$ (Fig. 6*D*). To a large extent, the differences in the amplitude ratios can be attributed to the fact that isoflurane, in contrast to halothane, had far less effect on $I_{Ba,p}$ whereas

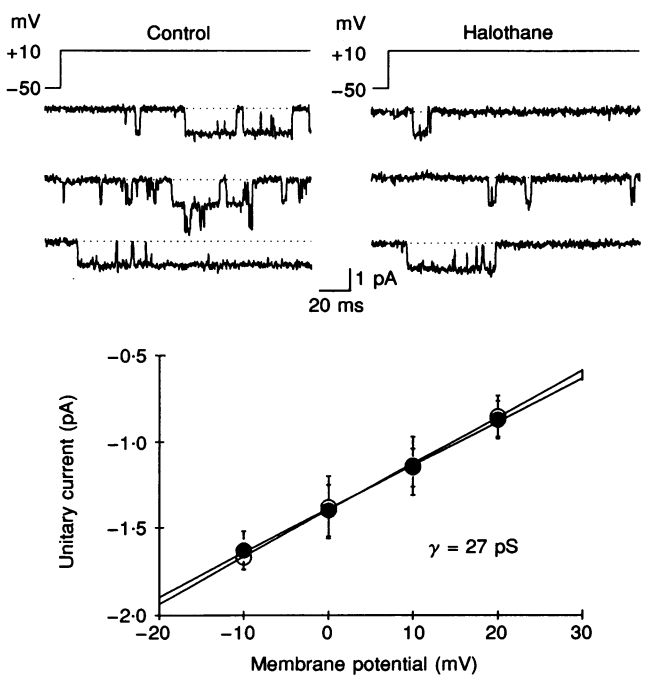

Figure 7. Halothane has no effect on single Ca^{2+} channel conductance

Three consecutive sweeps evoked by voltage steps from −50 to +10 mV from the same membrane patch under control conditions and after application of 1·4 mM halothane. Two Ca^{2+} channels were present in the membrane patch. The duration of Ca^{2+} channel opening was increased by including 5 μM Bay K 8644 in the patch pipette solution. Leak and capacitive currents have been subtracted; the downward deflections signify channel opening. Voltage steps from −10 to +20 mV were used to trigger Ca^{2+} channel openings to estimate channel amplitude at each potential and calculate single-channel conductance (γ). Data are given as means ± S.D. for at least 20 openings.

both anaesthetics increased the rate of I_{Ba} inactivation. Voltage steps well beyond the experimental reversal potential for I_{Ba} produced transient outward currents which appeared to be carried by Cs^+ flux through Ca^{2+} channels, due to the sensitivity of these currents to Cd^{2+} and nifedipine. For voltage steps to +110 mV, control k_{Ba} was $17{\cdot}55 \pm 0{\cdot}39\ s^{-1}$ ($n = 9$) increasing by $40 \pm 6\%$ ($n = 5$) and $41 \pm 11\%$ ($n = 4$) with halothane and isoflurane, respectively. Anaesthetic effects on the amplitude ratios at +110 mV were similar to those observed at less depolarized potentials.

Effects of halothane at the single Ca^{2+} channel level

Given the marked depression of I_{Ca} and I_{Ba} by halothane, further experiments focused on the effects of this anaesthetic at the single-channel level. Initial cell-attached patch-clamp measurements of Ca^{2+} channel activity were performed to verify that the effect of halothane was due to a change in P_O rather than a decrease in unitary conductance. For these experiments, 5 μM Bay K 8644 was added to the patch pipette solution to permit clear resolution of the open-channel current. Representative channel activity, evoked by

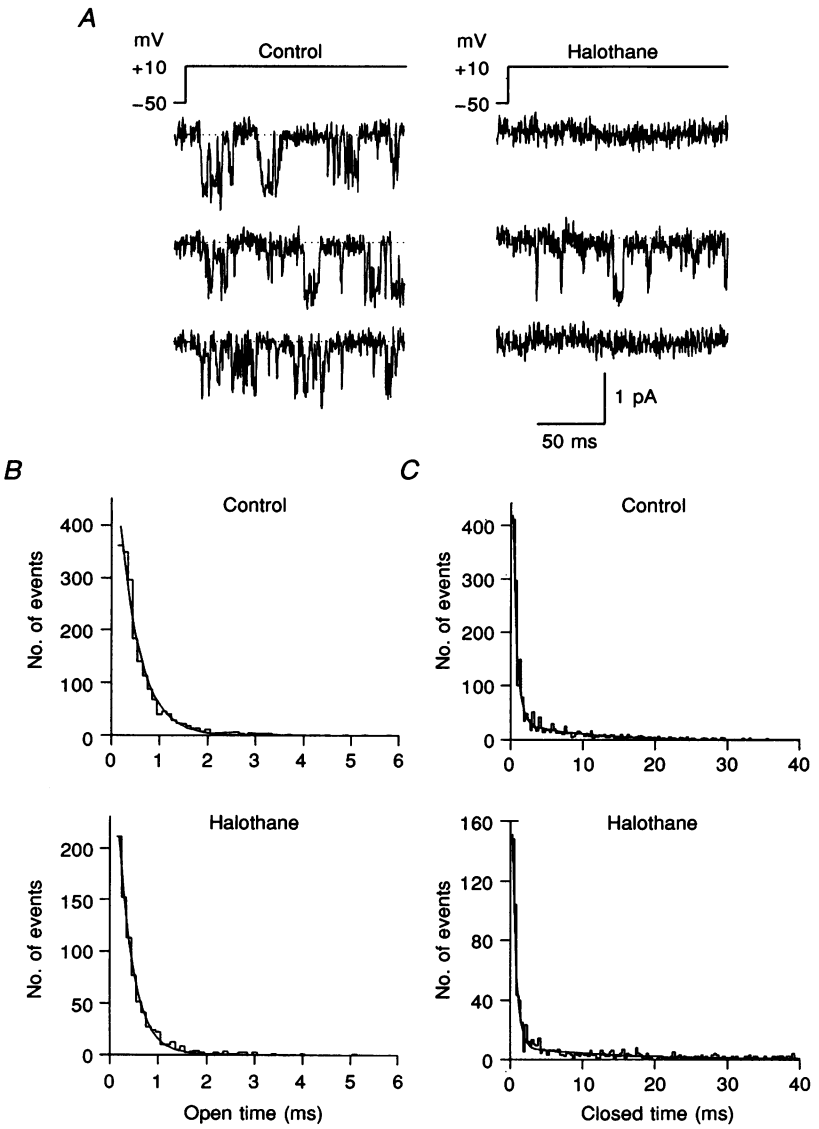

Figure 8. Effect of halothane on single-channel dwell times

A, three consecutive sweeps evoked by voltage steps from −50 to +10 mV from the same membrane patch with a single Ca^{2+} channel under control conditions and after application of 1·4 mM halothane. Leak and capacitive currents have been subtracted; the downward deflections signify channel openings. *B*, comparison of open-time distributions before (top) and after application of 1·4 mM halothane (bottom). *C*, comparison of closed-time distributions before (top) and after administration of 1·4 mM halothane (bottom). Halothane decreased the event frequency, the mean channel open time (τ_O) and increased the time constant of the slow closed-state component (τ_{CS}) with no effect on the time constant of the fast closed-state component (τ_{CF}). Nearly identical results were also observed in another single-channel patch recording.

Table 1. Summary of kinetic values derived for a Ca^{2+} channel under control and halothane-treated conditions

Condition	P_O	P_O(plat)	Null fraction	k_1 (s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	P_O(sim)
Control	0·02831	0·02951	0·4063	103	761	906	2500	0·02650
Halothane	0·00833	0·00968	0·5462	48	786	880	3333	0·00790

Values calculated according to Colquhoun & Hawkes (1981) for one of the single-channel patches. The ratio of the integrals of the fast to slow closures, equal to k_2/k_{-1}, was 1·19 for control *versus* 1·12 for 1·4 mM for halothane-treated data. Since $\tau_{CF}^{-1} = k_2 + k_{-1}$, these two rate constants can be determined directly. P_O(plat) was determined by calculating P_O after ignoring the first 30 ms of the data sweep to ensure that a plateau had been reached. After correcting P_O(plat) for the null fraction with the relationship: $P_O' = P_O$(plat) (1 + null fraction), the equation $P_O' = k_2k_1/(k_{-2}k_1 + k_2k_1 + k_{-2}k_{-1})$ was used to calculate k_1. The computer simulated result, P_O(sim), which shows excellent agreement with P_O, was generated with the three-state model parameters, along with two additional closed states to model nulls, using a computer simulation program (Pancrazio, 1995).

voltage steps from −50 to +10 mV, under control conditions and after application of 1·4 mM halothane are shown in Fig. 7. Halothane failed to alter the magnitude of single-channel current (i) at +10 mV; i was −1·17 pA for both control and halothane-treated conditions yielding a slope conductance of $\gamma = 27$ pS. Instead, P_O and the null fraction were affected by halothane. P_O decreased by $38 \pm 6\%$ ($n = 4$) from a control level of $0{\cdot}156 \pm 0{\cdot}060$ whereas the null fraction increased by $82 \pm 15\%$ from a control level of $0{\cdot}311 \pm 0{\cdot}106$. Interestingly, these changes in P_O and the null fraction for 1·4 mM halothane are nearly identical to those reported for 1·46 mM enflurane recorded under similar conditions (Takahashi, Puttick & Terrar, 1994). To compare the results from single-channel experiments with the whole-cell data, experiments were conducted without Bay K 8644 in the patch pipette solution. Voltage steps to +10 mV elicited Ca^{2+} channel openings which were quite brief, averaging ~0·4 ms (Fig. 8*A*). While halothane again had no effect on single-channel amplitude, both P_O and the null fraction were affected by the anaesthetic. P_O, which was $0{\cdot}047 \pm 0{\cdot}016$ ($n = 8$) under control conditions, fell by $61 \pm 5\%$, a magnitude depression which is virtually identical to that observed under whole-cell recording of I_{Ba} at this anaesthetic concentration. In addition, channel opening was far less frequent in the presence of the anaesthetic. Part of the reduction of P_O can be attributed to an increase in the null fraction; halothane elevated the null fraction by $32 \pm 6\%$ ($n = 5$) from a control level of $0{\cdot}393 \pm 0{\cdot}109$. In two instances, only a single Ca^{2+} channel was present in each membrane patch based on the lack of simultaneous openings in the data record and, more importantly, calculation of the probability, P_1, that a data record from a patch with N channels will exhibit only single openings. For both membrane patches for $N = 2$ channels, $P_1 \ll 0{\cdot}001$, suggesting that it is very unlikely that the data were due to two or more channels. Comparison of open-time histograms indicated that only one exponential decay function, thus one open state, was necessary to quantitatively describe the data under control and halothane conditions. The time constant of the open-time distribution (τ_O) decreased by 25–33% (Fig. 8*B*); a finding supported by data from two other patches with two channels present. In contrast, the closed-time distribution required the sum of fast and slow exponential decay functions with the time constants of the fast closed-state component (τ_{CF}) (0·6–0·7 ms) and the slow closed-state component (τ_{CS}) (7·6–8·8 ms), respectively. Halothane had no effect on the fast component, however τ_{CS} increased by 64–98% to 14·4–15·0 ms (Fig. 8*C*).

DISCUSSION

The present findings suggest that the different effects of halothane and isoflurane in myocardial function may be partially attributed to a differential suppression of I_{Ca} by these anaesthetics. The observed concentration dependence of the reduction in peak I_{Ca} by halothane agreed well with previous work by Bosnjak *et al.* (1991); however, a similarly large effect on peak I_{Ca} by isoflurane was not observed. The present results indicate that the blocking effects of halothane and isoflurane differ in potency; a view supported by earlier single microelectrode studies (Lynch *et al.* 1981; Lynch, 1986; Terrar & Victory, 1988*a*, *b*).

The anaesthetics alter I_{Ca} decay by selectively affecting a current component which can be separated at depolarized potentials by the slower rate of inactivation. This effect was independent of any change in the rate of recovery from inactivation, consistent with other recent work (Baum, Wetzel & Klitzner, 1994). A minor effect of halothane on steady-state inactivation, similar to that reported by Niggli and colleagues (Niggli, Maurer & Weingart, 1989), was also observed. This result differs from that of Baum *et al.* (1994) who reported that 1% halothane, ~0·6 mM in solution at room temperature, shifted the steady-state inactivation parameter V_n by 11 mV towards hyperpolarized potentials. However, this previously reported effect of halothane exhibited poor reversibility upon washout by only 4 mV, closer to the magnitude of the voltage shift observed in this study.

The AP voltage-clamp experiments offer some insights into how halothane and isoflurane might exert their differential effects on myocardial contractility. Previous work has identified an early and late phase of tension development in guinea-pig ventricle which are most prominent at high and low stimulation frequencies, respectively (Lynch, 1986, 1990). The rested state contraction (RSC), which is characterized by only delayed tension development presumably due to the depletion of activator Ca^{2+} from the SR, is depressed by both halothane and isoflurane (Lynch, 1986, 1990). Interestingly, the delayed tension development during an RSC appears to reach a peak at a time similar to the AP plateau current, which is suppressed by both anaesthetics (Fig. 3). In contrast, faster rates of stimulation permit the accumulation of activator Ca^{2+} in the SR and rapid tension development, which is depressed by halothane, and to a lesser extent, isoflurane (Lynch, 1986, 1990). This latter observation is consistent with the marked depressant effects of halothane, but not isoflurane, on the rapid inward Ca^{2+} current during the AP clamp upstroke observed in the present experiments. Clearly, anaesthetic effects on electrically evoked tension development depend on a number of other factors beyond Ca^{2+} channels, including the ryanodine receptor and/or Ca^{2+} release channel modulation (Connelly & Coronado, 1994; Lynch & Frazer, 1994) leading to a depletion of activator Ca^{2+} from intracellular stores (Wheeler *et al.* 1988; Wilde *et al.* 1991).

The inactivation phase of I_{Ca} required two exponential decay functions to achieve adequate fitting, an observation consistent with a number of past studies in intact heart cells (Richard *et al.* 1990; Balke & Wier, 1991; Richard *et al.* 1993) and in *Xenopus* oocytes expressing the α_{1C} cardiac Ca^{2+} channel subunit (Neely *et al.* 1994). Based on whole-cell recordings of I_{Ca} from rat ventricular myocytes, Richard *et al.* (1993) concluded that there are two separate pathways for Ca^{2+} channel gating where the channel can shift to one of two open states, O_1 or O_2. In their model, the two distinct rates of whole-cell inactivation arise from either the rapid transition from O_1 to I_1 or the slower transition from O_2 to I_2. Imredy & Yue (1994) have presented a model based on extensive quantitative analysis of single and multiple Ca^{2+} channel patches. In this model, a rise in $[Ca^{2+}]_i$ near or around the pore induces a rapid transition to a 'mode-Ca' gating pathway which is characterized by a very low probability of opening. The use of Ba^{2+} as a charge carrier was shown to abolish the shift to 'mode-Ca' (Imredy & Yue, 1994), consistent with the view of other groups that voltage-dependent inactivation can be isolated with the use of Ba^{2+}. The fact that (1) an anaesthetic-induced change in the fast component of inactivation was not observed, and (2) substitution of extracellular Ba^{2+} for Ca^{2+} failed to reduce the effectiveness of the anaesthetics, argues against a role for Ca^{2+}-dependent inactivation of I_{Ca} in the action of the anaesthetics under the experimental conditions of the present study. However, we cannot exclude the possibility that Ca^{2+}-dependent inactivation may be modulated by halothane in ventricular myocytes of other species. For example, application of the SR Ca^{2+}-release channel modulator ryanodine seems to markedly affect the inactivation of I_{Ca} in rat (Imredy & Yue, 1994) but not guinea-pig (Balke & Wier, 1991; J. J. Pancrazio and C. Lynch, unpublished data) ventricular myocytes recorded under similar experimental conditions.

The measurements of Ca^{2+} channel dwell times can be interpreted within the context of a kinetic model with two closed states, C_1 and C_2, followed by an open state, O.

$$C_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} O \qquad (7)$$

Using values derived from one of the single channel patches (Table 1), the major kinetic effects of halothane appeared to involve alterations in the rate constants k_1 and k_{-2}. To calculate k_1, equations derived for a three-state stationary process were used (Colquhoun & Hawkes, 1981). Two adjustments were made to the experimentally observed P_O to permit application of the previously derived equations. First, P_O was measured 30 ms after the onset of the command pulse to ensure that a plateau had been reached yielding P_O(plat). Second, P_O was corrected for the observed null fraction under each condition. The resulting kinetic parameters were then modelled using a computer simulation program (Pancrazio, 1995). An additional two closed states preceding the three-state model shown above were included to account for the null sweeps.

$$\begin{array}{l} C_O \longrightarrow C_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} O \\ \downarrow \\ C_N \end{array} \qquad (8)$$

This step was critical since the three-state model, even with the kinetic parameters derived for the halothane case, predicted that only 2% of the 160 ms duration sweeps would be nulls in contrast to the 54·6% that was observed. The simulated results showed excellent agreement with the experimental findings. Moreover, the importance of each of the halothane-induced kinetic effects on P_O can be assessed through modelling each effect independently. Based on the simulation, the increases in the null fraction and k_{-2} cause 32 and 35% decreases in P_O, respectively, whereas the decrease in k_1 results in a 55% decrease in P_O, suggesting that the halothane effect on the transition from C_1 to C_2 is the most important of the effects at the channel level.

From the whole-cell records it is apparent that one or more inactivated states should be introduced to the above model to explain the increase in the rate of inactivation of I_{Ba} by the anaesthetics. Note that the rate constants between state O and an inactivated state I, which should sum to k_{Ba}, are much slower than the other rate constants. Therefore, the presence of one or more inactivated states would have little or no impact on the values reported in Table 1. A previous model for Ca^{2+} channel inactivation derived from cardiac myocytes incorporated two inactivated states, I_1 and I_2

(Kass & Sanguinetti, 1984). The observed rise in k_{Ba} along with the decrease in $I_{Ba,ss}$ during application of halothane or isoflurane argues for an increase in the transition rate from states O to I_1. In fact, the increase in the rate of inactivation explains the majority of the effect of isoflurane, in agreement with a recent report (Hirota, Fujimura, Wakasugi & Ito, 1996). The slight effect of isoflurane on steady-state inactivation of I_{Ca} implies an additional effect of isoflurane, perhaps to increase the transition rate from I_1 to I_2. There is evidence to suggest that isoflurane and to a lesser extent halothane may reduce the membrane dipole potential which would be consistent with my observations concerning steady-state inactivation (Qin, Szabo & Cafiso, 1995).

The increase in the null fraction with halothane probably coupled with the marked reduction in peak I_{Ba} by halothane suggests the entry into a long-lived closed state, or perhaps mode 0, labelled C_N in the above model. In a study using biochemical methods, halothane but not isoflurane was shown to significantly inhibit the binding of [^{3}H]nitrendipine to purified bovine sarcolemma which was interpreted as a reduction in the maximal number of binding sites (Drenger, Quigg & Blanck, 1991). It can be speculated that halothane-induced entry into a long-lived closed state or mode 0 might explain the apparent 'loss' of sarcolemmal Ca^{2+} channels identified by binding of radio-labelled nitrendipine. In the only other study examining inhalational anaesthetic effects at the single Ca^{2+} channel level, it has been reported that the inhalational anaesthetic enflurane decreases cardiac Bay K 8644-enhanced L-type Ca^{2+} channel mean open time and increases the null fraction (Takahashi, Puttick & Terrar, 1994). Note that while the enflurane-induced changes in P_O and the null fraction agree well with the present data collected with Bay K 8644 present in the patch pipette solution, the absence of Bay K 8644 yielded different results indicating that there may be mode specific actions of the anaesthetics. Alternatively, the anaesthetic-induced depression observed in the presence of Bay K 8644 may represent inhibition of the dihydropyridine binding by the anaesthetics rather than a mode-specific effect. Further experiments will be necessary to clarify this issue.

The differential actions of halothane and isoflurane suggest that specific features of their chemical structures (e.g. ability to form hydrogen bonds) may have an important role in their actions. Such agent-specific behaviour is evident with ryanodine receptors, in which halothane clearly activates channel opening and ryanodine binding, while isoflurane does not (Connelly & Coronado, 1994; Lynch & Frazer, 1994). The common action in affecting the rate of slow inactivation of I_{Ca} may relate to the shared lipophilic characteristics of these agents. The differences between the anaesthetics in their effect on the SR ryanodine receptors and Ca^{2+} channels may contribute to the divergent actions of these anaesthetics on myocardial function.

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