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Effects of halothane on membrane currents associated with contraction in single myocytes isolated from Guinea-pig ventricle

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1 The effects of halothane on electrical activity and contraction were investigated in single myocytes isolated from guinea-pig ventricle.

2 Halothane depressed the plateau and shortened the duration of action potentials.

3 Halothane also reduced the amplitude of inward calcium currents and of additional inward current activated by cytosolic calcium under voltage-clamp conditions.

4 Contractions (measured by an optical technique) accompanying either action potentials or calcium currents were reduced by halothane. However, the extent of attenuation of contraction was greater than when a similar level of calcium channel blockade was induced by application of verapamil.

5 Actions of halothane on calcium-activated tail currents in double-pulse experiments were consistent with reduction by halothane of the cytosolic calcium transient, perhaps as a consequence of reduced uptake of calcium into sarcoplasmic reticulum stores.

6 It is concluded that the actions of halothane on inward currents contribute to its effects on action potentials. The reduction in contraction caused by halothane may result partly from a reduced influx of calcium to trigger contraction, and partly by a reduced release of calcium from sarcoplasmic reticulum stores.

Introduction

Halothane causes dose-dependent depression of function in isolated cardiac muscle (Brown & Crout, 1971), in the intact hearts of experimental animals (Prys-Roberts *et al.*, 1972) and man (Sonntag *et al.*, 1975). The mechanism of the negative inotropic effect of halothane is unclear but it has been suggested that inhibition of the contractile proteins could depress function (Merin *et al.*, 1974; Pask *et al.*, 1981). However, the anaesthetic concentrations used in these studies are an order of magnitude higher than that required to cause a significant reduction in contractility. A second possibility is that halothane may reduce the calcium transient which activates contraction. Inhibition of the aequorin light signal recorded during contraction of cat papillary muscle (Bosnjak & Kampine, 1986) supports this possibility. A reduced calcium transient might arise from inhibition of calcium influx and it has been suggested that halothane inhibits the second inward current in heart muscle (Lynch *et al.*, 1981; Ikemoto *et al.*, 1985). A further action of halothane inhibiting reuptake of calcium into the intracellular stores may indirectly reduce calcium release during an action potential (Su & Kerrick, 1979). However, other experiments investigating this possibility have yielded conflicting results (Lain, *et al.*, 1968; Blanck & Thompson, 1981).

The experiments described in this paper examine the effects of halothane in single heart cells on ionic currents such as the second inward current and the calcium-activated tail current which are thought to be associated with contraction (Mitchell *et al.*, 1987). The influence of halothane upon these currents can be compared with the effect on contraction. Preliminary observations have been communicated to the Physiological society (Terrar & Victory, 1986; 1987).

Methods

Cells were isolated from guinea-pig ventricles by collagenase and elastase digestion (Powell *et al.*, 1980;

Mitchell *et al.*, 1985). Aliquots of cell suspension were mounted on the surface of an agar-coated coverslip in a perspex organ bath. The superfusing solution contained (mM): NaCl 118.5, $NaHCO_3$ 15.4, KCl 4.2, KH_2PO_4 1.2, glucose 11.1, $CaCl_2$ 2.5, $MgSO_4$ 1.2, bovine serum albumin 2 mg ml^{-1}. All solutions were bubbled with 95% O_2:5% CO_2 (pH 7.4, 36–37°C) before delivery to the bath. This concentration of oxygen may not be essential for adequate oxygenation of single cells but was used to facilitate comparison with results from multicellular preparations which are oxygenated with this mixture. A tap at the inflow tube permitted rapid changeover to a solution pre-equilibrated for at least 45 min with halothane (ICI, BDH) from an Abingdon vaporiser. Halothane concentration in the bath was assayed by a gas-liquid chromatography method (Miller & Gandolfi, 1979). The concentrations determined were: 1.2%: 0.75 mM; 1.6%: 1.04 mM; 2%: 1.16 mM; 2.4%: 1.57 mM. Up to 3% inspired halothane may be used to induce anaesthesia, while 0.8–1.2% is used for maintenance of the anaesthetic state.

Heart cells were impaled with glass microelectrodes containing 0.5 M K_2SO_4 (resistance 15–25 MΩ) in the majority of experiments. Electrodes containing 3 M CsCl were used to suppress outward potassium current (Matsuda & Noma, 1984). Buffering of cytosolic Ca at a low level was achieved by loading cells with EGTA from electrodes filled with 125 mM K_2SO_4 and 500 mM EGTA (pre-titrated to pH 7.4 with 4 M KOH).

Membrane potentials were recorded and voltage signals fed to a pre-amplifier incorporating a bridge circuit (Axoclamp 2). Membrane potential could be changed by application of a constant current through the recording electrode and action potentials could be elicited by brief depolarizing pulses (2 ms). Cells could also be voltage clamped with a single microelectrode system in which the function of the electrode was switched rapidly between current passing and voltage recording (Wilson & Goldner, 1975). The Axoclamp 2 system was used at a switching rate of 2–8 kHz. Cells were clamped at a holding potential of −40 mV to inactivate sodium current. Current and voltage signals were displayed on a digital storage oscilloscope (Gould 4020) and recorded on magnetic tape for later analysis. The second inward current was evoked by a step depolarization from −40 to 0 mV. The magnitude of the current was measured as the difference between the peak inward current and the steady current at the end of the pulse (Mitchell *et al.*, 1983).

Time course and extent of contraction was monitored by a photodiode in the eyepiece of the microscope (Mitchell *et al.*, 1983; 1987). Light in the field of view was restricted to the region of the cell. Changes in the output of the photodiode correspond to the extent of contraction which was measured in arbitrary units. Voltage clamp records in this paper were obtained in the steady state at a stimulation rate of 0.3 Hz. This particular frequency was chosen to allow prolonged study of each cell and reduce the inactivation of the second inward current. The occasions where higher frequencies are used are stated in the text.

These experiments were performed during an 18 month period and observations made in over 200 cells obtained from 68 guinea-pigs. Results are displayed and quoted as mean ± s.e. mean change evoked by administration of halothane. Values before and after administration of anaesthetic were compared (Student's paired *t* test).

Results

Halothane applied in the solution flowing over the ventricular cells reduced the amplitude of contraction; there was a shortening and depression of the plateau of action potentials simultaneously recorded from the same cells. These effects are illustrated in Figure 1a where the dose of halothane applied from a vaporiser was 2% (volume/volume) which corresponded to a (free) concentration of 1.16 mM in the bathing solution (see Methods). Figure 1b shows currents recorded under voltage-clamp conditions for a step depolarization from −40 to 0 mV in the same cell used for Figure 1a. Halothane reduced the amplitude and speeded the decay of the second inward current. This action of halothane on the second inward current has been observed consistently in over 200 cells and would be expected to contribute to the negative inotropic effect and to the modification of action potentials described above.

Voltage-clamp pulses to different membrane potentials were applied and a current-voltage curve constructed (Figure 2). The currents at the end of the step depolarizations to different membrane potentials are plotted as triangles, and peak currents are plotted as squares. The difference between these curves provides a measure of the amplitude of the second inward current, but this estimate is complicated by time-dependent outward currents, particularly at very positive potentials. Problems arising from outward time-dependent potassium currents were minimised in this cell and 7 others by intracellular application of Cs (Matsuda & Noma, 1984). Under these conditions halothane reduced the amplitude of the second inward current without substantial effect on the shape of the current voltage-curves.

The effects of halothane on outward currents were investigated in cells which were not loaded with Cs.

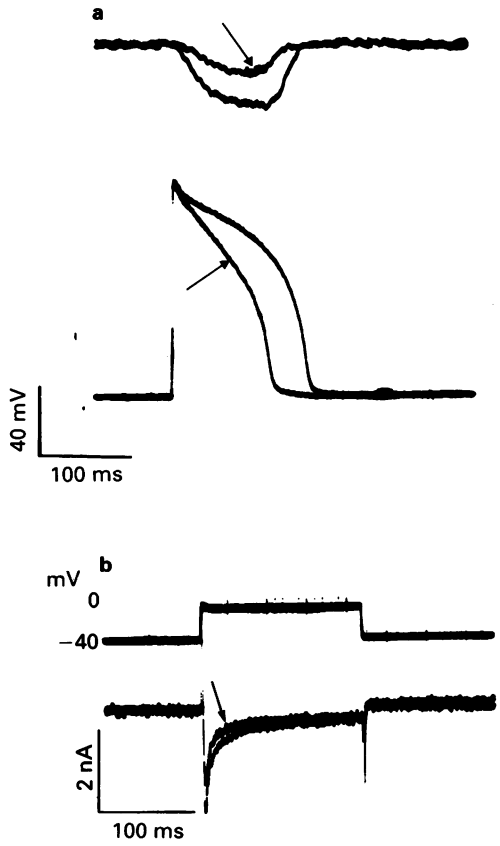

Figure 1 (a) Effects of 2% halothane (1.16 mM) on action potentials (lower trace) and contraction (upper trace). Contraction measured in arbitrary units. (b) Second inward currents (lower trace) evoked by a step depolarization (upper trace). Records in the presence of halothane at 90 s exposure, indicated by arrows. Stimulation frequency 0.3 Hz.

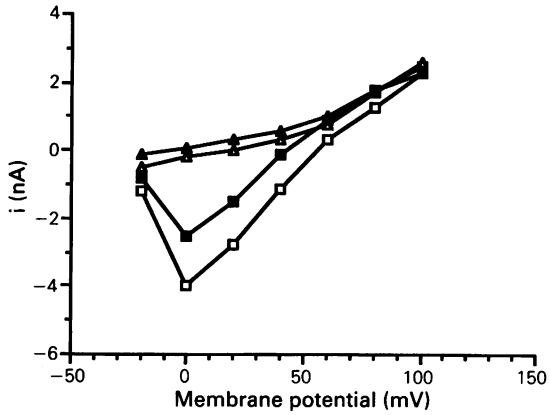

Figure 2 Current as a function of membrane potential during step depolarizations measured from a single cell loaded with Cs. For each depolarization current is measured at the end of the 200 ms pulse (triangles) or at the peak inward or outward current (squares). Records in the absence of anaesthetic are represented by unfilled symbols. Filled symbols show data points in the presence of 2% halothane. Stimulation frequency 0.3 Hz.

At the end of a step depolarization to +60 mV outward current was apparent. Administration of 2% halothane reduced this outward current by 57 ± 7% (measured in 8 cells relative to the holding current). This action of reducing outward current was also seen at less positive potentials such as +20 mV and +40 mV. A reduction of outward current might be expected to cause a lengthening of the action potential and the observed shortening described above indicates that effects of halothane on counterbalancing inward currents predominate.

Current carried by calcium is thought to contribute the major fraction of peak second inward current. However, additional inward current appears to be activated by the rise in cytosolic calcium which accompanies the second inward current (Mitchell *et al.*, 1984; 1987; Fedida *et al.*, 1987). This calcium-activated current can be recorded as a slow tail of inward current following repolarization to −40 mV after a brief (20 ms) depolarization to 0 mV. The effect of halothane (2%, 1.16 mM) was to reduce the tail current and the accompanying contraction as illustrated in Figure 3. These observations might be explained by a reduction in the cytosolic calcium transient which activates the tail current and contraction (see Discussion).

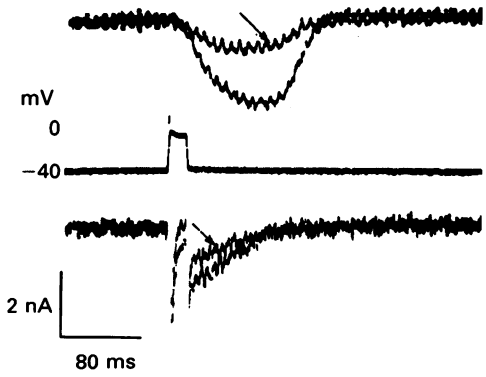

Figure 3 Superimposed records of contraction (upper trace, arbitrary units), membrane potential (middle trace) and current (lower trace) during 20 ms depolarizations from −40 mV to 0 mV before and 90 s after (arrows) the application of 2% halothane.

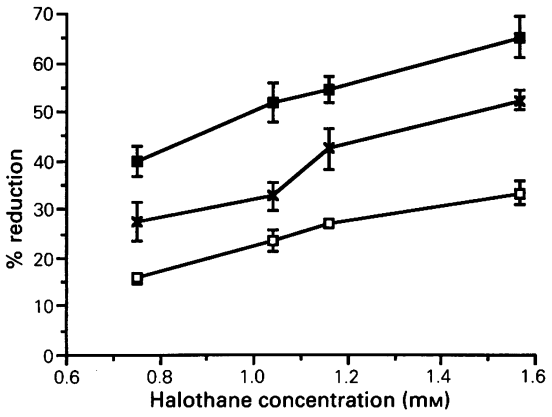

Figure 4 Dose-response relationship showing reduction (%) in contraction (□), second inward current (■) and tail current (X) plotted against halothane concentration (mM). Each data point shows the mean for 6–18 cells; s.e. mean indicated by vertical lines.

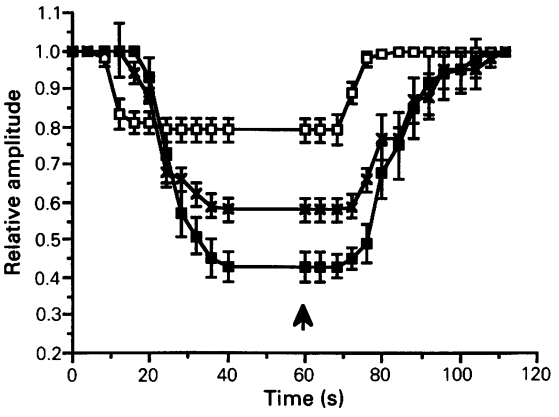

Figure 5 Time course of effects of 2% halothane at a stimulation frequency of 0.3 Hz. Contraction (■), second inward current (□) and tail current (X), shown as a fraction of the value at zero time. Values for 6 cells displayed as mean with s.e. mean indicated by vertical lines. Arrow indicates the washout of the anaesthetic.

The relationship between dose of halothane and the effects on peak amplitudes of contraction, tail current, and second inward current are illustrated in Figure 4. Effects of halothane were greatest on contraction, and least on second inward current, with the action on tail current being intermediate between the two.

The dose-dependence for the shortening of the action potential was also investigated, and measurements were made for the repolarization to 20% (APD_{20}) and 90% (APD_{90}) repolarization. These results are illustrated in Table 1. The depression of action potential duration is dose-dependent and greater at 20% repolarization.

The majority of experiments described in this paper were at a stimulation frequency of 0.3 Hz, but in 8 cells the actions of halothane on second inward current and contraction were measured at 3 Hz. In these cells halothane (2%, 1.16 mM) caused a greater depression of second inward current (to 50±4%, $P < 0.001$) and contraction (to 78±5%, $P < 0.001$) than at the lower stimulation frequency of 0.3 Hz. Therefore, the effect of halothane seems to be use-dependent.

Table 1 Depression of action potential duration expressed as a percentage reduction from control

	Halothane (%)			
	1.2	1.6	2.0	2.4
n	6	9	10	5
APD_{90}	10 ± 2*	13 ± 2**	28 ± 2**	32 ± 3**
APD_{20}	22 ± 2**	24 ± 4*	34 ± 3*	41 ± 5**

APD_{90} and APD_{20} are the times taken for 90% and 20% repolarization respectively.
Values are mean ± s.e. mean and *n* the number of cells.
* $P < 0.005$,** $P < 0.001$, compared to control.

When the time course of development of the effects of halothane were investigated, there appeared to be a discrepancy between the actions on tension and on second inward current. There was a delay in the action on contraction compared to that on second inward current; recovery of contraction was also delayed (Figure 5). In contrast the time course of the effect on the tail current paralleled the changes in contraction.

The observations shown in Figures 4 and 5, indicating a discrepancy between the actions of halothane on second inward current and contraction, might imply that the depression of tension involves mechanisms in addition to depression of second inward current. However, there could be a non-linear relation between the amplitude of second inward current and tension, with a small reduction in second inward current leading to a large reduction in tension. Observations on the effects of low doses of verapamil indicate a non-linearity between amplitude of second inward current and contraction in the opposite direction, i.e. a relatively large reduction in second inward current was required for a reduction in tension. This is illustrated in Figure 6, where it can be seen that halothane produced a greater reduction in contraction than verapamil for a given reduction in second inward current. It might be argued that verapamil exerts additional actions that

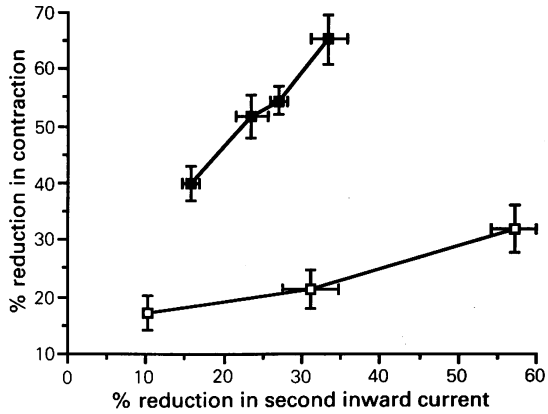

Figure 6 Effects of halothane (■) and verapamil (□) on second inward current and contraction evoked by step depolarizations to 0 mV from −40 mV for 200 ms. Reduction (%) in second inward current is plotted against the depression (%) of contraction produced by the dose of drug applied. The concentrations of verapamil used were 50 nM, 100 nM and 200 nM. Halothane concentrations were 1.2%, 1.6%, 2%, 2.4%. Stimulation frequency 0.3 Hz. All data points represent mean for 6–18 cells; s.e. mean shown by vertical and horizontal lines.

might affect the relation between the second inward current and contraction. However, it appears that this non-linear relation also holds true for nifedipine. In 8 cells exposed to 100 nM nifedipine a 61 ± 3% reduction in second inward current was required to produce a 37 ± 2% depression of contraction.

The possibility of an additional action of halothane on contraction was investigated in experiments using paired-pulse protocols. A brief (20 ms) 'test' depolarization to evoke second inward current and tail current with accompanying contraction was applied after various intervals following a longer (200 ms) 'conditioning' depolarization (Figure 7a). The conditioning depolarization led to substantial inactivation of calcium channels and perhaps to depletion of calcium available for release from intracellular stores. The rationale behind these experiments was to assess the extent of repriming of the mechanisms responsible for contraction after the conditioning depolarization. The action of halothane upon this repriming could then be investigated. At brief intervals between the pulses the amplitudes of second inward current, tail current and contraction were all depressed. The recovery of these amplitudes as the inter-pulse interval was increased is illustrated in Figures 7b, c and d. Halothane caused a substantial slowing of recovery of tail current and contraction amplitudes (Figure 7b and c), without a detectable effect on the recovery of second inward current (Figure 7d). In these graphs, the amplitudes of second inward current, tail current and contraction were plotted as a function of their amplitudes at 1000 ms, when recovery may have been incomplete. In other experiments (where a lower rate of stimulation of 0.2 Hz was used), the longest inter-pulse interval was increased to 2 s, and amplitudes expressed as a fraction of those at this interval; recovery of tail current and contraction continued up to this time, but a similar slowing of recovery by halothane was observed.

The mechanism of action of halothane was further investigated by examining more closely its effects on the decay of second inward current, mentioned above in relation to Figure 1. Semilogarithmic plots of the decay expressed as fraction of peak current (time zero) are shown in Figure 8a. Halothane (2%, 1.16 mM) quickened the decay. The effect of halothane might arise from a reduction in calcium-activated inward currents, as described above in the experiments on tail currents, and/or a speeding of inactivation of calcium channels. These possibilities were investigated in cells loaded with intracellular EGTA to suppress the calcium-activated tail currents. Buffering of the cytosolic calcium with EGTA abolished contraction, while the decay of second inward current was not affected. This is a surprising result as under these conditions less calcium-dependent inactivation of calcium channels would be expected to slow the decay. However, an opposing action to abolish calcium-activated inward currents which may contribute to the decay of the second inward current could also be involved. Further, it is unclear to what extent the calcium level is buffered near the surface membrane. In 10 cells loaded with EGTA the decay of the second inward current was still significantly quickened by halothane between 10–30 ms ($P < 0.05$), but the effects were smaller than those in unloaded cells (Figure 8b).

A possible effect of halothane on the inactivation of calcium currents was investigated in experiments with pre-pulses to different membrane potentials before a test pulse to activate second inward current. The relative amplitude of second inward current following a pre-pulse, expressed as a fraction of that during a test pulse alone, was plotted as a function of membrane potential during the pre-pulse (see Figure 9). Halothane caused an apparent enhancement of inactivation which was significant ($P < 0.05$) at those potentials where inactivation was incomplete in the absence of halothane.

Discussion

The main findings of this paper are that halothane (1) reduced the amplitude and speeded the decay of

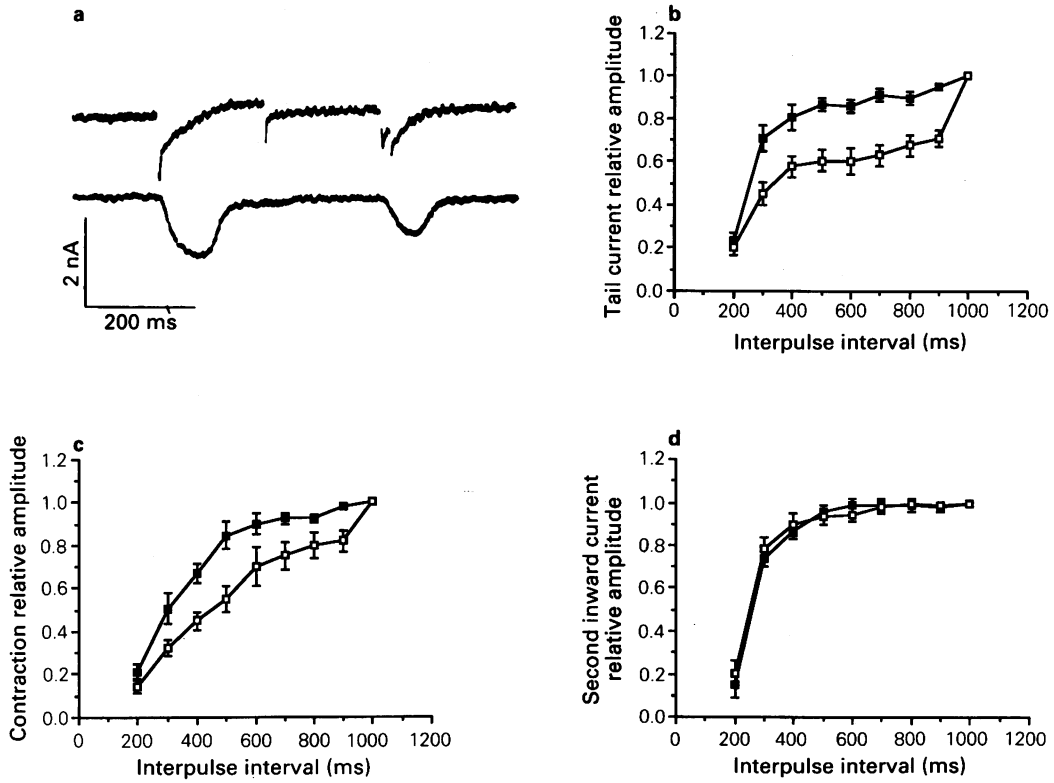

Figure 7 (a) The paired pulse protocol. Long depolarizations (200 ms) from −40 mv to 0 mV elicit the second inward current (upper trace) and contraction (lower trace). Brief depolarizations (20 ms) are applied at varying intervals after the long pulse to assess the recovery of the second inward current, tail current and contraction. Interpulse interval measured as the time between the start of each depolarization, was varied from 200 to 1000 ms. Stimulation frequency 0.3 Hz. Recovery of tail current (b), contraction (c) and second inward current (d) plotted as a fraction of its value at an interpulse interval of 1000 ms. Control recovery (□) and the recovery in the presence of 2% halothane (■) are shown as mean for data points from 8 cells; s.e. mean shown by vertical lines.

the second inward current, (2) reduced the amplitude of calcium-activated tail currents, (3) shortened the duration of action potentials and (4) reduced contractions accompanying either action potentials or step depolarizations under voltage-clamp conditions.

It is well known that calcium entry during the action potential triggers and controls contraction in cardiac muscle (Reuter, 1973; Fozzard, 1980). Evidence from voltage-clamp investigations in single-cell preparations supports the hypothesis that calcium carries the major part of the second inward current (Lee & Tsien, 1982; 1983; Matsuda & Noma, 1984; Mitchell *et al.*, 1983; 1984). Thus, the observed reduction of the second inward current supports the suggestion that halothane reduces the calcium influx which triggers contraction.

Inward 'tail' currents which are thought to be activated by cytosolic calcium (Mitchell *et al.*, 1985; 1987; Fedida *et al.*, 1987) were reduced by halothane. Such an effect might arise if halothane were to exert a direct effect on the membrane pathways for the calcium-activated inward current. The two possible pathways which have been suggested are calcium-activated current carried by (a) electrogenic sodium-calcium exchange (Mullins, 1980; Kimura *et al.*, 1986; Mechmann & Pott, 1986) and (b) current through non-selective cation channels (Colquhoun *et al.*, 1981). It is also possible that halothane may reduce the transient rise in cytosolic calcium which controls contraction, by a reduction of calcium influx and/or by a reduction of the calcium released from intracellular stores; this in turn would be expected to reduce the calcium-activated current. Evidence for an effect of low doses of halothane on calcium transients accompanying action potentials has been obtained by Bosnjak & Kampine (1986)

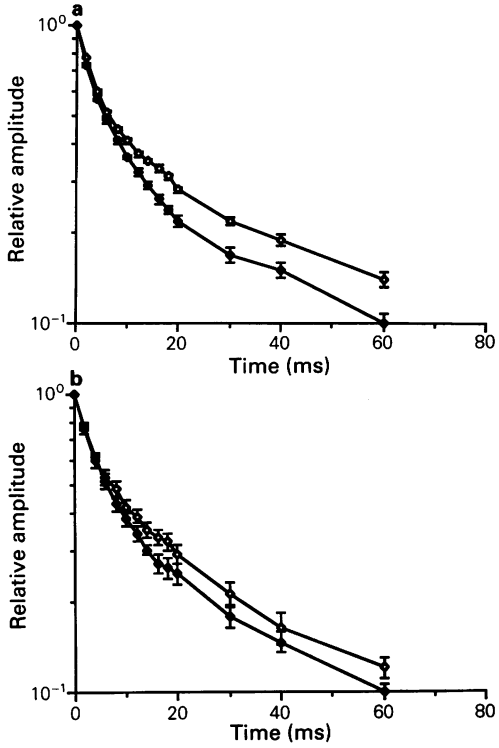

Figure 8 (a) Decay of second inward current (as a fraction of the peak value) plotted semilogarithmically against time (ms). Unfilled symbols show results (mean with s.e. mean shown by vertical lines) from 13 cells under control conditions. Filled symbols represent the effect of 2% halothane in the same cells. Stimulation frequency 0.3 Hz. (b) The same analysis of 10 cells loaded with EGTA.

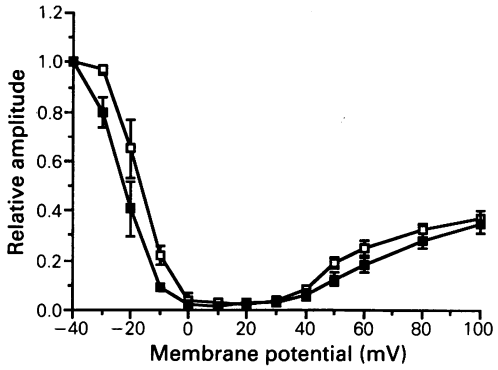

Figure 9 Inactivation of the second inward current as assessed by a prepulse protocol. The amplitude of the second inward current in response to a 'test' pulse from −40 to 0 mV applied 10 ms after a 'conditioning' prepulse is plotted as a fraction of its value in the absence of the prepulse. The membrane potential during the 'conditioning' prepulse was varied from −40 to +100 mV. Control (□); 2% halothane (■). Mean for data from 8 cells with s.e. mean shown by vertical lines. Values were significantly different in the presence of halothane ($P < 0.05$) except between 0 mV and +40 mV.

using aequorin as an indicator of cytosolic calcium. Any reduction in the cytosolic calcium transient would, of course, be expected to contribute to the negative inotropic effect of halothane.

The involvement of supplementary mechanisms in the negative inotropic effect of halothane is suggested by the different time courses of depression of second inward current and contraction. This idea is reinforced by the observation that halothane caused greater contractile depression than verapamil for equivalent reduction in calcium current. Su & Kerrick (1979) found that in rabbit skinned myocardial fibres, halothane exerted its effects when applied in the 'uptake phase', rather than in the 'release phase', and this was interpreted in terms of an effect of halothane on calcium reuptake into the sarcoplasmic reticulum, indirectly reducing the amount available for release; this view has, however, been challenged by Blanck & Thompson (1981) working on isolated sarcoplasmic reticulum.

Further evidence relevant to this discussion is provided by our observations of the effects of halothane during double-pulse voltage-clamp protocols. The action of halothane is to retard the recovery of the tail current and accompanying contraction after a prior depolarization, while an action on the recovery of the peak inward current was not detectable. One possible explanation of these results is that the magnitude of the tail current and contraction at a given interpulse interval represent the cytosolic calcium transient which may in turn depend on the extent of refilling of the intracellular stores by calcium. Halothane may inhibit the reuptake of calcium into the cellular calcium stores. However, a direct effect of halothane upon release of calcium from the stores is another possibility.

Calcium-activated currents and calcium channel inactivation may both play a role in controlling the decay of second inward current elicited by a step depolarization. A reduction of calcium-activated currents may be involved in the observed effect of halothane in speeding the decay of second inward current. When the cells were loaded with EGTA this speeding effect was reduced but not abolished. An additional effect may therefore be involved. Calcium channel inactivation is thought to be controlled by both voltage- and calcium-dependent mechanisms

(Lee *et al.*, 1985) and this may account for the typically U-shaped inactivation curves obtained for calcium current. Halothane enhanced the depression of second inward current at all potentials where inactivation of current was incomplete in control conditions. This result might be explained by a use-dependent block of the second inward current by halothane. Alternatively, halothane might exert a direct effect upon the state of calcium channel inactivation.

In summary it appears that the negative inotropic effect of halothane may not be completely attributable to a single mechanism. Actions upon calcium channels, calcium stores within the cell, and at the level of the contractile apparatus may all contribute to the overall depressant effect. Present evidence suggests that the effect of halogenated anaesthetics upon the contractile filaments may be small (Su & Kerrick, 1978; Rusy, 1975). Calcium current was reduced by halothane and a further action to reduce release of calcium from the intracellular stores also appeared to be important. These two actions might combine to reduce the level of calcium available to activate contraction. The similar time course of depression of calcium-activated tail currents and contraction are consistent with this hypothesis.

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