J. Physiol. (1987), **385**, *pp.* 565–589
With 12 *text-figures*
Printed in Great Britain

INWARD CURRENT RELATED TO CONTRACTION IN GUINEA-PIG VENTRICULAR MYOCYTES

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(*Received 21 April 1986*)

SUMMARY

1. A component of inward current has been identified in isolated guinea-pig ventricular cells that is closely correlated with the contraction of the cell and not with the rapidly activated calcium current. This is a delayed current most clearly seen as a current 'tail' after 50–200 ms depolarizing pulses. At 22 °C the delayed current has a maximum amplitude of ~0·5 nA at −40 mV (consistently 10–20% of the peak amplitude of the calcium current) and decays with a half time of ~150 ms.

2. Paired-pulse protocols show that at pulse intervals (300–400 ms) at which the calcium current is nearly fully reprimed, the delayed component is very small. It recovers over a time course of several seconds, as does the contraction. Adrenaline speeds the decay of the delayed current (~50%) and similarly accelerates cell relaxation. Adrenaline also shortens the recovery time of both the contraction and the delayed current.

3. During long trains of repetitive pulses, the delayed current amplitude follows that of the contraction 'staircase'. The half-time of the decay of the current 'tail' also matches that of contraction and suggests that both may reflect the time course of the underlying intracellular calcium transient.

4. The half-time of decay of the delayed current is only moderately voltage dependent over the potential range −80 to 0 mV. The amplitude of the delayed current normally reaches a minimum around −20 mV and increases at more negative potentials.

5. The voltage dependence and kinetics of decay of the current show that it should flow and decay largely *during* the action potential plateau and repolarization rather than during diastole.

6. Diffusion of high concentrations of EGTA into cells abolishes the delayed current and cell contraction. Under these conditions the fast calcium current is increased and its inactivation delayed.

7. When calcium is replaced by strontium, the delayed current amplitude is greatly reduced even though the contraction is larger and slower.

8. The results are consistent with the hypothesis that the delayed inward current is activated by the intracellular calcium transient. It may be carried by the sodium–calcium exchange process and/or by calcium-activated non-specific channels (especially when internal calcium is elevated by reduction of external sodium).

9. In the presence of 1 μM-ryanodine, the calcium current is greatly reduced, whereas the delayed current is not significantly altered.

INTRODUCTION

In recent years the nature and complexity of inward current mechanisms in cardiac tissues has greatly increased, due in a large part to the introduction of methods for the isolation and study of single adult cardiac cells (Kono, 1969; Powell & Twist, 1976). The initial characterization (Reuter, 1967; Rougier, Vassort, Garnier, Gargouil & Coraboeuf, 1969; Beeler & Reuter, 1970; Reuter & Scholz, 1977) of the slow inward current, i_{si}, in multicellular preparations identified a voltage-dependent gated calcium current following Hodgkin–Huxley kinetics considerably slower and smaller than the tetrodotoxin (TTX)-sensitive sodium current.

In isolated adult single ventricular cells a calcium current can still be identified that is thought to show voltage-dependent activation but which is much larger and activates and inactivates about an order of magnitude faster than multicellular work suggested (Isenberg & Klockner, 1982; Mitchell, Powell, Terrar & Twist, 1983; for review see Tsien, 1983). In this paper we shall refer to this rapidly activated current as $i_{Ca,f}$ (see Noble, 1984, for a discussion of terminology). This calcium current has a much higher selectivity than previously recognized and sodium ions do not contribute significant charge to the channel current (Lee & Tsien, 1984; Matsuda & Noma, 1984). Inactivation of the current no longer follows a single-exponential time course and is thought to be dependent on intracellular calcium (Kokobun & Irisawa, 1984), as in many other excitable cells (Eckert & Chad, 1984), with an added voltage-dependent component (Lee & Tsien, 1982; Lee, Marban & Tsien, 1985). Very slow or cumulative inactivation of the inward current has also been described in the heart (Kass & Scheuer, 1982; Fedida, Noble, Shimoni & Spindler, 1985*a*). At the same time evidence has accumulated on other types of calcium channels and currents that may contribute to the calcium current around contraction threshold (~ -50 to -30 mV) or form part of the current relaxation at more positive potentials. In different preparations activation of calcium-channel currents in the threshold range of voltages between about -55 and -25 mV is well described and thought in many cases to represent a discrete population of channels from those that carry $i_{Ca,f}$. Using conventional micro-electrode techniques Eckert & Lux (1975, 1976) have described such weak, persistent calcium currents in *Helix* neurones. In cardiac ventricular tissue Šimurda, Šimurdová, Bravený & Šumbera (1981) have described a component of second inward current present during weak depolarizations that was directly proportional to contractile force whereas the main component of second inward current present during large depolarizations was inversely proportional to force. These two components could be elicited virtually together during a two-step voltage-clamp pulse. In single guinea-pig ventricular cells Lee, Noble, Lee & Spindler (1984) have found a low-threshold maintained component of inward current which was relatively insensitive to cadmium and have presented evidence to suggest that it may be a separate calcium-channel mechanism from that responsible for $i_{Ca,f}$. Hume & Giles (1983) previously noted a similar net inward current at negative membrane voltages in bull-frog atrial cells but were uncertain of the ionic nature of the current.

In other tissues studies with conventional micro-electrodes have revealed multiple components of calcium-channel current in invertebrate egg-cell preparations

(Hagiwara, Ozawa & Sand, 1975; Fox & Krasne, 1984) and the ciliate, *Stylonychia* (Deitmer, 1984; for review see Miller, 1985). The introduction of the advanced technique of patch clamp has allowed functional properties of individual channels to be analysed in smaller cells and in greater detail than hitherto possible (Hamill, Marty, Neher, Sakman & Sigworth, 1981). The existence of more than one type of calcium channel in one cell is thus seen in recent whole-cell recordings from anterior pituitary cells (Armstrong & Matteson, 1985) and neuroblastoma cells (Yoshii, Tsunoo & Narahashi, 1985). Distinct threshold calcium channels and currents have been described in cultured rat and chick neurones (Carbone & Lux, 1984*a*, *b*; Nowycky, Fox & Tsien, 1984, 1985) using whole-cell voltage clamp and single-channel recording. Similarly in the heart in canine atrial cells, Bean (1985) has described a calcium-channel current (i_{fast}) most prominent at threshold potentials from negative holding potentials. A later, larger current (i_{slow}) predominates at more positive potentials and has many characteristics of the well-described cardiac $i_{Ca,f}$. The i_{slow} may be differentiated from i_{fast} in its voltage dependence, its sensitivity to β-adrenoceptor agonists and conductance modification by barium and cadmium ions. In guinea-pig ventricular cells two cardiac calcium channels have been described by Nilius, Hess, Lansman & Tsien (1985).

In addition to these channel mechanisms, slower inward currents have been described that are thought to be activated by intracellular calcium. Colquhoun, Neher, Reuter & Stevens (1981) first described a non-specific channel current in cultured rat heart cells. Slow inward currents have also been described in the rabbit sinoatrial node (Brown, Kimura, Noble, Noble & Taupignon, 1984), in isolated adult rat ventricular cells (Mitchell, Powell, Terrar & Twist, 1984*b*) and Arlock & Noble (1985) have described a current in ferret ventricular trabeculae. These currents, called $i_{si,2}$, have been attributed to activation of the sodium–calcium exchange process and are reproduced as such in the DiFrancesco–Noble (1985) model. This feature of the model is of crucial importance in enabling it to reproduce fast extracellular calcium transients (Hilgemann & Noble, 1986*a*).

An important advantage of using ordinary micro-electrode recording for single heart cells is that unlike whole-cell voltage-clamp recordings where intracellular calcium changes are buffered, the normal calcium transients during excitation may occur. In the present study we have identified a component of inward current that is apparent at negative potentials around threshold and is dependent on the occurrence of cell calcium transients. We have investigated the relationship between the cell contraction and this delayed component of inward current in an attempt to answer the question whether this current may be activated by calcium release or by the calcium entering through a calcium-channel mechanism.

METHODS

Guinea-pig ventricular cells were obtained by enzymic dissociation of adult hearts as described previously (e.g. Lee & Tsien, 1984). The activity of collagenase (Type I, Sigma, London or Worthington Type I; Lorne Diagnostics, U.K.) in the perfusate was kept constant at 175 u/ml. After separation cells were stored at room temperature in either a modified Tyrode solution containing calcium, 1 mM or a modified cell culture medium (Dulbecco's modified Eagle medium) with 10% added horse serum, penicillin (5000 u/100 ml) and streptomycin (5000 μg/100 ml) (Gibco Europe Ltd.).

During experiments cells were constantly perfused at room temperature, 18–22 °C. The solutions contained (in mM): NaCl, 137; KCl, 5·4; $CaCl_2$, 1·8 or 2·5; $MgCl_2$, 1; $NaHCO_3$, 12; NaH_2PO_4, 1; and glucose, 5. The solutions were constantly bubbled with a 95% O_2–5% CO_2 mixture. In experiments in which strontium replaced calcium direct substitution was employed. A low external sodium solution was obtained by partial or total replacement of NaCL by LiCl. This resulted in sodium contents of 46 and 13 mM (31% and 8·6%) of the control solution. Zero sodium solution contained (mM) LiCl, 150; KCl, 5·4; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 5; and HEPES, 5; gassed with 100% O_2. In experiments in which barium was substituted for calcium, the latter solution was used with NaCl instead of LiCl. The pH of external solutions was adjusted to 7·4 by HEPES–LiOH buffer (zero sodium) or HEPES–NaOH (barium).

Electrodes were normally filled with 3 M-KCl and bevelled from ~30 MΩ to between 8 and 15 MΩ before insertion using an alumina slurry method (Lederer, Spindler & Eisner, 1979). The use of low-resistance electrodes tended to introduce a leak conductance so that although all cells initially had a resting potential around −80 mV (we have previously shown action potentials from such cells; Fedida, Noble & Spindler, 1985*b*) some cells needed hyperpolarizing current at the holding potential later in the experiments. Many other cells had negative resting potentials but required little or no current at the holding potentials used in the present experiments. This probably reflects the marked N-shape of the background current–voltage relation in these cells that can lead to net inward current in the potential range −40 to 0 mV (Lee *et al.* 1984; see also Fig. 1). Such quantitative variation between cells did not alter the present findings. In experiments in which ethyleneglycol-bis(β-aminoethylether)*N*,*N'*-tetraacetic acid (EGTA) was injected into cells, electrodes were filled with 0·5 M-EGTA (pH 7·4 with KOH) and used as the clamp electrode. This was found to result in high-resistance electrodes relative to tip size and poor voltage-clamp control or difficulty with impalement. In a few experiments (e.g. Fig. 9) 0·5 M-EGTA was introduced through a second micro-electrode. Alternatively, various concentrations of KCl (150 mM to 1·5 M) were introduced into the electrode to improve the current-passing characteristics. No difference in results with EGTA was noticed between any of these methods.

The cells were voltage clamped using a single-electrode switching apparatus (Dagan Inc., model 3400) or in some experiments an Axoclamp 2A (Axon Instruments, U.S.A.). The contractions of the single cells were monitored using an optical method similar to that described by Kass (1981). In our experiments a photo-electric device was mounted in the eyepiece of the inverting microscope in conjunction with an iris diaphragm to reduce excess light. The data were collected on a PDP 11/34 computer at 10 kHz for subsequent analysis. In some cases data recorded after rapid initial changes in current had occurred were stored at a 10 × lower rate. This is apparent as a change in noise frequency on the current records in Figs. 5, 10 and 12 after about 40 ms. Data were also displayed on a digital oscilloscope (Gould OS4200) and could then be directly plotted onto a chart recorder. Altogether over 100 cells from more than fifty hearts yielded data for experiments described in this paper.

RESULTS

Fig. 1*A* shows records of contraction and membrane current during 3 s voltage-clamp pulses from a holding potential of −40 mV to various voltages between −35 and +8 mV. From this holding potential, the current records contain no contribution from the TTX-sensitive fast sodium current, which is fully inactivated. This is confirmed by the absence of fast inward current during the depolarizations to −35 and −28 mV.

The stronger depolarizations produce an inward current record similar to those found previously in isolated cardiac cells. Thus at +8 mV the inward current (attributed to calcium entry) activates rapidly after the capacity transient to reach a peak at about 8 ms. This is somewhat slower than observed in bovine (Isenberg & Klöckner, 1982) or rat myocytes (Mitchell *et al.* 1983) at 37 °C (see also legend to Fig. 8). This is attributable to the fact that we are working at lower temperature and accords well with whole-cell clamp data (Cavalié, McDonald, Pelzer & Trautwein,

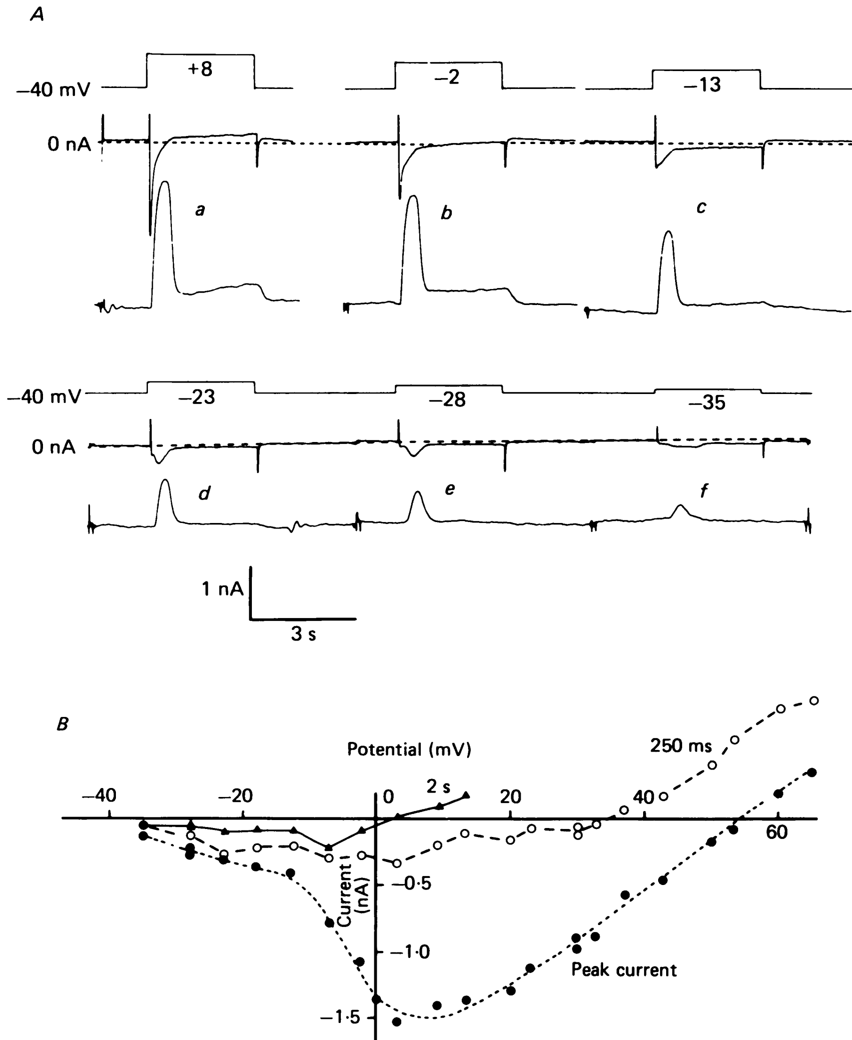

Fig. 1. *A*, second inward current and cell contraction. Holding potential −40 mV. Voltage recording (top trace); 3 s clamp steps from +8 to −35 mV (*a–f*). Membrane currents (middle) and cell contraction (below) recorded during voltage-clamp pulses. Continuous chart recording. *B*, current–voltage relation for the membrane current recorded during the depolarizing pulses at times indicated on graph. Same cell as in *A*. Peak inward current level, ●. Current 250 ms into pulse, ○. Current level 2 s into pulse, ▲. Note that in this Figure as in all others in this paper, contraction is in arbitrary units.

1985) and our own data (H. F. Brown, D. Fedida & M. J. Kilborn, unpublished observations). The current then inactivates with a complex time course. The present paper is concerned with the question whether all of this inactivation is attributable to $i_{Ca,f}$ channels or whether there are other slower current mechanisms involved.

The twitch that accompanies this current is shown below and consists of a large phasic response followed by a maintained phase of cell contraction. This is very similar to tension records obtained in multicellular preparations (e.g. Gibbons &

Fozzard, 1975; Eisner, Lederer & Vaughan-Jones, 1983). At more negative clamp potentials (Fig. 1*Ab* and *c*) a clear step appears on the relaxation phase of inward current and a clear separation is apparent (Fig. 1*Ad*, *e* and *f*) between the calcium current near threshold and a delayed component of inward current.

It should be noted that the inward currents near threshold (Fig. 1*Ae* and *f*; at −35 and −28 mV) are extremely slow, not only in their inactivation time course, but also in their activation. Thus, at −28 mV the peak inward current is reached only after 560 ms (at −35 mV, 1100 ms). The activation of contraction has a close temporal relationship to the slow inward currents near threshold (Fig. 1*Ad*, *e* and *f*). The peak of inward current is reached ~50 ms before the peak of contraction. The current–voltage relations for the inward currents in this cell are shown in Fig. 1*B*. It can be seen that there is a clear 'step' on the relation of peak inward current to potential at negative voltages. It can also be seen in the current–voltage relations recorded later in the pulse that there is maintained inward current at more negative potentials than ~ −15 mV.

Extremely slow inward currents with similar time courses have been recorded previously. Thus, Eisner, Lederer & Noble (1979) recorded a current with this time course in sheep Purkinje fibres. More recently, Brown *et al.* (1984) recorded a current with a similar envelope in rabbit sino-atrial node and Arlock & Noble (1985) have reported one in ferret ventricular trabeculae. In the latter two papers, the very slow current could sometimes (though not always) be recorded as a separate slow peak *after* the more rapidly activated calcium current. In our cells, this clear separation did not occur, but the same question arises: is the delayed inward current a manifestation of unusually slow kinetics of $i_{Ca,f}$ near its threshold, or some phenomenon that results from overlap of the activation and inactivation relationships of $i_{Ca,f}$. Alternatively it may be carried by another mechanism, such as an electrogenic sodium–calcium exchange (Mullins, 1979; Brown *et al.* 1984) or a non-specific cation-channel mechanism (Colquhoun *et al.* 1981) such as that proposed for the transient inward current in Purkinje fibres (Kass, Lederer, Tsien & Weingart, 1978). Both of these currents are activated by internal calcium and their time course would therefore follow the calcium transient.

Since, in our cells, the currents during depolarization do not clearly separate to form double peaks, larger fast calcium currents than seen in Fig. 1 will clearly tend to conceal the smaller delayed inward current. We have concentrated on analysing the tails of inward current that can be recorded on clamping back to the holding potential. This is a method similar to that used by Armstrong & Matteson (1985) to characterize fast- and slow-deactivating calcium-channel currents in pituitary adenoma cells. The calcium channel ($i_{Ca,f}$) itself is known to deactivate fairly rapidly on repolarization and we shall show later (see Fig. 9) that it does so in our cells. Any slower 'tails' of current might then be attributed to a different mechanism.

The basic protocol of the experiments is shown in Fig. 2, which shows currents during and after voltage-clamp pulses of different durations between 50 and 500 ms. It can be seen that, following the shortest depolarizations, a substantial inward tail is recorded, which requires several hundred milliseconds to decay. As the pulse duration is prolonged, the initial amplitude and the duration are both reduced until, after 500 ms, virtually no inward current tail is recorded.

A significant feature of these results is that the *absolute* time at which the inward tail decays to the base line is relatively independent of the pulse duration. This implies that the process concerned occurs at both voltages with a speed that is not strongly dependent on the duration of the depolarizing pulse (cf. Fig. 5). Clearly, the time course of the inward current tail is too slow to reflect deactivation of $i_{Ca,f}$ and inactivation of the current is expected to be complete after, at most, a 100 ms depolarizing pulse.

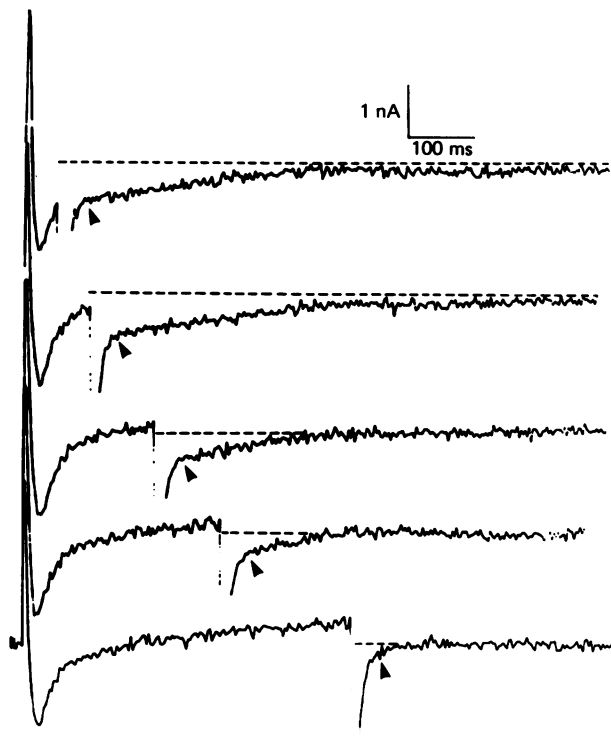

Fig. 2. Effect of pulse duration on a slow tail current. Recordings of membrane current reproduced from computer. Voltage-clamp pulses from −45 to +5 mV for 50–500 ms. The arrow in each record indicates the slow current tail that occurs on repolarization. Records displaced vertically for clarity.

In fact, in some cases, the slow phase of current decay *during* the pulse is almost indistinguishable from the continued decay following repolarization. A striking example of this kind of record is shown in Fig. 3. Clearly, in this case, if inactivation of $i_{Ca,f}$ is complete by 100 ms, both the amplitude and time course of current decay are not strongly voltage dependent. This experiment also shows another feature of major importance. A second pulse applied shortly after the decay of the delayed inward tail is itself followed by only a very small decay tail, even though the fast current has already recovered to within 15% of its initial value. The mechanism of the delayed current therefore also shows very slow repriming.

Since the contraction also shows slow repriming, we have used two types of pulse protocol that allow the amplitudes of the delayed tail and the contraction to be

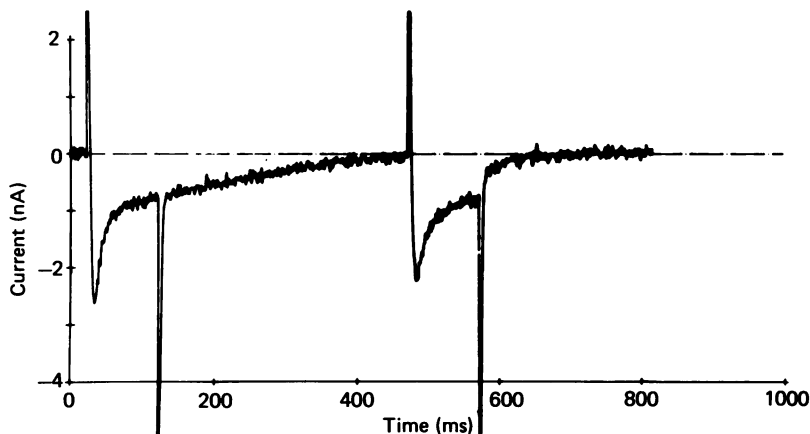

Fig. 3. Effect of a twin pulse on the reactivation of peak calcium current and the slow tail current. Membrane current recorded during two consecutive 100 ms voltage-clamp pulses from −45 to +5 mV. Note the absence of a slow current tail after the second pulse.

correlated under conditions in which the fast calcium current is not well correlated with contraction. The first protocol, illustrated in Fig. 4, uses long trains of pulses applied after rests. The contraction then shows variations that are characteristic of the well-known staircase phenomenon. At an interpulse interval of 1 s, 50 ms pulses to about −5 mV (Fig. 4*A*) give a large twitch in the first beat after a 3 min rest. The second twitch is small and subsequent contractions change little for up to 100 pulses. Rest reverses this effect. The delayed current tail shows identical changes in relative magnitude to the contraction changes throughout and is much reduced during repetitive pulsing. This is despite an *increase* in the peak of $i_{Ca,f}$ during the train (not shown in Fig. 4, but see Fig. 5 and Fedida *et al.* 1985). Pulses after similar rests to more positive membrane voltages (Fig. 4*B* and *C*) give rise to positive staircases of contraction and larger steady-state contractions (Gibbons & Fozzard, 1975). This last result is similar to the action potential and tension results of Reiter & Stickel (1968) on intact guinea-pig papillary muscles. The delayed inward current tails show excellent correlation with the changes in contraction while peak inward current *decreases* during pulses to +15 mV.

These results also demonstrate the pulse-voltage dependence of the time course of the inward current tail. After pulses to more positive voltages in Fig. 4*B* and *C* the rate of decay of contraction is markedly faster than in Fig. 4*A* and is associated with a faster decay of the inward current tail. In the steady state during stimulation the half-time of decay of the current tails and contraction decreased from 225 ms at −5 mV to less than 200 ms at +15 mV. This suggests that voltage-dependent processes affecting cell relaxation during stimulation may also affect the delayed current.

This effect is in direct contrast to the effect of pulse duration on the delayed inward tail during repetitive pulsing. In Fig. 5 an experiment is shown using data from another cell in which, after 3 min rests, 30 or 50 ms pulses were applied from −40 to −5 mV with a 1 s interpulse interval. The first and fortieth response in each train have been superimposed. In confirmation of data in Fig. 4 it can be seen that for both

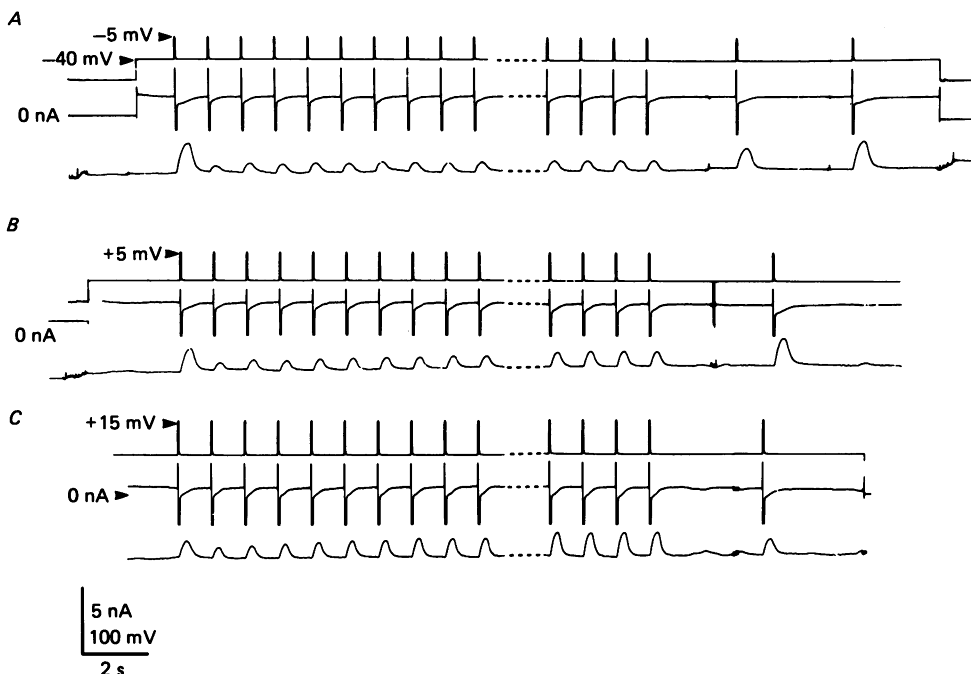

Fig. 4. Relationship of slow current tail to contraction. Each record (*A*, *B* and *C*) obtained from two sections of continuous chart record. Each panel shows membrane voltage (above), membrane current (middle) and cell contraction (below). In each case the cell was at rest for at least 3 min. The clamp was then switched on at a holding potential of −40 mV. Trains of 50 ms pulses with a 1 s interpulse interval were given to −5 (*A*), +5 (*B*), or +15 mV (*C*). The first ten pulses in each train are shown, and the last four pulses when the cell was in a steady state (thirty-six to forty in each train). The final records in *A* were obtained 15 and 30 s after the train had ended. In *B* and *C*, 30 s after the trains had ended. Note that the recorder speed was reduced between these final pulses. The inward current peak is off-scale in all panels.

pulse durations a *decline* in the magnitude of the delayed inward tail occurs (contraction not recorded) despite the fact that *more* inward current is present during the pulse to −5 mV after 30 ms. The essential finding shown in Fig. 5 is, however, that the rate of decay of the tail current is independent of pulse duration at constant pulse voltage, illustrated by the superimposition of tails in the left- and right-hand panels of Fig. 5.

The conclusion that may be drawn from data shown in Figs. 3–5 is that the delayed inward current tail mirrors the behaviour of cell contraction rather than $i_{\mathrm{Ca,f}}$. The calcium transient that underlies contraction and possibly the delayed tail is likely to be similar in activation and magnitude during 30 and 50 ms pulses.

The second protocol which allows us clearly to distinguish the properties of the delayed tail from that of the fast calcium current uses paired pulses as already shown in Fig. 3. Fig. 6 extends this approach by using pulses separated by different intervals. The idea of the experiment depended on the fact that the repriming of contraction (and therefore, presumably, of calcium release) is known to proceed very much more slowly than does the repriming of the calcium current (Gibbons & Fozzard, 1975).

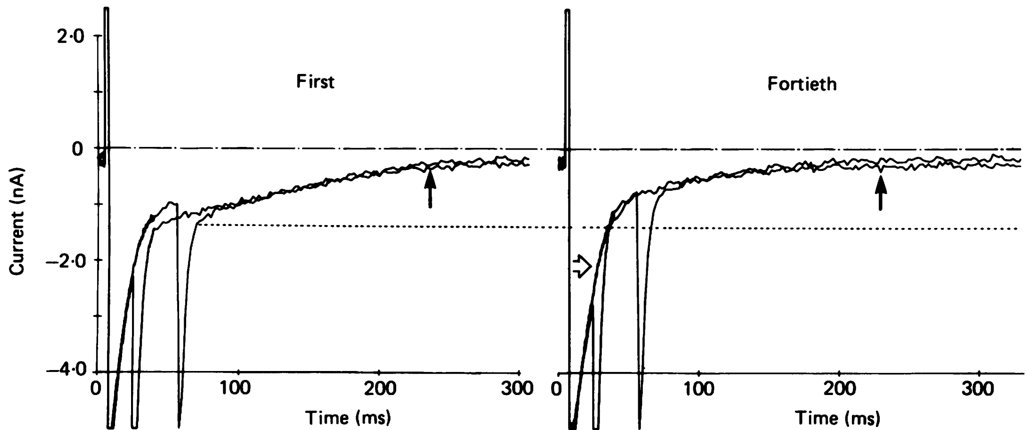

Fig. 5. Effect of pulse duration during repetitive stimulation on magnitude and decay of slow current tail. Membrane currents recorded during two separate trains of 50 and 30 ms pulses with a 1 s interval. Pulses in each case were given after a 3 min rest. Record obtained from computer, left-hand panel shows the first current response of the train of 50 ms pulses superimposed on the first obtained during the train of 30 ms pulses. Similarly the right-hand panel shows the fortieth pulse in each train superimposed. The filled arrow indicates the approximate time of decay of the current tail. The open arrow in the right panel indicates the inward current level during the first pulse of each train after 30 ms (from left panel) and indicates that relatively more inward current is present during the fortieth pulse although a smaller tail current is apparent on repolarization.

It therefore seemed possible to apply pulses at intervals at which the fast calcium current is fully reprimed but which did not permit full repriming of contraction.

Examples of such results are shown in Fig. 6*A*. Intervals longer than 540 ms allow full recovery of calcium current but it can be seen that repriming of the delayed tail is incomplete at both the 600 and 800 ms interpulse intervals. Moreover, slow reactivation of the current tail parallels the recovery of cell contraction. These findings are graphically represented in Fig. 6*B*. In this case 50 ms pulses (from −40 to +5 mV) were applied in pairs. At the shortest intervals (150–400 ms) an initially surprising result was obtained. Although the peak calcium current was reduced (as expected) the delayed tail current was *larger* at the shortest intervals. At intermediate intervals (400–800 ms) the peak calcium current is clearly fully reprimed (cf. Josephson, Sanchez-Chapula & Brown, 1984), in fact it usually shows enhancement at these intervals, whereas the current tail is not. The full repriming of the delayed current requires tens of seconds to reach completion. It is interesting to note that the minimum tail current amplitude is reached at a time when the peak calcium current recovery from inactivation is just about complete. This prominent delayed current at short interpulse intervals may be simply interpreted: after the control pulse of each pair the delayed current declines to zero over about 400 ms (Figs. 2, 3, 5 and 7). A second pulse applied during this time will exhibit a current tail on repolarization that reflects the remaining decay phase of the internal calcium transient and delayed current elicited by the preceding control pulse. Pulses applied at longer intervals than ~400 ms will then reflect the true repriming of the delayed current.

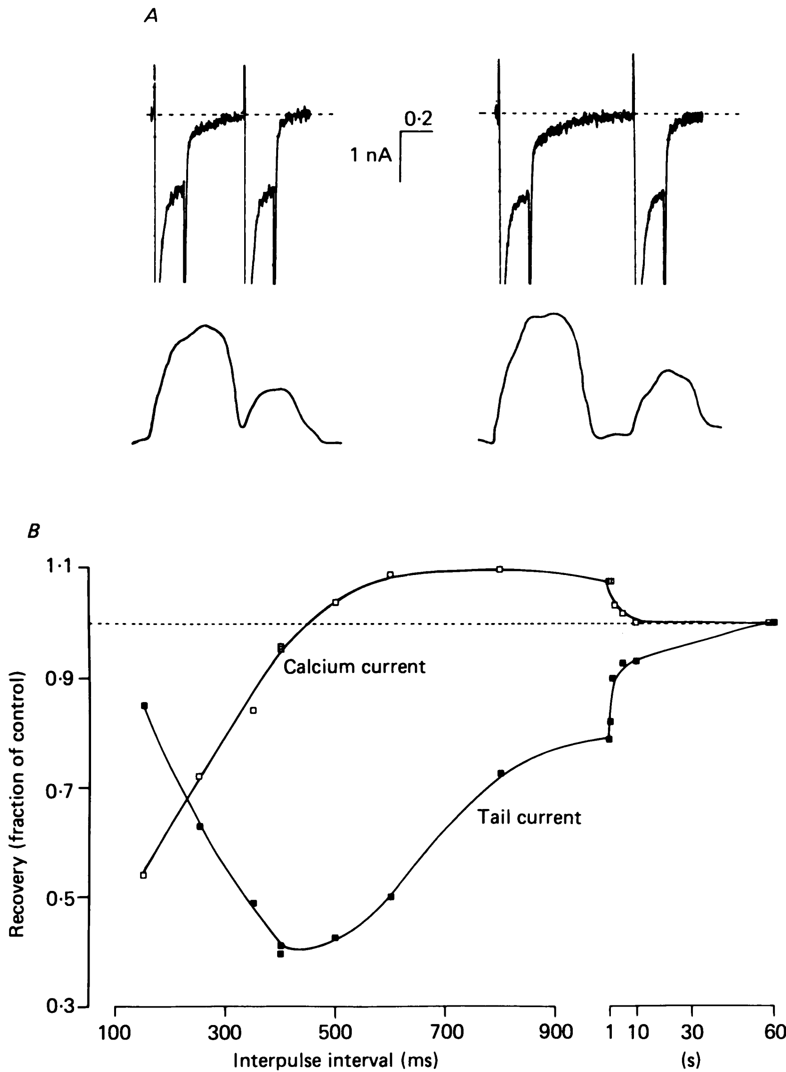

Fig. 6. Twin-pulse experiments to compare reactivation of calcium current, the slow current tail and cell contraction. *A*, records of membrane current (above) and contraction (below) for pairs of 200 ms voltage-clamp pulses from −45 to +5 mV. Interpulse intervals 600 (left panels) and 850 ms (right panels). Twin-pulse protocol given every 60 s. Peak inward calcium current (off-scale) is fully reprimed at these intervals (cf. *B*). During the second pulse of each pair the magnitudes of contraction and slow current tail are 0·46 and 0·44 (600 ms interpulse interval) of control-pulse magnitudes. At an interpulse interval of 850 ms recoveries of contraction and current tail are 0·56 and 0·60 of controls respectively. *B*, results from another cell showing recovery of peak calcium current (open squares) and tail current magnitude (filled squares) during a second, test, pulse as a fraction of the currents elicited during a preceding control pulse. Interpulse interval is shown on the abscissa. Pulses were 50 ms, −40 to +5 mV given in pairs once every 60 s. Control current values were: mean calcium current = 5·59 ± 0·06 nA (1 S.D., n = 14). Mean tail current size = 0·61 ± 0·02 nA (1 S.D., n = 14). Note change in time scale at longer interpulse intervals.

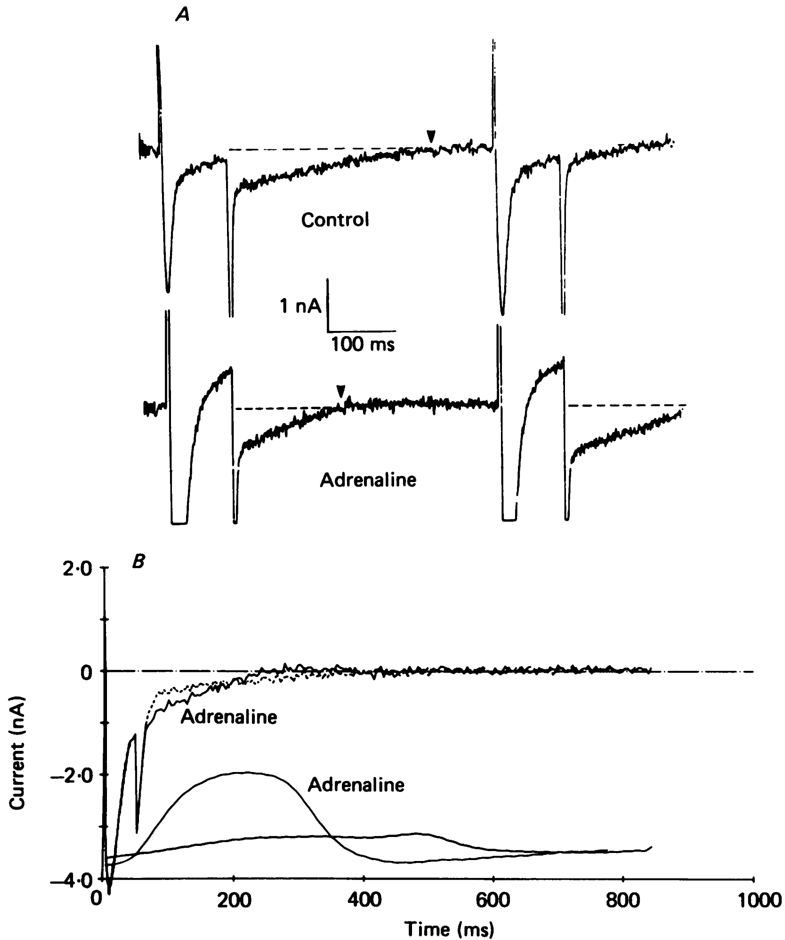

Fig. 7. Effect of adrenaline on slow current tail and current reactivation. *A*, membrane currents recorded during pairs of 100 ms depolarizing pulses (−40 to 0 mV) in the control situation above, and in the presence of 10^{-6} M-adrenaline, below. The half-time of decay of the slow current tail is 137 ms in control and 74 ms in adrenaline. *B*, membrane currents and contraction before, and in the presence of 5×10^{-7} M-adrenaline. In each case a single 50 ms depolarizing voltage-clamp pulse was given after a 3 min rest. Records superimposed by computer. The inward current during the pulse is largely off-scale in adrenaline and has been omitted for clarity. The filled arrows in Fig. 7*A* denote the approximate time taken for the current tail to relax.

Another way of demonstrating this correlation is to use adrenaline, which has been shown to speed the repriming of contraction (Shimoni, 1985). The top records in Fig. 7*A* show the result of paired pulses applied before adrenaline. The tail after the second pulse is small despite the fact that the calcium current during the pulse is actually larger (this is an example of the enhancement referred to above). The lower traces in Fig. 7*A* show the results in the presence of 10^{-6} M-adrenaline. The delayed tail current after the second pulse is now as large as that following the first pulse. Notice also that, although the tail current amplitude is enhanced by adrenaline, its decay is faster.

The addition of 10^{-6} M-adrenaline gives substantial support to the hypothesis that the delayed current is related to the rise in intracellular calcium that occurs during excitation. Fig. 7*B* correlates the effect of adrenaline on cell contraction and the delayed tail current. As shown in Fig. 7*A* the tail is somewhat larger in adrenaline, but the most striking effect is a speeding of the rate of decay of the current (half-time of decay, 172 ms in control, 93 ms in adrenaline). In association with this there is a larger but abbreviated contraction in the presence of adrenaline. These effects on contraction and the delayed current are consistent with the faster removal or sequestration of cytoplasmic calcium in the presence of adrenaline (Fabiato & Fabiato, 1975).

The results in Figs. 2–5 indicate that the potential during the voltage pulse, before the repolarization step that reveals the current tail, is important in determining the rate of relaxation of both the contraction and the delayed current. The repolarization voltage dependence of the tail current was investigated systematically in six cells by altering the potential on repolarization after a constant depolarizing pulse. In Fig. 8*A* records are shown of tail currents and contractions at a series of voltages from −65 to −8 mV after 50 ms pulses to +10 mV. It can be seen that less-negative potentials during repolarization give smaller tail currents (Fig. 8*A a*, *b* and *c*). This is despite the fact that the twitches elicited by the preceding pulse to +10 mV are similar in each case. In addition the half-time of decay of the delayed tail current (and contraction) is slower at the less-negative voltages.

At even less-negative potentials (Figure 8*A d* and *e*) the picture is more difficult to interpret. Steady-state activation of calcium current and time-dependent outward current may occur and overlap the delayed current. The tonic phase of contraction visible in Fig. 8*A e* suggests that maintained calcium release and/or entry via calcium current is occurring during the pulse to −8 mV.

Fig. 8*B* illustrates the complete voltage dependence of the tail current using results from another preparation. A minimum of delayed current was usually observed around −20 mV and progressive larger tail currents seen at more negative voltages.

If the delayed current tails are activated by the variations in intracellular calcium responsible for activating contraction, then they should be abolished when intracellular calcium is buffered. We therefore carried out experiments using electrodes containing high concentrations of EGTA. Fig. 9 shows trains of pulses in two cells showing typical results. The main panel records were obtained in a cell clamped with a 3 M-KCl electrode inserted in the centre of a long cell. The cell was then impaled with a second electrode containing EGTA (which also allowed an independent measure of membrane voltage to be made at the far end of the cell). The superimposed records of the first and fortieth response of a train reveal no delayed tail current (cf. Fig. 5 for control response). The inset panel record was obtained from another cell impaled with a single electrode containing 0·5 M-EGTA. On a slow time base the current response to a series of short pulses applied at 1 Hz after rest is shown. No delayed inward current tails are present. In all eighteen experiments of this kind we found, as in Fig. 9, that the contraction is abolished as soon as cells are impaled with an EGTA-containing electrode. So also are the delayed inward currents. By contrast, the calcium current is still very large. The rapidity of the decay on repolarization under these conditions confirms the rapidity with which the calcium current

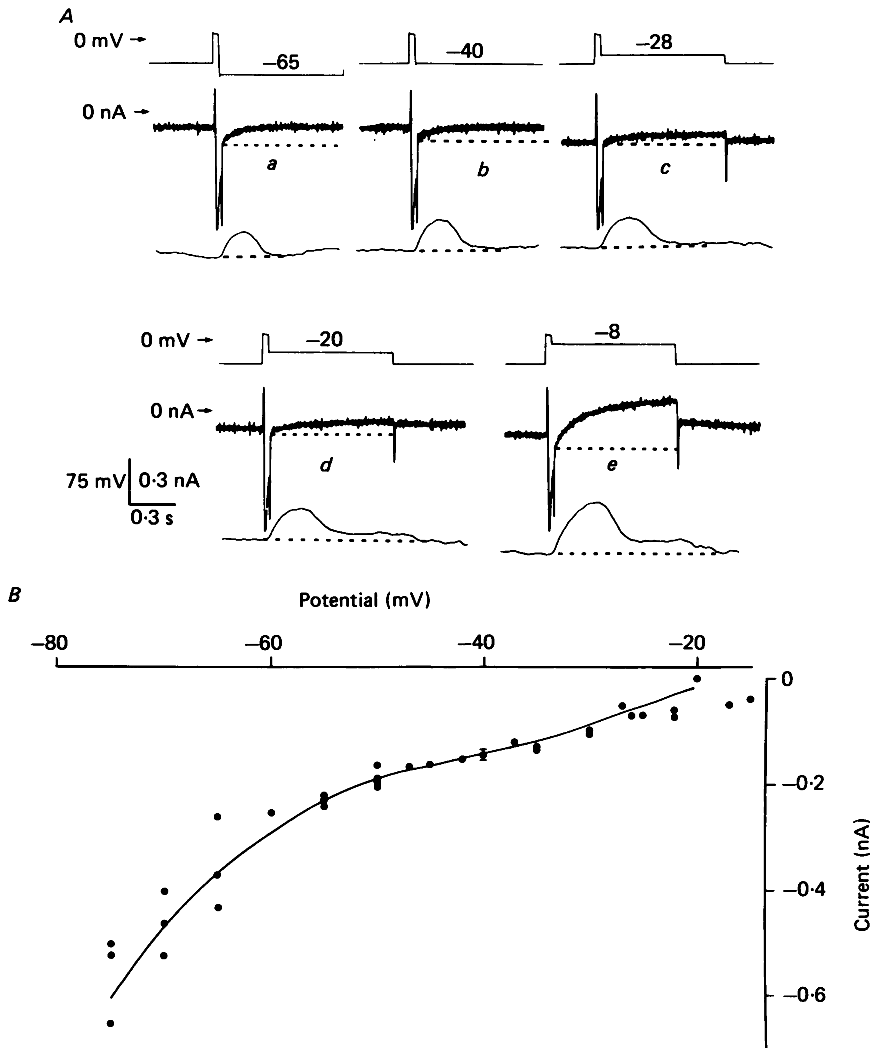

Fig. 8. Voltage dependence of the slow tail current. *A*, upper trace in panels *a*–*e* is membrane voltage. Holding potential −43 mV. 50 ms pulses to +10 mV are followed by 1 s pulses to various potentials from −65 to −8 mV as indicated. Middle traces show corresponding membrane currents and lower traces cell contractions. *B*, data from another cell illustrating the tail-current magnitude over a range of potentials using the protocol described in *A*. Holding potential −40 mV. 50 ms pulses to 0 mV followed by 1 s test pulses to a range of potentials. Peak $i_{Ca,f}$ during pre-pulses to 0 mV was 1·54 ± 0·07 nA (mean ± S.D., $n = 43$). Time to peak inward current 8·89 ± 1·88 ms (mean ± S.D., $n = 43$). At −40 mV mean tail-current magnitude = 0·14 ± 0·01 nA (mean ± 1 S.D., $n = 13$ measurements). Pulse protocol applied once every 60 s.

deactivates in these cells. The twin-electrode experiments confirm that this result is not an artifact created by the use of EGTA electrodes for passing current. The significance of this result in relation to whole-cell recording from cardiac cells is considered in the Discussion.

We have clearly shown the existence of a component of inward current related to

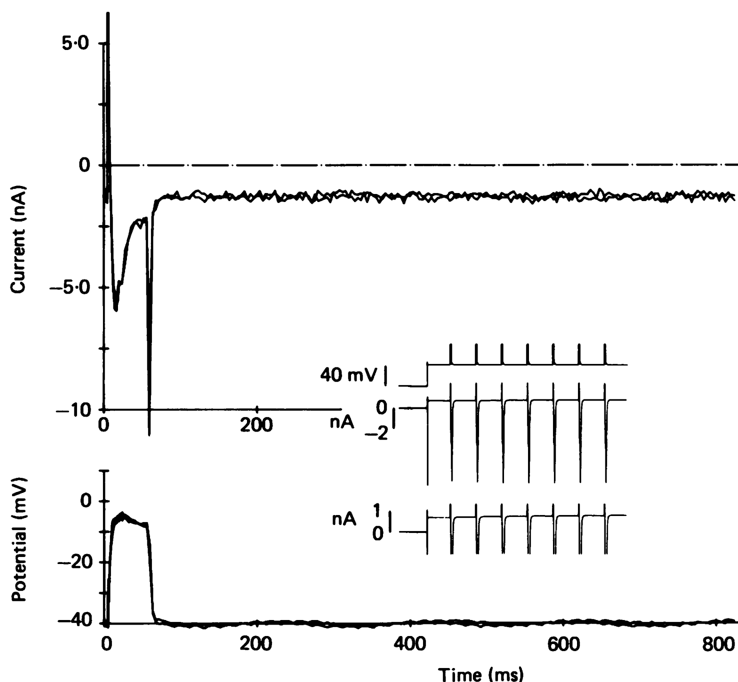

Fig. 9. Effect of internal EGTA injection on slow tail current. Main panel: membrane current (above) and voltage (below) recorded during a train of voltage-clamp pulses from −40 to 0 mV. Current electrode inserted at cell centre (3 M-KCl). EGTA electrode (0·5 M) inserted at end of cell provided a measure of membrane voltage. First and fortieth responses superimposed. Inset panel: membrane voltage and current (middle and lower traces show same current data at different gain) recorded from another cell with a single EGTA electrode (0·5 M) during a train of 50 ms voltage-clamp pulses from −40 to 0 mV.

contraction in guinea-pig myocytes. In the experiments illustrated in the next two Figures we have attempted to ascertain the possible nature of this current. It is possible that the current represents influx of sodium through a population of non-inactivating sodium channels. However, in four cells, exposure to 50 μM-TTX for some hours and the use of holding potentials in the range −90 to −40 mV failed to reduce or remove the delayed current tail elicited on repolarization after 50 ms depolarizing pulses. It is possible that the current represents cation influx through a slow voltage-activated 'calcium channel' around threshold similar to that described by Carbone & Lux (1984*a*) in rat neurones, or the very slow inward current described in this preparation (Lee *et al.* 1984). One constant feature of calcium-channel mechanisms is that the conductance to strontium and barium ions is usually at least that for calcium. Conversely, calcium-activated membrane mechanisms are usually poorly activated by strontium (Eckert & Chad, 1984). The effect of strontium replacement of calcium on the delayed current tail is illustrated in Fig. 10.

In this experiment, as in four others, strontium was unable to activate or support the delayed inward current although the main calcium current $i_{Ca,f}$ is preserved. The contraction in the presence of strontium is larger and slower to rise and relax. This

experiment therefore demonstrates a clear dissociation between the cell contraction mediated by strontium ions and the current tail, which is not. Further experiments in two other cells showed that barium also abolished the current tail but was unable to activate contraction.

Another candidate for the underlying mechanism of the delayed tail current is the sodium–calcium exchange mechanism. This has recently been characterized in guinea-pig myocytes by Kimura, Noma & Irisawa (1986). A potent test of this mechanism is to remove external sodium, in which case all remaining sodium–calcium exchange current is outward. A drawback of this experiment is that internal calcium is likely to rise quickly and produce irreversible contracture of the cell (Chapman, 1979).

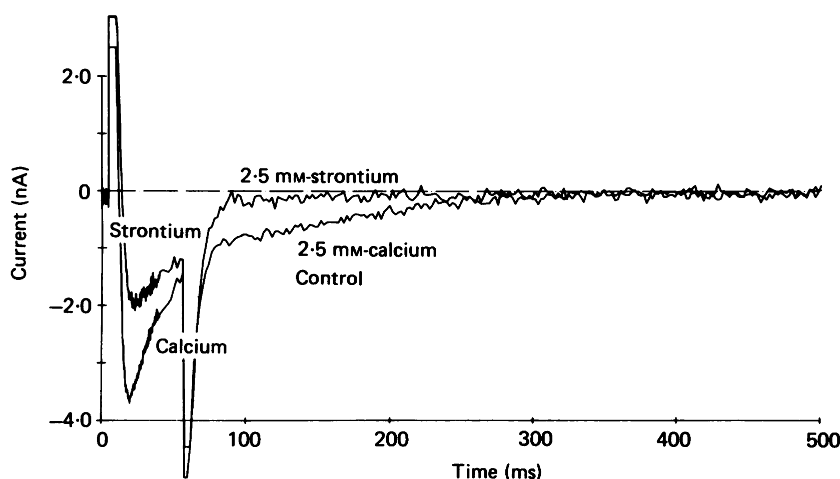

Fig. 10. Effect of strontium replacement of calcium on slow tail current. Current recorded in each case after a 3 min rest. Pulse for 50 ms from −40 to 0 mV. Control 2·5 mM-calcium and after exposure to 2·5 mM-strontium, zero calcium for 10 min. Records superimposed on computer.

In ten experiments external sodium was partially or totally replaced by lithium. The effects of > 90 % reduction in external sodium are shown in Fig. 11. Interestingly lithium ions are generally thought not to activate the sodium–calcium exchange mechanism (J. Kimura, personal communication; for review see Reeves, 1985), but may be carried via a non-specific conductance mechanism. Total sodium replacement by lithium resulted in the rapid development of oscillations of membrane current and contraction. There was an increase in the size of the delayed inward tail current but the cells quickly went into contracture. In seven further experiments in which total replacement of sodium by lithium was carried out in the presence of an EGTA electrode these changes were prevented. This suggests that cell contracture was caused by calcium overload concomitant on block of the sodium-dependent calcium extrusion. Reduction of external sodium to 8·6 % normal resulted in rather slower reproducible changes in five cells. Low external sodium perfusion resulted in an

increase in the magnitude of cell contraction and a corresponding increase in the size of the delayed inward tail current (Fig. 11*B*). Note that there is a small reduction in the inward calcium current during the pulse at this stage. This finding is consistent with a partial block of sodium-dependent calcium efflux, a subsequent rise in intracellular calcium and reduction in calcium-channel current (Matsuda & Noma, 1984). Long-term exposure to low-sodium solution resulted in a large inward shift of the holding-current level and virtual abolition of the delayed current tail and the calcium-channel current. At the same time (Fig. 11*C*) the contraction record shows fluctuating oscillations of varying size that are unrelated to the applied pulses.

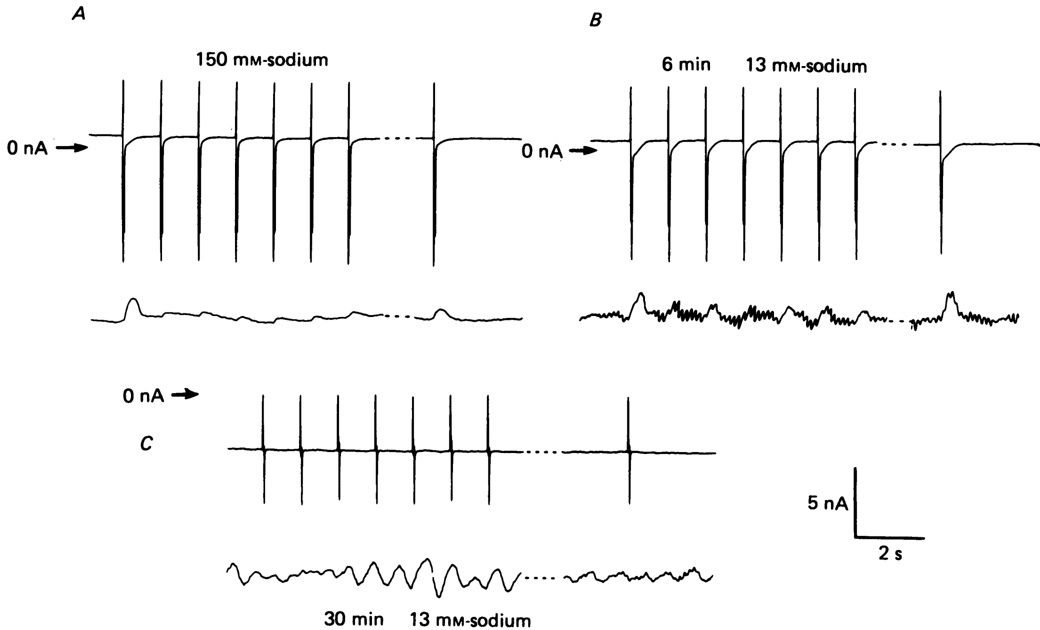

Fig. 11. Effect of replacement of 91 % external sodium by lithium (137 mM). Effect on slow tail current and cell contraction during pulse trains given after rest. Currents (above) in response to 50 ms pulses from −40 to 0 mV with accompanying contractions (below). Panels *B* and *C* show the effect of progressive exposure to 8·6 % external sodium (13 mM). In each record the trace shown after the dotted segment was obtained 15 s after the end of the pulse train. Record obtained from the chart recorder and pulses 8–40 of each of the trains have been omitted.

Finally, we also carried out experiments using ryanodine. This drug is thought to interfere with calcium release and/or sequestration and has been reported to abolish a slow inward current tail in rat cardiac myocytes (Mitchell *et al.* 1984*b*). A typical result is shown in Fig. 12. In the presence of 1 μM-ryanodine the calcium current during the pulse is greatly reduced. This effect usually took a number of minutes to develop. By contrast, the delayed tail is largely unchanged. A qualitatively similar result was regularly obtained with ryanodine in four other cells. It was also noted that contraction remained in ryanodine.

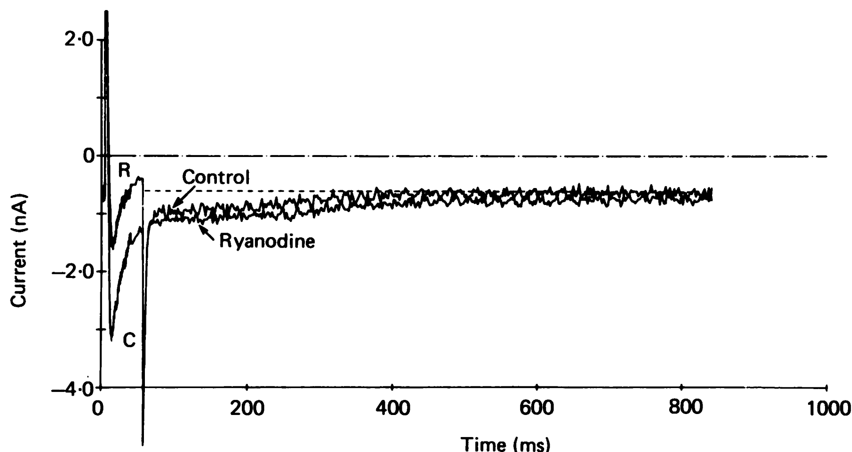

Fig. 12. Effect of 1 μM-ryanodine on the slow tail current. Membrane current recorded after a 3 min rest in each case in response to a 50 ms depolarizing pulse. Control (C) and response after exposure to ryanodine for 10 min (R). Note decrease in peak inward current *during* the pulse caused by ryanodine, consistently observed in all cells exposed to ryanodine.

DISCUSSION

The main conclusion of this paper is clear: the delayed inward tail currents in guinea-pig ventricular cells are strongly correlated with the contraction of the cells and not with the calcium current itself. The latter can be fully activated under conditions (e.g. too short an interval between pulses) where the delayed current is almost completely refractory (Figs. 3 and 6). In this behaviour, the tail-current repriming process resembles that of the contraction process, a resemblance which is made even more striking by the fact that adrenaline speeds both processes (Fig. 7 and Shimoni, 1985). Šimurda *et al.* (1981) have also shown that a 'contraction-dependent slow inward current, i_{sic},' can be separated from the total i_{si} in cat and dog ventricular muscle. In their experimental protocols small depolarizing pulses elicited a current component that was well correlated with contraction whereas larger depolarizing steps elicited a large i_{si} that was inversely related to contraction and which showed the known bell-shaped current–voltage relation for the second inward current.

The simplest interpretation of these results is that, like the contraction, the delayed current is activated by intracellular calcium release. This conclusion is strongly reinforced by our finding that the current is absent when EGTA is introduced into the interior of the cell (Fig. 9). The intracellular calcium transient in this situation is likely to be greatly reduced as evidenced by the complete absence of all cell movement.

This result is of great importance in the interpretation of results obtained with the whole-cell patch technique or when intracellular dialysis or perfusion is carried out. In these situations EGTA is invariably used to control the internal calcium levels and clearly they will be inappropriate for recording the slow component of inward current. Indeed, preliminary results indicate that this is the case using low-resistance

patch-clamp pipettes in whole-cell recording mode on guinea-pig ventricular cells. When the patch pipette contained 11 mM-EGTA plus 1 mM-$CaCl_2$ to buffer the intracellular calcium (pCa ~8·0) no inward tail current is observed; nor, of course, does the cell contract (H. F. Brown, D. Fedida & M. J. Kilborn, unpublished observation).

The experiments with EGTA electrodes also show that the influx of calcium via the calcium channels may not be sufficient to activate the delayed current. The calcium entering via the channels is clearly buffered under these conditions before it can activate the delayed current. This result is striking since the calcium influx is actually greater when EGTA is present since the calcium current then inactivates more slowly.

Does the delayed current represent current flow through a population of non-inactivating sodium channels or calcium channels at negative potentials? The failure of high concentrations of TTX to abolish the inward current tail renders the former possibility unlikely. Many reports have appeared recently of calcium channels that activate over a similar range of potentials to the delayed current in the present results (Fig. 1*B*; see Deitmer, 1984). It does not seem likely that the current described in the present study reflects ion flow through a population of calcium channels similar to those recently suggested to carry weak inward currents around threshold (for review see Reuter, 1985). The delayed inward tail current is present during pulses from both hyperpolarized and depolarized holding potentials unlike the second calcium-channel currents reported by Bean (1985) in canine atrial cells and Carbone & Lux (1984*a*) in mammalian neurones. The delayed current tail is poorly carried by both strontium (Fig. 10) and barium ions and is abolished by the presence of intracellular EGTA (Fig. 9). Finally, the delayed current tail, although clearly distinguished from the current carried by the fast calcium channels, $i_{Ca, f}$, is sensitive to the β-adrenoceptor agonists adrenaline and isoprenaline (Fig. 7).

The experiments of Nilius *et al.* (1985) and Nowycky *et al.* (1985) have revealed calcium channels similar to those described by Bean (1985) and Carbone & Lux (1984*b*) in excised membrane patches separated from the interior of the cell. The component of inward current described in the present work was shown to depend on a substantial rise in intracellular calcium derived from internal stores, and is therefore dependent on the intact structure of the cell for its appearance.

If the current is activated by internal calcium, what carries it? The two obvious candidates are the sodium–calcium exchange current (which has recently been recorded with patch electrodes in single guinea-pig cardiac cells; Kimura *et al.* 1986; Mechmann & Pott, 1986), and the non-specific cation channel identified in rat neonatal ventricular cells by Colquhoun *et al.* (1981). The voltage dependence of the delayed current tail (Fig. 8) gives little clue as to its identity other than for the fact that it would fit for both channel and exchange current. At the more critical potentials around 0 mV the delayed current tail cannot be clearly identified in our experiments. Our results also show that the current size, especially during depolarization, is quite small, around 0·25 nA (Figs. 1 and 8) although this will of course depend on the size of the calcium transient elicited inside the cell.

Although the recordings of ionic current described here, by themselves, do not unambiguously identify the mechanism of the contraction-related inward current,

they can be used in combination with measurements of trans-sarcolemmal calcium movements to discuss the two main possibilities. Hilgemann (1986*a*, *b*) has recently measured fast extracellular calcium transients during two-pulse stimulation in mammalian cardiac muscle. Although he used action potentials while we have used voltage-clamp pulses, the results (see his Fig. 8, Hilgemann, 1986*a*) are interesting to compare. First, during the first action potential of a pair, calcium depletion (attributable to calcium entry through the fast calcium channels) is followed by a large calcium re-accumulation. During the second action potential, if the interval is short enough for very little contractile repriming to occur, the calcium depletion still occurs but the calcium re-accumulation is greatly reduced or abolished. Thus, the calcium re-accumulation process behaves like the contraction-related current described in the present paper. This comparison, although between different species, nevertheless raises the possibility that a large part of the current tail described in the present experiments represents calcium exit from the cells via the sodium–calcium exchange process.

That the delayed current must flow as a part of the total current envelope during depolarization is clear, even though it cannot often be clearly separated from the fast calcium-channel current. This conclusion is derived from the results shown, for example in Fig. 2 showing that, when depolarizing pulses are longer than ~400 ms, the tail current is much smaller. Since the durations concerned are of the same order of magnitude as the action potential duration, this suggests that the majority of the delayed inward component flows during the plateau and repolarization phases rather than after it is terminated. This conclusion agrees entirely with the conclusion of Hilgemann & Noble (1986*a*) that calcium efflux via the exchange mechanism occurs largely during the action potential. Their work used the DiFrancesco–Noble (1985) computer model to reproduce Hilgemann's (1986*b*) recordings of fast extracellular calcium transients in rabbit atrium. Our results suggest that the same conclusion applies to guinea-pig ventricle. Moreover, the quantitative conclusions are very similar. Hilgemann & Noble (1986*b*) calculate that the peak amplitude of the sodium–calcium exchange current during the action potential should be about 7–10 % of the peak value of the calcium current. This is very similar to the amplitude of the delayed current recorded in our experiments (e.g. Figs. 1 and 8), though in the case of voltage-clamp pulses the precise ratio depends on the membrane voltage. Near −40 mV the contraction-related current can even dominate the current records (as it does in the DiFrancesco–Noble (1985) model at this voltage range (see DiFrancesco & Noble, Fig. 5, records a and b). This variation is also reflected in the role that the current may play in determining the shape and duration of the action potential. Thus, in preparations in which the action potential is short, such as rabbit atrium and rat ventricle the voltage dependence of the tail current may give rise to currents large enough to perturb the action potential time course. Schouten & ter Keurs (1985) and Mitchell *et al.* (1984*a*) have shown a late slow phase of the action potential in rat ventricle below −40 mV that they have attributed to a sodium–calcium exchange current.

Two other results in this paper support the view that the delayed current reflects mechanisms activated by the internal calcium transient. First, its decay is speeded up in the presence of adrenaline (Fig. 7). This is expected since adrenaline also speeds

up the decay of intracellular calcium by enhancing uptake by the sarcoplasmic reticulum. Secondly, the speed of decay of the current is only moderately voltage dependent (see Figs. 4, 5 and 8) either on the potential during the depolarizing pulse (Figs. 2, 4 and 5) or the potential on repolarization (Fig. 8).

Noble (1986) has recently discussed the possible voltage dependence of the sodium–calcium exchange current and has shown that it would be expected that, at one extreme, when the exchange process is the rate-limiting process in getting calcium out of the cell, the process would be expected to speed up to a moderate degree (3-fold increase per 60 mV) on hyperpolarization, which is what we find in Fig. 8. At the other extreme, when the rate-limiting process is internal sequestration or buffering, the current decay rate may also show little voltage dependence. This appears to be the situation in Fig. 4.

On the other hand perfusion of the cell with zero or very low external sodium (Fig. 11) replaced by lithium ions may be expected to abolish inward sodium–calcium exchange current. In this situation the internal calcium within the cell is expected to rise to a high level. The delayed inward tail current remains during this period and increases in magnitude (Fig. 11). This result is consistent with the idea that inward current channels are activated at high levels of internal calcium (Mechmann & Pott, 1986). However, the relative roles of calcium channels and the sodium–calcium exchange mechanism at normal levels of intracellular sodium and calcium cannot be assessed from such an experiment. Similar considerations apply to our results with strontium and barium. They support the view that the current is calcium activated but do not determine its precise mechanism. Strontium ions are carried by the sodium–calcium exchange mechanism in guinea-pig ventricular cells but the Michaelis constant (K_m) for strontium is about 7 mM externally applied rather than about 0·9 mM for calcium (J. Kimura, personal communication). Thus it is expected that strontium replacement of calcium will reduce a sodium–calcium exchange current. Strontium ions are also known to poorly activate calcium-activated mechanisms in general (e.g. see Eckert & Chad, 1984) so similarly strontium may fail to activate calcium-activated channel mechanisms. Such experiments highlight the difficulty in separating these components of inward current in situations where specific inhibitors for possible mechanisms do not exist.

Our experiments show that, although the guinea-pig ventricular action potential does not show the distinct phases of repolarization seen in the rat, an inward current probably activated by internal calcium is nevertheless involved in the later phases of the action potential. This current is often difficult to distinguish from the fast calcium current during depolarization. From a theoretical point of view, this issue of separability must depend on the relative speeds of calcium current inactivation and activation of the delayed current. If these are very different in speed, then separation of current peaks is likely to occur. If, however, the speeds are less disparate, the onset of the calcium-activated current may well be masked under most conditions by the calcium current itself. This current will then appear simply as a slower phase of decay of the total inward current, as suggested here.

The question remains though as to exactly what maintains the very high and long plateau in this preparation since at positive potentials, the calcium-activated inward current is likely to be very small (see the results in Fig. 8 of this paper, and the

current–voltage diagrams for the exchange current measured by Kimura *et al.* 1986). One possibility here is that a cadmium-resistant maintained inward current (Lee *et al.* 1984) may be involved. We also find a maintained inward current in our experiments (see Fig. 1*A*, records *c* to *f*). Further experiments are required to assess the quantitative contributions of the fast calcium current, the maintained current and the calcium-activated current to the maintenance of the plateau in cases like the guinea-pig where the plateau is high and long lasting. Our results emphasize the importance of doing these experiments in conditions which allow the normal intracellular calcium transient to occur instead of buffering calcium strongly with EGTA as in the great majority of work with patch electrodes.

Finally, it is worth commenting on the effect of ryanodine on guinea-pig myocytes. Mitchell *et al.* (1984*a*) have noted that at 36 °C guinea-pig action potentials and contraction are resistant to a short exposure to 1 μM-ryanodine, although some slowing of the activation of contraction is seen. In Fig. 12 we have shown that ryanodine does not affect the slow current tail. However, it does cause a marked slowing of the rate of activation of the fast calcium current and reduces the peak amplitude. We do not know what causes these effects under our experimental conditions but they may be related to the relative importance of calcium influx and sarcoplasmic reticulum calcium release to the generation of tension in this tissue (Bers, 1985).

This work was supported by grants from the British Heart Foundation, Wellcome Trust and the Medical Research Council. We thank Dr H. F. Brown and Dr D. Hilgemann for valuable discussion.

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