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USE-DEPENDENT REDUCTION AND FACILITATION OF Ca^{2+} CURRENT IN GUINEA-PIG MYOCYTES

BY D. FEDIDA*, D. NOBLE AND A. J. SPINDLER
From the University Laboratory of Physiology, Parks Road, Oxford OX1 3PT

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SUMMARY

1. Action potentials, calcium currents (i_{Ca}) and cell contraction have been recorded from single guinea-pig myocytes during periods of stimulation from rest. Voltage clamp was carried out using a single microelectrode. Cell contraction was measured optically. All experiments were performed at 18–22 °C.

2. An inverse relationship was observed between cell contraction and action potential duration or i_{Ca}. Mixed trains of action potentials and voltage clamp pulses preserved this relationship. Long voltage clamp pulses induced negative 'staircases' of i_{Ca} and positive 'staircases' of cell contraction. A facilitation of i_{Ca} was observed during repetitive stimulation with clamp pulses of 100 ms duration or less and was accompanied by a decrease in cell contraction.

3. The voltage dependence of inward current staircases was found to depend on Ca^{2+} entry rather than membrane voltage for long voltage clamp pulses and was not affected by 30 mM-TEA or 50 μM-TTX. Current reduction was greatest at 0 mV ($P < 0{\cdot}05$) when i_{Ca} was largest. Changes in cell contraction during pulse trains showed a similar voltage dependence. The time constant of current staircases was only mildly voltage dependent.

4. Interference with normal cellular mechanisms for Ca^{2+} uptake and release by strontium, 1–5 mM-caffeine and 1 μM-ryanodine increased current staircases and could abolish i_{Ca} facilitation with short clamp pulses.

5. Variations in the level of Ca^{2+}-dependent inactivation of i_{Ca} can explain many features of the changes in i_{Ca} during stimulation after rest. Long clamp pulses (or action potentials) may increase cell Ca^{2+} loading and inhibit i_{Ca}. Short clamp pulses reduce available Ca^{2+} for cell contraction and this may reflect a lowered myoplasmic Ca^{2+} level which allows facilitation of i_{Ca}.

INTRODUCTION

During stimulation at different rates a negative relation between peak tension and the action potential duration is a common finding in mammalian ventricular muscle. It has been observed in multicellular preparations of small mammals such as guinea-pig (Reiter & Stickel, 1968; Braveny & Sumbera, 1970) and cat papillary muscles

* Present address: Department of Medical Physiology, University of Calgary, School of Medicine, Calgary, Canada T2N 4N1.

(Allen, 1977). Changes in inward calcium current (i_{Ca} or i_{si}) during stimulation from rest (which we shall call 'staircases') have also been described previously and in cat ventricular muscle (Simurda, Simurdova, Braveny & Sumbera, 1976) these staircases are most often negative in direction. However, in frog atrium (Noble & Shimoni, 1981) and in sheep Purkinje fibres (Boyett & Fedida, 1988), for example, increasing inward currents or bidirectional changes during repetitive pulsing may be seen depending on the particular pulse protocol chosen.

A fall in the electrochemical potential for Ca^{2+} entry was suggested to underlie the decrease in inward current that accompanied an increase in force during stimulation (Reuter, 1973). An increase in internal Ca^{2+} that reduced i_{si}, which could cause action potential shortening, was also favoured by Simurda *et al.* (1976), although they also mentioned the possibility that Ca^{2+} conductance may vary with changes in internal Ca^{2+}.

In cardiac tissues Ca^{2+}- and voltage-dependent inactivation of Ca^{2+} current has been described in Purkinje fibres (Lee, Marban & Tsien, 1985) and ventricular myocytes (Hadley & Hume, 1987). In calf Purkinje fibres a slow inactivation of Ca^{2+} channels has been described that develops over a number of seconds (Kass & Scheuer, 1982). In the present experiments, we investigate the idea that changes in internal Ca^{2+} or Ca^{2+} entry itself can feed back to regulate membrane Ca^{2+} current. We have re-examined the relationship of Ca^{2+} current to cell contraction and action potential duration during trains of stimuli or voltage clamp pulses after rest. Both slow, use-dependent block and facilitation of i_{Ca} exist which are correlated with beat-to-beat changes in cell contraction. This relationship can be disrupted by modification of intracellular Ca^{2+} recycling with the agents strontium (Sr^{2+}), caffeine and ryanodine. Previous reports of some aspects of this work have been communicated (Fedida, Noble & Spindler, 1985).

METHODS

The techniques for cell isolation, maintenance and solutions for experimentation have been described in recent publications from this laboratory (Fedida, Noble, Rankin & Spindler, 1987). Only further details will be considered here.

The present experiments were carried out entirely at room temperature (18–22 °C). It was possible to maintain stable impalements for some hours with minimum interference from transient inward current, i_{TI} (cf. Mitchell, Powell, Terrar & Twist, 1985; Fedida *et al.* 1987). The time to peak of i_{Ca} for a single pulse from −40 to 0 mV after a 3 min rest was 9·05 ± 0·48 ms (mean ± S.E.M., $n = 70$ values). This is similar to a half-time of activation of 4·6 ms for i_{Ca} in guinea-pig myocytes, recorded with a switching voltage clamp by Josephson, Sanchez-Chapula & Brown (1984), and a time to peak inward current of between 8 and 10 ms in the steady state at 23 °C ('whole-cell' technique; Cavalié, McDonald, Pelzer & Trautwein, 1985). In several experiments the voltage control during peak i_{Ca} was evaluated using a second microelectrode directly sensing membrane voltage. Two electrodes in one cell invariably increased membrane leak current, but using a 12 MΩ 3 M-KCl electrode, the voltage recorded 50 μm from the current-passing electrode rose to the command potential in less than 4 ms (switch-clamp frequency 3 kHz). At 1, 7 and 20 kHz switch-clamp frequencies the rise times were less than 4·5, 20 and 27 ms respectively. Inward currents of up to 4 nA recorded under these conditions peaked at around twice the voltage rise time for clamp frequencies under 4 kHz which suggests that adequate control of i_{Ca} may be obtained at room temperature using a switching voltage clamp together with low-resistance bevelled microelectrodes (Wilson & Goldner, 1975).

The advantage of the switching voltage clamp was that dialysis of cell contents was minimized

and discontinuous feed-back can reduce errors from series resistance. Ca^{2+} current was also measured 40 ms after the clamp pulse was initiated (i_{t-40}) and findings we describe for peak i_{Ca} are supported by later data.

Solutions. A modified Tyrode solution containing 2·5 mM-Ca^{2+} was used. This was either bicarbonate or HEPES buffered to pH 7·4 at 20 °C (cf. Fedida *et al.* 1987). Tetrodotoxin (TTX, 20–50 μM) was used to block Na^+ channels as indicated. Tetraethylammonium chloride (TEA, 30 mM) was used to block i_K without correction for the increase in osmotic pressure. No attempt was made to block inwardly rectifying K^+ current. Adrenaline or isoprenaline (0·01–1 μM), caffeine (1–5 mM) and ryanodine (1 μM) were added to the superfusate as stated. All drugs were obtained from Sigma, Poole, Dorset, with the exception of ryanodine (kind gifts from Dr C. C. Ashley and Dr D. Hilgemann).

The number of cells studied in each case is stated in the text. Statistical data are expressed as mean ± one standard deviation (S.D.) or standard error (S.E.M.). Curves are fitted by eye and straight lines using a least-squares method. Fit of data to the line is expressed in terms of the correlation coefficient (r). The significance of differences between means of two samples is evaluated using Student's t test.

RESULTS

The relationship of action potential duration and cell contraction to i_{Ca}

In Fig. 1, action potentials and membrane currents at different stimulus rates are illustrated. After 4 min rest periods the cell was stimulated at rates between 6 and 30/min. There is a shortening of the action potential over some 30–60 s that it takes to reach a steady state. The degree of shortening is graded according to the stimulus frequency and amounts to 31% at 30/min, 23% at 12/min and 19% at 6/min. The voltage clamp protocol shown at the foot of Fig. 1 was applied to the same cell. After rest the preparation was clamped from the resting potential (RP) to −55 mV, then repetitively pulsed to 0 mV for 1 s at the rates indicated. The superimposed current records at each rate show a marked decline in the inward current during the train, with little change in the outward holding current level during the stimulation period. From such a negative holding potential, residual Na^+ current may distort the early current phases and for this reason in most experiments we used more positive holding potentials (−40 to −50 mV) or TTX to ensure abolition of i_{Na}. In the latter situation inward currents elicited on depolarization could be abolished with 10^{-5} M-D600 or 0·1–0·25 mM-Cd^{2+} to reveal little time-dependent outward current in the majority of cells (see later). In Fig. 1, at the higher rates, the reduction in inward current during trains of voltage clamp pulses is greater. The time course of this reduction is very similar to that seen for action potential shortening illustrated in the left panels.

During trains of voltage clamp pulses the minimum interpulse interval is 1 s (at 30/min) and an appreciable negative staircase of current is still apparent at an interpulse interval of 10 s (6/min). It is unlikely, therefore, that the inward current decline can be attributed to the incomplete recovery of i_{Ca} from inactivation between beats, a well-described cause of action potential shortening at high rates (Boyett & Fedida, 1988). In single guinea-pig myocytes, recovery from inactivation is complete within 500 ms for 200 ms pulses (Josephson *et al.* 1984; Hadley & Hume, 1987).

Changes in cell contraction, action potential duration and i_{Ca} during stimulation after rest are illustrated in Fig. 2. Trains of action potentials are shown in Fig. 2*A* at 60/min and in Fig. 2*B* at 30/min. At the higher rate, stimulation causes a progressive shortening of the action potential, excluding a slightly longer action

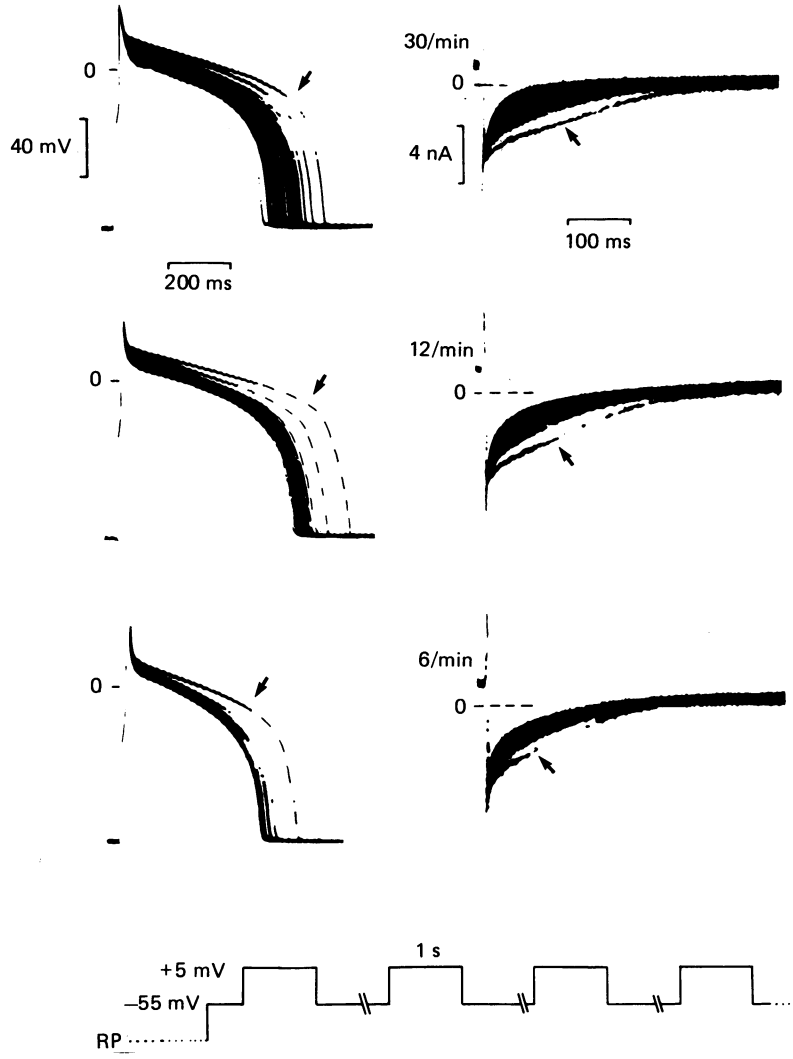
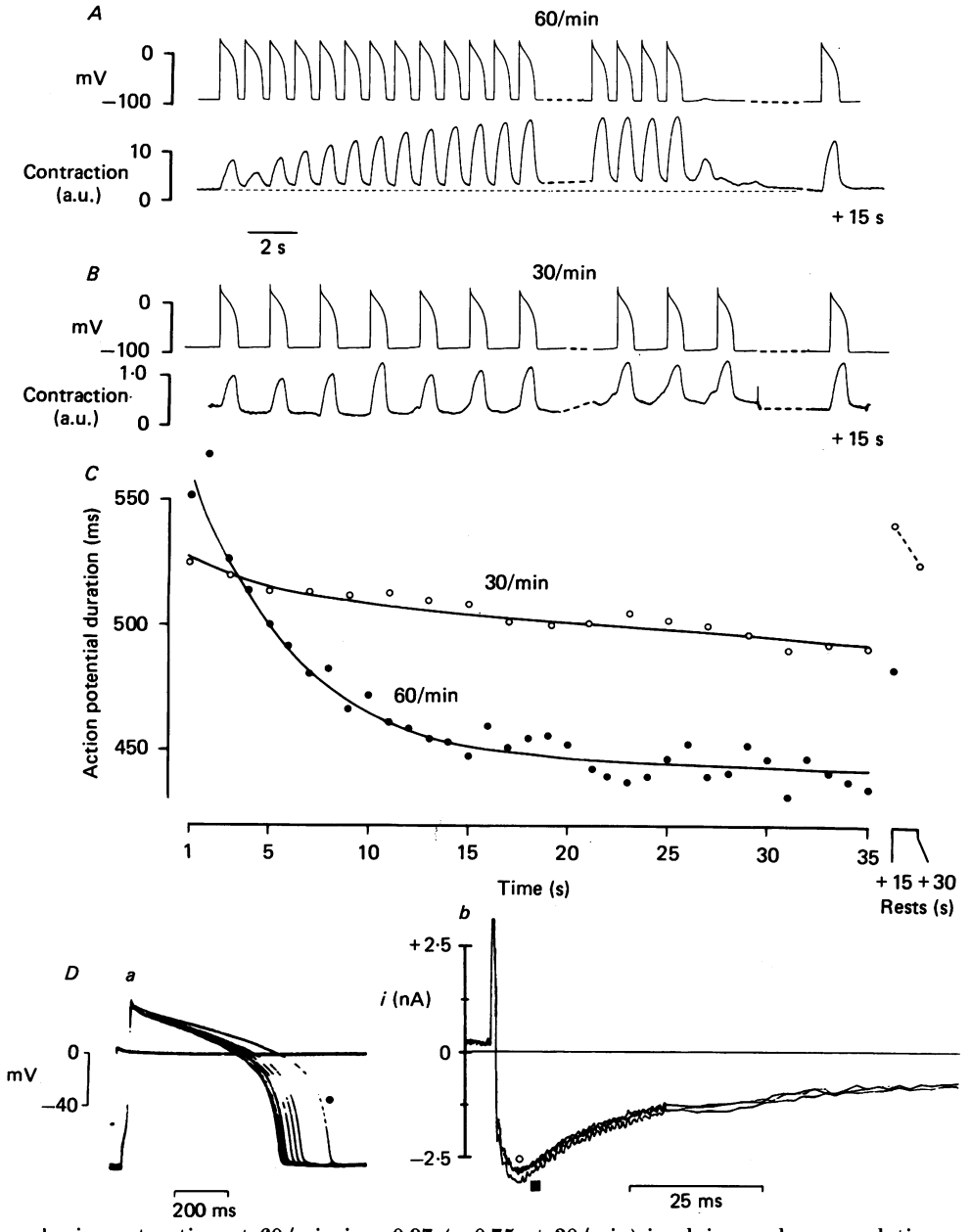


Fig. 1. The effect of stimulus rate on action potentials (left) and currents (right) after 4 min rests. First action potential and current of each train indicated by arrows. Currents recorded during 1 s pulses as indicated by protocol inset at foot of figure. Stimulus rates 30, 12 and 6/min. All records from the same preparation. Due to fade of the store facility on the oscilloscope during repetitive sweeps, the first one or two action potentials recorded at 12 and 6/min have been retouched. The calibration bars in the top panels also apply to records in the other panels. In between trains of voltage clamp pulses the cell was at the resting potential with the clamp switched off.

Fig. 2. Action potential and contraction changes on stimulation from rest. *A* and *B* show action potentials and contractions reproduced from chart recordings during periods of stimulation after 3 min rests at 60/min (*A*) and 30/min (*B*). Chart recordings have been interrupted to show only the first few responses, the steady-state responses and action potentials and contractions 15 s post-stimulation. *C*, the time course of changes in action potential duration at the two rates. These latter data were obtained using an electronic device to measure action potential duration from the upstroke until repolarization to −25 mV. The statistical correlation (r) between changes in action potential duration and



phasic contraction at 60/min is −0·97 (−0·75 at 30/min) implying a close correlation between changes in the two variables. *D*, effect of clamp pulses interposed during an action potential train. Panel *a* shows superimposed action potentials stimulated at 30/min from rest; the first action potential is indicated (●). Instead of the second stimulus the cell was pulsed from −50 to 0 mV for 1 s. This is seen as the step voltage change in panel *a* and corresponds to the current trace labelled ■ in panel *b*. Action potentials were then elicited again and clamps interposed at the seventh and thirteenth stimuli (currents recorded during these pulses shown in panel *b*, labelled ○). Note the gap in the action potential record corresponding to the current recorded in place of the second stimulus. Computer digitization rate reduced from 10 to 1 kHz after first 40 ms of current records. Data from another cell to that illustrated in *A–C*.

potential in the second beat after rest. This is best seen in the graph in Fig. 2*C*. The marked action potential shortening at 60/min is associated with a positive staircase of contraction. There is a larger increase in both the phasic and tonic contraction of the cell at 60/min than at 30/min. The effect of 15 or 30 s rest is to partially reverse these changes. There is a strong correlation between the time course of action potential shortening and the increase in cell contraction shown in the lower traces of panels *A* and *B*.

In Fig. 2*D* is a direct demonstration of changes in i_{Ca} during a train of action potentials. Voltage clamp pulses and stimuli to generate action potentials have been mixed during a single period of stimulation. After a 4 min rest a current pulse stimulates the first action potential, labelled ● in panel *a*. Voltage clamp pulses were interpolated periodically to record i_{Ca} (current traces in panel *b*). It is difficult to compare the relative action potential shortening and reduction in Ca^{2+} current as the first Ca^{2+} current after rest is not recorded using this protocol. However, the action potential shortens from the third to steady-state beats by about 10%. This is the same as the reduction in peak i_{Ca} from the second to thirteenth beats, and occurs over the same time course.

The results above confirm the inverse relation between action potential duration and contraction in single myocytes and show that a reduction of Ca^{2+} current sufficient to cause the action potential shortening occurs over a similar time course. The effect of altering the duration of standard voltage clamp pulses on contraction and i_{Ca} is shown below.

Effect of voltage clamp pulse duration on cell contraction and i_{Ca} during pulse trains

All voltage clamp protocols are based on an interpulse interval of 1 s. This value was chosen to allow i_{Ca} to recover from inactivation caused by the preceding pulse (see earlier). The direction of the Ca^{2+} current staircase is critically dependent on the duration of the activating voltage clamp pulses and this observation is illustrated in Fig. 3*A*. In the upper set of records (*A a*) the protocol was as follows. After a 3 min rest the preparation was clamped at a holding potential of −40 mV and repetitively pulsed to 0 mV for 1 s at a rate of 30/min. On a slow time base, the continuous record shows the cell contraction during stimulation. The phasic contractions become larger and reach a plateau. There is a similar rise in the baseline level of cell contraction. At the end of the stimulation period the cell was rested. Pulses triggered 15 and 30 s later show that the phasic and tonic cell contraction have diminished somewhat. Membrane currents during the pulse to 0 mV were recorded at the times indicated and show a diminution in inward current from beat 1 to beat 3. The Ca^{2+} current is smaller still by beat 20. This reduction in current is partially reversed by a 30 s rest. A more negative staircase could either be achieved by increases in the clamp rate or by an increase in the duration of the pulse to 0 mV, with a constant interpulse interval. The time course of each current trace is little changed during the train of pulses. This was the most commonly seen result but is in contrast to records shown in Fig. 1.

Conversely, a shortening of the pulse duration at 0 mV reduced the size of the negative staircase provided that the interpulse length remained constant. Pulse durations of 50 ms or less consistently gave a positive current staircase and

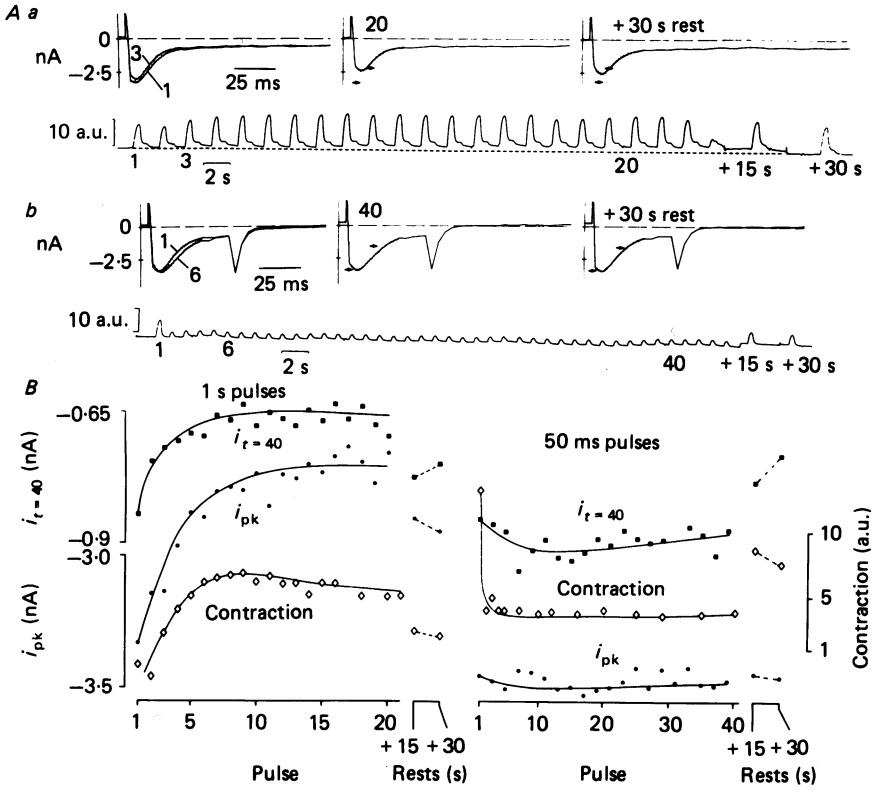


Fig. 3. The time course of changes in inward current and contraction during trains of long and short voltage clamp pulses after rest. *A a*, upper tracings are membrane currents recorded at different times during a train of 1 s pulses from −40 to 0 mV with a 1 s interpulse interval, applied after a 3 min rest. Currents reproduced from computer on a fast time base (only first 100 ms of each record shown); note reduction of digitization from 10 to 1 kHz after first 40 ms of record explains reduction in resolution in later part of traces. The first and third responses are superimposed in the left panel, the twentieth response is shown in the middle panel, and current recorded after a 30 s rest is shown in the right panel. The arrows in the middle and right panels indicate peak inward and later current levels of the first current trace. The lower trace shows a continuous record of cell contraction (reproduced from the chart recorder; arbitrary units (a.u.)) during the train of 1 s pulses and two rested beats as indicated to the right. The numbers under the chart record correspond to the current tracings in the upper panel. *A b*, currents (above) and contractions (below) during a train of 50 ms pulses from −40 to 0 mV after a 3 min rest. Interpulse interval is 1 s. Currents recorded at times indicated on lower chart trace. Note that spike of current on repolarization is not faithfully represented due to decrease in digitization rate. Note the slow time base in the chart recordings and that the recorder speed was reduced at the end of the period of stimulation. This gives small jumps in the record before the tracings of the rested beats which were obtained after 15 and 30 s rests. *B*, graphical representation of changes in current and contraction during the 'staircases' shown in the upper panels. Current measured from computer as peak inward current (i_{pk}, ●) and current 40 ms after the start of the clamp pulse ($i_{t=40}$, ■). Contraction (◇) is in arbitrary units (a.u.). The same ordinate scales apply to both graphs, with increasing inward current downwards. The data from the rested beats are separate from data collected during the train and joined by dashed lines. The left graph illustrates changes during the train of 1 s pulses and the right graph changes in current and contraction during the train of 50 ms pulses.

characteristic changes in cell contraction as shown in Fig. 3*Ab*. In this cell most of the change in current occurs during the relaxation phase of current; there is a slowing of inward current inactivation evoked by the short pulse that becomes apparent in the second beat. The arrows indicate the time course of the current during the first pulse and show that increased inward current is still present at the fortieth beat and that it is removed when the cell is rested. The cell contraction is large in the first beat; subsequent contractions are much smaller and change very little during stimulation. Rest also reverses the effect of stimulation on cell contraction.

The time course of these events from the same cell is illustrated in Fig. 3*B*. Currents have been measured at peak inward current level and at $t = 40$ ms. During stimulation with 1 s pulses the inward current declines over the first 10–12 beats and is closely paralleled by the increase in contraction. These changes are reversed when the preparation is rested. In Fig. 3*Aa* the increase in the resting level of cell contraction occurs over a similar time course. For the short pulses, on stimulation there is a small increase in the peak inward current and a larger increase at $t = 40$ ms. The current increase is immediately lost on resting the preparation. The contraction shows an instantaneous drop on short-pulse stimulation that is maintained throughout the train and reversed on rest.

In Fig. 4*A* the traces illustrate current staircases recorded from a single cell at two extremes of pulse duration. Five second pulses with a 1 s interpulse interval cause a marked reduction in i_{Ca}. In contrast to this is the increase in i_{Ca} that results from trains of 30 ms pulses with a 1 s interpulse interval (left panel). The first current in the train is clearly seen (●). The second current has a similar peak inward current, but a delayed phase of inactivation. Repetitive pulses result in an increase in the peak inward current and delayed current relaxation. Cumulative data on the effect of varying pulse duration and holding potential on i_{Ca} are shown in Fig. 4*B*. The data fall into two natural groups. One second or longer pulses (i.e. where 50% or greater of the cycle time is spent depolarized) cause a progressively greater reduction in i_{Ca}. Pulses shorter than 50 ms give positive current staircases.

The current change at the end of the clamp pulse is shown as half-filled symbols (mean data with error bars). In all cases the facilitation of current during short pulse staircases is more marked at the end of the clamp pulse than at the time of peak inward current. Thus, an important effect of repetitive short pulsing is to slow Ca^{2+} current inactivation. Mean data indicate that the facilitation of i_{Ca} is greatest for 20 ms pulses.

Voltage dependence in staircases of inward current and contraction

The effect of increased voltage clamp pulse duration to produce negative current staircases could reflect either a voltage-dependent effect from the increase in time spent depolarized or an increase in Ca^{2+} channel inactivation resulting from the increased Ca^{2+} entry during long voltage clamp pulses. The latter hypothesis is favoured by the i_{Ca} facilitation that occurs when cell contraction falls. Voltage-dependent inactivation usually becomes larger and faster at more positive membrane potentials (Hadley & Hume, 1987), whereas Ca^{2+}-dependent effects reflect the voltage dependence of Ca^{2+} entry and changes in cell contraction (Eckert & Chad, 1984).

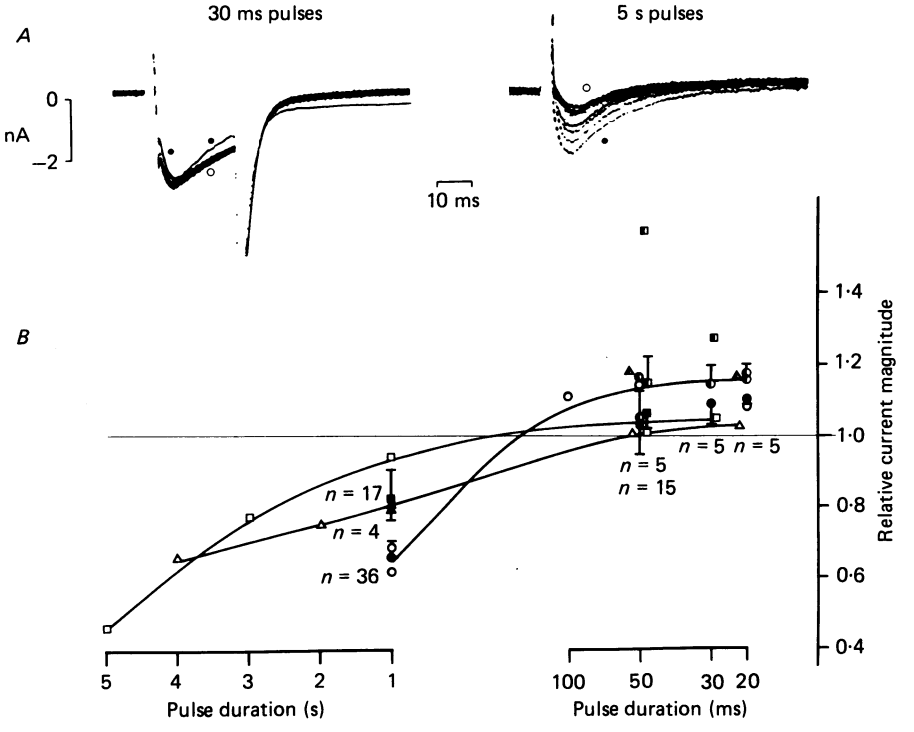


Fig. 4. Effect of pulse duration on inward current staircases: cumulative data. *A*, superimposed current records during repetitive 30 ms (left) or 5 s (right) voltage clamp pulses from −50 to 0 mV. Pulses applied after a 3 min rest with a constant 1 s interpulse interval. First (●) and steady-state responses (○) in each train are marked. Data correspond to open-square data in panel *B*. *B*, normalized data for staircases of inward current plotting relative change in peak inward current during trains of pulses of different durations (abscissa) with a constant 1 s interpulse interval. A relative current magnitude greater than 1·0 signifies a positive current staircase, less than 1·0 indicates inward current decrease during repetitive stimulation. Individual data for three cells from different holding potentials are indicated by the open symbols joined by separate curves: −40 to 0 mV, ○; −45 to +5 mV, △; −50 to 0 mV, □. Open symbols refer to change in peak inward current during staircases. Filled symbols represent mean data for peak current and have bars indicating one standard deviation (S.D.). Half-filled symbols represent relative changes in inward current measured at the end of short voltage clamp pulses and those with error bars are mean data for these later current measurements. Circles represent data for pulses from −40 to 0 mV, squares represent data for −50 to 0 mV, and triangles data for pulses from −45 to +5 mV. Note therefore that for staircases of pulse durations of 50 ms or less, staircases are predominantly positive and that inward current measured towards the end of the clamp pulse (half-filled symbols) increases more than the peak inward current level. The values for n adjacent to the mean values indicate the number of staircases and for shorter pulse durations refer to both peak current and later current measurements (half-filled symbols). The error bar for 1 s pulses from −40 to 0 mV = 0·5 S.D. for clarity. One second pulses from −40 to 0 mV reduce peak i_{Ca} in the steady state to 0·66±0·027 (mean±S.E.M) of that in the first pulse after rest. This reduction is significantly greater than for pulses from −50 to 0 mV (0·812±0·026) or −45 to +5 mV (0·802±0·027; $P < 0·001$). The t value for the difference of means for i_{Ca} reduction during trains from holding potentials of −45 and −50 mV is 5·72 ($P < 0·001$). No significant difference was seen between short-pulse data from holding potentials of −40 mV (circles in Fig. 4*B*; mean±S.E.M. = 1·03±0·028) or −50 mV (squares, 1·06±0·016).

Current–voltage relations of Ca^{2+} current staircases with 1 s pulses are shown in Fig. 5*A* and *B* along with the accompanying cell contraction staircases (Fig. 5*C*). The current–voltage relations formed by the peak inward currents in the first and twentieth pulses are plotted. The Ca^{2+} current is largest around 0 mV and there is no shift in this voltage dependence during repetitive pulsing. The *magnitude* of the staircase (the change in current from the first to twentieth pulse) is a maximum at the potential at which i_{Ca} is greatest (i.e. around 0 mV) and thus where Ca^{2+} entry is maximal. There is little change in the current levels at 800 ms which suggests that no summation of i_K occurs during repetitive 1 s pulses to shift Ca^{2+} currents in the outward direction. High concentrations of TEA, given to block i_K, reduced i_{Ca} during long exposure but the voltage dependence of the current staircase is unchanged from that shown in Fig. 5*A*, so that negative staircases of current were largest at potentials at which i_{Ca} was largest and smaller at more positive and negative potentials.

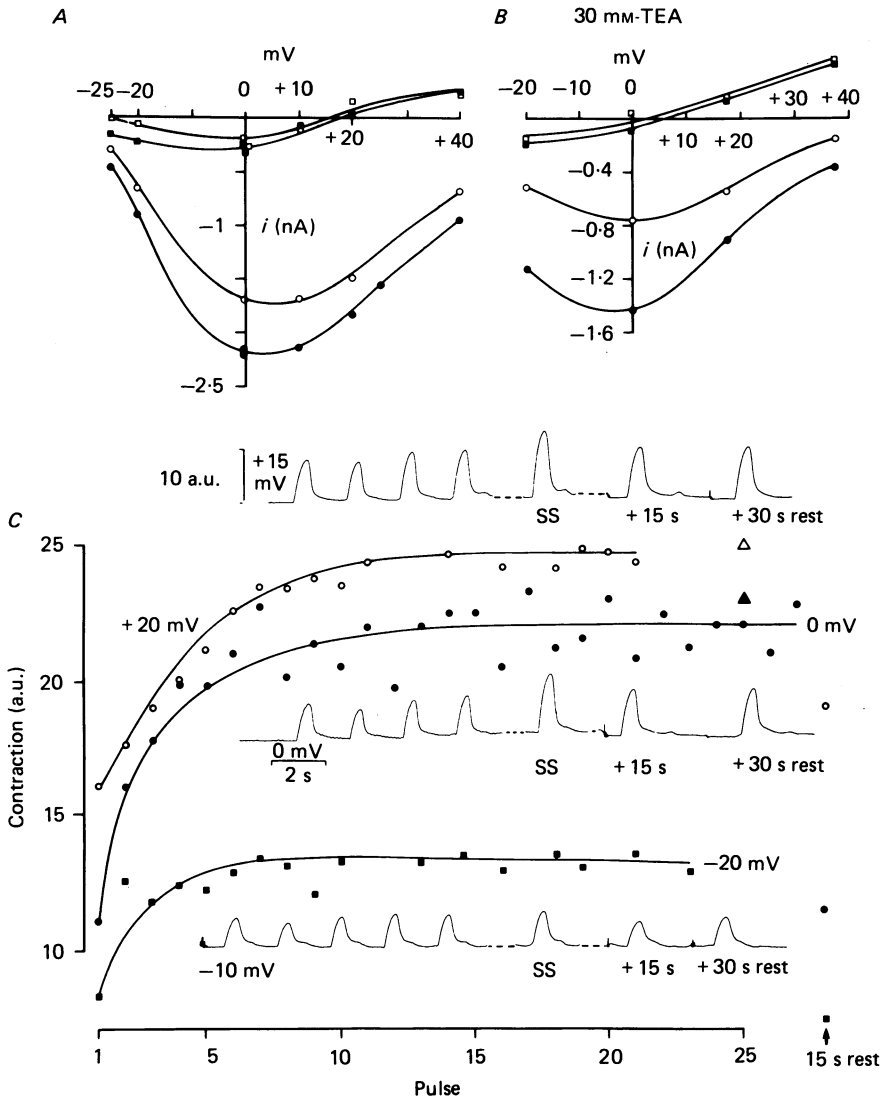
The lower panel (Fig. 5*C*) illustrates the effect of voltage on contraction staircases in two cells (plotted data from the same cell as data in Fig. 5*A*). The cell contraction in the first beat after rest increases with the voltage step from the holding potential, as does the contraction in the steady state. Note that the filled and open triangles indicate the steady-state contraction for pulses to +10 and +40 mV respectively. The change in cell contraction during the pulse train is largest for pulses to 0 mV (see both plotted and original contraction data) in Fig. 5*C*. Thus, the largest *increase* in cell contraction occurs at potentials at which Ca^{2+} entry is greatest and when negative staircases of inward current are largest. Current–voltage relations of staircases as shown in Fig. 5 were obtained in ten cells from holding potentials between −55 and −35 mV and in four cells exposed to 30 mM-TEA, with accompanying contraction recordings in four more cells.

In eight cells holding potentials in the range −100 to −60 mV were used to investigate inward current staircases produced by pulses in the negative voltage range −50 to 0 mV. TTX (20–50 μM) was present in these experiments to block i_{Na}. Trains of 1 s pulses with a 1 s interpulse interval always resulted in negative current staircases, as at more positive potentials.

The time courses of current staircases are well fitted by a single-exponential process. The time constants ranged between 10 and 16 s in eight cells in the voltage range −20 to +60 mV and did not show a marked voltage dependence; nor were they affected by exposure to 30 mM-TEA in four cells (not shown).

The changes in i_{Ca} and cell contraction during trains of short clamp pulses to different potentials are shown in Fig. 6. In this cell, after 4 min rests, the preparation was pulsed from −40 mV to a test potential in the range −5 to +25 mV for about 40 beats. This process was repeated for the entire range of test potentials. The current (Fig. 6*A*, increasing inward current plotted upwards) increases during the second pulse at each potential. Subsequently, however, this increase is only

Fig. 5. Current–voltage relations for staircases of inward current in response to 1 s pulses. *A*, standard situation: peak inward current during first pulse after rest (●) and steady current at 800 ms (■); peak inward current during twentieth pulse (○) and current at 800 ms (□). Absolute current level (no leak subtraction) is plotted against the pulse



potential. The graph thus represents cumulated data from eight current staircases in one cell. Holding potential is −40 mV throughout. The mean normalized reduction in i_{Ca} during current staircases from −40 to −10 mV was 0·678 ± 0·24 (± S.D., $n = 5$), to 0 mV 0·659 ± 0·16 ($n = 36$), to +10 mV 0·694 ± 0·13 ($n = 19$), and to +20 mV 0·738 ± 0·19 ($n = 4$). These differences are statistically significant between −10 and 0 mV ($P < 0·05$, paired data), 0 and +10 mV ($P < 0·01$), and 0 and +20 mV ($P < 0·02$, paired data). *B*, another cell after exposure to TEA for 90 min. Symbols as in panel *A*: holding potential is −40 mV. *C*, changes in contraction in two cells during trains of 1 s pulses after rest to different potentials. Plotted data show contraction increases for clamp pulses to −20 mV (■), 0 mV (●) and +20 mV (○). In addition, steady-state contraction for pulses to +10 mV (▲) and +40 mV (△) is shown; data from same cell as panel *A*. Inset records of cell contraction from another cell from the same heart as plotted data show similar results. First four contractions after rest, steady-state (SS), and contraction after 15 and 30 s rests are shown. Contraction is given in arbitrary units (a.u.).

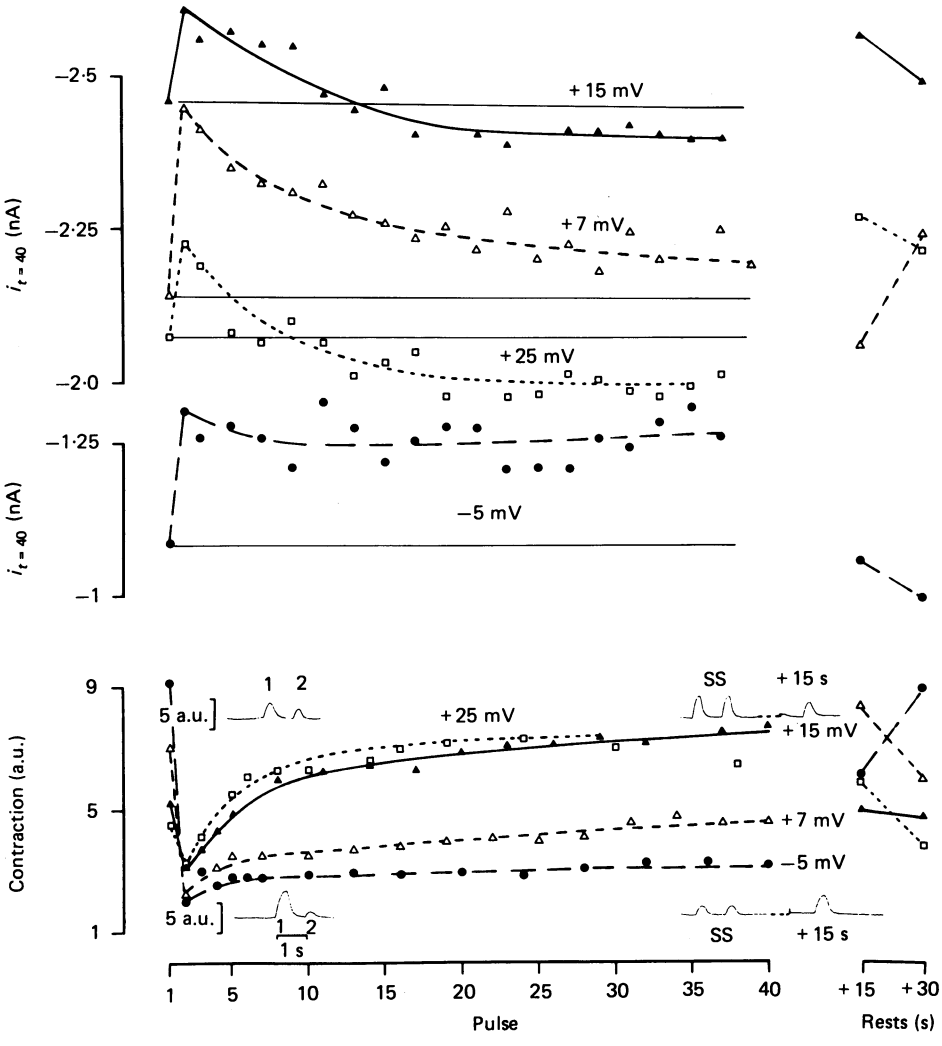


Fig. 6. Effect of pulse potential on inward current staircases and cell contraction elicited by trains of 50 ms pulses. Upper panels show current recorded (increasing upwards) at $t = 40$ ms into each pulse. Trains applied after 3 min rest from −40 mV to a range of potentials from −5 to +15 mV as indicated. The horizontal lines on the graph highlight the inward current level of the first beat after rest; thus values falling below this line during a train indicate a net negative staircase of inward current. Values obtained 15 and 30 s after the train ended are shown to the right. The lower graph shows changes in cell contraction during the current staircases in the upper panel. Symbols used in the lower panel are the same as those used in the upper panel. Pulse voltages are also indicated next to each result. Note that there is always a fall in cell contraction from the first beat to the second and that contraction recovery during the train increases for more positive clamp potentials. Rested beats are shown to the right. The inset panels show original contraction data for staircases to −5 and +15 mV. Contraction is given in arbitrary units (a.u.).

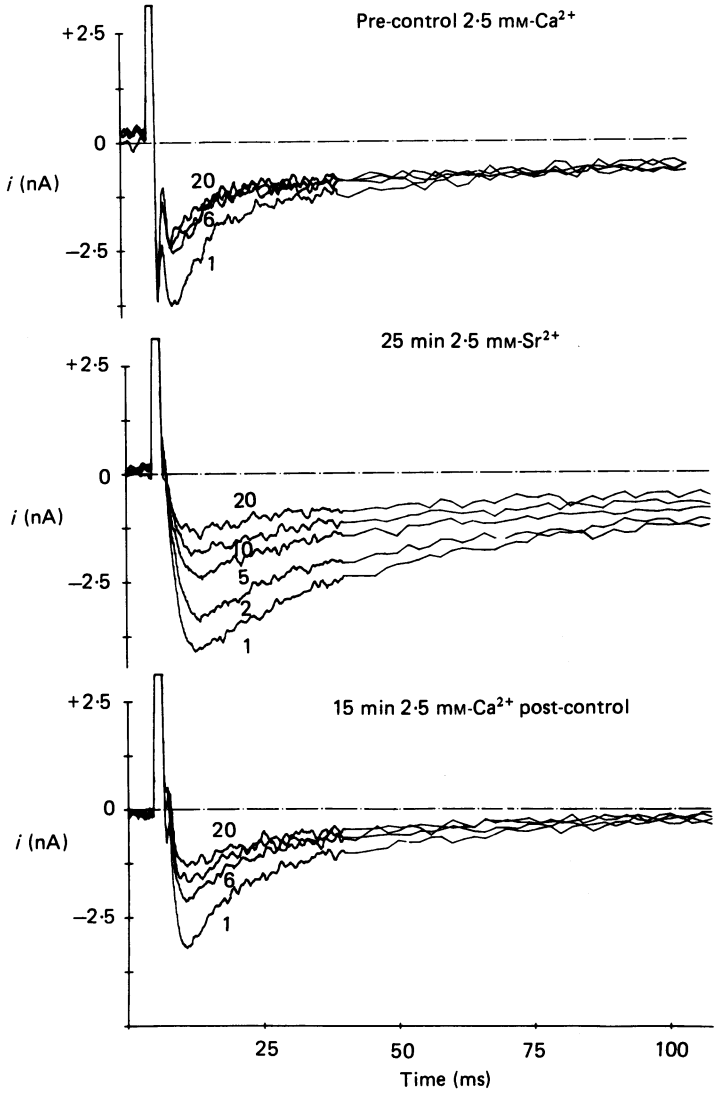


Fig. 7. Effect of replacement of bath Ca^{2+} by Sr^{2+} (2·5 mM) on inward current staircases in response to 1 s clamp pulses from −40 to 0 mV at 30/min after rest. Note slowing of current inactivation in the presence of Sr^{2+} (middle panel). The position of each trace in its respective train is indicated by the numbers next to the traces. The time constant (τ) for the negative current staircase in control solution before Sr^{2+} exposure was 10·2 s; after 6 min in Sr^{2+} τ was 11·2 s and after 25 min in Sr^{2+} τ was 12·5 s. Fifteen minute exposure to normal-Ca^{2+} Tyrode solution did not reverse this change: τ = 13·6 s.

maintained at a test potential of −5 mV. At more positive potentials the inward current declines again. At +15 and +25 mV the current eventually falls below the inward level recorded during the first pulse. These changes in inward current are reversed on rest. Coincident changes in cell contraction are shown in Fig. 6*B* and bear an inverse relation to the current. A fall in contraction occurs in the second beat in

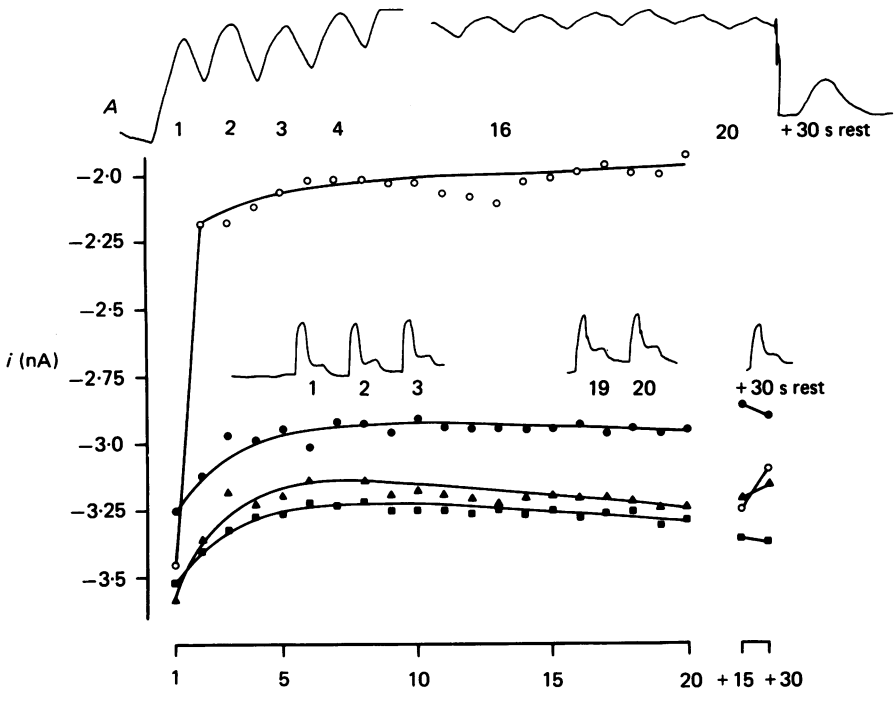
each train. There is then a voltage-dependent rise in cell contraction which is negligible at −5 mV but more prominent at more positive voltages. Trains of pulses to +15 or +25 mV eventually gave rise to contractions that exceeded the magnitude of the first contraction after rest (see inset panels for original data). The voltage dependence of current staircases with short pulses and accompanying contraction proved more variable than for 1 s pulses but current changes were always inversely correlated with cell contraction.

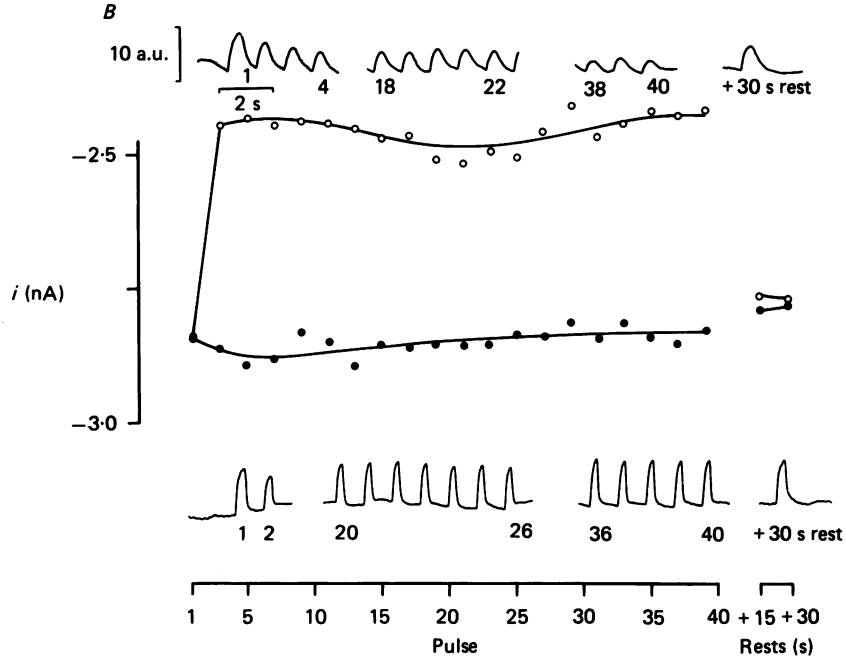
Interestingly, an absolute inverse relation between current and contraction is not seen. For example, steady-state contraction increased up to quite positive voltages whereas peak inward current in the steady-state declined at more positive potentials according to the current–voltage relation for i_{Ca} described previously. In five cells in which current–voltage relations of contraction and voltage were obtained, contraction did decline at more positive potentials than about +40 mV.

Effects of modification to cellular Ca^{2+} recycling

We have shown a close relationship between cell contraction and the direction, magnitude and pulse potential dependence of inward current staircases. It is possible that the correlation between changes in these variables reflects changes in intracellular Ca^{2+} levels, and in particular Ca^{2+} release from the sarcoplasmic reticulum (SR) at each beat. Changes in internal Ca^{2+} may alter the inactivation of Ca^{2+} current over a rather prolonged time course. Ca^{2+} substitutes such as Sr^{2+} and Ba^{2+} are known to be poor mediators of Ca^{2+} current inactivation, in both cardiac (Kohlhardt, Herdey & Kubler, 1973) and other preparations (Eckert & Chad, 1984). Also Sr^{2+} slows the speed of contraction and relaxation in ventricular muscle (de Hemptinne & Weyne, 1967; Fedida *et al.* 1987), which suggests that Sr^{2+} and Ba^{2+} ions are transported less efficiently by intracellular systems for Ca^{2+} transport (Ahmed & Connor, 1979). In Fig. 7, in the presence of Sr^{2+} there is an increase in i_{Ca} and a marked slowing of the time course of inward current relaxation. There is

Fig. 8. Effect of exposure to 2·5 mM-caffeine on inward current and contraction during 1 s and 50 ms pulse trains. *A*, currents recorded during trains of 1 s pulses from −40 to 0 mV. Three control staircases of current prior to caffeine exposure shown as ■, ● and ▲; data after rest to the right. Control contraction changes shown inset above current data. Data for i_{Ca} after exposure to caffeine for 25 min is shown as ○. Note current reduction from first to second beat. Corresponding contraction changes at top of panel *A* show development of sustained cell contraction during train of pulses; numbers show the position of beats in the pulse train. *B*, currents and contractions recorded during trains of 50 ms clamp pulses. Control current data before caffeine show a small increase in i_{Ca} during train (●). Contraction changes inset below data. Ca^{2+} current data after exposure to 2·5 mM-caffeine for 20 min are shown as ○. Contraction changes (arbitrary units) for 50 ms pulses during exposure to caffeine are at the top of panel *B* and the calibration bars shown on this contraction record also apply to all other contraction records in this figure. Note that for all original traces of cell contraction the first few beats after rest at the start of each pulse train are shown (beat Nos. 1–4). There is then a break in the record and a section of contraction towards the middle of the train (beat Nos. 18–22) is shown. Another break precedes contraction data recorded at the end of each train (beat Nos. 37–40) and finally contractions recorded 30 s after the end of each train are shown. All data in the figure were obtained from the same cell.


A
1 2 3 4 16 20 +30 s rest
−2·0
−2·25
−2·5
−2·75
−3·0
−3·25
−3·5
i (nA)
1 2 3 19 20 +30 s rest
1 5 10 15 20 +15 +30


B
10 a.u.
1 4 18 22 38 40 +30 s rest
2 s
−2·5
−3·0
i (nA)
1 2 20 26 36 40 +30 s rest
1 5 10 15 20 25 30 35 40 +15 +30
Pulse
Rests (s)

a 40% decrease in the peak inward current during the control staircase and a 66% reduction in the presence of Sr^{2+} ions. The time to peak was lengthened from 4·5 to 6·6 ms by Sr^{2+} (post-control 5·9 ms, values for first current recorded after rest), which reflects a slower inactivation in the presence of Sr^{2+} although an effect on surface potential by Sr^{2+} replacement of Ca^{2+} cannot be excluded.

The time constants of the inward current staircases were slower in Sr^{2+} than in control Ca^{2+}-containing solutions, but were still an excellent fit to a single exponential ($r > 0·95$ for all data in Fig. 7). The important finding was that although the inactivation of individual Ca^{2+} currents was slowed when extracellular Ca^{2+} was replaced by Sr^{2+}, larger negative staircases were seen in the presence of Sr^{2+}. When the cell is rested at the end of the train Sr^{2+} does not prevent the recovery of current to control levels (not shown).

Current staircases in Sr^{2+} produced by trains of 50 ms voltage clamp pulses were similar to those described for control solutions. In one of five cells positive current staircases in control 2·5 mM-Ca^{2+} were converted to negative staircases. In three further cells replacement of Ca^{2+} with 2·5 mM-Ba^{2+} ions had similar effects to Sr^{2+} on current staircases resulting from 1 s and longer clamp pulses.

Caffeine exerts a number of complex effects in cardiac tissues including a direct effect on myofilament sensitivity to Ca^{2+}. It releases Ca^{2+} from many SR preparations (Endo, 1977) and raises intracellular cyclic AMP levels by inhibiting phosphodiesterase (Korth, 1978). Ten cells were exposed to caffeine. Three cells exposed to 5 mM-caffeine showed rapid reduction in i_{Ca} and deterioration. In seven cells exposed to 1 or 2·5 mM-caffeine results were similar although slower in onset with the lower concentration. A typical result with 2·5 mM-caffeine is shown in Fig. 8. Trains of 1 s pulses during caffeine exposure reduce i_{Ca} dramatically from the level in the first pulse of the train. Almost complete recovery of i_{Ca} occurs after a 15 s rest. Cell contraction changes during caffeine exposure are also dramatic. After periods of rest, the first contraction is large and long-lasting and the cell fails to relax completely before the next clamp pulse. Subsequently there is a partial fusion of contractions and the cell does not relax again until the train of clamp pulses has ended.

During trains of 50 ms voltage clamp pulses (Fig. 8*B*) caffeine again reduces i_{Ca} between the first and second clamp pulses and abolishes the facilitation seen in the control situation. There is little further change in i_{Ca} during the pulse train and, as in Fig. 8*A* for the 1 s pulses, recovery of i_{Ca} is rapid and reaches the control level after only 15 s rest.

The cell contraction record for short pulses is anomalous. In the control situation, contraction changes are reciprocal to current, but in the presence of caffeine there is a reduction in both cell contraction and i_{Ca} during the pulse train that is reversed when the cell is rested. The normal inverse relation between i_{Ca} and contraction is therefore disrupted by caffeine during trains of short voltage clamp pulses (see Discussion).

An attempt was made to block SR Ca^{2+} release with the alkaloid ryanodine at a concentration of 1 μM (Sutko & Kenyon, 1983). In six cells long exposure to this concentration reduced i_{Ca} and markedly slowed the time to peak inward current. Staircases of inward current with 1 s pulses were not greatly altered in magnitude. In four out of five cells, facilitation of i_{Ca} during trains of 20, 30 and 50 ms pulses was abolished and negative staircases of current occurred.

DISCUSSION

Do changes in membrane current during repetitive activity reflect changes in i_{Ca}?

The results in this study suggest that the inward Ca^{2+} current activated during stimulation from rest changes in different directions depending on the protocols used. Could inward current decline represent increases in an outward current?

Time-dependent outward current (i_K). This could summate during clamp pulses and apparently produce a negative staircase of peak inward current, especially during long voltage clamp pulses. It is unlikely that i_K is contaminating i_{Ca} measurements. First, i_K was small in cells at room temperature, the absence of i_K tails on repolarization to -40 mV and constant holding current at potentials positive to E_K throughout pulse trains (Figs 1, 3, 4, 5 and 7) supports this. Second, 30 mM-TEA added to block i_K gave qualitatively similar results (Fig. 5). Third, greater activation of i_K at more positive potentials should increase current staircases, which is the reverse of what is observed (Fig. 5). Fourth, the kinetics of i_K are too slow to affect peak inward current or even measurements at $t = 40$ ms as long as deactivation of i_K between pulses is complete. Finally, decreases of inward current are also seen at potentials too negative to activate i_K.

The voltage-activated transient outward current (i_{to}). This is known to be strongly rate dependent and decreases on repetitive activation after rest (Boyett, 1981). i_{to} is not described in guinea-pig myocytes but a holding potential of -40 mV used during pulse trains will inactivate i_{to} to a large extent.

Currents activated by intracellular Ca^{2+}. These change in parallel with the intracellular calcium transient so may be difficult to differentiate from genuine Ca^{2+} current changes that correlate with cell contraction. The Ca^{2+}-activated transient outward current in the heart is abolished by Sr^{2+} ions (Coraboeuf & Carmeliet, 1982) unlike current changes that we describe (Fig. 7). For a given change in internal Ca^{2+}, Ca^{2+}-activated K^+ current ($i_{K(Ca)}$) will increase at more positive potentials, whereas data in Fig. 5 suggest that current staircases decrease. $i_{K(Ca)}$ is virtually eliminated in snail neurones by internal EGTA (Meech, 1974), as are non-specific cation channel current and Na^+–Ca^{2+} exchange current (Kimura, Miyamae & Noma, 1987) but current staircases persist (Fedida *et al.* 1988). The latter currents would affect inward current staircases in different directions either side of their reversal potentials and produce the smallest current changes around 0 mV. No evidence of staircase reversal was found. Staircases also tend to be largest around 0 mV (Fig. 5).

In conclusion, then, none of these contaminating currents appear to contribute greatly to the current changes that we observe.

Do calcium current staircases and the inverse relation of action potential duration to cell contraction reflect changes in Ca^{2+}-dependent inactivation of i_{Ca}?

We have shown, under non-steady-state conditions, a strong inverse correlation between cell contraction and action potential duration (Fig. 2). There is a reduction in i_{Ca} during stimulation that can reduce the duration of an action potential appreciably and reflects the delicate balance of ionic current flowing during the plateau of the cardiac action potential (Noble, 1984). Ca^{2+} current is also inversely related to cell contraction under many experimental situations (Figs 3 and 6). A

reduction of i_{Ca} in response to a rise in internal Ca^{2+} could constitute a negative feedback mechanism for prevention of cell Ca^{2+} overload during normal activity (Allen, 1977).

(i) A decline in i_{si} on stimulation from rest cannot be attributed to an intracellular accumulation of Ca^{2+} reducing the driving force for i_{Ca} as, on the basis of constant field theory, even millimolar accumulations of Ca^{2+} near the mouth of the channel will only reduce i_{Ca} by about 10% (Hagiwara & Byerly, 1981). In rabbit atrium external Ca^{2+} depletion can occur during high-rate stimulation (Hilgemann & Noble, 1987). However, elevation of external Ca^{2+} *increases* the magnitude of negative current staircases, which is the opposite of that expected if external Ca^{2+} depletion were important (Fedida *et al.* 1988).

(ii) Ca^{2+} current may not recover from inactivation. On depolarization, i_{Ca} in guinea-pig cells inactivates relatively rapidly and is dependent both on Ca^{2+} and voltage (Hadley & Hume, 1987). Recovery from voltage-dependent inactivation occurs over a time course of hundreds of milliseconds (Lee *et al.* 1985) whereas recovery from Ca^{2+}-dependent inactivation may depend on many factors such as a decrease in free intracellular Ca^{2+} or phosphorylation of Ca^{2+} channels (Chad & Eckert, 1986). Thus stimulation rates faster than 2 Hz will reduce i_{Ca} due to a failure of recovery from voltage-dependent inactivation. This could overlap and obscure any slower changes in i_{Ca} (Boyett & Fedida, 1988). Evidence from the present experiments using long interpulse intervals supports an important role for Ca^{2+}-dependent inactivation of i_{Ca}.

Effects of pulse duration on i_{Ca}. After rest the first contraction in response to long voltage clamp pulses or an action potential is small. The Ca^{2+} content of the sarcoplasmic reticulum is low as during rest net removal of Ca^{2+} reflects a predominance of Ca^{2+} extrusion over entry (Allen, Jewell & Wood, 1976). Subsequent beats load the cytoplasm and thus the SR via membrane channels or changes in the activity of exchange mechanisms (Hilgemann & Noble, 1987) to sustain a positive contraction staircase. This increase in cell Ca^{2+} is associated with a decrease in i_{Ca}. Fabiato (Fig. 6 of Fabiato, 1985) has shown in skinned cardiac cells that long pulses of activator Ca^{2+} are required to produce positive force staircases, while brief pulses give negative staircases. Our experiments on intact cells agree. Longer pulses enable greater net Ca^{2+} entry and allow progressive SR and myoplasmic Ca^{2+} loading. This increases tonic and phasic components of cell contraction and Ca^{2+}-dependent inactivation of i_{Ca} (Fig. 3). Clamp pulses of 50 ms duration or less evoke small contractions after rest that decline during stimulation (Figs 3, 6 and 8). No increase in a tonic component of cell contraction was seen with short voltage clamp pulses (e.g. Fig. 3). We postulate that myoplasmic Ca^{2+} levels are lowered in the second and subsequent pulses as cell Ca^{2+} entry is insufficient to maintain the level of SR Ca^{2+} release. Beat-dependent facilitation of i_{Ca} and slowing of inactivation may then reflect a reduction in Ca^{2+}-dependent inactivation of i_{Ca}.

Ca^{2+} current is largely inactivated at the end of a 50 ms pulse (Figs 2 and 3) so the origin of net Ca^{2+} entry during longer clamp pulses is of interest. Ca^{2+} extrusion by Na^{+}–Ca^{2+} exchange occurs at predominantly negative potentials in guinea-pig ventricular cells (Kimura *et al.* 1987) so that a higher ratio of time spent at depolarized potentials will bias net Ca^{2+} flux towards entry. Ca^{2+} entry itself may

continue at positive potentials: Ca^{2+}-dependent inactivation of i_{Ca} predicts a maintained Ca^{2+} influx during prolonged depolarization and non-inactivating Ca^{2+} channels may exist (Eckert & Chad, 1984). An estimate of Ca^{2+} entry during clamp depolarizations predicts a sixfold increase during a 1 s ($2{\cdot}8 \times 10^{-15}$ mol) compared with a 50 ms pulse ($4{\cdot}4 \times 10^{-16}$ mol; D. Fedida, D. Noble & A. J. Spindler, unpublished data).

Finally, Na^+–Ca^{2+} exchange, at voltages positive to its reversal potential, may allow continued Ca^{2+} entry during long voltage clamp pulses as suggested from Ca^{2+} indicator studies (Barcenas-Ruiz & Weir, 1987) and voltage clamp experiments (Terrar & White, 1987).

Effects of voltage on inward current staircases. Voltage-dependent inactivation is usually monotonic becoming greater and faster at more positive voltages (although see Meves, 1978), whereas Ca^{2+}-dependent inactivation reflects Ca^{2+} entry and has a bell-shaped relation to membrane potential. For 1 s clamp pulses current staircase magnitude closely follows the voltage dependence of Ca^{2+} entry (Figs 5 and 6), becoming smaller at more positive potentials. The time courses were single exponentials and showed little voltage dependence, a finding unaffected by the presence or absence of 30 mM-TEA or 50 μM-TTX. Cell contraction staircases at different potentials have a similar voltage dependence.

The facilitation of i_{Ca} seen with short clamp pulses seemed even more clearly related to changes in cell contraction. When cell contraction rises during a train as at more positive potentials (when Ca^{2+} entry is expected to be less) the facilitation is lost (Fig. 6). This suggests that a rise in intracellular Ca^{2+}, either via increased SR release or reduced extrusion from the myoplasm, is important in the slow inactivation of i_{Ca}. Current staircases from a holding potential of -40 mV were significantly more negative than those from -45 or -50 mV. This may reflect the relative voltage dependence of Ca^{2+} uptake and extrusion mechanisms within the cell or across the sarcolemma or entry through Ca^{2+} channels open at this level of depolarization. A rise of internal Ca^{2+} could contribute to a steady Ca^{2+}-dependent inactivation of i_{Ca} during a train of pulses.

Effect of alterations to SR function. Strontium increased peak inward current and markedly slowed the time course of inward current relaxation (Kohlhardt *et al.* 1973; Josephson *et al.* 1984). This suggests a partial removal of a cation-dependent inactivation. However, the negative current staircases under these conditions are larger and slower to reach steady state than in control solution. The diminished affinity of the Na^+–Ca^{2+} exchange for Sr^{2+} ions (Kimura *et al.* 1987) may lead to a slow intracellular accumulation of Sr^{2+} and larger current staircases without affecting the relaxation of individual current traces. Current facilitation with short clamp pulses is still present in Sr^{2+}, but here the situation may be complex as short pulses allow little time for increased Sr^{2+} accumulation despite delayed current relaxation. There is also a slower onset of contraction in guinea-pig cells in the presence of Sr^{2+} ions (Fedida *et al.* 1987) so that the intracellular Sr^{2+} transient may be delayed.

Caffeine strikingly reduced i_{Ca} from the first to second beat of the train for both long and short pulses (Fig. 8). Contractions resulting from long pulses formed a partially fused tetanus indicating that a large rise in internal Ca^{2+} occurred at the

first beat of the train which presumably inactivated i_{Ca} to a large extent during subsequent pulses. Higher doses produced a greater effect. In agreement, similar doses of caffeine reduce a component of the intracellular Ca^{2+} transient attributable to Ca^{2+} entry (Hess & Weir, 1984). Caffeine (2·5 mM) only partially inhibits Ca^{2+} uptake by the SR as rest brought about recovery of i_{Ca} and the twitch (Fig. 8). The facilitation of i_{Ca} during short clamp pulses was abolished although there was no increase in cell contraction during trains of 50 ms pulses. This effect of caffeine may be related to its ability to raise cyclic AMP levels within the cell (cf. Fedida *et al.* 1988).

Ryanodine, an agent thought to block SR Ca^{2+} release in some tissues (Sutko & Kenyon, 1983), is known to have species-dependent effects. In guinea-pig cells 1 μM-ryanodine only partially suppresses phasic contractions (Fedida *et al.* 1987; Mitchell, Powell, Terrar & Twist, 1987). Current staircases remained although current facilitation was often abolished. Ryanodine (1 μM) may impair SR function sufficiently to increase Ca^{2+}-dependent inactivation of i_{Ca}, perhaps via an increase in myoplasmic Ca^{2+} levels. Such an effect of ryanodine on intracellular Ca^{2+} has recently been described in ferret ventricular cells (Hansford & Lakatta, 1987).

Conclusion

These experiments show clearly that the use dependence of i_{Ca} is closely paralleled by changes in cell contraction and therefore that changes in myoplasmic calcium levels may set a level of Ca^{2+}-dependent inactivation of i_{Ca}. The pulse duration, and voltage and SR dependence of these effects, strongly support the modulating role of intracellular Ca^{2+} rather than membrane voltage *per se*.

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