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MECHANISM OF THE USE DEPENDENCE OF Ca^{2+} CURRENT IN GUINEA-PIG MYOCYTES

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SUMMARY

1. The mechanism of the use-dependent reduction and facilitation of the calcium current (i_{Ca}) in single guinea-pig myocytes described by Fedida, Noble & Spindler (1988) has been examined by varying $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ and i_{Ca}.

2. Moderate enhancement of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ changes produced by increasing $[Ca^{2+}]_o$ reduces i_{Ca} and enhances the use-dependent reduction.

3. Intracellular calcium overload, produced by reducing $[Na^+]_o$, greatly reduces i_{Ca} and almost totally eliminates the use-dependent variations.

4. Use-dependent reduction of i_{Ca} is also smaller after substituting external Ba^{2+} ions for Ca^{2+} ions.

5. When $[Ca^{2+}]_i$ is buffered by intracellular EGTA sufficient to eliminate other $[Ca^{2+}]_i$-dependent processes, such as contraction and Na^+–Ca^{2+} exchange, some use-dependent reduction of i_{Ca} remains, although the effect is smaller. Use-dependent facilitation of i_{Ca} is more prominent in the presence of internal EGTA.

6. The facilitation of i_{Ca} is abolished by Ba^{2+} replacement of Ca^{2+} and by the β-adrenoceptor agonist isoprenaline. This suggests that the facilitation is mediated by Ca^{2+} entry itself rather than membrane voltage. Facilitation is evident as a delay of current relaxation, even in the presence of internal EGTA.

INTRODUCTION

In the previous paper (Fedida, Noble & Spindler, 1988) we described both a facilitation and reduction of calcium current (i_{Ca}) in single ventricular muscle cells when stimulated from rest. The conclusion reached was that slow changes in the level of i_{Ca} during pulse trains under different conditions could be explained by changes in a beat-to-beat level of Ca^{2+}-dependent inactivation of i_{Ca}, and other mechanisms did not need to be invoked.

The inactivation of i_{Ca} in guinea-pig myocytes has been extensively studied (Lee, Marban & Tsien, 1985; Hadley & Hume, 1987) and there appears no doubt that inactivation of individual current transients is dependent on both intracellular Ca^{2+} and voltage. Thus, it was a great advantage to use guinea-pig cells in the present study.

The aims are to test three hypotheses: (a) that i_{Ca} is inhibited by increases in

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$[Ca^{2+}]_i$, (b) that use-dependent reduction of i_{Ca} may depend on the entry of Ca^{2+} through the channel as well as on the level of $[Ca^{2+}]_i$, and (c) that use-dependent facilitation of i_{Ca} should be more evident when the use-dependent inhibition that may mask it is removed. Such a facilitation has been described in cardiac tissues in frog atrial muscle (Noble & Shimoni, 1981), in sheep Purkinje fibres (Boyett & Fedida, 1984, 1988) and in rabbit sino-atrial node cells (Brown, Kimura, Noble, Noble & Taupignon, 1984). It is also seen in other tissues, in *Aplysia* neurones (Eckert & Ewald, 1983) and in bovine chromaffin cells (Fenwick, Marty & Neher, 1982; Hoshi, Rothlein & Smith, 1984).

METHODS

These are described in the previous paper (Fedida *et al.* 1988) and by Fedida, Noble, Shimoni & Spindler (1987).

RESULTS

Enhancement of use dependence by increasing external calcium, $[Ca^{2+}]_o$

Since increased $[Ca^{2+}]_o$ increases the $[Ca^{2+}]_i$ transient and cell contraction, this manoeuvre would be expected to increase the use dependence.

The effect of increasing $[Ca^{2+}]_o$ is shown in Fig. 1. Raising $[Ca^{2+}]_o$ from 2·5 to 5 or 7·5 mM results in an increase in the size of negative current staircases with 1 s pulses. This is illustrated in the original records in Fig. 1*A*. The cell was repetitively pulsed after rest from −40 to −10 mV and in the control situation a slight negative staircase of inward current results, with recovery on rest (cf. Fedida *et al.* 1988). After exposure to 5 mM-bathing Ca^{2+} for 20 min a decline in the magnitude of i_{Ca} is seen but now there is a large increase in the size of the negative current staircase. Again, recovery is seen on resting the cell. Note also that at 2·5 mM $[Ca^{2+}]_o$ there is a steady level of inward current during each pulse, which is eliminated when $[Ca^{2+}]_o$ is increased. This may also reflect $[Ca^{2+}]_i$-dependent inactivation of i_{Ca} since the effect is abolished in the presence of intracellular EGTA (see Fig. 6).

The effect of 5 mM $[Ca^{2+}]_o$ on 50 ms voltage clamp pulses is shown in the graph in Fig. 1*B*. Here, litte change is seen in the size of i_{Ca} during repetitive pulsing in the control situation. After exposure to 5 mM-Ca^{2+} for 25 min, however, a marked negative staircase of i_{Ca} is seen, again with rapid recovery after rest. When external Ca^{2+} was lowered to 1 or 0·5 mM there was a decrease in the magnitude of inward i_{Ca} (not shown). There was still a negative staircase of inward current during 1 s pulses but they were smaller in size. Normalized changes in peak inward current during staircases in 1 mM-external Ca^{2+} were similar to current changes in 2·5 mM-external Ca^{2+}. Similar results with various external Ca^{2+} concentrations were observed in eight cells altogether.

Saturation of use dependence in calcium overload

If the use dependence of i_{Ca} is attributable to beat-to-beat changes in $[Ca^{2+}]_i$ then one way to reduce or eliminate the effect should be to raise $[Ca^{2+}]_i$ massively to saturate the $Ca^{2+}{}_i$-dependent site responsible. In the experiments illustrated in Fig. 2 we have reduced external Na^+ to raise internal Ca^{2+} to a very high level via a

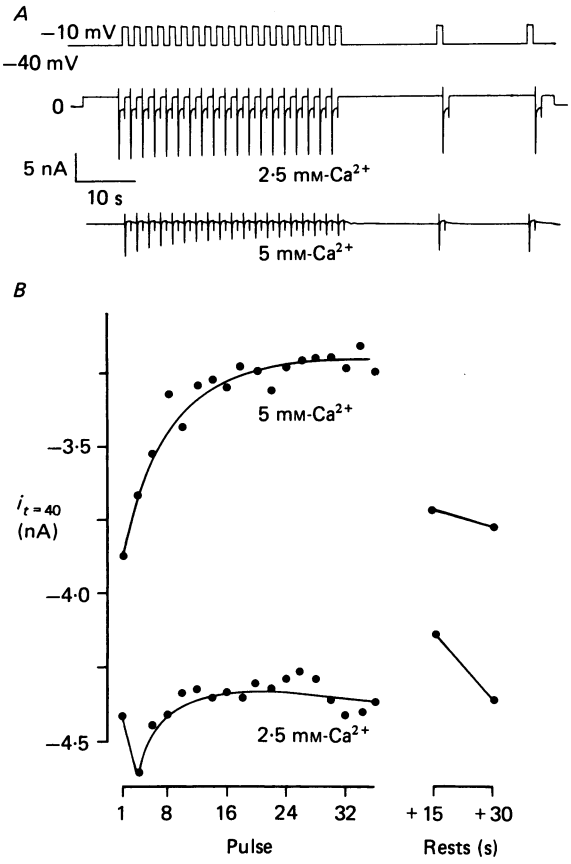

Fig. 1. Effect of elevation of external Ca^{2+} on inward current staircases, 3 M-KCl electrode. *A*, top trace, membrane voltage showing 1 s clamp pulses at 30/min from −40 to −10 mV; middle trace, control situation in 2·5 mM-external Ca^{2+} showing inward current in response to clamp pulses; lower trace, inward current staircase after exposure to 5 mM-Ca^{2+} for 20 min. *B*, effect of Ca^{2+} elevation on short 50 ms voltage clamp pulses. In control situation as indicated there is little net change in inward current during the stimulation period. After exposure to 5 mM-Ca^{2+} for 25 min there is a marked decrease in inward current during the staircase. Recovery after 15 or 30 s rest is shown on the right of the graph.

reduction of the ionic gradient available to the Na^+–Ca^{2+} exchange mechanism. Figure 2*A* shows the effect of lithium replacement of Na^+ on staircases of action potential duration during stimulation after 3 min periods of rest. In the control situation there is a large negative staircase of duration and subsequent recovery when the cell is rested. After exposure to 40 mM-Na^+ for 15 min there was a marked reduction in the magnitude of the staircase and recovery after rest is abolished. Note also that compared with the control situation the absolute action potential duration is much reduced during superfusion with reduced-Na^+ solution. This is expected as a consequence of a reduction of i_{Ca} secondary to cell Ca^{2+} overload in low-Na^+ solution. Reperfusion with Tyrode solution containing 150 mM-Na^+ produces some return of a negative staircase and recovery afterwards. Further recovery was

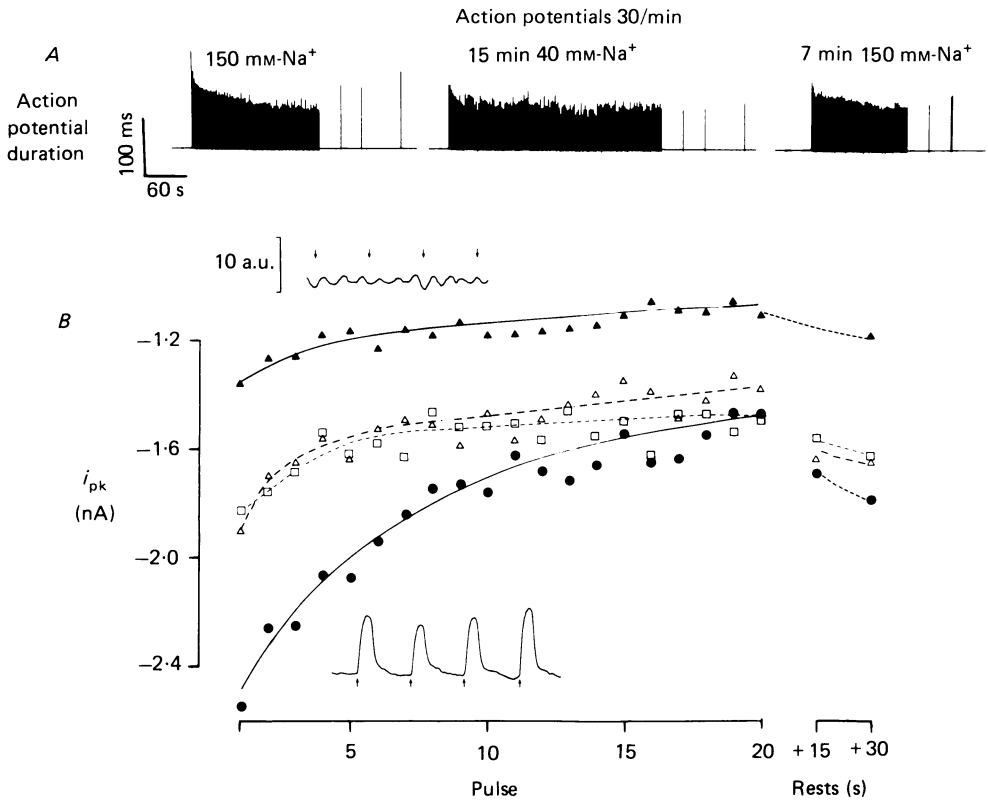

Fig. 2. *A*, effect of lowered external Na^+ (46 mM) on action potential staircases. Action potential duration measured at −25 mV. Stimulation at 30/min after 3 min rest. The vertical height of each spike represents the duration of an action potential from upstroke until repolarization to −25 mV. There is an offset at the baseline on the device of 250 ms in the left panel and 100 ms in the middle and right panels. The vertical spikes to the right of each dark section of trace are the durations of action potential recorded after rests. *B*, effect of Na^+ reduction to 8 mM on peak inward current during trains of 1 s voltage clamp pulses. ●, control prior to Na^+ reduction (inset panel shows cell contraction in response to first four voltage clamp pulses from −40 to 0 mV at 30/min); △, 6 min in low Na^+; □, 13 min in low Na^+; ▲, 20 min in low Na^+ (inset panel shows cell contraction in arbitrary units (a.u.) during first four pulses of train after 20 min exposure to 8 mM-Na^+). Arrows in inset panels denote timing of clamp pulses at 30/min, and the same ordinate applies to both inset panels. Beats to the right were recorded after 15 or 30 s rest at end of pulse train.

apparent after 15 min superfusion with 150 mM-Na^+ solution (not shown). After return to normal-Na^+ solution there is not much absolute increase in the action potential duration although the staircase reappears. The loss of the staircase in low-Na^+ solution cannot therefore be attributed simply to the reduction in action potential duration that accompanies superfusion with 40 mM-Na^+.

In Fig. 2*B* the effect of reducing external Na^+ on current staircases resulting from 1 s pulses is shown. The graph shows the peak inward current recorded during each beat of the train. In the control situation a negative staircase of i_{Ca} is seen. Perfusion with low-Na^+ solution (in this case 12 mM, 8%) progressively reduces the size of i_{Ca}

and with longer exposure to the reduced-Na^+ Tyrode solution the staircases in response to 1 s pulses are much reduced in size. The changes of inward current were accompanied by a loss of the normal phasic cell contraction, there was a rise in the tonic level of cell contraction and small contractile oscillations could be observed on this elevated baseline. The inset panels of Fig. 2 show the changes in cell contraction on exposure to reduced-Na^+ solution. After 17 min exposure it can be seen that the stimulated contractions can barely be distinguished from the oscillatory contractile behaviour of the cell in 8 mM-external Na^+. Similar results were seen in fourteen cells in which the bath Na^+ concentration was reduced to 46, 40 or 12 mM-external Na^+ ($n = 3$, 5 and 6 cells respectively). Lowered external Na^+ also consistently abolished i_{Ca} facilitation seen with short voltage clamp pulses.

Increased use dependence resulting from increased i_{Ca}

Adrenaline or isoprenaline was used to increase i_{Ca} and thus raise Ca^{2+} entry during short pulses. The results are shown in Fig. 3.

In the control situation it can be seen that by beat 38 there is a marked increase in the inward current which is reversed by a 30 s rest. This is accompanied by a moderate fall in contraction during the pulse train. After exposure to adrenaline there is a large increase both in inward current and in contraction. The contractions relax extremely rapidly but now show a *positive* staircase during stimulation. In parallel with this a marked negative staircase of current is seen in both peak inward i_{Ca} and current levels after 40 ms. Resting the cell partially reverses these changes, as in control solutions. Similar results with adrenaline or isoprenaline (5×10^{-7} M or 10^{-6} M) were seen in six cells.

Effect of intracellular injection of EGTA

The results described so far are consistent with the hypothesis that increased $[Ca^{2+}]_i$ reduces i_{Ca} and that the use dependence of i_{Ca} reflects progressive changes either in $[Ca^{2+}]_i$ transient or in Ca^{2+} entry or, perhaps, in both. We therefore decided to attempt to eliminate the $[Ca^{2+}]_i$ transient to see whether effects due simply to Ca^{2+} entry could be observed.

Intracellular EGTA injection is a method of lowering the intracellular Ca^{2+} level and buffering the subsequent Ca^{2+} fluctuations. Cells were impaled with electrodes containing either 3 mM-EGTA + 150 mM-KCl (Josephson, Sanchez-Chapula & Brown, 1984) or EGTA alone in concentrations from 100 mM to 0·5 M (Mitchell, Powell, Terrar & Twist, 1984; Fedida *et al.* 1987). On impalement with the bevelled electrode all cell movement ceased immediately. When the cell was stimulated, large long action potentials were observed but there was no sign of any cell contraction under these conditions, consistent with leakage of EGTA from the electrode and consequent buffering of intracellular Ca^{2+} at a low level. This procedure also eliminates Ca^{2+}-activated currents, such as the Na^+–Ca^{2+} exchange current tails (Fedida *et al.* 1987).

Leakage from a 70 MΩ electrode filled with 400 mM-EGTA has been estimated at approximately 1 fmol/s (Mitchell *et al.* 1984) so that after 1 min the internal concentration of EGTA in a cell of internal volume 20 pl would rise to 1·5 mM. Impalement with a lower-resistance electrode such as those used in the present

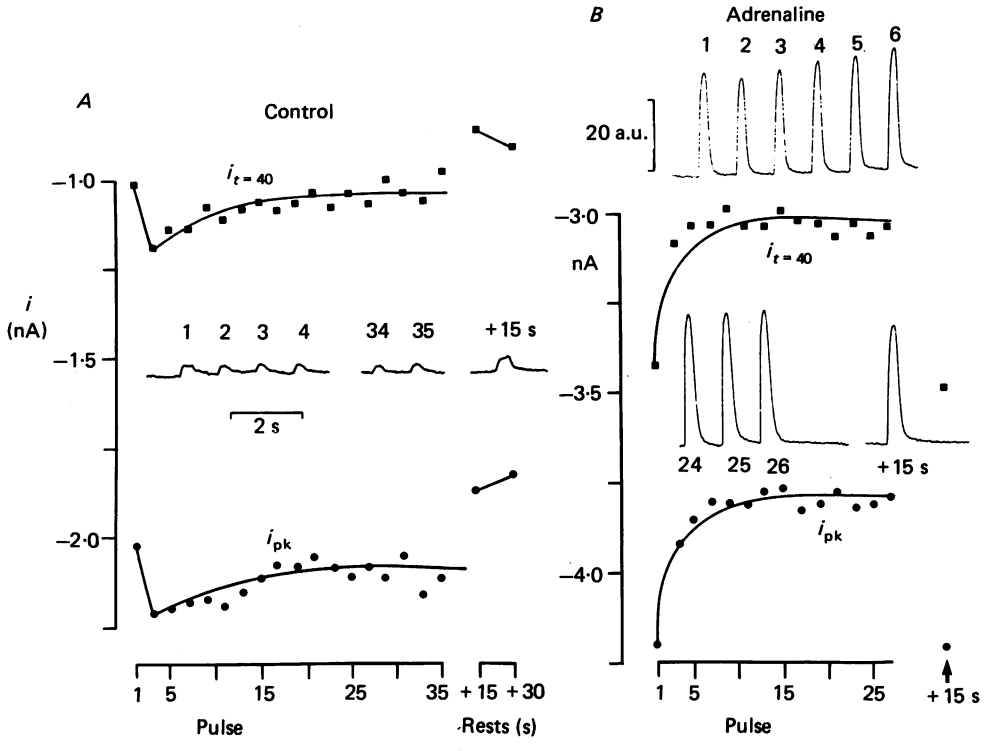

Fig. 3. Effect of adrenaline on staircases of i_{Ca} during short voltage clamp pulses. *A*, control: peak inward current (i_{pk}) and i_{t-40} for 50 ms voltage clamp pulses from −35 to −5 mV with a 1 s interpulse interval. Currents also recorded after 15 and 30 s rest as indicated. *B*, currents in response to 50 ms voltage clamp pulses after exposure to 10^{-6} M-adrenaline for 15 min. The single current value indicated by the arrow on the abscissa was recorded after a 15 s rest. The original records of cell contraction in arbitrary units (a.u.) inset into both panels depict the first few contractions (1–4 in *A*; 1–6 in *B*) of each train, steady-state contractions towards the end of each train (34 and 35 in *A*; 24–26 in *B*) and contractions recorded 15 s after the end of the stimulation period. All records of cell contraction are at the same gain and time base. All data from a single cell.

experiments should lead to a rapid rise in the internal EGTA concentration sufficient to keep internal Ca^{2+} below 1 nM.

In Fig. 4, the effect of internal EGTA on action potential staircases in single cells is shown. The left-hand panels show two staircases on slow (above) and fast (below) time bases. Action potentials in cells loaded with EGTA are very long, presumably reflecting slowing of Ca^{2+}-dependent inactivation of i_{Ca} in these cells. However, it can be seen that stimulation after rest nevertheless results in a large-duration staircase when stimuli are given at 30/min. Recovery is seen on rest. The traces of membrane potential and cell contraction show little or no change throughout the stimulation period. On a faster time base in the lower panels the first four beats of a 34/min train are shown. Again there are long action potentials which shorten, but there is no cell contraction. The right-hand panels of Fig. 4 illustrate data from another cell in which the voltage dependence of current staircases with 1 s pulses was investigated. Interestingly, the bell-shaped relation of the current staircase magnitude as a

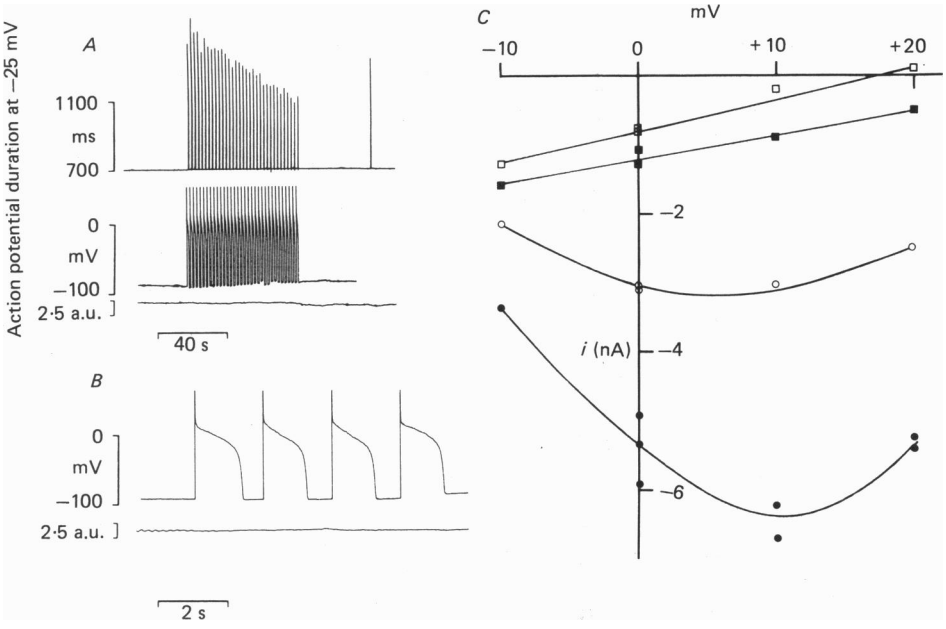

Fig. 4. Effect of intracellular EGTA on action potential trains and current staircases at different potentials. *A*, upper panel shows action potential duration measured from upstroke until repolarization to −25 mV during stimulation at 30/min after 3 min rest; middle panel shows membrane potential during train; lower panel shows changes in cell contraction in arbitrary units (a.u.) at high gain. Note slow time bar. *B*, the first four action potentials from another train recorded in the same cell as in *A*, with cell contraction below. Stimulation rate 30/min after 4 min rest. Cell in *A* and *B* impaled with 20 MΩ electrode filled with 0·5 M-EGTA. *C*, effect of pulse potential on staircases of inward current. One second clamp pulses from a holding potential of −40 mV; interpulse interval 1 s. current-passing electrode contained 3 M-KCl, 12 MΩ; second electrode contained 0·5 M-EGTA. ●, peak inward current; ■, steady-state current at 700 ms; both for the first pulse of train. ○ and □, peak and steady-state currents for twentieth pulse of train. Pulses from −45 to 0 mV.

function of pulse potential is still present, despite the loading of cells with internal EGTA (cf. Fedida *et al.* 1988). The largest current staircases still occur at the potential at which i_{Ca} is greatest (~ +10 mV). There is now also a greater shift in the steady-state current in the outward direction from the first to the twentieth pulse.

Facilitation of i_{Ca} on stimulation from the rested state was much more prominent than in the normal situation. This is clearly seen in Figs 5, 7 and 8. In Fig. 5 the effect of EGTA on i_{Ca} and the effect of stimulation from rest is shown. The left panel shows three current traces. There is an increase in i_{Ca} during stimulation after rest to the steady-state response (●). Most of the change in current occurs later in the current relaxation (which is minor at 50 ms) and often little or no change was seen in peak inward current. The effect of 30 s rest was to reverse these current changes as shown by the superimposition of the first and rested current traces. The right panels show similar results for 100 ms pulses and illustrate the very slow decay of i_{Ca} in EGTA-loaded cells. The relaxation of current is so slow that tail currents are seen on

repolarization. These are recorded at −45 mV and also show the facilitation of i_{Ca} during pulse trains. Note that at this potential changes in tail currents are additional evidence that the facilitation involves i_{Ca} rather than an overlap with K^+ currents.

The changes in current during longer-duration pulses are very different from those described in cells without internal EGTA (Fedida *et al.* 1988). In most cases there was virtually no change in peak inward current during the trains. The changes are largely in the inactivation time course following the peak current.

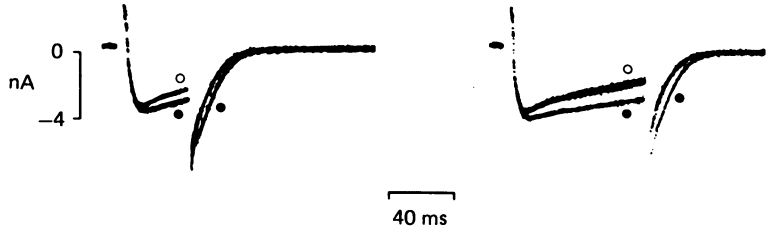

Fig. 5. Facilitation of inward current in cells impaled with EGTA electrodes. Membrane currents recorded during 50 ms (left) and 100 ms (right) pulse trains from −45 to 0 mV. The steady-state current trace (●) in each case shows a delayed phase of relaxation compared with the first pulse of each train (○) and an increased tail current. Currents recorded after a 30 s rest are also shown and overlay the first current trace of the train.

The top panel of Fig. 6 illustrates the effect of raising $[Ca^{2+}]_o$ from 2·5 to 5 mM and then reduction to 2·5 mM again. In 2·5 mM-Ca^{2+} a negative staircase of inward current is still present, even when intracellular Ca^{2+} is buffered to a low level. When external Ca^{2+} was elevated to 5 mM a small increase in i_{Ca} was consistently seen. In this situation the size of the current staircase in 5 mM-external Ca^{2+} was similar to that seen in lower external Ca^{2+}. This is also apparent in the recovery of current when the cell is rested at the end of the stimulation period. Return to 2·5 mM-Ca^{2+} reduced the size of inward currents again but had little effect on the magnitude of the inward current staircase. In Fig. 6*B* the effect of elevation of bath Ca^{2+} to 10 mM is shown on the positive staircases of inward current that result from trains of 50 ms pulses. In this situation an increase in i_{Ca} is seen in 10 mM-Ca^{2+} and the facilitation of Ca^{2+} current during the pulse train is preserved (compare Fig. 1). Also, the late decay of the facilitation during the later phase of repetitive pulsing seen in the pre-control situation seems reduced in the presence of 10 mM-Ca^{2+}. These changes in current in high-external-Ca^{2+} solution are readily reversible on return to lower external Ca^{2+} again (Fig. 6*B*, lower panel). These results are quite different from those seen in non-EGTA-loaded cells (Fig. 1). The low level of internal Ca^{2+} in the presence of EGTA results in an increase in peak inward current when external Ca^{2+} is increased (Kokobun & Irisawa, 1984) which reflects an increased driving force in the 10 mM-Ca^{2+} solution. There is no increase in the negative current staircase with long pulses despite an increased Ca^{2+} entry. With the short pulses, however, there is an increase in the facilitation of inward current in 10 mM-Ca^{2+}. The data for peak inward current compared with that obtained at 40 ms reveal that 10 mM-Ca^{2+} also induces a further slowing of i_{Ca} relaxation so that the inward current increased more at 40 ms than at peak inward current when exposed to 10 mM-Ca^{2+}.

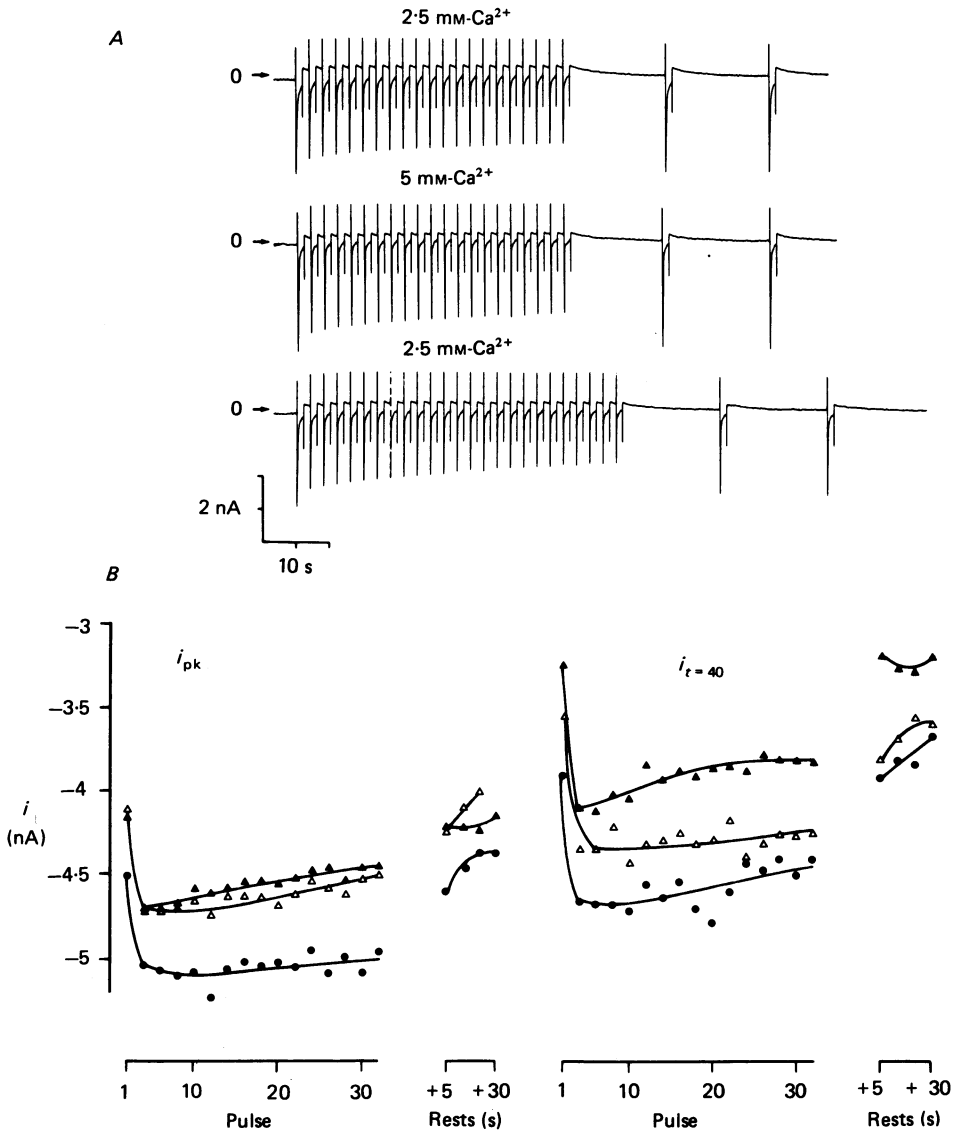

Fig. 6. Effect of elevation of external Ca^{2+} on current staircases in cells impaled with EGTA electrodes. *A*, current traces on a slow time base for 1 s clamp pulses at 30/min from −40 to −10 mV. Top trace, control in 2·5 mM-Ca^{2+}; middle trace, after 20 min in 5 mM-Ca^{2+}; bottom trace, after return to 2·5 mM-Ca^{2+}. The traces to the right were recorded after 15 or 30 s rest. *B*, effect of 10 mM-Ca^{2+} on positive current staircases with 50 ms voltage clamp pulses from −45 to 0 mV; different cell to panel *A*; constant 1 s interpulse interval. The left panel shows current changes of peak inward current. The right panel shows changes at $t = 40$ ms. ▲, current changes before exposure to 10 mM-Ca^{2+}; ●, during 10 mM-Ca^{2+} exposure; △, after return to 2·5 mM-Ca^{2+}. Current changes recorded after 5 and 30 s rest at the end of each train are shown to the right.

In EGTA-loaded cells the increase of i_{Ca} during short-pulse-duration trains is lost when Ca^{2+} ions are replaced by Ba^{2+} ions (Fig. 7). In this cell three staircases of current in response to 50 ms pulses are shown in the control situation. Prior to Ba^{2+} exposure eight trains of 50 ms pulses in 2·5 mM-external Ca^{2+} over 3 h gave similar positive current staircases to those shown. The effect of Ba^{2+} replacement of Ca^{2+} at

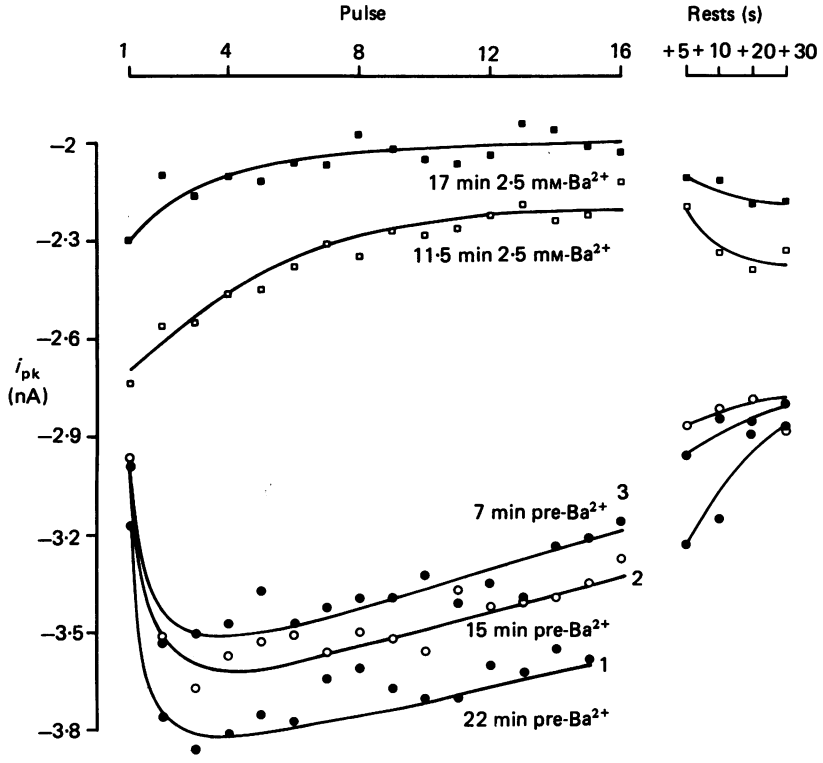

Fig. 7. Effects of 2·5 mM-Ba^{2+} replacement of Ca^{2+} ions in bath solution. Three control staircases of current shown prior to Ba^{2+} exposure in 2·5 mM-Ca^{2+} as ● or ○; 50 ms pulses from −45 to 0 mV with 1 s interpulse interval. Note facilitation of current on stimulation after rest with some later fall-off during train; recovery of current after rest shown to right. Current changes after exposure to 2·5 mM-Ba^{2+} for 11·5 or 17 min shown as □ and ■ respectively. All currents measured at peak inward level.

12 and 17 min exposure is illustrated. The facilitation of i_{Ca} in Ca^{2+} is abolished by Ba^{2+} ions. There is some subsequent current decrease at this pulse duration in this cell and there is no suggestion that Ba^{2+} ions augment this phase. Certainly the abolition of the facilitation by Ba^{2+} is not brought about by an increase in a negative current staircase. After 12 min Ba^{2+} exposure there is still an effect which reduces current levels, as shown by the poor recovery of i_{Ca} on rest. This is not the case after 17 min Ba^{2+} and it appears that not only is the facilitation of inward current by Ca^{2+} abolished, but the later phase of current decline seen on continued stimulation in the presence of Ca^{2+} ions is reduced in the presence of Ba^{2+} ions. Similar results have been seen in five cells impaled with EGTA electrodes and exposed to Ba^{2+} ions.

The effect of isoprenaline on short pulse staircases of current using EGTA electrodes is shown in Fig. 8. Control current staircases for pulses of 30, 50 and 100 ms duration are shown. Interestingly the facilitation is greater at the shorter-pulse durations and a later phase of current decline becomes apparent during stimulation with 100 ms pulses. After exposure to 5×10^{-7} M-isoprenaline, there is an increase in the size of i_{Ca} at all pulse durations but now there is a decrease in peak

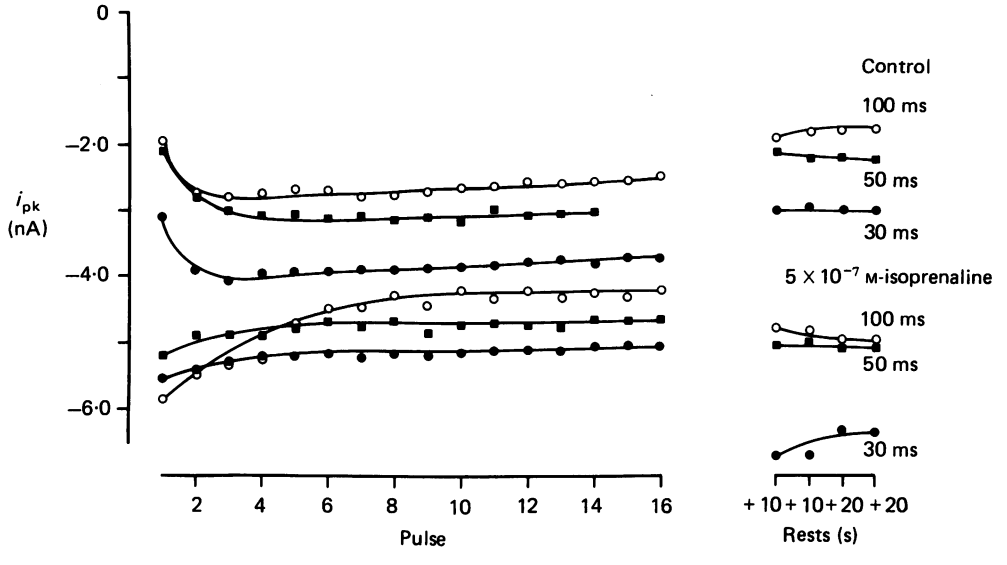

Fig. 8. Effect of 5×10^{-7} M-isoprenaline on positive current staircases with short voltage clamp pulses. Current changes for 30 ms (●), 50 ms (■) and 100 ms pulses (○) from −45 to +5 mV applied after 3 min rests with 1 s interpulse interval. Top three sets of data are control situation. This is indicated adjacent to data recorded after rests which in each case show return of current to rested level. Lower three sets of data recorded during exposure to 5×10^{-7} M-isoprenaline after 10–20 min. Note that in control situation all currents show facilitation during stimulation whereas during exposure to isoprenaline current changes at all pulse durations are negative. All currents measured at peak inward level.

inward current during the pulse trains at all pulse durations. This decrease is pulse-duration dependent and largest for 100 ms pulses in Fig. 8. For both control current staircases and those after exposure to isoprenaline all current changes during trains of pulses are readily reversible on resting the cell. Similar results were seen in four other cells.

DISCUSSION

The results are all consistent with the hypotheses outlined in the Introduction. They also show that the use-dependent changes in i_{Ca} can be saturated during Ca^{2+} overload. Increased $[Ca^{2+}]_i$ therefore has a biphasic effect. Moderate increases sufficient to enhance contraction increase the use-dependent changes. Very large increases, sufficient to generate large tonic contraction and almost abolish the twitch, greatly inhibit i_{Ca} but also remove the use-dependent changes.

Sarcolemmal or sarcoplasmic reticulum control of current changes

Administration of internal EGTA abolished cell movement rapidly and indefinitely (Fig. 4); cells lasted many hours and proved resistant to levels of external Ca^{2+} (10 mM) and zero external Na^+ that killed cells under normal conditions (Fedida *et al.* 1988). We have also previously shown that the levels of EGTA used here are sufficient to remove totally the delayed current tails attributable to Na^+–Ca^{2+} exchange (Fedida *et al.* 1987). Moreover, no significant difference was observed between the results with 3 mM-EGTA and higher concentrations of between 100 and 500 mM. These results imply that the levels of EGTA we used kept intracellular Ca^{2+} levels very low and abolished sarcoplasmic reticulum (SR) Ca^{2+} release. This is important since EGTA is a relatively slow buffer and it also liberates protons, which themselves inhibit contraction. The guinea-pig ventricular cell was thus converted into a tissue essentially without a sarcoplasmic reticulum.

In these conditions i_{Ca} was larger and its inactivation was slowed (Fig. 5). This is a well-described phenomenon (e.g. Josephson *et al.* 1984), and may reflect the removal of a portion of inactivation of i_{Ca} normally mediated by myoplasmic Ca^{2+} or the intracellular Ca^{2+} transient. However, the phenomena of negative staircases of inward current in response to long voltage clamp pulses and positive staircases of inward current in response to short voltage clamp pulses were not abolished by EGTA. Failure of injected EGTA to fully suppress Ca^{2+}-mediated inactivation during current flow has been cited as evidence of a component of voltage-dependent inactivation in *Helix* neurones (Brown, Morimoto, Tsuda & Wilson, 1981). However, failure of EGTA to fully block inactivation appears to be insufficient evidence that the residual inactivation is insensitive to Ca^{2+}. As the binding constant for Ca^{2+} to EGTA is relatively slow (Thomas, 1982), Ca^{2+} binding by EGTA almost certainly lags behind Ca^{2+} entry. In addition, steady entry of Ca^{2+} during current flow may load much of the EGTA present near the inner openings of the channels, allowing free Ca^{2+} to rise in these regions (Brehm & Eckert, 1978; Lee *et al.* 1985). The conditions of our experiments therefore allow elimination of changes attributable to the bulk myoplasmic Ca^{2+} concentration and to Ca^{2+} release from the SR, but not those attributable to Ca^{2+} entry via the channels themselves.

This idea is reinforced by the fact that, when Ca^{2+} is buffered even more severely than we have done here (e.g. as in the experiments with suction electrodes reported by Lee (1987) and in those of Lipp & Pott (1986), Ca^{2+}-dependent inactivation can be eliminated. In Lipp & Pott's work on atrial cells i_{Ca} even ceases to show inactivation.

Do negative current staircases reflect Ca^{2+}- or voltage-dependent inactivation of i_{Ca}?

Despite the abolition of the SR contribution to myoplasmic Ca^{2+} levels during pulse trains, the voltage dependence of negative current staircases was essentially unaltered (Fig. 4). There is a slight shift of the relation to more positive voltages but we cannot exclude the possibility that reduction of internal positive surface charge may be responsible for this. A bell-shaped relation was still observed which suggested that Ca^{2+} entry was the major determinant of the magnitude of negative current staircases rather than voltage itself.

It was noted by Mitchell, Powell, Terrar & Twist (1985) that the magnitude of inward current reduction after stimulation from rest at rates of 180/min to 240/min (200 ms interpulse interval in the former situation) was much reduced by the injection of intracellular EGTA in rat myocytes. This was cited as evidence for a predominantly Ca^{2+}-dependent inactivation process. In our experiments, however, we note an overlapping *facilitation* of i_{Ca} that is increased in the presence of internal EGTA. This is well illustrated in Fig. 8. After beat-dependent facilitation has been abolished, there is still a slow inactivation of inward current that is larger for longer pulse durations. Ba^{2+} ions seem best able to reduce this slow current reduction (Fig. 7), although, as for low-Na^+ solutions, the effect is not complete.

We conclude therefore that current reduction during trains of long voltage clamp pulses reflects primarily a Ca^{2+}-dependent process that can be modified by SR Ca^{2+} release (Fedida *et al.* 1988). Our experiments do not exclude the possibility that a small component of current reduction resistant to both Ba^{2+} replacement of Ca^{2+} and internal EGTA may reflect a slow, purely voltage-dependent process.

Effects of elevated external Ca^{2+} and isoprenaline

In normal cells these two manoeuvres produce quite different results since increased $[Ca^{2+}]_o$ reduces i_{Ca} (probably by increasing $[Ca^{2+}]_i$-dependent inhibition), whereas isoprenaline increases it. In cells loaded with intracellular EGTA both methods increase i_{Ca}. Not surprisingly the effects on use-dependent inhibition are then changed. Thus, elevation of external Ca^{2+} to 10 mM (Fig. 6) increases i_{Ca} but the negative current staircase is no longer larger with 1 s pulses than in 2·5 mM-Ca^{2+}. This may be explained by the fact that *facilitation* of i_{Ca} is much increased by 10 mM-Ca^{2+} (as evidenced by the effect of short-pulse stimulation in Fig. 6*B*) and will antagonize the effect of increased pulse duration. When the inward current facilitation is abolished by isoprenaline (Fig. 8; see below) then the effect of longer pulses to increase inactivation is clearly seen.

The facilitation of inward current during trains of short voltage clamp pulses

Facilitation of i_{Ca} is greater in cells loaded with internal EGTA. In normal cells it is often concealed by Ca^{2+}-dependent inactivation effects (Fedida *et al.* 1988; Figs 1 and 3 of this paper) except in special stimulation conditions. Facilitation of inward current is probably widespread in cardiac preparations but usually masked for the reason mentioned. Interestingly it is most often seen in tissues which are known to possess a more rudimentary sarcoplasmic reticulum. For example it is well seen in rat atrium (Payett, Schanne & Ruiz-Ceretti, 1981), in frog atrium (Noble & Shimoni, 1981) and sheep Purkinje fibres (Boyett & Fedida, 1984, 1988). This observation is entirely in keeping with our previous conclusion that SR Ca^{2+} release is an important factor in negative staircases of inward current during repetitive stimulation (Fedida *et al.* 1988).

Facilitation of i_{Ca} is also abolished by Ba^{2+} replacement of Ca^{2+} and exposure to the β-adrenoceptor agonist isoprenaline. The failure of facilitation by Ba^{2+} ions suggests that Ca^{2+} ion entry is important rather than simply divalent ion entry, unlike the facilitation of Ca^{2+} current in bovine chromaffin cells which was supported by Ba^{2+} ions (Hoshi *et al.* 1984). The facilitation of i_{Ca} is associated with a slowing

of the time course of current inactivation both in the control situation (Fedida *et al.* 1988; Fig. 4) and in cells loaded with EGTA (Fig. 5).

This is similar to the effect of β-adrenergic agonists on cardiac cells (Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofman, 1982; Bean, Nowycky & Tsien, 1984). We suggest that facilitation of i_{Ca} may therefore reflect a change in channel phosphorylation. This would explain why isoprenaline, which maximally phosphorylates the channels, abolishes the beat-to-beat facilitation seen after rest. Eckert & Chad (1984; also Chad & Eckert, 1986) propose that Ca^{2+} current inactivation is mediated enzymatically. A proportion of Ca^{2+} channels are maintained in the phosphorylated condition in the low-internal-Ca^{2+} rested state. During the flow of i_{Ca} dephosphorylation of regulatory sites is responsible for inactivation of Ca^{2+} conductance. A generalized rise in internal Ca^{2+} will result in a rise in steady inactivation by increasing the proportion of channels in the dephosphorylated state.

This model explains our results for non-EGTA-loaded cells, and explains why facilitation is more clearly seen in EGTA-loaded cells when the steady level of internal Ca^{2+} is lower and kept lower. It does not explain why short-pulse facilitation, which after all represents Ca^{2+} entry, in the presence of intracellular EGTA, seems to mimic the effect of channel phosphorylation.

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