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TRANSIENT INWARD CURRENT IN GUINEA-PIG ATRIAL MYOCYTES REFLECTS A CHANGE OF SODIUM–CALCIUM EXCHANGE CURRENT

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SUMMARY

1. Emzymatically isolated, cultured myocytes from hearts of adult guinea-pigs were voltage clamped with a whole-cell patch-clamp technique. The pipette-filling solution for internal dialysis contained 65 mM-citrate and 50 μM-EGTA as Ca^{2+}-chelating agents and 20 mM-Na^+. Potassium channel currents were blocked by replacing this ion on both sides of the membrane by Cs^+.

2. In the above conditions myocytes develop spontaneous transient inward currents (I_{ti}) at constant negative membrane holding potentials. At a given membrane potential I_{ti} can be recorded with constant amplitude and frequency for periods of up to *ca*. 40 min. A membrane current with similar properties can be evoked by superfusion of the cell with caffeine-containing (5–10 mM) solution.

3. Depolarization results in a reduction of I_{ti} amplitude and a prolongation of its duration. After a step change of the membrane potential to *ca*. −10 mV or a less-negative level only one inward current change is observed. Thereafter the membrane current remains inward with regard to the instantaneous current at this membrane potential. Complete relaxation of I_{ti} then is only observed after repolarization to a more-negative membrane potential.

4. The current change caused by sarcoplasmic Ca^{2+} release is inward in a range of membrane potentials between −90 and +75 mV. A reversal of I_{ti} was never detected.

5. Both the instantaneous current–voltage (I–V) relation and voltage dependence of peak I_{ti} display distinct outward rectification. Both I–V relations can be described by a formalism suggested for a membrane current caused by electrogenic Na^+–Ca^{2+} exchange ($I_{Na,Ca}$) assuming a 3:1 stoichiometry and a single energy barrier in the electric field of the membrane.

6. An increase of the time integral of I_{ti} at the holding potential is observed after depolarizations to positive membrane potentials, where the outward-rectifying current component is prominent. This supports the view that the outward current represents $I_{Na,Ca}$ in the 'reverse mode', carrying Ca^{2+} ions into the cell.

7. After prolonged cell dialysis a run-down of I_{ti} is observed. Since strong depolarizations in this condition still can cause inward currents upon repolarization,

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run-down is likely to reflect an impairment of sarcoplasmic reticulum function rather than an effect of cell dialysis on the exchanger.

8. We conclude that under the present conditions a membrane current is measured, which to a large extent determines the 'passive' I–V curve of the myocytes. This current is modified by a rise in Ca_i^{2+} following sarcoplasmic Ca^{2+} release. The properties of this current, its voltage dependence, and the effect of an intracellular Ca^{2+} transient on the voltage dependence, are highly compatible with electrogenic Na^+–Ca^{2+} exchange as a charge-carrying mechanism.

INTRODUCTION

Transient graded elevations of free intracellular Ca^{2+} activity (Ca_i^{2+}) are the key signals determining contractile force of cardiac muscle (for reviews see Chapman, 1983; Rüegg, 1986). This role of Ca_i^{2+} as second messenger requires transport mechanisms, which regulate its level at rest and during activity.

One major mechanism which contributes to keeping diastolic Ca_i^{2+} at a low level is a counter-transport which uses the inwardly directed Na^+ gradient across the cell membrane to extrude Ca^{2+} ions from the cytoplasm. Such a mechanism was postulated from the finding that both ions act antagonistically on the contraction of the frog heart (Lüttgau & Niedergerke, 1958). Cardiac Na^+–Ca^{2+} exchange has first been demonstrated in guinea-pig atria (Reuter & Seitz, 1968; Glitsch, Reuter & Scholz, 1970). These authors demonstrated both an Na_o^+-dependent Ca^{2+} efflux and an Na_i^+-dependent Ca^{2+} influx, suggesting Na^+–Ca^{2+} exchange to operate in both directions across the sarcolemma. A Na^+ dependent Ca^{2+} transport system was also identified in other excitable tissues (Baker, Blaustein, Hodgkin & Steinhardt, 1969). Theoretical considerations led to the suggestion that movement of more than two Na^+ ions is linked to the transport of one Ca^{2+} ion (e.g. Blaustein & Hodgkin, 1969; Mullins, 1976). For any stoichiometry greater than 2 Na^+ : 1 Ca^{2+} each transport cycle moves one or more positive charges across the cell membrane. Thus Na^+–Ca^{2+} exchange was postulated to be electrogenic and to be dependent on membrane potential. In cardiac preparations the exchanger has been studied extensively during recent years (for reviews see Eisner & Lederer, 1985; Philipson, 1985; Reeves, 1985; Sheu & Blaustein, 1986). As for the stoichiometry, evidence for 3 Na^+ : 1 Ca^{2+} has been accumulated. Identification of a membrane current caused by the exchanger in intact cardiac cells has been hampered by the fact that separation of such a current from other contaminating membrane currents is difficult without specific inhibitors (e.g. Mentrard, Vassort & Fischmeister, 1984). Furthermore the magnitude and, possibly, the direction of such a current might change in conventional voltage-clamp experiments in an unknown way, since Ca_i^{2+}, which is one determinant of the current, is allowed to change dynamically.

Using single perfused myocytes from guinea-pig ventricular muscle a convincing isolation of $I_{Na,Ca}$ and a quantitative description of its voltage and ion dependence based on a previously published model have been presented recently (Kimura, Noma & Irisawa, 1986; Kimura, Miyamae & Noma, 1987; for the theoretical background compare DiFrancesco & Noble, 1985). A different approach was used by Mechmann & Pott (1986), who measured transient inward currents (I_{ti}) in dialysed atrial

myocytes caused by sarcoplasmic Ca^{2+} release. The voltage and ion dependence of I_{ti} was found to be highly compatible with $I_{Na,Ca}$ as the charge-carrying mechanism. In the present investigation we have studied transient inward currents over an extensive range of membrane potentials. I_{ti} did not reverse its direction between -90 and up to $+75$ mV, which excludes an ion channel as charge-carrying mechanism. I_{ti} can be described as an inward change of a 'background current', which under the conditions of the present study shows distinct outward rectification. Evidence will be presented that this background current reflects $I_{Na,Ca}$ and that I_{ti} represents a change of this current caused by a shift of the thermodynamic driving force due to a rise in Ca_i^{2+}. Some preliminary results have been published previously (Lipp & Pott, 1986; Pott, 1986).

METHODS

Voltage-clamp experiments were performed on cultured myocytes from hearts of adult guinea-pigs by means of patch-clamp pipettes (whole-cell mode; Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The conditions for cell isolation and culture have been described in detail previously (Bechem, Pott & Rennebaum, 1983). Briefly, Ca^{2+}-tolerant atrial cells from guinea-pigs of either sex (200–250 g) were obtained by a modified Langendorff perfusion with a solution containing (mM): NaCl, 140; KCl, 5·4; $MgCl_2$, 1·0; HEPES, 10·0; adjusted with NaOH to pH 7·4. During the first 5 min of perfusion this solution contained additionally 10^{-4} M-EGTA. This was followed by an enzyme solution without EGTA containing added $CaCl_2$ (20 μM), collagenase (Worthington CLS II, 1 mg/ml) and elastase (Serva 2039, 10 μl suspension/ml). After 20–40 min of enzyme treatment the atria were cut off, placed in a dish containing enzyme solution and dispersed by gently agitating the tissue. In order to remove the proteolytic enzymes the cells were carefully washed with culture medium (M 199, Gibco) buffered with 20 mM-HEPES and supplemented with 1–2% fetal calf serum (Gibco) and Gentamycin (25 μg/ml). The cells were plated in tissue culture dishes (Falcon, 35 mm diameter) at a density of a few thousand cells per dish. The dishes were placed in an incubator at 37 °C, 90% humidity and 0·5% CO_2. For the experiments spherical myocytes with a diameter between 15 and 25 μm were used, which were in culture for periods of 3–14 days. The membrane capacity of the myocytes ranged from 12 to 50 pF; in the majority of measurements it was between 20 and 30 pF.

One hour before an experiment the culture medium was replaced by a solution of the following composition (mM): NaCl, 140; CsCl, 2·0; $MgCl_2$, 1·0; $CaCl_2$, 2·0; HEPES–NaOH, 10·0; adjusted to pH 7·4. The solution contained additionally the Ca^{2+} channel blocking substance D600 (2–5 μM; Knoll AG). The dish containing the cells was placed on the stage of an inverted microscope. Measurements were performed at room temperature (21–23 °C).

Patch-clamp pipettes were fabricated from Pyrex glass and were filled with the following solution (mM): caesium citrate, 65; NaCl, 20; EGTA, 0·05; HEPES–CsOH, 10; pH 7·4; Mg–ATP, 1–2; cyclic AMP, 0·1. The rationale for the composition of this solution was (i) to block K^+ channel currents by Cs^+, (ii) to impose a constant Na^+ load (20 mM) to the cells and (iii) to have a rapid Ca^{2+}-buffering system, which, however, has a low buffering capacity. Cyclic AMP was added to the internal solution, since run-down of transient inward current seemed to be retarded as compared to solutions not supplemented by the cyclic nucleotide. The voltage-dependent properties of the current to be studied were not affected by cyclic AMP. The DC resistance of the pipettes filled with this solution ranged from 2 to 6 MΩ. Voltage and current measurements were performed by means of a patch-clamp amplifier (List LM/EPC 7). Signals were stored on analog tape (Racal 4DS) and later analysed using an IBM PC equipped with an AD-board (Data-Translation DT-2801 A) at appropriate sampling rates. In order to minimize contamination of the currents to be studied by leak components, experiments were continued only if (i) the seal resistance in the cell-attached configuration was $\geqslant 20$ GΩ and (ii) after rupture of the membrane under the tip of the pipette and equilibration with the dialysing fluid for about 1 min the holding current at -50 mV did not exceed -10 pA.

RESULTS

Spontaneous transient inward currents

Upon dialysis with the pipette-filling solution listed in the Methods, guinea-pig cardiac myocytes develop spontaneous transient inward currents (I_{ti}) at constant negative holding potentials. Figure 1*A* shows a sequence of 38 s in duration of membrane current recorded from a cell (holding potential: -50 mV) which had been dialysed for *ca.* 6 min. Spontaneous transient inward currents occurred fairly regularly with intervals of *ca.* 5 s and with an amplitude of 50 pA.

Figure 1*B* displays a single transient inward current on a faster time-scale to illustrate its characteristic time course. The current maximum is reached within 100–200 ms. This is followed by a slow phase of relaxation which may last for about 1 s to more than 10 s. Finally there is always a distinct faster component of relaxation, the beginning of which has been marked by the arrow.

From the observation that spontaneous I_{ti} is always accompanied by a strong contraction of the cell, visible through the microscope, Ca^{2+} release from the sarcoplasmic reticulum was assumed to be the primary event causing this current. This hypothesis has been tested by using caffeine, a substance known to release Ca^{2+} from the sarcoplasmic reticulum (SR) of cardiac and skeletal muscle when applied extracellularly in millimolar concentrations (e.g. Weber & Herz, 1968). The result of a representative experiment using caffeine is shown in Fig. 1*C*. Rapid superfusion of the cell with the solution containing the substance (5 mM) evokes a transient inward current with an amplitude identical to spontaneous I_{ti} but displaying much slower and incomplete relaxation. In the presence of this substance spontaneous I_{ti} is abolished. Additionally, opening events of an ion channel with large unitary conductance ($\geqslant 220$ pS) are regularly observed following administration of caffeine. In most experiments activity of this type of channel could be detected occasionally also during spontaneous I_{ti}. The induction of a current change similar to spontaneous I_{ti} by caffeine and its subsequent suppression clearly demonstrate that cyclic Ca^{2+} release from the SR is involved in causing this current. The caffeine-evoked inward current incompletely relaxes towards a level which is inward with regard to the initial holding current and corresponds to the current level of the transition between the two components of the decay of I_{ti} shown in Fig. 1*B*. Thus, I_{ti} obviously is not only elicited by sarcoplasmic Ca^{2+} release, but the time course of its decay is – at least partly – dependent on a functional sarcoplasmic reuptake mechanism.

After achieving access to the interior of the cell by destroying the membrane under the tip of the recording pipette, there is first a period of quiescence lasting from a few seconds to about 2 min until the first I_{ti} is detected. The shape of the current transient changes with increasing duration of cell dialysis. A typical example is shown in Fig. 2.

In this cell the first I_{ti} was detected 8 s after the beginning of cell dialysis. In the experimental conditions used during a period of several minutes, consecutive current events are more and more prolonged until a steady state is reached, which may last for various periods of time. Inward currents of nearly constant amplitude and time course could be measured at a given membrane potential for up to *ca.* 40 min. In this

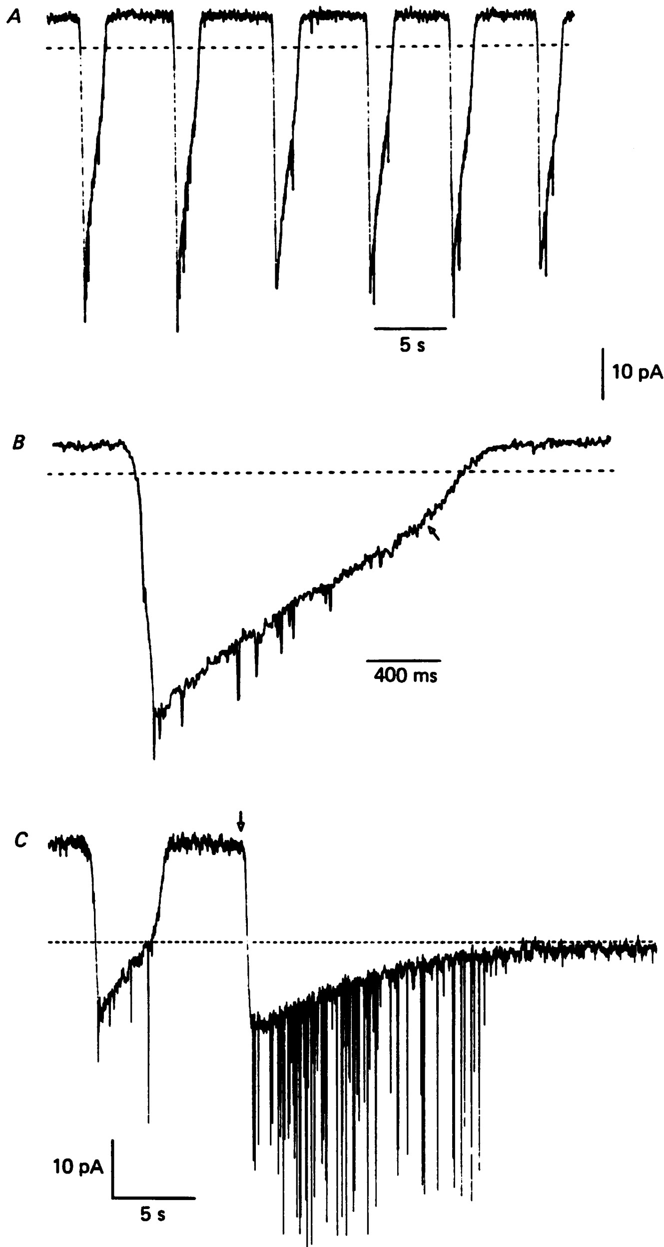

Fig. 1. *A*, spontaneous transient inward currents. The cell was dialysed with intracellular solution for 6 min. Membrane potential was held at -50 mV throughout. *B*, expanded single transient (from *A*). The dashed line denotes the zero-current level. The arrow marks the transition between the two phases of relaxation. *C*, inward current evoked by caffeine. At the time marked by the arrow caffeine-containing solution was puffed onto the cell. Caffeine (5 mM) dissolved in extracellular solution was put in a micropipette (tip diameter *ca.* 10 μm), which was positioned at a distance of 50 μm from the cell. The dashed line denotes the holding current in the presence of caffeine, which was -12 pA (holding potential: -45 mV). The rapid downward deflections represent openings of the large ion channel.

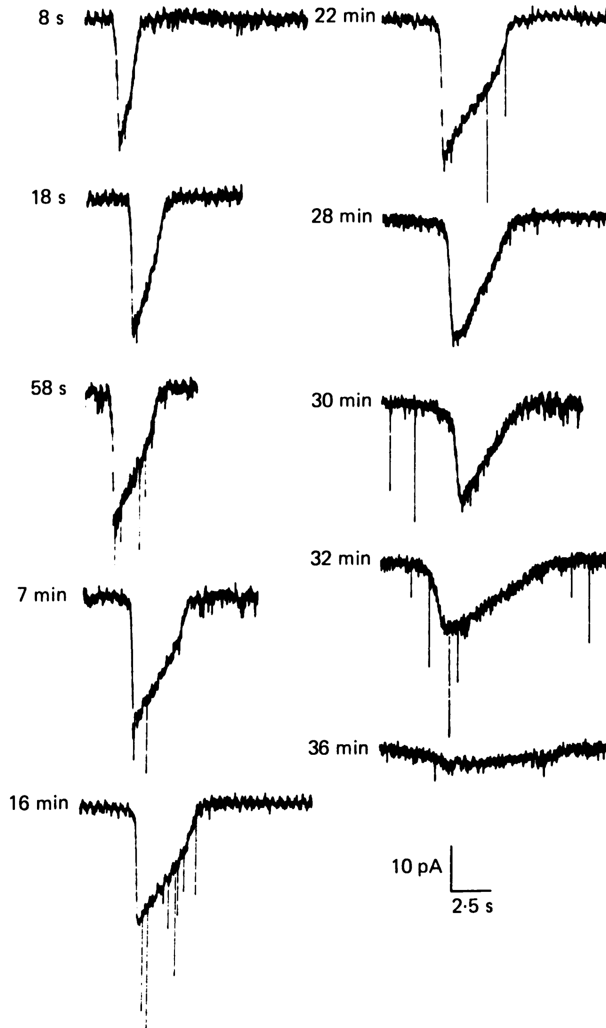

Fig. 2. Change of I_{ti} during prolonged cell dialysis. Transient inward currents were recorded at the times indicated on the left of the traces. At $t = 0$ the membrane under the tip of the recording pipette had been ruptured by a suction pulse. Membrane holding potential was -50 mV throughout. The mean holding current was *ca.* $+6$ pA from 18 s to 28 min. Thereafter it slowly changed in the inward direction to about -10 pA after 36 min.

cell I_{ti} remained constant with regard to its amplitude and time course for a period of about 20 min. Thereafter a rapid 'run-down' of this current was observed, which was consistently found to include a reduction of the amplitude, a prolongation of time-to-peak and a small inward shift of the holding current, by *ca.* 16 pA in that experiment. In the final stage, spontaneous I_{ti} activity completely ceases.

The occurrence of spontaneous I_{ti} depends critically on the composition of the dialysing fluid in the recording pipette, particularly with regard to its Ca^{2+}-buffering properties. Spontaneous I_{ti} was not observed if this solution in addition to 65 mM-citrate contained EGTA at a concentration of 1–2 mM. In cells loaded with such a

solution, however, a membrane current with properties similar to those of the transient inward current investigated in the present paper can be evoked by loading the cell with Ca^{2+} via long-lasting or repetitive activation of the transmembrane Ca^{2+} current (Lipp, Mechmann & Pott, 1987). On the other hand, dialysis with 65 mM-citrate without additional EGTA resulted in current recordings with irregular I_{ti} activity. We assume the major prerequisite for recording regular I_{ti} activity to be Ca^{2+} buffering at a low level ($< 10^{-7}$ M) but with a low buffering capacity, in order to permit changes in Ca_i^{2+} due to both Ca^{2+} entry across the cell membrane and Ca^{2+} release from the SR.

From the long periods of time over which this current can be reproducibly recorded and from the fact that it was found in nearly all cells (more than 200) studied under the conditions described in the Methods section, we conclude that I_{ti} does not reflect a state of cell damage, but represents a physiological mechanism, which, however, is likely to be altered in its properties by the experimental conditions. The frequency and duration of I_{ti} varied considerably in different cells. In the steady state the current could display a duration of approximately 1 s up to 10 s at the standard holding potential of -50 mV. The amplitude of the current at the normal holding potential was very variable in different myocytes and was not correlated to the size of the cell. In the majority of cells the amplitude of I_{ti} at -50 mV was between 10 and 50 pA. The current densities ranged from 0·5 to 7 pA/pF.

Spontaneous transient inward currents with the properties to be described were not a peculiarity of cultured atrial myocytes but could be identified also in freshly isolated guinea-pig atrial and ventricular cells as well as in Purkinje cells from rabbit and sheep if studied under otherwise identical experimental conditions.

Voltage dependence of I_{ti} relaxation

In order to obtain information on the charge-carrying mechanism of I_{ti}, its voltage dependence was studied. Since this current is not an event triggered by a change in membrane potential, step changes were applied from a negative holding potential (usually -70 to -50 mV) the duration of which was manually adjusted to capture one or several events at the depolarized voltage level. The general features of the effect of changes in membrane potential on I_{ti} are illustrated in Fig. 3. As has been shown previously (Mechmann & Pott, 1986), the amplitude of the inward current change caused by sarcoplasmic Ca^{2+} release is reduced, and its duration is prolonged, if the cell is depolarized from -50 to -30 mV. A further reduction in membrane potential, to -10 mV in the experiment shown, results in incomplete relaxation of the current. After one single release event the current remains inward with regard to the instantaneous (pre-release) level. Whenever this happens, spontaneous I_{ti} generation ceases. Full relaxation of the current and subsequent cyclic activity is only observed after stepping back to a more-negative membrane potential. Under the conditions of the present study the membrane potential where this behaviour was first observed ranged from -30 to $+10$ mV.

Since the primary event causing I_{ti} is a Ca^{2+} release from the SR, i.e. a rise in Ca_i^{2+}, this behaviour suggests some voltage-dependent process of Ca^{2+} removal from the cell to determine, or at least to contribute to, the decay of the intracellular Ca^{2+} transient. From the type of experiment illustrated in Fig. 3 one cannot conclude

unequivocally that I_{ti} itself represents this voltage-dependent Ca^{2+}-transport mechanism. It is likewise possible that an ion channel, activated by a rise in Ca_i^{2+}, is the charge-carrying mechanism of I_{ti}. In that case the effect of voltage on the duration of the Ca^{2+} transient could be caused by an electrically silent process of Ca^{2+} removal from the cell.

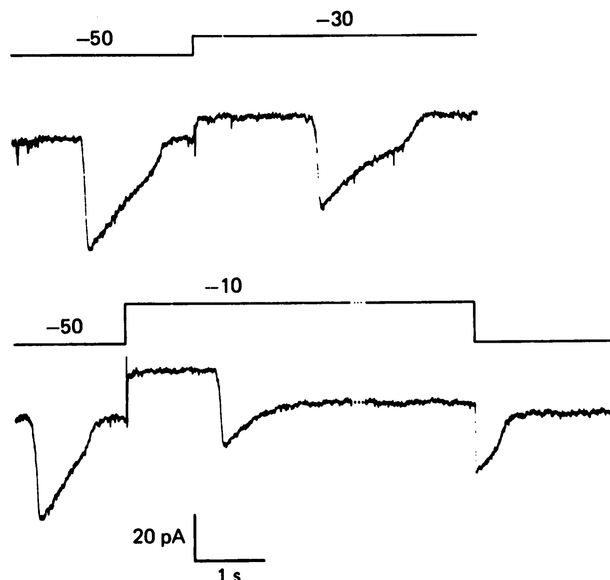

Fig. 3. Slowing of I_{ti} by depolarization. Membrane potential was stepped, as indicated, from the holding potential (−50 mV) to −30 and −10 mV respectively. At −10 mV the trace is interrupted for 5 s.

Lack of a reversal potential of I_{ti}

If an ionic channel is the elementary event of I_{ti} one should detect a reversal of the current at a membrane potential depending on the selectivity of such a channel for the ions involved in the system under study.

The voltage dependence of I_{ti} determined in a range of membrane potentials between −87 and +75 mV is illustrated in Fig. 4. After stepping from −50 to −87 mV only a single I_{ti} was observed. This was consistently found in all experiments of this kind: spontaneous I_{ti} activity ceased at membrane potentials negative to −75 to −80 mV.

At −2 mV the behaviour already shown in Fig. 3 can be detected, namely an incomplete relaxation of the current after one single I_{ti}. Increasing depolarization further diminishes the amplitude of the current change caused by Ca^{2+} release. At +75 mV a small but reproducible inward deflection can be seen but no outward change with regard to the pre-release current level was observed. A reversal of I_{ti} was never observed in the present study. Both the instantaneous current and peak I_{ti} display distinct outward-rectifying properties. Thus, if the absolute current levels are considered, I_{ti} might well reflect a reduction of a membrane current, which is outward at positive membrane potentials.

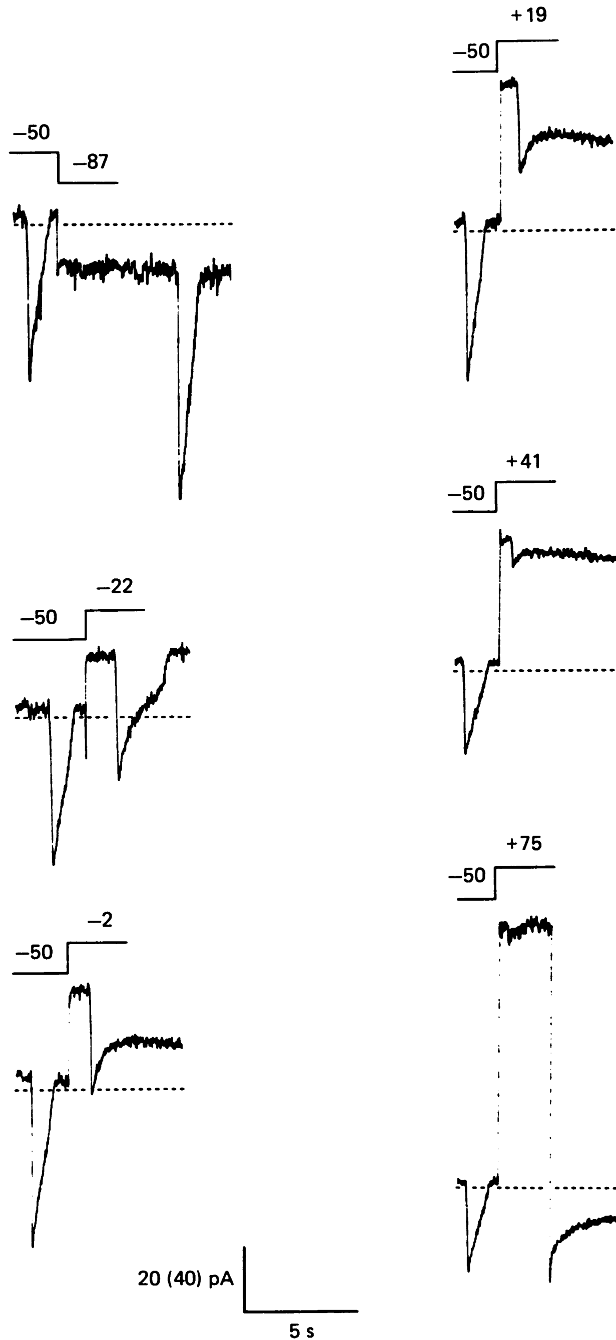

Fig. 4. Voltage dependence of instantaneous current and I_{ti}. Membrane potential was changed stepwise as indicated from a holding potential of -50 mV. The scaling in parentheses applies to the voltage steps to $+41$ and $+75$ mV. The dashed line represents the zero-current level. After repolarization from $+75$ mV a slowly decaying tail current is recorded (compare Figs 7 and 8).

Is I_{ti} a 'Ca²⁺-activated' current?

From the results so far described I_{ti} is unlikely to be carried by a Ca_i^{2+}-activated ion channel. The lack of a current reversal is compatible with the previous suggestion that I_{ti} is carried only by Na^+–Ca^{2+} exchange (Mechmann & Pott, 1986). This does not imply that the exchange current does not possess a genuine reversal potential. Assuming a 3 Na^+ : 1 Ca^{2+} stoichiometry (e.g. Reeves & Hale, 1984; for further

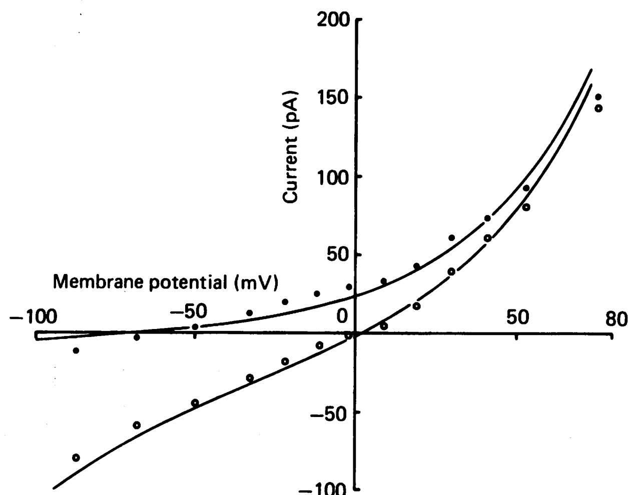

Fig. 5. Current–voltage relation of instantaneous current and I_{ti}. Instantaneous current (●) and peak transient inward current (○) from the experiment illustrated in Fig. 4 have been plotted against membrane potential. The curves were calculated using eqn (2) with the following parameters: $Ca_i^{2+} = 6 \times 10^{-8}$ M (●), or $9{\cdot}5 \times 10^{-7}$ M (○); $Na_o^+ = 150$ mM, $Na_i^+ = 16$ mM. The latter value yielded a better fit than 20 mM, which might be due to incomplete dialysis of the cell. For σ a value of 0·68 was used. In order to account for the reversal potential at low Ca_i^{2+}, which is *ca.* 17 mV more positive than the theoretical one with the above parameters, a linear leak conductance of 200 pS with a reversal potential at 0 mV was added to the calculated curves.

literature see Eisner & Lederer, 1985; Sheu & Blaustein, 1986) the equilibrium potential ($E_{Na,Ca}$) is given by:

$$E_{Na,Ca} = 3E_{Na} - 2E_{Ca}, \tag{1}$$

with E_{Na} and E_{Ca} denoting the Nernst potentials of the respective ion species. Since a rise in Ca_i^{2+} following Ca^{2+} release always causes a change of E_{Ca} to less-positive membrane potentials, the resulting change in the driving force for Na^+–Ca^{2+} exchange can only cause a current change in the inward direction, although the absolute level of the exchange current during peak I_{ti} may be outward. This prediction is valid, if the driving force is considered as the only determinant for the direction of $I_{Na,Ca}$. With certain assumptions about the kinetic properties of the exchanger it is also possible, however, to account for an apparent reversal of a Na^+–Ca^{2+} exchange current transient caused by a rise in Ca_i^{2+} (Eisner & Lederer, 1985).

Since at present there are no inhibitors of the exchanger available with a defined

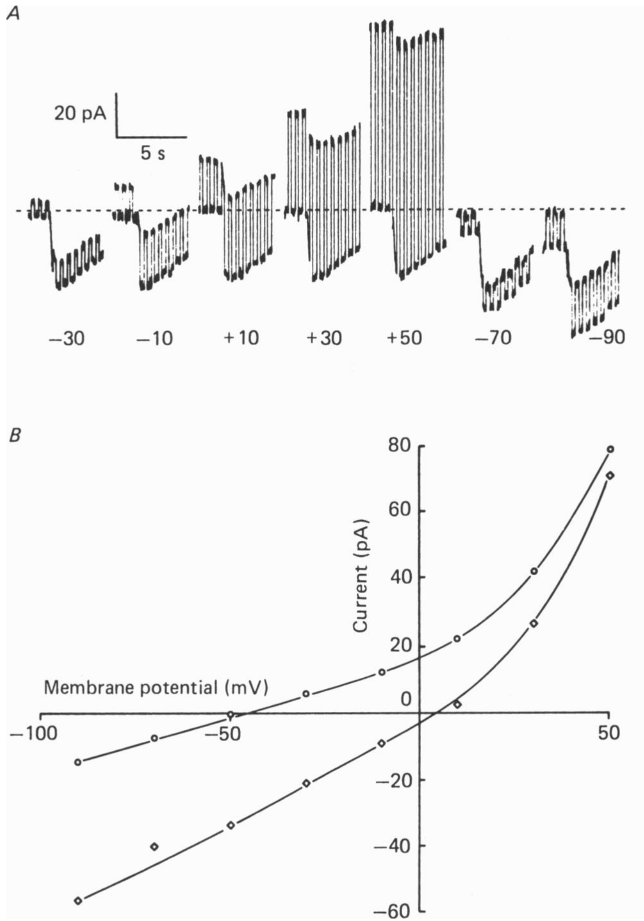

Fig. 6. Measurement of current–voltage relations of instantaneous current and I_{ti} using brief voltage steps. *A*, voltage-clamp steps of 300 ms in duration were applied to the membrane potentials indicated from a holding potential of −50 mV. For reasons of clarity only the beginning of the transient inward currents with superimposed pulse-evoked current changes have been traced. The dashed line corresponds to the zero-current level. *B*, plot of the current level before I_{ti} (○) and during peak I_{ti} (◇) against membrane potential. The curves were calculated using eqn (2) with the following parameters: $Na_i^+ = 17$ mM; Ca_i^{2+} (low) $= 5 \times 10^{-8}$ M; Ca_i^{2+} (high) $= 1{\cdot}2 \times 10^{-6}$ M; $\sigma = 0{\cdot}67$; input conductance 220 pS.

specificity, a straightforward identification of a component of current caused by this transport mechanism to the current–voltage relations is not possible. The voltage dependencies of the instantaneous current (●) and of peak I_{ti} (○) from the experiment illustrated in Fig. 4 are shown in Fig. 5. The absolute current values have been plotted in this Figure against membrane potential.

Both the curve representing the instantaneous (pre-release) current and that describing peak I_{ti} are characterized by an outward-rectifying component at positive membrane potentials. The reversal potential of the total membrane current is shifted in the positive direction by 68 mV after Ca^{2+} release. In twenty-four experiments analysed in this way the difference of the reversal potentials for the two I–V curves ranged from 52 to 71 mV ($64{\cdot}8 \pm 4{\cdot}6$ mV; see also Fig. 6). The absolute reversal

potentials were more variable. These are likely to be determined by the outward-rectifying current and a leak current, which we assume to have ohmic properties and a reversal potential close to 0 mV. Contribution of the electrogenic Na^+ pump to the background current should be negligible in the present conditions, since Cs^+ is a very weak activator ion. The leak current, which in turn depends on the seal resistance and the passive input resistance of the cell, may vary in different measurements, causing variable contributions to the total I–V curve. Therefore, as has been stated in the Methods section, only data from experiments were evaluated, where the holding current at −50 mV did not exceed an arbitrarily chosen value of 10 pA in the inward direction. The voltage dependence of the instantaneous current and peak I_{ti} suggests the latter to represent a reduction of an outward current at membrane potentials positive to *ca.* 0 mV, and to represent a current which transiently changes from net outward to net inward between the reversal potential of the instantaneous I–V curve and 0 mV. However, a quantitative separation of the outward-rectifying current from leak current is not possible without the availability of a specific inhibitor for either of the two.

An alternative experimental protocol which was used to study the voltage dependence of I_{ti} is shown in Fig. 6*A*. In this experiment, from the holding potential (−50 mV) brief (300 ms) depolarizing or hyperpolarizing clamp pulses were given at a frequency of $2\ s^{-1}$.

This permits one current transient to be studied at two different membrane potentials. Like in the experiments described in Figs 5 and 6, the voltage dependence of the 'background current' (before I_{ti}) and of peak I_{ti} display outward rectification (Fig. 6*B*). Again, no reversal of the current change caused by Ca^{2+} release is detected in the entire range of membrane potentials covered in that experiment (−90 to +50 mV). The reversal potentials of the two I–V curves representing instantaneous current and peak I_{ti} differed by 56 mV in this experiment (−49 and +7 mV respectively).

A current displaying outward rectification – at least under certain sets of ionic gradients – is predicted by the exponential formalism suggested recently (Di-Francesco & Noble, 1985, see also Jack, Noble & Tsien, 1975; Noble, 1986):

$$I_{Na,Ca} = k\{Ca_o^{2+}(Na_i^+)^3 \exp(\sigma EF/RT) - Ca_i^{2+}(Na_i^+)^3 \exp(-(1-\sigma)EF/RT)\}. \quad (2)$$

This is a simplified version which does not take into account the possibility of different affinities for Na^+ and Ca^{2+} on both sides of the membrane, nor does it consider that at extreme positive or negative membrane potentials the carrier-mediated current is likely to saturate. Apart from the concentrations (or activities respectively) of Na^+ and Ca^{2+} on both sides of the membrane and the membrane potential, this equation contains a 'partition factor' (σ), which accounts for an asymmetrical location of a rate-limiting voltage-dependent step within the field of the membrane. In order to simulate the measured current–voltage relations we assumed an intracellular resting Ca^{2+} activity of 5 to 6×10^{-8} M. This order of magnitude had been determined in the pipette-filling solution by means of a Ca^{2+} electrode. However, it is close to the detection limit of the electrode available to us, and therefore should be considered only as an estimate. As for the intracellular Ca^{2+} activity following Ca^{2+} release from the SR, we assumed an order of magnitude of

10^{-6} M. This seems to be reasonable, since in all cells a strong contraction accompanying I_{ti} could be observed through the microscope. Thus, using eqn (2), we can calculate I–V curves, one for the low (resting) Ca_i^{2+} and one for Ca_i^{2+} after release. The other concentrations involved were fixed by the compositions of the extracellular solution ($Na_o^+ = 140$ mM, $Ca_o^{2+} = 2$ mM), and the pipette-filling solution respectively ($Na_i^+ = 20$ mM). For Ca_o^{2+} an activity coefficient of 0·35 was used. For Na^+ inside and outside the cell identical activity coefficients were assumed.

The curves in Figs 5 and 6*B* have been calculated using eqn (2). Although the data points are satisfactorily fitted by the model, this should be regarded under qualitative aspects. The experimental current–voltage relations are unlikely to be determined solely by Na^+–Ca^{2+} exchange current because of contamination by at least a leakage current of unknown magnitude and voltage dependence. In each experiment both curves could be fitted by eqn (2) with (i) the same value for σ, which in sixteen experiments evaluated under this aspect ranged from 0·67 to 0·75, and (ii) identical scaling for the simulated low-Ca^{2+} and the high-Ca^{2+} I–V curve. The only parameter which had to be changed in order to account for the shift of the I–V curve following Ca^{2+} release is Ca_i^{2+}.

If the outward current at positive membrane potentials represents electrogenic Na^+–Ca^{2+} exchange, it should carry Ca^{2+} ions into the cell and therefore cause a rise in Ca_i^{2+} in addition to Ca^{2+} release. This additional Ca^{2+} load in turn should affect the membrane current during the positive voltage pulse and after repolarization to the holding potential. Such effects of strong depolarizations can indeed be detected. In Fig. 7 the effect of imposing positive voltage steps of increasing amplitude on membrane current is illustrated. At membrane potentials $\geqslant +18$ mV after the release-evoked inward current, which partly relaxes, a slow change of the membrane current in the inward direction is observed (*A*). The slow tail current after repolarization to -50 mV is prolonged as compared to the spontaneous I_{ti} at this membrane potential (*B*).

If I_{ti} represents the exchange current, its time integral is a direct measure for the number of Ca^{2+} ions transported out of the cell. The time integrals of the tail currents shown in Fig. 7*B* have been plotted against the membrane potential of the preceding positive voltage step in Fig. 7*C*. The dashed line in this plot denotes the mean time integral of the spontaneous I_{ti} at the holding potential. A value below the dashed line indicates that a fraction of the Ca^{2+} ions released during the depolarization must have left the cell when it was repolarized. On the other hand, for data above the dashed line Ca^{2+} must have entered the cell in addition to Ca^{2+} released from the SR. The intersection of the dashed line and the curve connecting the data points corresponds to a membrane potential where apparently no net transmembrane Ca^{2+} movement occurs after Ca^{2+} release. In the experiment illustrated this membrane potential was $+17$ mV. If Na^+–Ca^{2+} exchange were the only mechanism of transmembrane Ca^{2+} removal, this would represent the reversal potential of the exchanger at high Ca_i^{2+}. Since, however, we do not know to what extent an ATP-driven Ca^{2+} pump is operating in these cells under our experimental conditions (cf. Caroni & Carafoli, 1980; Barry, Rasmussen, Ishida & Bridge, 1986) and at which rate Ca^{2+} is removed by diffusional equilibration with the solution in the recording pipette, this membrane potential can only be regarded as an estimate

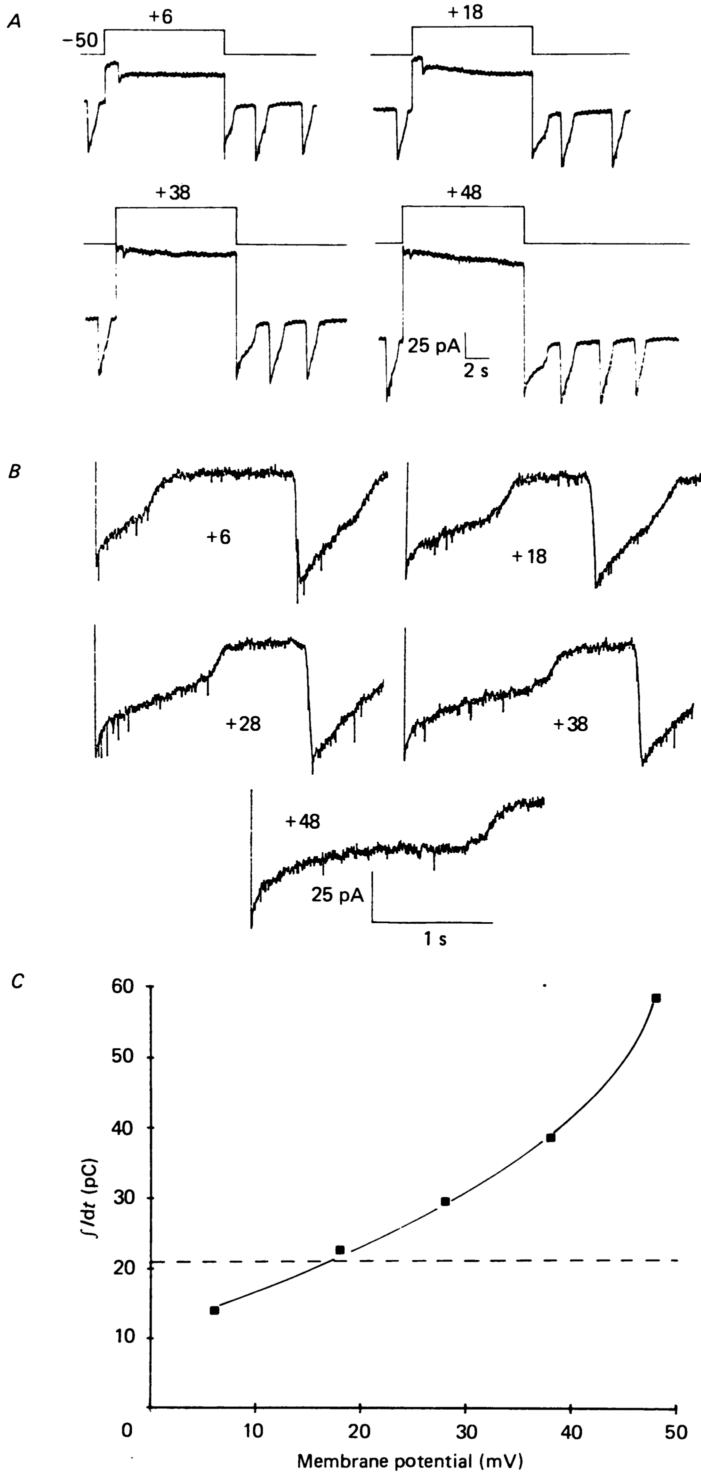

Fig. 7. For legend see opposite.

of $E_{Na,Ca}$: the true value is likely to be more positive, but it is unlikely to be less positive. Assuming a free Ca^{2+} concentration in the cell of 10^{-6} M after release, the calculated $E_{Na,Ca}$ is -13 mV, i.e. the value determined by the estimate in Fig. 12 at least is very close to the theoretical one for a 3:1 stoichiometry. This consideration is valid only if the prolongation of I_{ti} following a strongly positive voltage step does not reflect an increase in Ca^{2+} release as compared to spontaneous release at the holding potential. Figure 8 presents evidence that the prolongation of the tail current is unlikely to be due to such a mechanism but is caused by additional Ca^{2+} entry at positive membrane potentials. In this experiment a voltage step of constant amplitude (from -50 to $+40$ mV) from 1 to 8 s in duration was applied (*A*).

The tail currents upon repolarization were compared to the spontaneous transient inward current at the holding potential. The prolongation of the tail current, or the increase in its time integral respectively, seems to be linearly related to the duration of the positive voltage step (*B*). If the straight line is extrapolated to a duration of 0 s, it intersects at a charge which is identical to that of spontaneous I_{ti} at the holding potential (indicated by the arrow). Since Ca^{2+} release (i.e. the rapid inward change in membrane current) is always detected within the first second of the depolarization, the increase of the time integral with duration is unlikely to reflect an effect of membrane potential as such on sarcoplasmic Ca^{2+} release. Note that in Figs 7 and 8 the amplitude of the current after repolarization from positive voltage steps never exceeds that of spontaneous I_{ti} at -50 mV. This was found consistently and is likely to reflect saturation of $I_{Na,Ca}$ by the free Ca^{2+} concentration. The value of *ca.* 10^{-6} M assumed to fit the experimental data using eqn (2) therefore has to be considered a saturating level; the true Ca_i^{2+} following Ca^{2+} release might be even higher.

What causes cyclic Ca^{2+} release?

The data so far presented leave no doubt that cyclic release of Ca^{2+} ions from intracellular stores is the primary event underlying spontaneous I_{ti}. The mechanism of this cyclic Ca^{2+} release, however, at present is not understood.

It occurs spontaneously in a condition where classical Ca^{2+} current has been blocked and other novel Ca^{2+} current pathways should be inactivated (cf. Bean, 1985; Nilius, Hess, Lansman & Tsien, 1985). Thus, Ca^{2+} entry via Ca^{2+} channels is not involved in causing Ca^{2+} release from the SR under the conditions of the present investigation. The free Ca^{2+} concentration in the recording pipette, however,

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Fig. 7. Effect of strong depolarizations on subsequent I_{ti} relaxation. *A*, step changes in membrane potential were applied as indicated (duration 10 s). In that range of membrane potentials only one single inward current is observed, which relaxes incompletely (compare Fig. 3). Note the slow inward shift of the membrane current at $+38$ and $+48$ mV. The membrane currents after repolarization from the levels indicated are traced in *B*. Note the prolongation of the inward current transient at -50 mV as compared to the spontaneous I_{ti} at this membrane potential with increasing amplitude of the depolarization. *C*, plot of the time integral of the current transients measured after repolarization against membrane potential. The dashed line denotes the integral of spontaneous I_{ti} at -50 mV. The calculated charge represents the mean of the five transient inward currents preceding the five voltage pulses.

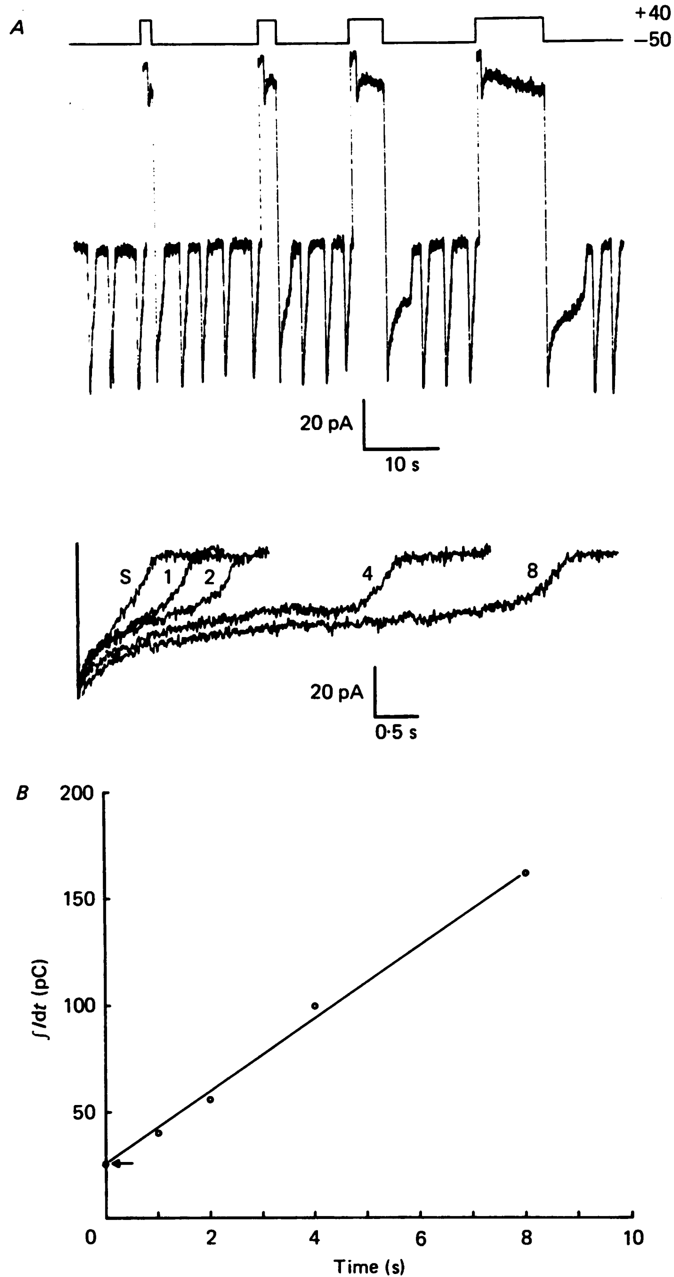

Fig. 8. Effect of duration of a positive voltage step on subsequent I_{ti} relaxation. The clamp protocol is illustrated in *A* (upper panel). The current transients after repolarization from voltage steps to +40 mV of increasing duration (1–8 s) and spontaneous I_{ti} (S) at the holding potential, unaffected by a change in membrane potential, are superimposed in the lower panel of *A*. *B*, plot of time integral of inward currents from *A* against duration of the voltage step to +40 mV. The charge transported by spontaneous I_{ti} (no preceding depolarization, i.e. duration = 0) has been marked by the arrow. The straight line was fitted by eye.

(*ca.* 5×10^{-8} M) is in a range where Ca^{2+}-induced Ca^{2+} release is unlikely to occur (Fabiato, 1985*a, b, c*).

Therefore one has to assume a source of continuous Ca^{2+} entry, the magnitude of which depends on membrane potential. Figure 9 illustrates the effect of membrane potential on I_{ti} over longer periods of time. As shown before, the amplitude of the

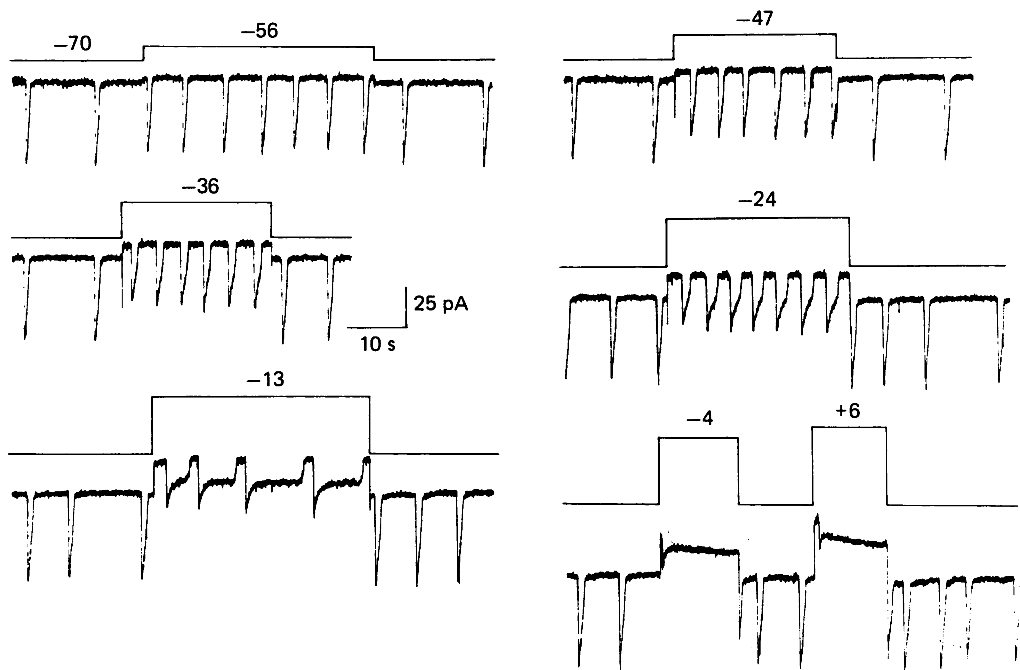

Fig. 9. Effect of membrane potential on I_{ti} frequency. Membrane potential was changed as indicated from a holding potential of -70 mV (holding current: -12 pA).

current is reduced upon depolarization, and its duration is prolonged. Furthermore, there is a distinct effect of the membrane potential on the time interval between two successive release events. This interval becomes shorter with increasing depolarization between -56 and -13 mV. At less-negative membrane potentials, as shown above, only one single I_{ti} occurs, which relaxes incompletely. Note that the interval to the second I_{ti} after repolarization is also reduced as compared to the steady-state interval at -70 mV, suggesting that the effect of membrane potential on Ca^{2+} release does not represent a genuine voltage effect but a mechanism outlasting the clamp step by several seconds. From these results we conclude that the dependence of the release interval on membrane potential reflects a voltage dependence of Ca^{2+} entry. Since (i) Ca^{2+} current has been blocked by D600 and (ii) a Ca^{2+} entry via a leak should be reduced by depolarization, it is likely that Na^{+}–Ca^{2+} exchange is the mechanism of Ca^{2+} entry in the present experimental condition. If Ca^{2+} is continuously flowing into the cell between two successive transient inward currents via Na^{+}–Ca^{2+} exchange, one would expect a corresponding rise in free Ca_i^{2+}, which in turn should be detectable as a decreasing outward current, because of the linear dependence of $I_{Na,Ca}$ on Ca_i^{2+} (compare with eqn (2)). In most

experiments the current trace appeared to be completely flat between two successive inward currents (compare Figs 1, 2 and 7). In a few cells, however, a slow change in holding current preceding the rapid rising phase of I_{ti} could be detected. An example of this behaviour is shown in Fig. 10. The cell was held at −50 mV, where transient inward currents occurred regularly with a frequency of 0·26 s^{-1}. From the end of one I_{ti} until the beginning of the rapid rising phase of the subsequent one (arrow) the

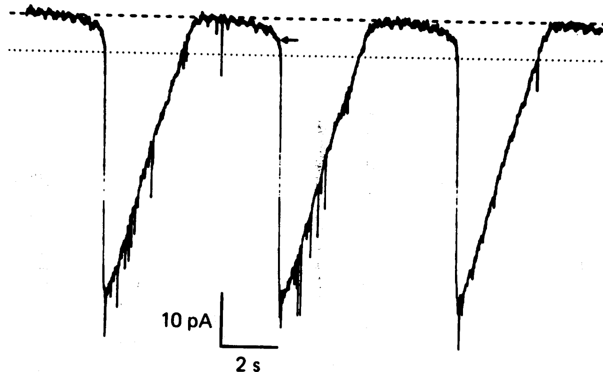

Fig. 10. Slow change of holding current. Membrane current recorded from a cell at −50 mV. The dotted line marks the zero-current level. The dashed line was drawn to make clear the slow change of the holding current in the inward direction between two successive transient inward currents. The arrow marks the transition between the slow change and the beginning of I_{ti}.

holding current is not constant but slowly changes in the inward direction by *ca.* 5 pA.

We interpret this different behaviour as follows: in all cells studied there is a continuous Ca^{2+} entry via Na^{+}–Ca^{2+} exchange. Simultaneously Ca^{2+} is pumped into the SR. In the myocyte investigated in Fig. 10 Ca^{2+} entry exceeds sarcoplasmic Ca^{2+} uptake, resulting in a slow rise in Ca_i^{2+}, which causes a corresponding change of $I_{Na,Ca}$. In most cells, however, a Ca^{2+} uptake either balances or exceeds Ca^{2+} entry throughout, or at least up to a point of time close to the next release. This may result in a superposition of the slow inward change due to Ca^{2+} entry and the fast-release-dependent I_{ti}, which cannot be separated from each other.

If, as has been rendered likely above, the major pathway of Ca^{2+} entry is also Na^{+}–Ca^{2+} exchange, which corresponds to an outward current, only part of the I_{ti} at the holding potential is in fact a net inward current. Since the frequency and amplitude of I_{ti} at a constant membrane potential remain rather constant over long periods of time, the net balance between Ca^{2+} entry and Ca^{2+} removal must be zero. This balance in total is not affected by the SR, which under the present conditions only acts as a temporary buffer. This means that on average the same amount of Ca^{2+} which had been released and transported out of the cell during one I_{ti} must have entered the cell during the period of time after the preceding I_{ti}. By means of the procedure illustrated in Fig. 11 we have attempted to isolate the inward and outward components of current at one membrane potential. In this experiment comparatively

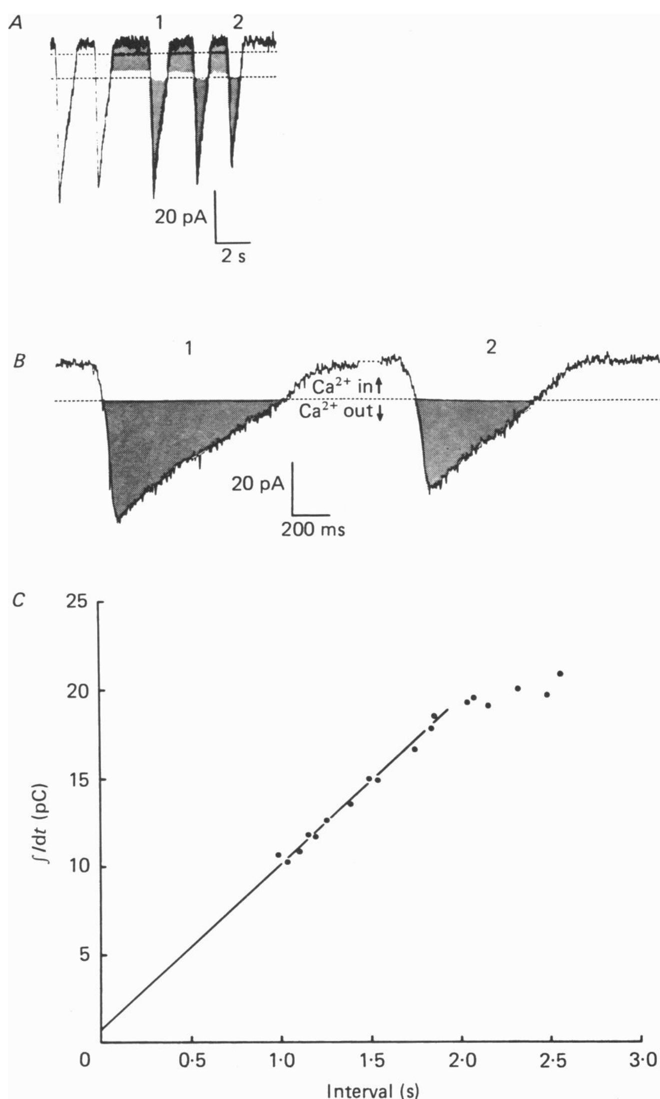

Fig. 11. Estimate of outward and inward component of I_{ti}. *A* shows a sequence of 13 s in duration with transient inward currents of variable amplitude (holding potential: −50 mV). The two current transients marked 1 and 2 are shown in *B* on an expanded time-scale. The transition between the two components of I_{ti} relaxation was visually estimated and marked by the dashed line. The current below this line was assumed to reflect net inward $I_{Na,Ca}$, i.e. outward transport of Ca^{2+}. The time integral of this current (shaded area) therefore was assumed to be proportional to the number of Ca^{2+} ions extruded from the cell by an electrogenic pathway. Assuming that the same amount of Ca^{2+}, which is extruded from the cell during I_{ti} flow, must have entered the cytoplasm before being released from the SR, the time integral of the inward current was fitted into the area framed by the holding current, the individual transient inward current analysed and the preceding one (*A*). Three examples for this procedure are denoted by the shaded areas in *A*. *C*, plot of the time integral of the inward current as defined in *B* against the preceding interval. A continuous current recording of 55 s in duration was evaluated as explained above (identical experiment as *A*). The straight line was calculated by linear regression for the data up to an interval of 2 s.

large variations in the amplitude of I_{ti} were found (*A*). As in the previous Figures relaxation of I_{ti} occurred in two phases.

These two phases were more pronounced at depolarized membrane potentials (e.g. Figs 2 and 9), sometimes resulting in a plateau current lasting for several seconds, before the final relaxation occurred in an all-or-none fashion. The apparent stability of this current level at more depolarized membrane potentials and the fact that the inward current evoked by caffeine only relaxed to a level which is equal or close to the transition between slow and fast relaxation (Fig. 1*C*), led us to the assumption that this transition represents the zero-current level of the exchanger. In experiments like the one illustrated in Fig. 11, where distinct variations in I_{ti} amplitude could be detected, it was found that an I_{ti} of comparably large amplitude was preceded by a long interval, whereas after shorter intervals transient inward currents of smaller amplitude are observed. Assuming the transition between the two phases of relaxation to reflect zero $I_{Na,Ca}$, i.e. a change from inward to outward current, the current below this level was integrated (shaded area in Fig. 11*A* and *B*). With the above assumption, the time integral of this current should correspond to the number of charges (i.e. Ca^{2+} ions in the case of a 3:1 stoichiometry) transported out of the cell. The integration was done for a sequence of eighteen inward currents at this membrane potential. In Fig. 11*C* the charge obtained by this procedure has been plotted against the duration of the interval before each I_{ti}. The resulting relation is linear for intervals up to 2 s in duration saturating at higher values. The extrapolated intersection with both the horizontal and vertical axis is close to zero. In Fig. 11*A* the time integrals of three successive inward currents have been matched to the assumed 'outward current' before each I_{ti}. Apart from a small gap between two corresponding shaded areas, which might result from an error in the visual estimate of the transition between the two phases of relaxation, the charge due to outward current closely corresponds to the charge translocated by the subsequent inward current. This result clearly suggests that I_{ti} studied in the present investigation reflects an inward component due to outward movement of Ca^{2+} and a steady outward component, the latter carrying Ca^{2+} into the cell. At present we suggest this model as a working hypothesis. In order to quantitatively support this hypothesis experimentally, it would be necessary to specifically inhibit $I_{Na,Ca}$. Preliminary studies using 3,4-dichlorobenzamil, a substance with some antagonistic potency for Na^+–Ca^{2+} exchange (Siegl, Cragoe, Trumble & Kaczorowski, 1984), but with yet unsatisfying selectivity (Bielefeld, Hadley, Vassilev & Hume, 1986), indeed revealed an inward shift of the holding current at −50 mV by this drug and an inhibition of I_{ti} (Lipp & Pott, 1987).

Creep currents

In Fig. 2 it has been shown that after long periods of dialysis spontaneous I_{ti} activity ceases. This is accompanied by an inward shift of the holding current of a few picoamperes. The run-down of I_{ti} during cell dialysis can either be due to a time-dependent decrease of the exchange current or a gradual loss of the capability of the SR to accumulate and/or release Ca^{2+} ions. In Figs 7 and 8 evidence was presented that strong depolarizations cause a Ca^{2+} load via Na^+–Ca^{2+} exchange which results in a prolonged transient inward current upon repolarization. This SR-independent

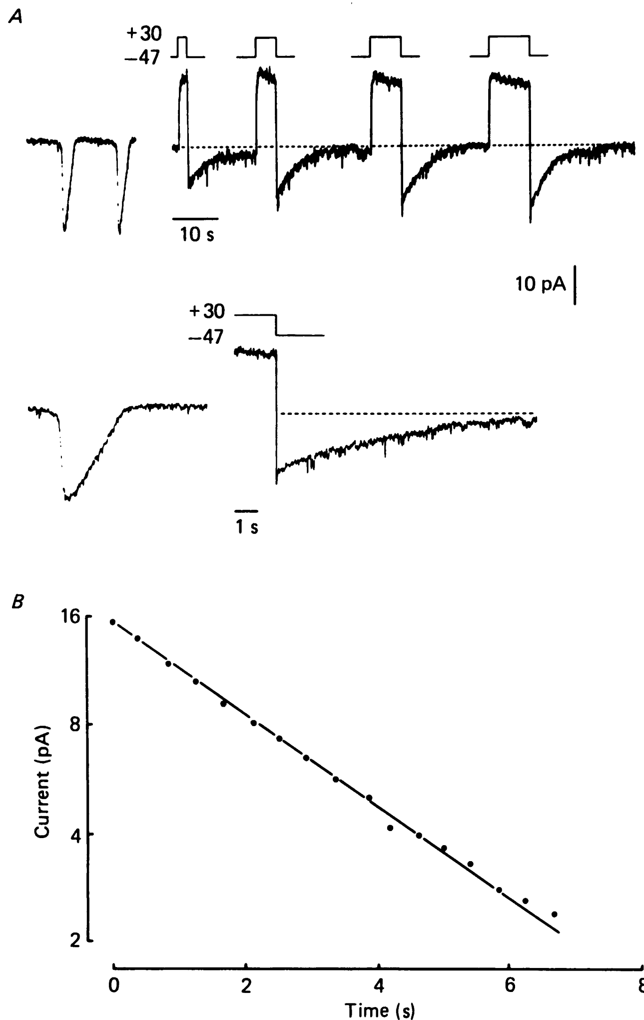

Fig. 12. Inward creep currents. The cell was depolarized from a holding potential of −47 to +30 mV for 2, 4, 8 and 10 s. The continuous trace (top) starts after 44 min of cell dialysis, when spontaneous I_{ti} activity had completely ceased. The two transient inward currents on the left were recorded at the same membrane potential about 8 min earlier. The lower traces displayed expanded recordings of a spontaneous I_{ti} and the creep current after repolarization from the 8 s step to +30 mV. *B*, plot of creep current (logarithmic scale) after repolarization from 8 s positive voltage step against time. The regression line yields a time constant of 2·76 s.

component of I_{ti} should not be affected in the case of run-down being due to an impaired SR function. In the experiment shown in Fig. 12, after complete cessation of I_{ti} activity, which was detected after 44 min of cell dialysis, strong depolarizations (from −50 to +30 mV) of increasing duration were imposed. This causes a slowly decaying 'creep current' (time constant: 2·76 s, Fig. 12*B*) when the cell is repolarized. The amplitude of this current increases with the duration of the depolarizing pulse.

Note, however, that at +30 mV the decrease in outward current with time, which is likely to reflect the change of $I_{Na, Ca}$ due to the Ca^{2+} load, is not symmetrical to the current change at holding potential. This is in accordance with the model calculations, which predict a smaller effect of changes in Ca_i^{2+} on $I_{Na, Ca}$ at positive membrane potentials than in the negative voltage range (compare Figs 5 and 6*B*). From this observation, which was similarly made in six other cells studied for sufficiently long periods of time, it can be concluded that cessation of spontaneous I_{ti} is not caused by run-down of Na^+–Ca^{2+} exchange current but is likely to reflect a loss of SR function. Considering the slow development of I_{ti} run-down, the impairment in SR function is likely to be caused by diffusional loss of at least one component of high molecular weight necessary for the uptake and/or release mechanism.

DISCUSSION

Spontaneous cyclic Ca^{2+} release

In the present investigation a membrane current has been studied in single heart cells, which is caused by a rise in Ca_i^{2+} due to Ca^{2+} release from the SR. This current occurs either spontaneously, i.e. without imposing changes in membrane potential, or it can be evoked on a once-only basis by extracellular application of caffeine, a substance known to release Ca^{2+} ions from, and to inhibit their reaccumulation into, the SR (Weber & Herz, 1968; Jundt, Portzig, Reuter & Stucki, 1975). Under conditions where I_{ti} does not occur spontaneously (e.g. using an intracellular solution with a higher Ca^{2+}-buffering capacity), I_{ti} can be evoked by voltage-clamp depolarizations activating I_{Ca} (Pott & Mechmann, 1986; Lipp *et al.* 1987). A membrane current which is inward at negative membrane potentials, and which is related to contraction or Ca^{2+} release, has recently been described in guinea-pig ventricular myocytes (Fedida, Noble, Shimoni & Spindler, 1987). This current could be unambiguously distinguished from inward current through classical Ca^{2+} channels, since it is found after repolarization from voltage steps eliciting I_{Ca} and can be separated from the latter by various means, e.g. by loading the cell with EGTA to prevent changes in free Ca_i^{2+}. An inward current flowing upon caffeine-evoked Ca^{2+} release has been investigated in aggregates of cultured embryonic chick myocytes (Clusin, 1983; Clusin, Fischmeister & De Haan, 1983). This current has some properties in common with I_{ti} studied here: it lacks a reversal potential, and it is slightly prolonged if the preparation is depolarized, although the voltage dependence was less pronounced than in the present study.

In mammalian cardiac muscle, Ca^{2+}-induced Ca^{2+} release seems to be the likely physiological mechanism of coupling excitation to contraction. This has been extensively studied in most-elegant experiments on skinned cardiac cells (Fabiato, 1983, 1985*a*, *b*, *c*). According to these studies the amount of Ca^{2+} released by a trigger Ca^{2+} surge does not only depend on its amplitude but to a large extent on its rate of rise. Apart from the physiological Ca^{2+}-induced Ca^{2+} release, a second type of Ca^{2+} liberation from the SR is distinguished in those studies. This 'cyclic' Ca^{2+} release occurs under conditions of Ca^{2+} overload (free Ca^{2+} concentration: $\geqslant 3 \times 10^{-7}$ M). Both types of Ca^{2+} release, however, have several properties in common, and the transfer of Ca^{2+} ions across the SR membrane is likely to proceed through the same

type of Ca^{2+}-permeable channels (e.g. Rousseau, Smith, Henderson & Meissner, 1986; Meissner & Henderson, 1987).

Ca^{2+} overload is not *a priori* a condition inherent to the present study: the free Ca^{2+} concentration of the dialysing fluid is well below 10^{-7} M ($5–6 \times 10^{-8}$ M). On the other hand, at a constant membrane potential of -50 mV a rapid increase of the intracellular Ca^{2+} concentration, via whatever sarcolemmal pathway, is unlikely to occur. The condition under which Ca^{2+} release is observed in the present study therefore is not clearly consistent with either of the two conditions defined as being different as to the Ca^{2+} release mechanism in skinned cells. In intact (i.e. non-dialysed) cardiac cells and multicellular preparations spontaneous Ca^{2+} release has been described to occur also under non-Ca^{2+}-overloading conditions (Stern, Kort, Bhatnagar & Lakatta, 1983; Capogrossi, Kort, Spurgeon & Lakatta, 1986*a*; Capogrossi, Suarez-Isla & Lakatta, 1986*b*). This type of spontaneous release is particularly observed in quiescent multicellular and single-cell preparations of the rat. As these authors discuss, a definition of this phenomenon in terms of physiological or unphysiological mechanisms seems to be inappropriate, since quiescence as such is an unphysiological situation for any type of cardiac cell. In a recent study on rat myocytes using the fura-2 digital-imaging technique (Wier, Cannell, Berlin, Marban & Lederer, 1987) it was shown that cells displaying occasional spontaneous mechanical activity have a slight but significantly higher resting Ca_i^{2+} than cells which are quiescent throughout (*ca.* $1 \cdot 3 \times 10^{-7}$ M *vs. ca.* $2 \cdot 7 \times 10^{-7}$ M). It might be possible that the variations in Ca_i^{2+} are due to corresponding variations in resting potential and/or intracellular Na^{+} activity, resulting in slightly different equilibrium conditions for Na^{+}–Ca^{2+} exchange. The level of Ca_i^{2+} of the cells displaying spontaneous contractile activity again does not correspond to a significant Ca^{2+} overload. These observations suggest that spontaneous Ca^{2+} release does not require a massive cellular Ca^{2+} overload but reflects a physiological or near-physiological situation of cardiac sarcoplasmic reticulum.

The charge-carrying mechanism of I_{ti}

The current change does not reverse, i.e. no outward transient is observed in a voltage range between -90 and $+75$ mV. The voltage-dependent reduction of I_{ti} upon depolarization is always accompanied by a prolongation of its duration or – at membrane potentials positive to -20 to -10 mV – even incomplete relaxation. Both a reduction in Na_o^{+} or an increase in Ca_o^{2+} have been shown to be qualitatively equivalent to a depolarization with regard to I_{ti} amplitude and duration (Mechmann & Pott, 1986). This clearly suggests the existence in the membrane of cardiac cells of a mechanism of Ca^{2+} removal from the cytoplasm, which depends on membrane potential and the transmembrane gradients for Na^{+} and Ca^{2+}. The transient inward current does not necessarily have to be identical to this transport system, but might simply reflect the time course of the Ca_i^{2+} transient, which is controlled by a different, electrically silent mechanism. In that case, however, the hypothetical charge-carrying system is hardly compatible with any known ion channel – apart from a strictly Ca^{2+}-selective one – because such a Ca^{2+}-activated channel should possess a reversal potential positive to $+70$ mV (Figs 7 and 8). Any contribution, however,

of Ca^{2+} channels to I_{ti} can be excluded because of (i) the insensitivity to Ca^{2+}-antagonistic drugs and (ii) its apparent activation by a rise in Ca_i^{2+}.

The 'passive' current–voltage characteristics of the myocytes, measured under the present experimental condition, are characterized by a distinct outward-going rectification. Since K^+ ions on both sides of the membrane are replaced by Cs^+, and furthermore, this behaviour is seen in the instantaneous current upon a voltage step, this is unlikely to reflect a classical outward-rectifying conductance pathway. An exponential dependence of outward current on membrane potential is predicted by a recently suggested model for $I_{Na,Ca}$, which assumes a single energy barrier located at a fractional distance (σ in eqn (2); $0 \leqslant \sigma \leqslant 1$) from the surface of the membrane (DiFrancesco & Noble, 1985; Noble, 1986; see also Jack *et al.* 1975). This formalism (cf. eqn (2)) satisfactorily describes the voltage and ion dependence of a current identified experimentally as $I_{Na,Ca}$ in perfused guinea-pig ventricular myocytes (Kimura *et al.* 1986, 1987). Furthermore, inward creep currents, which are observed in Na^+-loaded frog myocytes after periods of Ca^{2+} entry, have also been found to be compatible with such a formalism (Hume & Uehara, 1986*a*, *b*). This model and any other model, which assume the direction of $I_{Na,Ca}$ to be governed solely by the thermodynamic gradients of the two ion species involved (cf. Mullins, 1979; Eisner & Lederer, 1985), predict a shift of the I–V curve in the inward direction, if the intracellular Ca^{2+} concentration rises. For this particular scheme the inward shift should be more pronounced at negative membrane potentials as compared to the positive voltage region. This is what is observed experimentally: both the instantaneous I–V curve and the curve describing the dependence of peak I_{ti} on membrane potential approach each other with increasing depolarization. The theoretical fit by eqn (2) critically depends on three assumptions, namely (i) the intracellular Ca^{2+} activity, (ii) the partition coefficient (σ), and (iii) the stoichiometry. For the latter a value of 3 Na^+:1 Ca^{2+} now seems to be commonly accepted and experimentally supported (Sheu & Fozzard, 1982; Reeves & Hale, 1984; for further literature as to controversial determinations of Na^+–Ca^{2+} stoichiometry see Eisner & Lederer, 1985; Sheu & Blaustein, 1986).

Reasonable assumptions can be made as to Ca_i^{2+}. The resting level corresponds to that of the dialysing fluid (5–8×10^{-8} M) whereas after Ca^{2+} release, it is likely to be in the order of magnitude of 10^{-6} M, because I_{ti} is accompanied by a strong contraction. In all experiments, which allowed the current to be studied over a broader range of membrane potentials, the optimum fit was obtained by setting σ to a value close to 0·7. In a given cell both curves measured could be fitted using identical figures for σ and identical scaling. Thus, the shift of the I–V curve following Ca^{2+} release can be completely accounted for by the variation of the transmembrane Ca^{2+} gradient. These results support the view of I_{ti} being a change of the current, which dominates the 'passive' I–V curve, instead of being a Ca^{2+}-activated current. The current, which is altered in its voltage-dependent properties, apart from its compatibility with theoretical models, shows additional symptoms of reflecting electrogenic Na^+–Ca^{2+} exchange: depolarization to positive levels of membrane potential, where the outward-rectifying current is prominent, results in a prolongation of the subsequent I_{ti} as compared to the spontaneous I_{ti} at the holding potential. The prolongation is increased with increasing amplitude and duration of

the clamp pulse, but it is only seen if the depolarization exceeds a certain level. Below this level the current tail after repolarization is always shorter and smaller in amplitude than the corresponding I_{ti} at the holding potential. Saturation of I_{ti} amplitude is likely to result from saturation of the carrier under the present condition. This points to the possibility that the value for Ca_i^{2+} after Ca^{2+} release inserted into eqn (2) might be lower than the level of free Ca^{2+} which is actually reached. 10^{-6} M then would simply correspond to an intracellular Ca^{2+} activity which is saturating for the carrier. A more graded dependence of the inward exchange current is observed in the present investigation after long periods of cell dialysis, which result in a 'run-down' of the SR uptake and/or release mechanism. Note, however, that the decay of the slow 'creep' current after depolarization is mono-exponential and much slower than under conditions of cyclic Ca^{2+} uptake and release by the SR. This points to the fact that I_{ti}, although carried by Na^{+}–Ca^{2+} exchange, is not the only mechanism controlling the duration of the Ca^{2+} transient. Apart from diffusional equilibration with the solution in the recording pipette (which has been determined to proceed with a half-time of 50–100 s for small molecules, and thus does not affect the Ca^{2+} transient to a large extent; Bechem & Pott, 1985) there is a considerable contribution by the sarcoplasmic uptake. This is also in line with previous investigations on oscillatory electrical and/or mechanical activity in mammalian cardiac preparations under conditions of Ca^{2+} overload. Caffeine in small concentrations reduces the amplitude of both electrical and mechanical oscillations and increases their frequency, whereas higher concentrations abolish oscillatory activity (Glitsch & Pott, 1975; Vassalle & DiGennaro, 1984; Eisner & Valdeolmillos, 1986) resulting in smooth creep currents and smooth tonic tension.

In the present study cyclic Ca^{2+} release from SR is not caused by Ca^{2+} overload but is obviously caused by a condition of constant Ca^{2+} influx, via Na^{+}–Ca^{2+} exchange. For the usual set of ionic gradients (Na_o^{+} = 140 mM; Na_i^{+} = 20 mM, identical activity coefficients; Ca_o^{2+} = 2 mM, activity coefficient 0·33; $Ca_i^{2+} = 5 \times 10^{-8}$ M), and a 3:1 stoichiometry, the equilibrium potential for the exchanger is calculated as −86 mV. This coincides rather well with the finding that at membrane potentials negative to −75 to −80 mV cyclic I_{ti} activity was not observed. At membrane potentials positive to this value a net Ca^{2+} influx is generated by this mechanism. The resulting rise in Ca_i^{2+} should shift $E_{Na,Ca}$ to less-negative values, which in turn should result in a change of $I_{Na,Ca}$ in the inward direction. In a small number of experiments a slow inward change of the holding current between two successive inward current transients has been observed. In most of the cells studied, however, the current trace remains completely flat, suggesting that the myoplasmic free Ca^{2+} concentration remains constant between two successive I_{ti}. This is assumed to result from continuous Ca^{2+} uptake by the SR, balancing Ca^{2+} entry. If the cell is slightly depolarized, this does not cause a rise in resting free Ca_i^{2+}, but a rise in I_{ti} frequency. Such a transfer of Ca^{2+} entering the cell to the sarcoplasmic reticulum provides a mechanism which counteracts the development of a contracture. A free intracellular Ca^{2+} activity can be maintained, which is lower than the level that is thermodynamically possible via the exchanger. Since the storage capability of the SR is limited, Ca^{2+} ions have to be released from time to time. This causes a rapid reversal of the driving force for Na^{+}–Ca^{2+} exchange now favouring Ca^{2+} extrusion.

Without any other component of Ca^{2+} removal the level of Ca_i^{2+} after release will approach the equilibrium value of the exchanger, which is given by:

$$Ca_i^{2+} = Ca_o^{2+}(Na_i^+/Na_o^+)^3 \exp(EF/RT). \quad (3)$$

At −50 mV membrane potential and the standard set of ionic conditions, eqn (3) yields a value of $2{\cdot}3 \times 10^{-7}$ M. We assume that Ca^{2+} uptake by the SR in most cells balances or even exceeds Ca^{2+} entry via the exchanger. If the open-state probability of the SR release channels is assumed to be Ca^{2+} dependent (e.g. Rousseau *et al.* 1986), after a release the SR remains leaky for a certain period of time, depending on the velocity of Ca^{2+} removal. Lowering Ca_i^{2+} will decrease the SR permeability to Ca^{2+} resulting in a growing net uptake by this system. By a sudden Ca_i^{2+}-dependent complete closure of the release channels Ca_i^{2+} can be shifted to a level below the equilibrium for the exchanger according to eqn (3). If, however, this equilibrium level is too high to allow for closure of SR release channels, Ca_i^{2+} is arrested at or close to that concentration. This in turn prevents further regenerative release. Such a behaviour would account for the incomplete relaxation of I_{ti} at depolarized membrane potentials. A typical membrane potential, where incomplete relaxation is first observed (*ca.* −10 mV), corresponds to an equilibrium free Ca_i^{2+} of *ca.* 1 μM. At this Ca^{2+} concentration continuous opening activity of Ca^{2+}-release channels in isolated rabbit cardiac SR vesicles has been demonstrated recently (Rousseau *et al.* 1986). In Fig. 11 we have attempted to separate the two components of I_{ti} reflecting a decreasing outward current (carrying Ca^{2+} into the cell) and an inward current, corresponding to outward movement of Ca^{2+}. The idea behind this evaluation is reasonable, namely that the total balance between Ca^{2+} entry and Ca^{2+} removal has to be zero, since the storage capacity of the SR is finite. At present, however, this should be considered merely as a scheme to illustrate that I_{ti} is likely to reflect a current modulated by the intracellular Ca^{2+} activity.

Relation to previously studied transient inward currents

In various types of mammalian cardiac preparations oscillatory electrical and/or mechanical activity has been described under conditions causing intracellular Ca^{2+} overload (Glitsch & Pott, 1975; Lederer & Tsien, 1976; Kass, Lederer, Tsien & Weingart, 1978*a*; Eisner & Lederer, 1979). The electrical activity (after-depolarizations or corresponding transient inward currents in voltage-clamp measurements) is not generated by the normal action potential mechanism (Lederer & Tsien, 1976). The latter, however, can be initiated if an after-depolarization is above threshold for the inward current systems involved in the cardiac action potential. Therefore transient inward currents are suspected to be responsible for triggered cardiac arrhythmias (e.g. Ferrier & Moe, 1973; for further literature see Wit & Rosen, 1986). It is generally accepted that after-depolarization or transient inward currents and the accompanying oscillations in contractile force are caused by spontaneous Ca^{2+} release from the SR (Glitsch & Pott, 1975; Lakatta & Lappé, 1981; Kass & Tsien, 1982). Corresponding oscillations of intracellular Ca^{2+} concentration have been directly verified by means of aequorin (Orchard, Eisner & Allen, 1983; Eisner & Valdeolmillos, 1986). The charge-carrying mechanism of the current oscillations, however, is being discussed controversially. Two different current pathways have

been suggested: (i) a Ca^{2+}-activated non-selective cation channel (Kass *et al.* 1978; Colquhoun, Neher, Reuter & Stevens, 1981; Cannell & Lederer, 1986) and (ii) a Ca^{2+}-dependent change of a current generated by electrogenic Na^{+}–Ca^{2+} exchange (Karagueuzian & Katzung, 1982; Arlock & Katzung, 1985; see also Noble, 1984). The major arguments in favour of a Ca^{2+}-activated cation channel result from observations of a reversal potential in the quoted study by Kass *et al.* (1978). More recently Canell & Lederer (1986) observed a reversal of I_{ti} at about -40 mV in isotonic $CaCl_2$ solution. Although their conclusion that under such a condition Na^{+}–Ca^{2+} exchange is absent needs to be substantiated, a contribution of $I_{Na,Ca}$ to I_{ti} under that condition is indeed very unlikely. In the present investigation ionic conditions were used that virtually block all other membrane conductances. Therefore it cannot be excluded that in a more physiological situation more than one current might contribute to I_{ti}. From a previous study on interactions of Ca^{2+} current and sarcoplasmic Ca^{2+} release it might even be possible that in a certain range of membrane potentials Ca^{2+}-dependent inactivation of I_{Ca} contributes to the current changes underlying I_{ti} (Lipp *et al.* 1987). Since in mammalian cardiac muscle Ca^{2+} release from the SR is triggered during each action potential, the current(s) underlying I_{ti} are likely not to be only of pathophysiological significance but to contribute to normal cardiac excitation (Noble, 1984; DiFrancesco & Noble, 1985; Hilgemann & Noble, 1987). From simulations of interactions between I_{Ca}, sarcoplasmic Ca^{2+} release and $I_{Na,Ca}$ these authors propose considerable effects of the latter on the configuration of cardiac action potentials, particularly under non-steady-state conditions such as post-rest stimulation. In order to substantiate these ideas, further experimental work as to the dynamic interactions between the sarcolemmal and sarcoplasmic Ca_i^{2+}-regulating transport systems is necessary.

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