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VOLTAGE DEPENDENCE OF SODIUM–CALCIUM EXCHANGE CURRENT IN GUINEA-PIG ATRIAL MYOCYTES DETERMINED BY MEANS OF AN INHIBITOR

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SUMMARY

1. Spontaneous transient inward currents (I_{ti}) caused by cyclic release of Ca^{2+} ions from the sarcoplasmic reticulum were studied in cultured atrial myocytes from hearts of adult guinea-pigs. K^+ channel currents were blocked by replacing K^+ on both sides of the membrane by Cs^+; the L-type Ca^{2+} current was inhibited by D600.

2. The voltage dependence of peak I_{ti} and the background current displayed distinct outward-going rectification. The I–V curves for both currents approach each other at strongly positive membrane potentials but do not intersect.

3. 3′-4′Dichlorobenzamil (DCB) causes a concentration-dependent inhibition of peak I_{ti} and a shift of the holding current (at -60 to -40 mV) in the inward direction. Inhibition of I_{ti} is half-maximal at a concentration of 30 μM.

4. DCB reduces the outward-rectifying component of both peak I_{ti} and the background current. The I–V curves of the control and DCB-inhibited currents intersect at *ca.* $+10$ mV (peak I_{ti}) and negative to -75 mV (background current), suggesting the reversal potential of the DCB-inhibited current to be shifted by *ca.* 85 mV in the positive direction if Ca_i^{2+} rises following Ca^{2+} release.

5. The voltage dependence of the DCB-inhibited currents is highly compatible with the concept of Na^+–Ca^{2+} exchange being the charge-carrying mechanism of the outward-rectifying background current. Ca^{2+} release from the SR alters the I–V curve of this current according to the shift of the thermodynamic driving force.

INTRODUCTION

One major mechanism regulating intracellular Ca^{2+} concentration (Ca_i^{2+}) and thereby contractile strength developed by cardiac muscle cells is Na^+–Ca^{2+} exchange, a counter-transport which moves Na^+ and Ca^{2+} in opposite directions across the sarcolemma (for reviews see Eisner & Lederer, 1985; Sheu & Blaustein, 1986). This transport system, which was discovered in cardiac muscle about 20 years ago (Reuter & Seitz, 1968), is not directly linked to hydrolysis of energy-rich phosphates. The magnitudes and possibly the directions of the net ionic fluxes via this system depend

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on the transmembrane gradients of the two ion species involved and membrane potential (E_m). The latter results from the stoichiometry which is commonly accepted as $3(Na^+):1(Ca^{2+})$ (e.g. Reeves & Hale, 1984). For any coupling ratio different from $2(Na^+):1(Ca^{2+})$, activity of the exchanger causes a net flow of charges across the cell membrane: it is electrogenic. Thus, Na^+–Ca^{2+} exchange is not only of relevance as a mechanism of ion regulation but also as a pathway of electric current flow (Mullins, 1979; Noble, 1986). Since two determinants of the driving force of the exchanger, namely Ca_i^{2+} and E_m, undergo dynamic changes during the cardiac cycle, there should be a varying contribution of the exchange current to the membrane currents underlying electrical activity of cardiac cells. In order to understand this function of the exchange current, the knowledge of its dependence on membrane potential and its modulation by variations in Ca_i^{2+} is necessary. Measurements of the voltage dependence of the exchange current in mammalian (Kimura, Miyamae & Noma, 1987; Lipp & Pott, 1988) and frog (Hume & Uehara, 1986) myocytes have been published recently. In these studies an outward rectification of $I_{Na,Ca}$ has been demonstrated. Furthermore in the investigation by Lipp and Pott it was shown that a rise in Ca_i^{2+} following Ca^{2+} release from the sarcoplasmic reticulum causes a transient inward curent, which can be accounted for by a change of $I_{Na,Ca}$ (see also Mechmann & Pott, 1986). A precise determination of the I–V curve of $I_{Na,Ca}$, particularly with regard to the reversal potential, requires a separation from the passive background current. In the present investigation this was attempted by means of 3′-4′dichlorobenzamil, a substance with some antagonistic potency for cardiac Na^+–Ca^{2+} exchange (Siegl, Cragoe, Trumble & Kaczorowski, 1984).

Some preliminary results have been published previously (Lipp & Pott, 1987).

METHODS

Voltage clamp experiments were performed on cultured atrial 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). For the experiments, spherical myocytes ('cardioballs') 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 38 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) on the cells and (iii) to have a rapid Ca^{2+} buffering system, which however has a low buffering capacity (Bechem & Pott, 1985). 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 with the cyclic nucleotide. 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

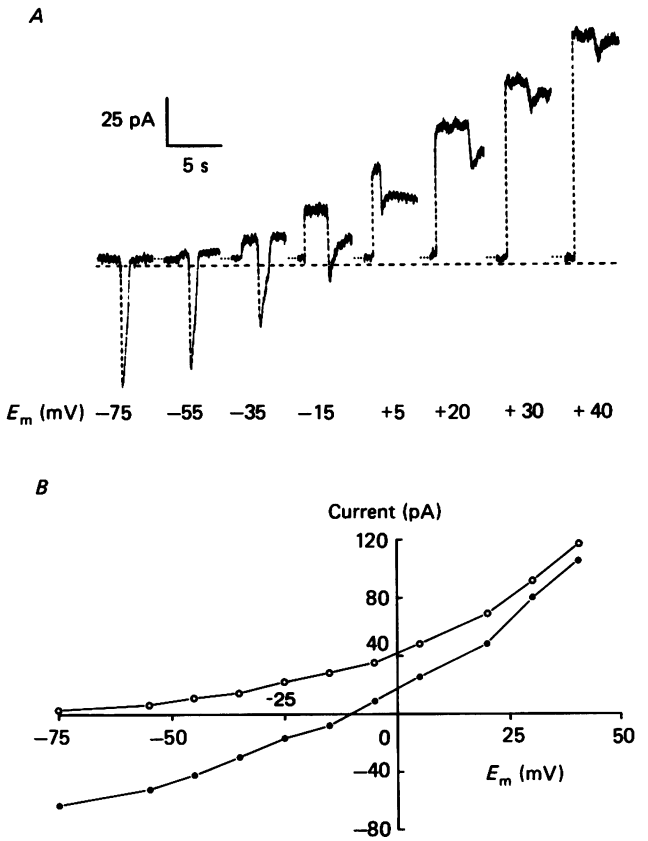

Fig. 1. Voltage dependence of instantaneous current and I_{ti}. *A*, sections of current recordings obtained during clamp steps to the membrane potentials (E_m) indicated (holding potential, −75 mV). The sections show the holding current, the instantaneous current following the voltage step (the fast upstroke has been dashed for graphical reasons), and the beginning of the first I_{ti} at that potential. The dashed horizontal line denotes zero current level. Note the prolongation of I_{ti} with increasing depolarization (cf. Mechmann & Pott, 1986). *B*, plot of instantaneous current (○) and peak I_{ti} (●) from *A* against membrane potential. The lines connecting the data points were drawn by eye.

resistance in the cell-attached configuration was ⩾ 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

Upon dialysis with a solution of the composition listed above atrial myocytes display spontaneous transient inward currents at constant negative membrane holding potentials with usually constant amplitude and frequency for periods of up to 40 min (Lipp & Pott, 1988). Figure 1*A* shows spontaneous I_{ti} recorded at different membrane potentials. The traces illustrate the change of the holding current following a voltage step to the levels indicated and the first I_{ti} after the voltage step.

As we have shown previously the current change is reduced with increasing

depolarization. The current–voltage relations of both the instantaneous current and peak I_{ti} display distinct outward-going rectification and the reversal potential of the total membrane current is shifted from a value negative to −75 mV in that particular experiment to *ca.* −10 mV, i.e. by at least 65 mV, following Ca^{2+} release.

Based on the fact that the two *I–V* curves illustrated in Fig. 1*B* can be accounted for by a simple model for Na^+–Ca^{2+} exchange current (Lipp & Pott, 1988; compare

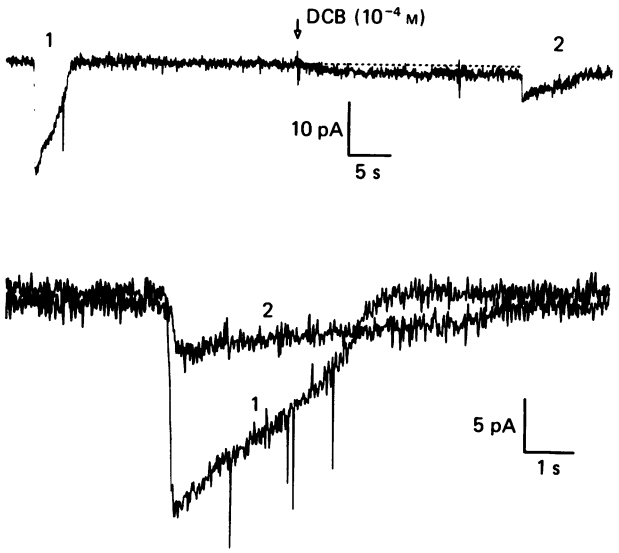

Fig. 2. Effect of DCB (10^{-4} M) on I_{ti} and holding current. The upper trace represents membrane current recorded from a myocyte at a holding potential of −50 mV. At the time indicated by the arrow the substance dissolved in normal extracellular solution was 'puffed' onto the cell from a microcapillary. In the lower panel the two transient inward currents marked 1 and 2 have been superimposed.

DiFrancesco & Noble, 1985) we concluded the background current in the present conditions to be dominated by this current. The shift of the *I–V* relationship following sarcoplasmic Ca^{2+} release can then be predicted by assuming Ca_i^{2+} to rise from a resting level of $5–6 \times 10^{-8}$ M (which corresponds to the Ca^{2+} activity measured in the pipette-filling solution) to a level in the order of magnitude of 10^{-6} M. The theoretical reversal potential ($E_{Na,Ca}$) of the exchange current is given by:

$$E_{Na,Ca} = 3E_{Na} - 2E_{Ca}, \quad (1)$$

with E_{Na} and E_{Ca} denoting the Nernst potentials of the two ion species. For the resting condition ($Ca_i^{2+} = 5–6 \times 10^{-8}$ M) eqn (1) yields a reversal potential between −85 and −89 mV. A shift of the current reversal to −10 mV (Fig. 1*B*) corresponds to a Ca_i^{2+} of $1{\cdot}1 \times 10^{-6}$ M.

Assuming the above consideration to be adequate, application of a compound inhibiting $I_{Na,Ca}$ should have the following results: (i) The outward-rectifying component of the background current should be reduced. At the standard holding potential (−50 mV) the current therefore should be shifted in the inward direction.

(ii) The amplitude of I_{ti} should be reduced. (iii) Since I_{ti} is assumed to reflect an outward transport of Ca^{2+} ions, a reduction of its amplitude should result in a prolongation of its duration. The effect of superfusion of a myocyte with DCB (10^{-4} M) at −50 mV holding potential is illustrated in Fig. 2. (In order to permit a rapid onset of the drug it was puffed onto the cell from a microcapillary, tip diameter *ca.* 10 μm, which was positioned in a distance of about 20 μm from the cell. This may result in effective concentrations not exactly corresponding to those in the capillary.

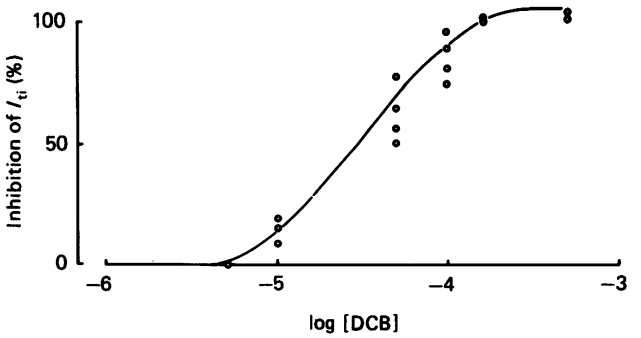

Fig. 3. Concentration–response curve of the inhibition of I_{ti} by DCB. The reduction of I_{ti} amplitude has been plotted against the concentration of DCB in the flushing pipette.

In control experiments performed in the context of a different study it was found that rapid superfusion of a cell using the above device could result in a small ($\leqslant$ 4 pA) apparent shift of the holding current in either direction (four out of seven measurements). Despite these disadvantages we preferred this method of drug application because of its advantage of a very fast onset of action.)

Upon administration of the substance the holding current is shifted in the inward direction by about 3 pA. This change is small and in the range which could also be seen with a drug-free solution, but an inward shift was found consistently in all fifteen cells studied in this way (see also Figs 4, 5 and 6). The amplitude of the transient inward current in the presence of DCB is reduced by 78%. Superposition of I_{ti} in normal external solution and I_{ti} in the presence of DCB reveals a prolongation from 4·3 s to 6·7 s by the drug. In four experiments using DCB at a concentration of 10^{-4} M the prolongation ranged from 1·33- to 1·9-fold.

The concentration dependence of the inhibitory action of DCB on I_{ti} is illustrated in Fig. 3. Although – due to the reduction of both I_{ti} amplitude and an outward current component (see below) – the action of the drug is more complex, we used the reduction of the size of the current transient as a measure to derive the concentration–response curve. The EC_{50} determined this way is *ca.* 30 μM, which is very similar to the EC_{50} found for the inhibition of Na^+-dependent Ca^{2+} uptake in sarcolemmal vesicles (Siegl *et al.* 1984).

The effect of DCB (10^{-4} M) on the current–voltage characteristics of a cell is illustrated in Fig. 4. In *A* the instantaneous currents following voltage steps to various membrane potentials and the beginning of I_{ti} at these membrane potentials

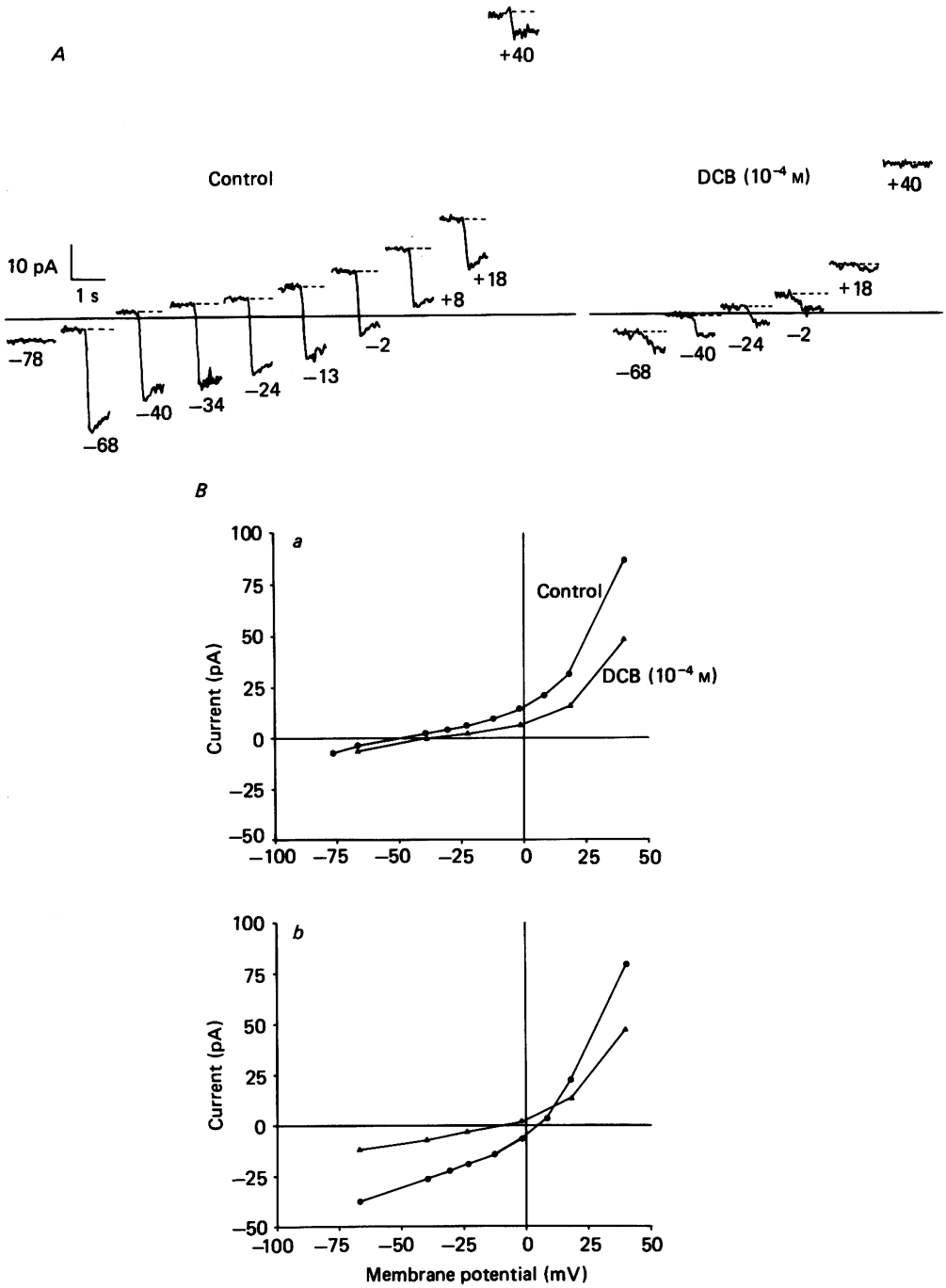

Fig. 4. *A*, effect of DCB on the current–voltage characteristics of a dialysed myocyte. The instantaneous current levels and the beginning of transient inward current at the membrane potentials indicated have been traced (holding potential was −40 mV). At −78 mV (left) no spontaneous I_{ti} occurred in this cell. The continuous line represents zero current level. The traces marked DCB were recorded during continuous superfusion of the cell with the drug. *B*, plot of instantaneous currents (*a*) and peak I_{ti} (*b*) from the experiment illustrated in *A* (●, control; ▲ in presence of DCB). The curves were drawn by eye.

have been traced. After superfusion of the cell with DCB (10^{-4} M) I_{ti} is reduced at each membrane potential. The outward-rectifying component of membrane current is considerably reduced. The I–V curves of peak I_{ti} intersect at a membrane potential of +11 mV (Fig. 4*B*). This membrane potential should correspond to the reversal potential of the current inhibited by DCB. This is valid for one condition, namely a high intracellular Ca^{2+} activity due to sarcoplasmic Ca^{2+} release. For the instantaneous current the two I–V curves approach each other at negative membrane potentials, suggesting a reversal potential negative to −75 mV.

On the basis of previous work (Lipp & Pott, 1988) it is assumed that the instantaneous I–V curve in the present experimental conditions is determined by Na^+–Ca^{2+} exchange current plus a leak current with presumably ohmic properties but unknown magnitude. Since currents through K^+ channels are effectively blocked due to the ionic conditions on both sides of the membrane, classical ('L-type') Ca^{2+} channels are inhibited by D600, and the fast Na^+ current is inactivated at the standard holding potential, DCB is likely to inhibit specifically the current generated by the exchanger. In that case the differences between the control currents and those in presence of the drug should reflect the pure exchange current, uncontaminated by the leak component. This should be valid despite the fact that, as in Fig. 4, the I–V curve in the presence of the drug bears a residual unblocked fraction of $I_{Na,Ca}$, which still causes outward rectification. The difference currents (peak I_{ti} and instantaneous current) have been plotted against membrane potential in Fig. 5. Both difference currents are characterized by their outward-rectifying properties.

The voltage dependence of the current inhibited by DCB is very similar to the voltage dependence of the total membrane current of the myocytes in the particular experimental conditions of the present study, suggesting the latter to be greatly dominated by the DCB-sensitive pathway.

An outward-rectifying current and a positive shift of the reversal potential upon a rise in Ca_i^{2+} are predicted by the formalism, which has been used recently to account for the voltage dependence of Na^+–Ca^{2+} exchange current (DiFrancesco & Noble, 1985; Kimura, Noma & Irisawa, 1986; Kimura *et al.* 1987; Lipp & Pott, 1988):

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

where F/RT has its usual thermodynamic meaning, E denotes membrane potential, k is used as a scaling factor, and σ denotes the fractional distance through the membrane of the limiting energy barrier.

Equation (2) has been used to fit the data in Fig. 5. For the instantaneous current (low Ca_i^{2+}) all the ion activities should correspond to those in the external solution and the dialysing fluid respectively. Thus the data were fitted using: $Na_o^+ = 140$ mM; $Ca_o^{2+} = 2$ mM (the concentration of 2 mM had been multiplied by an activity coefficient of 0·35); $Na_i^+ = 20$ mM (identical activity coefficients for Na^+ on both sides of the membrane); and $Ca_i^{2+} = 6 \times 10^{-8}$ M. The latter value had been determined in the pipette filling solution by means of a Ca^{2+} electrode. In accordance with previous studies (Kimura *et al.* 1987; Lipp & Pott, 1988) for the partition factor a value of *ca.* 0·7 was chosen ($\sigma = 0{\cdot}72$ in the experiment illustrated). The reversal potential for this set of ionic activities is −87 mV. This value is difficult to verify experimentally, because the I–V curves in this voltage range are extremely flat. In order to account

for the current–voltage relationship following Ca^{2+} release, only Ca_i^{2+} has to be changed in eqn (2). The value to be used can be read from the intersection of the two curves in Fig. 4. According to eqn (1), a reversal potential of +10 mV corresponds to an intracellular Ca^{2+} activity of about 3×10^{-6} M. (A slightly better fit was in fact obtained by using $2{\cdot}7 \times 10^{-6}$ M for Ca_i^{2+}.)

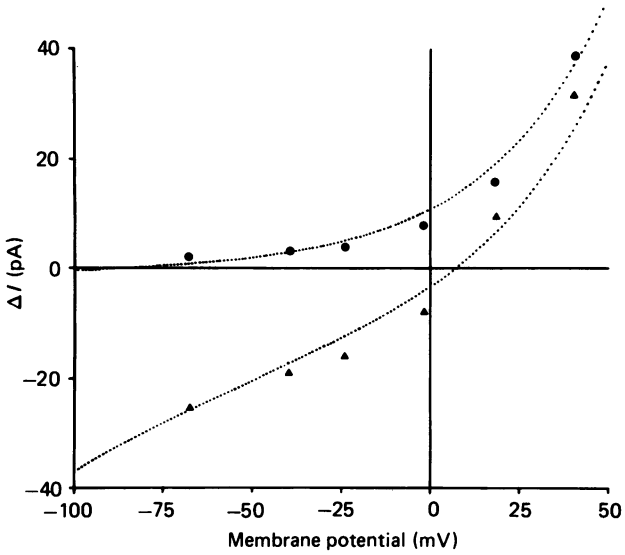

Fig. 5. Current–voltage characteristics of DCB-sensitive current components. The difference currents (control minus current in the presence of the drug) have been plotted *vs.* membrane potential (●, instantaneous current; ▲, peak I_{ti}; same experiment as in Fig. 4). The dotted curves have been calculated using eqn (2). For details see text.

As has been argued previously (Lipp & Pott, 1987), the current named I_{ti} is not a pure inward current but reflects a change of $I_{Na,Ca}$. Thus, I_{ti} can be a current which changes from net outward to net inward at a given membrane potential (e.g. between −75 and +10 mV, according to Fig. 5).

The change from the inward to the outward component of I_{ti} has been suggested to correspond to the transition between the two phases of its relaxation. In that case a complete inhibition of $I_{Na,Ca}$ at the standard holding potential should result in a shift of the holding current towards that current level. Figure 6 illustrates the result of an experiment, where extracellular application of DCB (2×10^{-4} M) caused complete cessation of cyclic I_{ti} activity. After starting superfusion of the cell with the compound, one single spontaneous inward current of much smaller amplitude is recorded. This inward current partly relaxes. The stable holding current after administration of DCB exactly corresponds to the current level where the transition between the two phases of relaxation had occurred in the control situation. Such a correspondence confirms our hypothesis that this current level reflects zero current of the exchanger. In all experiments where I_{ti} did not relax following DCB administration after a short period of time more and more large-conductance ion channels of the type described previously (Mechmann & Pott, 1986; Lipp & Pott, 1987) became active (Fig. 6*B*). This rendered the measurement of the remaining

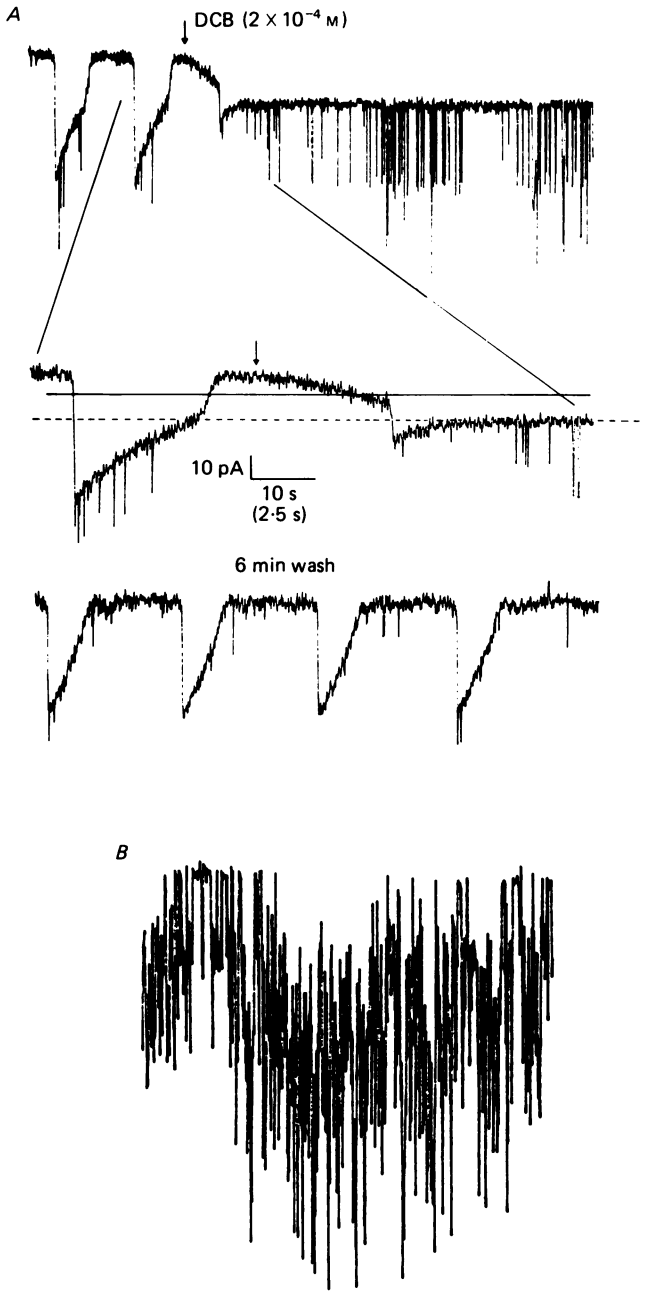

Fig. 6. *A*, complete inhibition of I_{ti} activity by DCB. Membrane current recorded from a myocyte at a holding potential of −60 mV. At the time indicated by the arrow DCB (2×10^{-4} M) was applied as described. The rapid downward deflections represent openings of a large-conductance ion channel, which are inadequately resolved. *B*, increased activity of this channel is illustrated in the trace, which begins 15 s after the end of the top trace of *A*. The continuous line through the expanded (middle) trace represents zero current level. The dashed line was drawn in order to demonstrate the identity of the transition between the two phases of I_{ti} relaxation and the stable current level during superfusion with the inhibitor. The bottom trace was recorded 6 min after removal of the drug-containing pipette from the bath.

(leak) I–V curve impossible. In the experiment shown in Fig. 6 the difference between zero $I_{Na,Ca}$ and the absolute zero current level at −50 mV was 12 pA. Assuming this difference to reflect the pure leak conductance of the cell, the latter is in the order of magnitude of 250 pS.

DISCUSSION

In agreement with previous studies (Mechmann & Pott, 1986; Lipp & Pott, 1988) spontaneous transient inward currents caused by cyclic Ca^{2+} release from the SR can be shown to reflect changes of $I_{Na,Ca}$. The change of $I_{Na,Ca}$ can be quantitatively accounted for by a corresponding shift of the thermodynamic driving force. In the experimental conditions of the present study the 'background' current–voltage characteristics of the cardiac cells is dominated by the exchange current contaminated by a leak conductance, the latter being dependent on the properties of the cell membrane after dialysis with the artificial internal solution and the patch seal resistance. Under optimal recording conditions the contribution of the leak pathway to the total I–V curves is small and does not affect its shape very much. It causes, however, an error in the estimate of the reversal potential of the exchanger and of its shift following Ca^{2+} release. One way to measure the reversal potential of $I_{Na,Ca}$ for a given set of ionic conditions is by partial inhibition of this current. The inhibited fraction of current should bear the voltage-dependent properties of $I_{Na,Ca}$, including the reversal potential.

Inhibitory actions on Na^+–Ca^{2+} exchange have been described for various substances. DCB, an analogue of amiloride, has been described as inhibiting the exchanger in cardiac sarcolemmal vesicles with an EC_{50} of 17 μM. In a recent investigation of the action of DCB in isolated frog myocytes a slightly lower EC_{50} was found (Bielefeld, Hadley, Vassilev & Hume, 1986). In that study it was also shown that DCB in the same range of concentrations blocks the classical Ca^{2+} current and the delayed outward K^+ current. Since in our experiments K^+ channel currents are effectively blocked by replacing K^+ on both sides of the membrane by Cs^+, and L-type Ca^{2+} current is inhibited by D600, effects due to an action of DCB on these current systems can be safely excluded. As an analogue of amiloride DCB is likely to inhibit Na^+–H^+ exchange, which is supposed to be a major mechanism for controlling intracellular pH (pH_i, e.g. Deitmer & Ellis, 1980). Activity of this exchange transport depends on intracellular pH. It is apparently 'switched off' at levels of intracellular pH $\geqslant$ 7·2 (Kaila & Vaughan-Jones, 1987). Thus, in the present conditions (pH_i = 7·4) changes of intracellular Na^+ caused by inhibition of Na^+–H^+ exchange are very unlikely. A reduction of I_{ti} amplitude would be observed if DCB interfered with the sarcoplasmic Ca^{2+} release mechanism reducing the intracellular Ca^{2+} transient. Such an effect, however, would hardly account for the increased duration of I_{ti} nor would it explain the shifts of the I–V curves observed in the present study.

The current–voltage relation of both the 'background' current and of peak I_{ti} have recently shown to reflect $I_{Na,Ca}$ at two levels of Ca_i^{2+}, namely $5–6 \times 10^{-8}$ M, which corresponds to the Ca^{2+} activity of the dialysing fluid, and *ca.* 10^{-6} M following Ca^{2+} release from the sarcoplasmic reticulum. This assumption is confirmed by the

present results. The DCB-inhibited current can be described by the same formalism which has been used previously (see also Kimura *et al.* 1987). Since eqn (2) describes the current for both levels of Ca_i^{2+} only on the basis of the thermodynamic driving forces, without an additional term to account for the Ca^{2+} affinity of an intracellular binding site (cf. DiFrancesco & Noble, 1985), the system seems to operate close to V_{max} conditions for Ca_i^{2+}. This is in agreement with the recent investigation by Kimura and co-workers, who found a maximal stimulation of the outward exchange current (carrying Ca^{2+} into the cell) by raising Ca_i^{2+} from $\leqslant 10^{-9}$ M to about 8×10^{-8} M. On the other hand in cardiac sarcolemmal vesicles much lower affinities have been determined. The lowest K_D values for Ca_i^{2+} reported in those studies are in the micromolar range (for literature see Philipson, 1985; Carafoli, 1987). Assuming a K_D of 1 μM, the current carried by the exchanger at the resting Ca_i^{2+} of the present investigation ($5–6 \times 10^{-8}$ M) should be only *ca.* 10% of the corresponding current determined by the driving force only. After a step change of membrane potential to a level where the driving force is outward also for increased levels of Ca_i^{2+}, a sudden rise of Ca_i^{2+} should cause a transient outward change in membrane current by such a catalytic mechanism (compare Eisner & Lederer, 1985). The fact that at strongly positive membrane potentials Ca^{2+} release never evoked a change of the membrane current in the positive direction further argues against a K_D as high as 1 μM in the conditions used here. At present we have no explanation for this discrepancy. It might point to the possibility of a regulatory mechanism, acting on the affinities of the exchanger for the ions involved, which is lost in vesicular preparations. A complex Ca^{2+}–calmodulin-dependent regulation has in fact been described as influencing Ca^{2+} affinity of the exchanger in sarcolemmal vesicles (Caroni & Carafoli, 1983). The lowest K_D found in that study, however, is still in the micromolar order of magnitude.

Although DCB and related substances can be used to evaluate the voltage-dependent properties of the exchange current under conditions where all other currents relevant to cardiac excitation are effectively blocked, its use as a tool is limited. Due to its lack of specificity (e.g. Bielefeld *et al.* 1986) it cannot be employed in order to obtain information as to the contribution of $I_{Na,Ca}$ to normal cardiac electrical activity. To study this problem, and to test experimentally recent elaborate models of cardiac action potentials (DiFrancesco & Noble, 1985; Hilgemann & Noble, 1987), a highly selective inhibitor is strongly desired.

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