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TURNOVER RATE OF THE CARDIAC Na^+–Ca^{2+} EXCHANGER IN GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. Single guinea-pig ventricular myocytes were voltage clamped using the whole-cell configuration of the patch-clamp technique and membrane current generated by the Na^+–Ca^{2+} exchange mechanism recorded.

2. Rapid increases in cytosolic free calcium ($[Ca^{2+}]_i$) evoked by flash photolysis of either nitr-5 or DM-nitrophen resulted in current relaxations, arising from a redistribution of exchanger carrier conformations induced by the changes in $[Ca^{2+}]_i$.

3. Relaxation time constants were temperature dependent with a temperature coefficient over a 10 °C range (Q_{10}) of ~ 3 and also voltage dependent, decreasing on hyperpolarization for membrane potentials in the range +40 to −80 mV.

4. The experimental results are consistent with consecutive exchange models having electrogenic Na^+ translocation steps, together with a site density and turnover rate similar to that for the Na^+–K^+ pump.

INTRODUCTION

The sarcolemmal Na^+–Ca^{2+} exchanger (Allen, Noble & Reuter, 1989; Nicoll, Longoni & Philipson, 1990) supports a wide variety of cellular processes by extruding cytosolic free calcium ($[Ca^{2+}]_i$) following Ca^{2+} influx associated with membrane excitation. However, quantitative analysis of exchange function has been hindered by the lack of experimental data on exchanger turnover rates. Here we report direct measurements of carrier kinetics by recording exchanger current relaxations induced by rapid increases in cytosolic free calcium ($[Ca^{2+}]_i$) evoked by flash photolysis of caged calcium (Gurney, Charnet, Pye & Nargeot, 1989; Kaplan, 1990) in voltage-clamped heart cells. We provide evidence that the observed current relaxations arise from a redistribution of carrier conformations induced by the rapid changes of $[Ca^{2+}]_i$. Relaxation time constants (τ_r) were temperature dependent with a temperature coefficient over 10 °C (Q_{10}) of ~ 3 and also voltage dependent, with τ_r^{-1} showing an e-fold change per 117 mV. Our results are consistent with consecutive exchange models in which the primary electrogenic step is associated with Na^+

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translocation (Hilgemann, Nicoll & Philipson, 1991) and from our relaxation τs we conclude that the sum of ion transfer rates is $< 75\ s^{-1}$ at -40 mV and 26 °C, with a site density of $> 1235\ \mu m^{-2}$. These observations suggest that the turnover number of the Na^+–Ca^{2+} antiporter is similar to those of ion-motive ATPases and related membrane transporters.

METHODS

Ventricular myocytes were isolated from guinea-pig hearts, obtained from animals killed by cervical dislocation after stunning, by established procedures involving retrograde perfusion with collagenase and protease (Powell, Terrar & Twist, 1980). Cells were stored at room temperature in Dulbecco's modified Eagle's medium, as detailed previously (Noma, Shioya, Paver, Twist & Powell, 1991). Membrane current and voltage were recorded with a voltage-clamp amplifier (Axopatch 1-*C*, Axon Instruments, Foster City, CA, USA) using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981); currents were stored at 20 kHz on a DAT recorder and subsequently sampled at 7 kHz for computer analysis. Pipette solution contained (mM): tetraethylammonium chloride, 5; aspartate, 71; MgATP, 10; $MgCl_2$, 1·8; Hepes buffer, 5; pH 7·2 with CsOH. To this was added the tetrasodium salt of nitr-5 (5 mM) or DM-nitrophen (3·6 mM). Superfusing solution contained (mM): NaCl, 140; $CaCl_2$, 2; $MgCl_2$, 2; $BaCl_2$, 2; Hepes buffer, 5; nicardipine, 0·004; ouabain, 0·05; ryanodine, 0·01; pH 7·4 with NaOH.

The experimental protocol involved holding the cell at -80 mV for 5–10 min for adequate cell dialysis to occur and then applying 40 ms pulses at 5 s intervals to the required test potential from -40 mV until stable Na^+–Ca^{2+} exchange current ($I_{Na,Ca}$) was recorded. As shown previously (Noma *et al.* 1991), under these experimental conditions steady-state $[Ca^{2+}]_i$ is determined by the exchanger and at -40 mV was calculated to be $\sim 1\ \mu M$ for nitr-5 and ~ 420 nM in the case of DM-nitrophen. Using the known binding constants for these caged compounds (Adams, Kao, Grynkiewicz, Minta & Tsien, 1988; Kaplan, 1990), nitr-5 will then be 84% loaded with Ca^{2+} and 2·7% loaded with Mg^{2+}, whereas the corresponding values for DM-nitrophen are 39% for Ca^{2+} and 61% for Mg^{2+}. Photolysis was effected by a xenon flashlamp coupled to the rear port of an inverted microscope and directed to the cell under study through a microscope objective (×40, numerical aperture 1·25) via a 400 nm dichroic mirror (Kozlowski, Twist, Brown & Powell, 1991). We estimated $[Ca^{2+}]_i$ following photolysis of nitr-5 by using ramp pulses to measure the reversal potential of $I_{Na,Ca}$ (determined as Ni^{2+}-sensitive current; Kimura, Miyamae & Noma, 1987) 300 ms after a flash, and in five cells mean (±S.E.M.) postflash $[Ca^{2+}]_i$ was $2{\cdot}42 \pm 0{\cdot}77\ \mu M$. Care was taken to minimize electrical artifacts generated by the flashlamp, but on occasion transients of variable magnitude and duration were observed (see the Ni^{2+} trace in Fig. 1*A* for an example). We compared difference currents obtained as described above with those derived from net currents after flashes with and without Ni^{2+} and in a minority of cells discarded records exhibiting large transients. This may explain in part why we have not observed the conformational currents reported using the caged Ca^{2+} probe DM-nitrophen, together with the fact that such currents are measured optimally at 20 °C in Mg^{2+}-free solutions after treating cells with 3′,4′-dichlorobenzamil (Niggli & Lederer, 1991).

RESULTS

When an assembly of membrane-embedded ion pumps is perturbed by a sudden change of substrate concentration, the state variables of the system approach a new steady-state distribution and the transient current associated with this relaxation process contains information on the microscopic parameters of the transport cycle (Läuger, 1991). To exploit this approach, single guinea-pig ventricular myocytes were loaded with the photolabile Ca^{2+} chelators nitr-5 or DM-nitrophen, via a perfused pipette under the whole-cell patch-clamp recording configuration and in a bathing solution designed to block membrane current components other than that generated by the Na^+–Ca^{2+} exchanger ($I_{Na,Ca}$; see Methods). Equilibration of the intracellular medium with the pipette solution was monitored by the gradual

development of a Ni^{2+}-sensitive membrane current and once a steady state had been reached a flash of UV light induced a rapid inward shift of the holding current (Fig. 1*A*).

Theoretically, assuming that the cardiac Na^+–Ca^{2+} exchanger moves Na^+ and Ca^{2+} in separate steps, both of which have been reported to be electrogenic (Hillgeman *et*

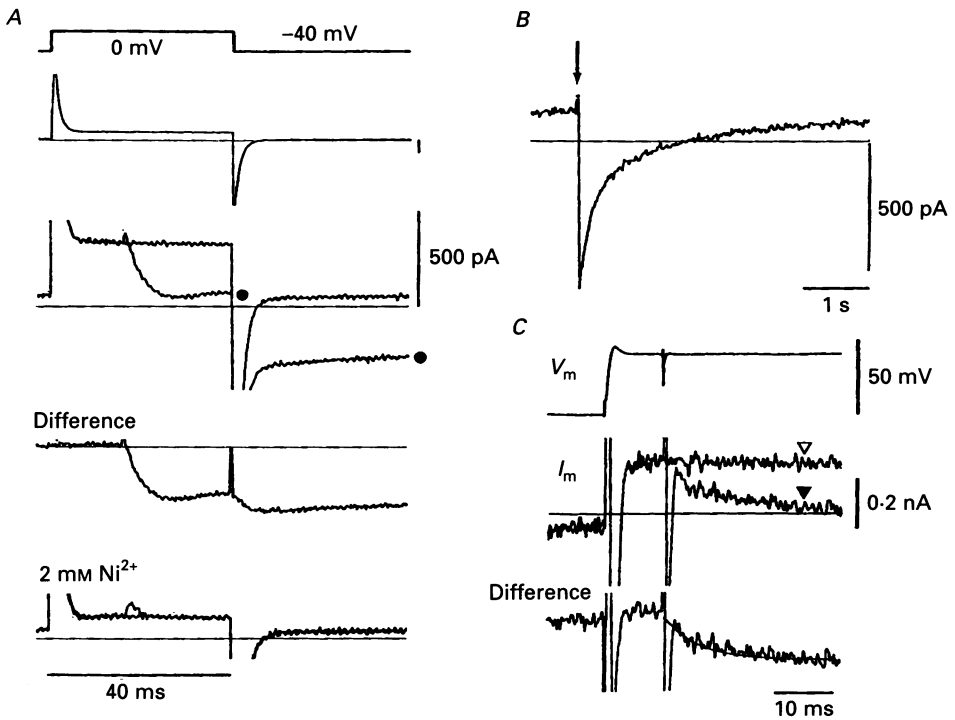

Fig. 1. Voltage-clamp records at fast (*A*) and slow (*B*) time bases during flash photolysis of nitr-5 in guinea-pig heart cells at 26 °C. *A*, the top records are membrane potential and current as a 40 ms test potential was applied to 0 mV from a holding potential of −40 mV. This procedure evoked capacitative transients shown in the second record. Below this, at a much higher gain, are shown two current records, one during a control pulse and the other (●) a typical response to photolysis of nitr-5 evoked 20 ms after the onset of the test pulse. Subtraction of these records resulted in a difference current which develops with a τ_r of 4·6 ms to an amplitude of 330 pA. The final panel shows superimposed currents recorded using the same protocol as above, but in the presence of 2 mM Ni^{2+}. Cell capacitance, 87 pF. *B*, this record was taken from the same cell as in *A* and the current decayed with a multi-exponential time course following photolysis (at arrow), the major initial component having a τ_r of 0·8 s on repolarization to the holding potential. *C*, dual-pipette voltage-clamp experiment. Two patch electrodes were attached to a single cell, one for voltage recording (V_m) and the other for current injection (I_m). Photolysis was effected as described in *A* and caused a rapid transient voltage artifact. Subtraction of the evoked current record (▼) from that recorded under control conditions (▽) resulted in a difference current (bottom record), which could be fitted by a single exponential having a τ of 5·8 ms.

al. 1991; Niggli & Lederer, 1991), relaxation currents should arise following photorelease of Ca^{2+} as exchanger binding sites re-orientate and generate current as net charge moves through the membrane electrical field. In the example shown in

Fig. 1*A*, the holding current at 0 mV increases rapidly inward from the preflash level by some 330 pA, before declining slowly ($\tau \sim 0{\cdot}8$ s; Fig. 1*B*) on returning to the holding potential of −40 mV. The time integral for the flash-induced current indicated that the amount of Ca^{2+} pumped out of the cell following photolysis of nitr-5 was $3{\cdot}98 \pm 0{\cdot}31$ fmol (mean ± S.E.M., $n = 57$). Assuming a specific membrane capacitance of 1 μF cm^{-2} and an external surface/volume ratio of 0·47 μm^2 μm^{-3} for cardiac myocytes (Severs, Slade, Powell, Twist & Warren, 1982) then since mean cell capacitance in these experiments was measured as 151 ± 42 pF ($n = 65$), the total Ca^{2+} released is ~ 120 μM. Corresponding values for DM-nitrophen were $0{\cdot}32 \pm 0{\cdot}03$ fmol ($n = 26$) and 10 μM, respectively. It is likely that the less efficient release of Ca^{2+} from DM-nitrophen is due to the significant affinity of this caged compound for Mg^{2+}, resulting in reduced Ca^{2+} binding (see Methods). Since we did not wish to use Mg^{2+}-free solutions and, in addition, there is the possibility of Ca^{2+} spikes being generated by partly loaded DM-nitrophen (Zucker, 1993), the majority of our experiments were conducted using nitr-5. However, peak $I_{Na,Ca}$ was similar following photolysis of either nitr-5 (225 ± 18 pA, $n = 54$) or DM-nitrophen (212 ± 21 pA, $n = 29$), indicating that the current amplitude is saturated.

Subtracting the ion-jump-induced current from that recorded during the voltage-clamp pulse preceding photolysis revealed a difference current, which relaxed with a τ_r of ~ 5 ms (middle panels, Fig. 1*A*). Since such a response was not observed either when nitr-5 or DM-nitrophen was omitted from the dialysing solution or in the presence of 2 mM Ni^{2+} (bottom panel, Fig. 1*A*) we conclude that the change in current is due to rapid activation of $I_{Na,Ca}$ evoked by photoreleased Ca^{2+}. In the presence of Ni^{2+} it was also possible to check the electrical noise level generated by the UV flash and it was clear (Fig. 1*A*) that the artifact was small compared to the amplitude of the flash-induced increase in $I_{Na,Ca}$. To exclude the possibility of inadequate voltage control, the photolysis protocol was repeated using a dual-pipette voltage clamp, employing separate electrodes for potential recording and current injection. Under these conditions, $I_{Na,Ca}$ relaxations were observed similar in time course to those measured with only a single patch pipette (Fig. 1*C*).

The difference currents shown in Fig. 2*A* reveal that the Ca^{2+}-induced relaxations were dependent on both membrane potential and temperature. Representative records indicate that, on photorelease of Ca^{2+}, $I_{Na,Ca}$ relaxes with a single τ_r under a variety of experimental conditions. At 26 °C, $I_{Na,Ca}$ relaxed with a τ_r of $\sim 2{\cdot}5$ ms at a test potential of −80 mV and ~ 5 ms at 0 mV. On warming to 30 °C, $I_{Na,Ca}$ kinetics became faster at both membrane potentials with τ_rs of ~ 1 and ~ 3 ms, respectively. Data obtained from forty-eight cells are summarized in Fig. 2*B* and illustrate that τ_r is highly temperature dependent, having a Q_{10} of 2·6 at 0 mV and 3·3 at −80 mV, similar to values reported elsewhere (Kimura *et al.* 1987) for steady-state $I_{Na,Ca}$.

A stringent test can be applied to our experimental results by adopting a specific model for the exchanger, since simulations can then be performed to determine the effects of Ca^{2+} concentration jumps and quantitative predictions can be made concerning the relationship between relaxation and turnover rates. We used a simple consecutive 'two-step' transport model in which Ca^{2+} and Na^+ translocations arise through serial molecular rearrangements involving two membrane-crossing transitions (Fig. 3*A*) with the assumptions that the ion-binding reactions are

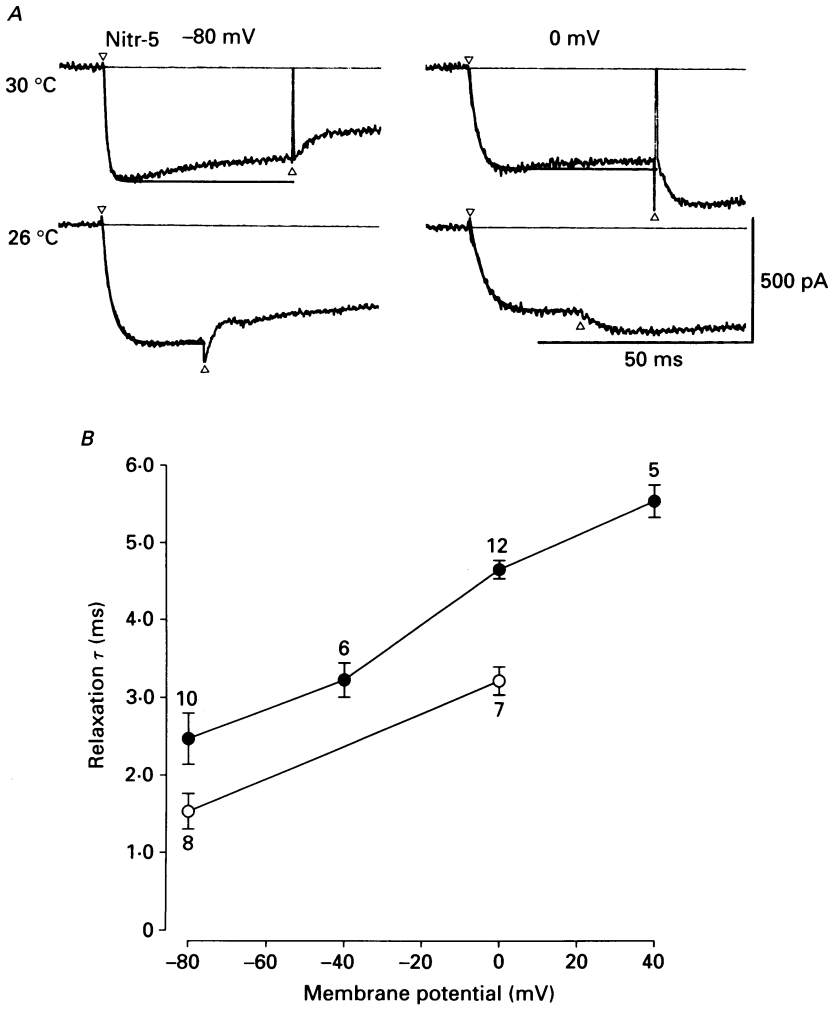

Fig. 2. Responses of cardiac $I_{Na,Ca}$ to intracellular ion jumps of Ca^{2+}. *A*, records are difference currents obtained separately from 4 cells as outlined in Fig. 1, with test potentials of −80 mV shown in the left column and 0 mV on the right. Photolysis occurred as indicated (▽) and cells were returned (△) to the holding potential (−40 mV) after 40 or 25 ms. Zero current is shown as the thin horizontal line and superimposed on each record is the least-squares fit used to calculate τ_r. Photolysis of nitr-5 at 30 °C (top panels) evoked activation of $I_{Na,Ca}$ with a τ_r of 1·03 ms at −80 mV to a final amplitude (I_p) of 451 pA in a cell of capacitance (C_m) 145 pF. At 0 mV, τ_r increased to 2·88 ms in another cell (I_p = 402 pA, C_m = 114 pF). On cooling to 26 °C (bottom panels), τ_r was 2·54 ms (I_p = 476 pA, C_m = 92 pF) and 4·92 ms (I_p = 335 pA, C_m = 115 pF) at the two test potentials. *B*, voltage and temperature dependencies of $I_{Na,Ca}$ relaxation kinetics following photorelease of Ca^{2+}. Mean values (±S.E.M.) are shown for τ_r recorded using nitr-5 at 26 (●) and 30 °C (○), with the number of cells given for each condition.

instantaneous (Hilgemann *et al.* 1991) and an exchanger stoichiometry of $3Na^+:1Ca^{2+}$. Under these conditions, the exchanger can be represented by a lumped two-state model (Fig. 3*B*) with forward and backward rate constants α and β such

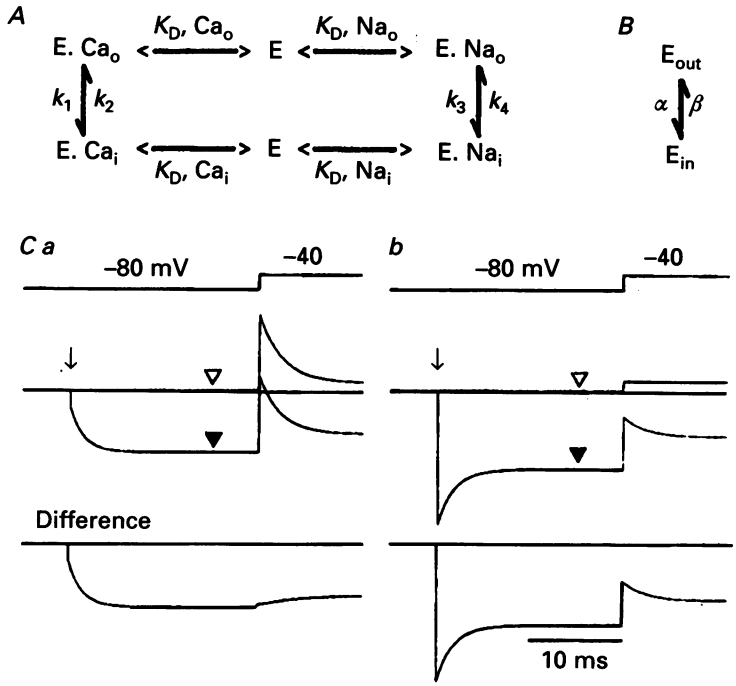

Fig. 3. *A*, a consecutive model of the Na^+–Ca^{2+} exchanger. We assumed that binding of Ca^{2+} or Na^+ to the carrier (E) is virtually instantaneous, with the dissociation constants ($K_{D,X_{o,i}}$ for ion X) indicated, and that the membrane-crossing transitions are rate limiting. E_{in} and E_{out} represent ($E.Ca_i + E + E.Na_i$) and ($E.Ca_o + E + E.Na_o$), respectively, while the *k*s are the rate constants for the Ca^{2+} and Na^+ transfer steps. Binding site occupancy ($P_{ENa_{i,o}}$, $P_{ECa_{i,o}}$) is given by terms of the form

$$P_{ENa_o} = 1/(1+(1+Ca_o/K_{D,Ca_o})(K_{D,Na_o}/Na_o)^3),$$

$$P_{ECa_o} = 1/(1+(1+(Na_o/K_{D,Na_o})^3(K_{D,Ca_o}/Ca_o)).$$

Assuming the Na^+ translocation step is electrogenic,

$$I_{Na,Ca} = (Ne)(k_4 E_{in} P_{ENa_i} - k_3 E_{out} P_{ENa_o}) = Ne\,k_T,$$

where *e* is the elementary charge, *N* the total number of exchangers per cell and k_T the turnover rate. *B*, lumped two-state model based on that shown in *A*, in which a pair of inward and outward rate constants (α, β) are given by

$$\alpha = k_3 P_{ENa_o} + k_1 P_{ECa_o} = h_3 + h_1,$$

$$\beta = k_4 P_{ENa_i} + k_2 P_{ECa_i} = h_4 + h_2,$$

and the relaxation time constant $\tau_r = (\alpha+\beta)^{-1} = (h_1+h_2+h_3+h_4)^{-1}$, where all $h_i > 0$. Under these conditions, $k_T = (h_1 h_4 - h_2 h_3)\tau_r$, whose upper bound is

$$k_T < (K/(1+K)^2)\tau_r^{-1} < (4\tau_r)^{-1},$$

where $K = (h_1+h_3)/(h_2+h_4) = (E_{in}/E_{out})$. *C*, theoretical time courses of Ca^{2+} relaxations evoked by an instantaneous jump of $[Ca^{2+}]_i$ induced at the arrow (↓). In *Ca* are shown simulations of $I_{Na,Ca}$ responses when Na^+ translocation is electrogenic (see *B* above). The difference current (lower panel) was obtained by subtracting a control response (▽) from that following photolysis (▼). The simulations in *Cb* were obtained by assigning electrogenicity to the Ca^{2+} translocation step.

that $\tau_r = (\alpha+\beta)^{-1}$. Using this model, we could satisfactorily simulate the experimental results by assuming that photorelease of Ca^{2+} produced a rapid increase in the Ca^{2+}-bound form of the carrier ($E.Ca_i$, see Fig. 3*A*) and by attributing electrogenicity to the Na^+ translocation step (Fig. 3*Ca*). With these assumptions it

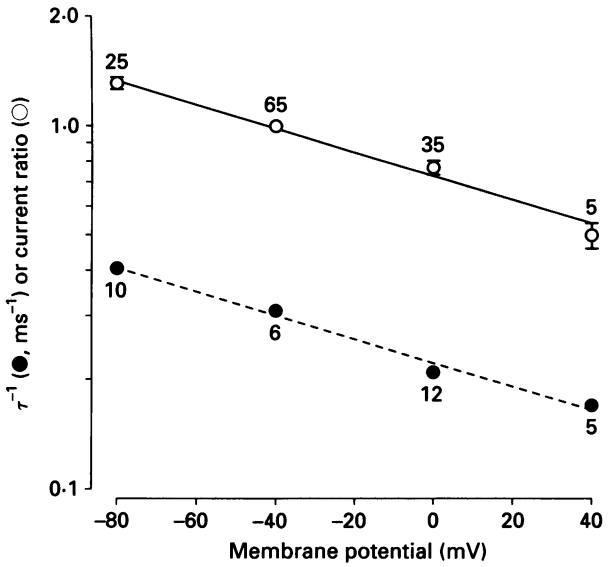

Fig. 4. Variation of steady-state $I_{Na,Ca}$ and τ_r^{-1} with membrane potential. Experimental values (means ± S.E.M.) for $I_{Na,Ca}$ at the test potentials indicated were normalized to those at −40 mV (○) and the data analysed by linear regression and one-way analysis of variance. Normalized current ($\bar{I}$) showed a highly significant ($P < 0{\cdot}01$) regression with voltage (V) such that $\log \bar{I} = (-0{\cdot}0032 \pm 0{\cdot}0012)\,V - (0{\cdot}133 \pm 0{\cdot}014)$ as represented by the continuous line. The values of τ_r^{-1} (●) were calculated from the means of the data shown in Fig. 2*B* for nitr-5 at 26 °C and show an excellent fit to the dashed line, which was drawn with a slope identical to that calculated for normalized $I_{Na,Ca}$.

is possible to estimate the turnover rate (k_T) of the exchanger from the experimental relaxation τ_rs, since an upper bound for k_T is given by $k_T < (4\,\tau_r)^{-1}$. Alternatively, if the assumption is made that Ca^{2+} translocation is the primary electrogenic step, an instantaneous jump of $[Ca^{2+}]_i$ results in a temporary accumulation of $E.Ca_i$ states, which in this case produces a rapid transient current followed by an exponential decay (Fig. 3*Cb*), a response which is not observed experimentally. Our results are thus not consistent with the conclusion that Ca^{2+} translocation involving net negative charge movement (Niggli & Lederer, 1991) is the major mechanism generating $I_{Na,Ca}$.

Analysis of the data shown in Fig. 2*B* for τ_r at 26 °C reveals a voltage dependence such that τ_r^{-1} increases e-fold per 117 mV hyperpolarization, which correlates almost identically with the variation of k_T with potential, estimated by taking the ratio of current amplitudes of the steady-state flash-induced $I_{Na,Ca}$ during and after stepping to the required test potential (Fig. 4). According to Eyring rate theory, the product of charge moved (z) during the presumed Na^+ translocation step following Ca^{2+} release and δ, the partition parameter representing the position of the energy barrier

in the electric field, is $z\delta = 0{\cdot}23$. This is consistent with either $+0{\cdot}46$ charges moving over a symmetric energy barrier (Niggli & Lederer, 1991) or one charge moving in a rate-limiting step possibly located at the extracellular end of the Na^+ translocation pathway (Hilgemann *et al.* 1991).

DISCUSSION

Understanding of the physical basis of the electrogenicity of the cardiac Na^+–Ca^{2+} exchanger has progressed rapidly since the demonstration that it was possible to obtain electrophysiological measurements of exchanger current components (Kimura *et al.* 1987). More recently, outward current transients during rapid application of cytoplasmic Na^+ in giant excised patches containing either the cloned or native cardiac exchanger have been interpreted as partial electrogenic reactions through movement of positive charge through the membrane field during outward Na^+ translocation (Hilgemann *et al.* 1991). These results contrast with those of Niggli & Lederer (1991), who suggested that an inward current transient following flash photolysis of caged calcium in guinea-pig ventricular myocytes reflects movement of negative charge through the membrane field during outward Ca^{2+} translocation by the exchanger. The data presented here are clearly consistent with the notion that electrogenicity is associated primarily with Na^+ translocation, although we cannot rule out the possibility that there might be a minor component of voltage dependency arising from Ca^{2+} occlusion/deocclusion, as described recently by Matsuoka & Hilgemann (1992).

Our major purpose in carrying out the present work was to exploit the use of caged compounds for evoking intracellular concentration jumps of Ca^{2+}, in order to estimate both the site density and turnover rate of the exchanger. At -40 mV, τ_r is $3{\cdot}23 \pm 0{\cdot}18$ ms ($n = 6$) at 26 °C (Fig. 2*B*), so that $k_T < 75\ s^{-1}$. Peak flash-induced $I_{Na,Ca}$ at this membrane potential was 225 pA for cells with a mean surface area of $1{\cdot}51 \times 10^4\ \mu m^2$, so that there must be at least 1235 exchangers μm^{-2}, a value similar to that reported for the Na^+–K^+ pump (Jorgensen, 1980). However, if more than one Ca^{2+} ion is exchanged per cycle at a constant $3Na^+:1Ca^{2+}$ stoichiometry, the site density would be reduced proportionately. Using the experimental values for τ_r shown in Fig. 2 with the measured Q_{10}s, we calculate that exchanger turnover rates at -80 mV and 36 °C are $< 300\ s^{-1}$, again of a similar magnitude to those reported for the Na^+–K^+ pump and related ion-motive ATPases (Läuger, 1991).

That our measurements contrast with Na^+–Ca^{2+} exchanger turnover rates of $\sim 2500\ s^{-1}$ estimated from conformational currents in myocytes under Mg^{2+}-free conditions (Niggli & Lederer, 1991) or maximum rates of $\sim 5000\ s^{-1}$ calculated from charge movements generated by the deregulated exchanger in giant excised patches (Hilgemann *et al.* 1991), may reflect subtle control of the exchanger in the intact cell (Hilgemann, 1989, 1990; Collins, Somylo & Hilgemann, 1992). Indeed, it may well be that the description of exchanger cycling in terms of a single rate constant is now misleading, since the electrogenicity of the cardiac exchanger can be broken down into multiple steps involving rearrangement of binding sites, with some charge movement remaining 'hidden' in *I–V* relations (Niggli & Lederer, 1991; Matsuoka & Hilgemann, 1992).

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