Voltage Dependence of Na-Ca Exchanger Conformational Currents

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ABSTRACT Properties of a transient current (I_{conf}) believed to reflect a conformational change of the Na-Ca exchanger molecules after Ca^{2+} binding were investigated. Intracellular Ca^{2+} concentration jumps in isolated cardiac myocytes were generated with flash photolysis of caged Ca^{2+} dimethoxynitrophenamine, and membrane currents were simultaneously measured using the whole-cell variant of the patch-clamp technique. A previously unresolved shallow voltage dependence of I_{conf} was revealed after developing an experimental protocol designed to compensate for the photoconsumption of the caged compound. This voltage dependence can be interpreted to reflect the distribution of Na-Ca exchanger conformational states with the Ca^{2+} binding site exposed to the inside of the cell immediately before the flash. Analysis performed by fitting a Boltzmann distribution to the observed data suggests that under control conditions most exchanger molecules reside in states with the Ca^{2+} binding site facing the outside of the cell. Dialysis of the cytosol with 3′,4′-dichlorobenzamil, an organic inhibitor of the Na-Ca exchange, increased the magnitude of I_{conf} and changed the voltage dependence, consistent with a parallel shift of the charge/voltage curve. This shift may result from intracellular DCB interfering with an Na^+-binding or Na^+-translocating step. These observations are consistent with I_{conf} arising from a charge movement mediated by the Na-Ca exchanger molecules after binding of Ca^{2+}.

INTRODUCTION

The Na-Ca exchanger is a transport protein located in the cell membrane and plays an important role in the Ca^{2+} homeostasis of many different cell types (for review see Allen et al., 1989). Extrusion and uphill transport of Ca^{2+} are performed by the Na-Ca exchange using the inwardly directed Na^+ gradient. Depending on the prevailing electrochemical gradients, the Na-Ca exchange can also run "backward" and import Ca^{2+} into the cell while removing intracellular Na^+. Cells relying on extensive Ca^{2+} signaling across the cell membrane typically exhibit a high degree of Na-Ca exchange function, and such cells have been used as experimental model systems to investigate the properties of this transporter. Examples are cardiac muscle cells and vesicles or giant patches derived from their cell membrane (Kimura et al., 1987; Reeves and Hale, 1984; Philipson, 1985; Hilgemann et al., 1992a), the squid giant axon (Baker et al. 1969; DiPolo and Beaugé, 1990), the outer segment of retinal rods (Yau and Nakatani, 1984; Cervetto et al., 1989), and barnacle muscle fibers (Rasgado-Flores et al., 1989). In cardiac muscle cells, the Na-Ca exchange plays an important role during the relaxation by removing the Ca^{2+} that entered with the Ca^{2+} current (Bridge et al., 1990; Bers and Bridge, 1989). Recently, an important role of the Na-Ca exchange during more rapid events of cardiac excitation-contraction coupling has also been suggested (Leblanc and Hume, 1990; Lederer et al., 1990; Niggli and Lipp, 1993b; Lipp and Niggli, 1994).

A number of biochemical and biophysical methods have been employed to assess the activity of the Na-Ca exchange including radioactive tracer flux measurements (e.g., Baker et al. 1969; DiPolo and Beaugé, 1990; Reuter and Seitz, 1968) and detection of Ca^{2+} concentration changes with fluorescent indicators (e.g., Crespo et al., 1990; Beuckelmann and Wier, 1989; Bers et al., 1990). From these and other studies it is known that the cardiac Na-Ca exchange is electrogenic and has a stoichiometry of 3 Na^+:1 Ca^{2+} (for review see Eisner and Lederer, 1985). The Na-Ca exchanger of rod outer segments was recently found to be a different protein with a stoichiometry of 4 Na^+:1 Ca^{2+} + 1 K^+ (Cervetto et al., 1989; Schnetkamp and Szerencsei, 1991). Making use of the electrogenicity of the exchanger, membrane currents associated with exchange activity have been recorded with the whole-cell variant of the patch clamp technique in isolated cells (e.g., Kimura et al., 1987; Lipp and Pott, 1988; Niggli and Lederer, 1993) as well as in giant membrane patches derived from cardiac myocytes (Hilgemann et al., 1992b; Doering and Lederer, 1993). These techniques provided insight into the cellular function and the steady-state kinetics of the exchanger in different preparations and under a variety of conditions. However, details about the molecular function of the Na-Ca exchanger are still largely unknown.

In principle, information about individual molecular reaction steps of any biochemical reaction sequence can be obtained if the distribution of relevant conformational states can be disturbed rapidly and if at least one parameter reflecting the reequilibration can be measured (Läuger, 1984). The observed transient-state kinetic events then contain information about molecular reaction steps. Given that the Na-Ca exchange encompasses a transport cycle with at least one voltage-dependent rate constant current transients are expected to occur after rapid voltage changes, i.e., immediately after voltage-clamp steps. In combination with a specific inhibitor this approach has revealed detail about molecular reaction steps of the Na^+/K^+ ATPase in cardiac sarcolemma (Nakao and Gadsby, 1986) and in *Xenopus* oocytes (Rakowski, 1993). However, application of this method to the Na-Ca exchange has been difficult for two

Received for publication 4 November 1994 and in final form 30 June 1994.
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0006-3495/94/10/1516/09 $2.00

reasons: 1) no specific inhibitor is available for the Na-Ca exchange that would allow the separation of Na-Ca exchange transients from other transients occurring after a voltage step (e.g., large capacity currents); 2), the Na-Ca exchange apparently has a very fast turnover rate, challenging the temporal resolution of conventional patch-clamp amplifiers used with the whole-cell preparation (Hilgemann et al., 1992a).

At least one rate constant of the Na-Ca exchange cycle is expected to depend on the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ and with rapid intracellular concentration jumps of Ca^{2+} it appeared possible to disturb the steady-state distribution of the molecular states and to induce current transients at fixed membrane potentials (Niggli and Lederer, 1991b). Let us consider, for example, a consecutive transport mechanism for the Na-Ca exchanger that includes two voltage-dependent membrane crossing transitions (E′Ca↔E″Ca and E′3Na↔E″3Na; see Fig. 1 *A*). In addition, the model comprises several Na^+ and Ca^{2+} binding reactions, each lumped into a single step (e.g., E′↔E′3Na). In such a consecutive transporter the concentration-dependent Ca^{2+} association rate would instantaneously increase after a Ca^{2+} concentration jump, whereas all other partial reactions would not change initially. This in turn would lead to a transient increase of Ca^{2+}-bound exchanger states (E′Ca), which enter a membrane-crossing transition. Therefore, a transient current would be predicted, provided the membrane-crossing transition entered by the Ca^{2+}-bound states actually moves charge (as indicated by the negative charge in Fig. 1 *A*) or leads to charge movement in a subsequent step.

We have observed such a transient membrane current after jumps of $[Ca^{2+}]_i$ induced by flash photolysis of the caged Ca^{2+} compound dimethoxynitrophenamine (DM-nitrophen) (Niggli and Lederer, 1991b,c). This transient current (I_{conf}) has been interpreted to reflect a molecular conformational rearrangement after binding of intracellular Ca^{2+} and to involve a membrane-crossing partial reaction of the Na-Ca exchanger other than the rate-limiting transition. This interpretation was based on the observation that in steady-state experiments the voltage dependence of the Na-Ca exchange current was much less steep than expected for a single elementary charge, which is translocated across the entire electrical field in one rate-limiting step (Niggli and Lederer, 1993). From the shallow voltage-dependence of the inward Na-Ca exchange current obtained in this earlier study, we estimated that the apparent charge moved during the rate-limiting step corresponded to only +0.44 elementary charges. An obvious possibility is that the exchange exhibits two (or more) distinct and consecutive membrane crossing transitions that each move part of the total charge, but only one is rate limiting for cycling. Both simultaneous as well as two-step consecutive transport models satisfy this requirement. We have therefore proposed that the remaining charge (−0.56) is moved during a second, more rapid (i.e., not rate-limiting) membrane-crossing transition giving rise to I_{conf} (Niggli and Lederer, 1991b).

In this article we report on a previously unresolved voltage dependence of I_{conf} revealed by implementing a protocol that enabled us to correct for the photoconsumption of DM-nitrophen. Furthermore, addition of intracellular 3′,4′-dichlorobenzamil (DCB), an organic inhibitor of the Na-Ca exchange, had a dramatic effect on the observed voltage dependence. Preliminary results have been presented to the Biophysical Society (Niggli, 1992; Niggli and Lipp, 1993a).

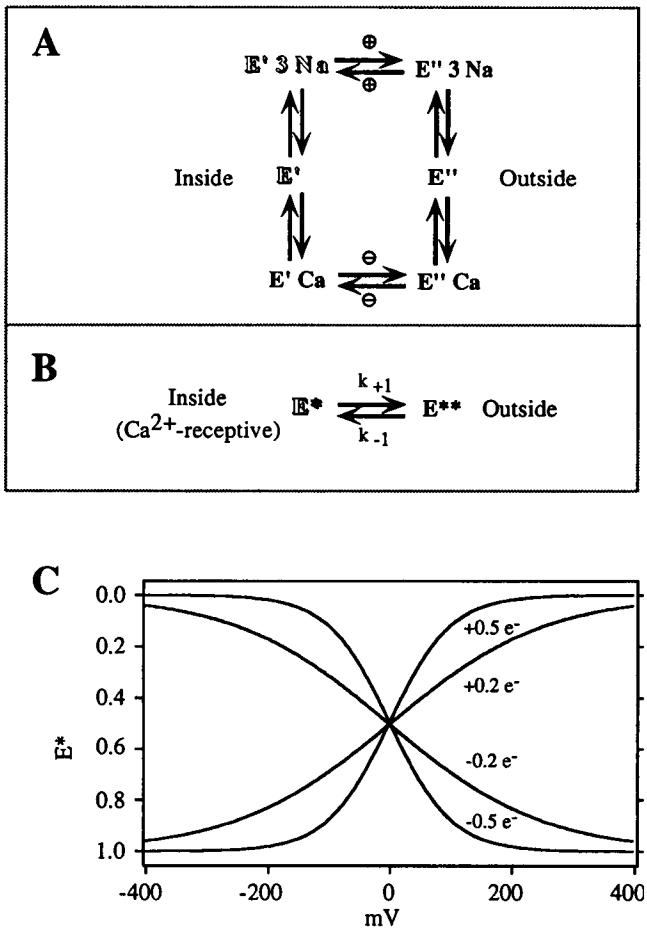

FIGURE 1 Model predictions. (*A*) Consecutive transport model for the Na-Ca exchange. States with the Ca^{2+} binding sites facing the inside (E′) are either $[Ca^{2+}]_i$ receptive (outlined characters) or have already bound Ca^{2+}. None of the states with the binding sites facing the outside are immediately receptive for intracellular Ca^{2+}. Positive charge is moved with the Na^+-translocating step, whereas the charge translocated with Ca^{2+} is negative. (*B*) The model presented in (A) is further simplified into inside $[Ca^{2+}]_i$-receptive states (E*) and outside (E**) states. The two states are connected with voltage-dependent forward (k_{+1}) and backward (k_{-1}) rate constants. (*C*) The predicted voltage dependence of the fraction of E* states is shown for different "virtual" charges (see text for details).

MATERIALS AND METHODS

Voltage clamp

Cells were dialyzed with a filling solution containing DM-nitrophen using patch-clamp pipettes pulled from filamented borosilicate glass (GC 150F, Clark Electromedical Instruments, Reading, UK) on a horizontal puller (BB-CH, Mecanex, Geneva, Switzerland) to a series resistance of 0.8–1.2 MΩ. Membrane currents were measured with a voltage-clamp amplifier with the low-pass filter set to 10 kHz (Axopatch 1D, Axon Instruments, Foster City, CA). The experiments were controlled and data acquired at a sampling rate of 50 kHz with an Apple Macintosh IIcx computer equipped with an NB-MIO-16-H9 multifunction board (National Instruments, Austin, TX).

Custom-made software to drive the board was developed by us with LabView (National Instruments, Austin, TX).

Flash photolysis

Light from a xenon short-arc flash lamp (230 Ws, duration of flash ≈350 μs) was used to photolyze intracellular DM-nitrophen in an epiillumination arrangement (for details see Niggli and Lederer, 1991a). Ultraviolet wavelengths (330–390 nm) were selected by a sequence of three dichroic mirrors in the optical path. The cells were imaged and flashed through a Zeiss Neofluar lens (63x; N.A. 1.25; Carl Zeiss, Zürich, Switzerland). Special care was taken to suppress the electromagnetic interference artifact arising from the capacitor discharge. This required multiple shielding of the experimental setup as well as the flash lamp. Fiber optics was used to trigger the flash, and a liquid light guide (Oriel, Stratford, CT) collected the light and directed it into the patch-clamp setup. The power line was interrupted briefly during the flash to avoid the transmission of voltage spikes through the power line. After the flash, Ca^{2+} is released with a τ of around 30 μs (Vergara and Escobar, 1993); thus, the specified flash duration of ≈350 μs (full width at half-maximal amplitude) is relatively long lasting and becomes rate limiting for the Ca^{2+} concentration jump and for the decay of the current transient of I_{conf} in our setup. During the loading with DM-nitrophen (≥5 min) and between the flash experiments at different test potentials, the cells were held at a membrane potential of 0 mV to elevate the resting $[Ca^{2+}]_i$ slightly (≈200 nM if the Na-Ca exchanger is at equilibrium). This ensures complete loading of DM-nitrophen with Ca^{2+} and eliminates rebinding of photoreleased Ca^{2+} to unphotolyzed DM-nitrophen. Every 30 s (time required to recharge the capacitors in the flash lamp), the cells were clamped to the control potential (−40 mV) or to the test potential. The voltage change was started 500 ms before the flash was triggered and was maintained for 1 s. For details of the experimental protocol see also Fig. 2 *A*.

Cell preparation and solutions

Cardiac ventricular myocytes were isolated from guinea pig hearts with enzymatic methods, as described previously (Niggli and Lederer, 1993). Briefly, guinea pigs of either sex were heparinized (30 U/kg) and killed by cervical dislocation. The heart was quickly removed and perfused with a nominally Ca^{2+}-free solution for 5 min. Collagenase 0.5 mg/ml (Boehringer, Rotkreuz, Switzerland) and protease 0.1 mg/ml (Sigma, Buchs, Switzerland) were added for another 5 min. The ventricular tissue was then cut in small pieces and placed in a gently rotating shaker at room temperature. Cells were harvested from the supernatant. The experimental superfusion solution had the following composition (in mM): NaCl (145), KCl (4), $CaCl_2$ (1), HEPES (10), glucose (10), pH 7.4. To measure I_{conf} CsCl (1), $BaCl_2$, (0.5), and $NiCl_2$ (8) were added. The pipette filling solution contained (in mM): Cs-gluconate (120), TEA-Cl (20), HEPES (10), Na_4-DM-nitrophen (2), $CaCl_2$ (0.5), K-ATP (5), pH 7.2. Reduced glutathione (GSH), (2 mM) was used to protect cellular proteins from the possible adverse effects of photolytic byproducts (Nichols et al., 1990). DCB (1 mM) was added to the pipette filling solution where indicated. Mg^{2+} was omitted from the pipette filling solution to minimize Mg^{2+} binding to DM-nitrophen (Kaplan, 1990). All experiments were performed at room temperature (20–23°C).

Data analysis

Data were analyzed on an Apple Macintosh IIcx computer using Igor software (Wavemetrics, Lake Oswego, OR). The I_{conf} peak amplitude was calculated by determining the mean current of five data points around the most negative sample in the record (measuring interval corresponding to 80 μs). The charge moved was determined (after baseline subtraction) by numerically integrating from the beginning of the current to the point where the baseline was reached again. Boltzmann functions $Q = Q_{max}/(1 - \exp[(V - V_h)/k])$ were fitted to the measured normalized data using the least-squares fitting routine built into Igor without any constraints on the parameters, except where indicated.

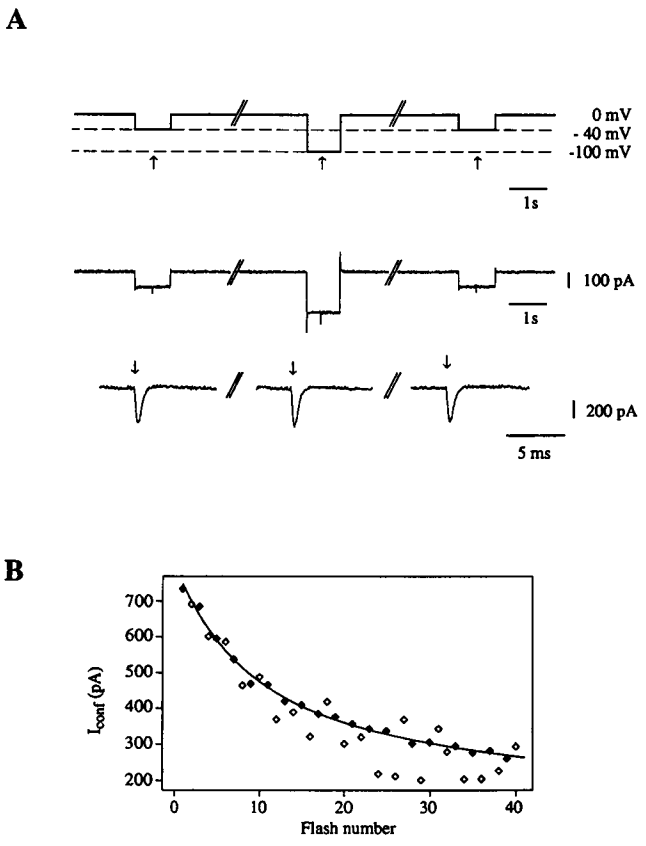

FIGURE 2 Experimental protocol. (*A*) This protocol was used to normalize the current records for cell size and to correct for the photoconsumption of DM-nitrophen. Test flashes at various potentials were bracketed by control flashes at −40 mV. The cell was held at 0 mV. Every 30 s, the membrane potential was clamped to the control potential (−40 mV) or to a variable test potential (−100 mV in the example shown). A flash (↓) was triggered 500 ms after the voltage step, which was maintained for another 500 ms. At higher temporal resolution, I_{conf} is resolved (lower traces).(*B*) Normalization of the peak current was performed by linear interpolation. The line represents a double-exponential function fitted to the control data (◆). Open symbols (◇) show flashes at test potentials. (A) and (B) show data recorded in the presence of intracellular DCB.

RESULTS

Model predictions

Provided the charge movement producing I_{conf} is the result of a conformational change of the Na-Ca exchanger molecules after Ca^{2+} binding, the charge measured during I_{conf} is predicted to reflect the number of Na-Ca exchanger molecules available to bind intracellular Ca^{2+} (Ca^{2+}_i-receptive states) in the steady-state immediately before the flash. As outlined in the introduction I_{conf} is consistent with either a consecutive or a two-step simultaneous transport model, because both types of transporters can move charge across the membrane in two consecutive steps. We have preferred the consecutive over the two-step simultaneous model primarily because it can more easily be reconciled with homoexchange (i.e., Na^+-Na^+ and Ca^{2+}-Ca^{2+} exchange). With the consecutive transport model outlined in Fig. 1 *A* the "empty" inside (E′) states as well as the inside states with Na^+-bound (E′3Na) are rapidly available for binding of intracellular Ca^{2+} because the

exchanger has a low binding affinity for Na^+. Given that preflash intracellular and extracellular Na^+ and Ca^{2+} concentrations do not vary significantly from flash to flash, the amount of charge moved during I_{conf} would correlate with the fraction of Na-Ca exchanger molecules with the Ca^{2+}-binding sites facing the inside of the cell membrane. These Ca^{2+}_i-receptive states are represented with outlined characters in Fig. 1 *A*.

To facilitate the presentation and discussion of the experimental results we reduced the consecutive model further (Fig. 1 *B*). In this simplified two-state scheme the voltage-dependent forward (k_{+1}) and backward (k_{-1}) rate constants connect Ca^{2+}_i-receptive (inside, E*) states with outside states (E**) that cannot bind intracellular Ca^{2+}. The rate constant k_{+1} comprises both transitions moving Na-Ca exchanger binding sites outward (i.e., E′3Na→E″3Na lumped together with E′Ca→E″Ca), whereas k_{-1} includes the two membrane-crossing transitions moving binding sites inward (E″3Na→E′3Na lumped together with E″Ca→E′Ca). It is important to realize that both "simplified" rate constants (k_{+1} and k_{-1}) are composed of two individual "real" rate constants that each have opposite voltage dependencies (because one moves positive charge but the other moves negative charge; see Fig. 1 *A*). Given that the voltage dependence of the steady-state distribution is opposite for these two "real" membrane-crossing transitions, the resulting overall voltage dependence of the steady-state distribution (as modeled by the two "simplified" rates) is impossible to predict without knowing the actual kinetics of the "real" membrane-crossing reaction steps and the population of the states adjacent to these rate constants. The overall voltage dependence can even have a negative slope when the distribution of exchanger states is largely determined by a reaction-translocating negative charge. This phenomenon is illustrated in Fig. 1 *C* where the voltage-dependent distribution predicted by this model is plotted for −0.5, −0.2, +0.2, and +0.5 apparent elementary charges, respectively. In principle, it is also possible that the opposing effects of a voltage change on the two membrane-crossing transitions may counterbalance each other in a limited voltage range and eliminate a detectable voltage dependence of the distribution. In this case, the apparent charge would be 0. Therefore, in a transporter with negative and positive charge moving across the membrane in two parallel steps (i.e., a consecutive transporter in which cycling is inhibited), the voltage-dependence of the steady-state distribution of charge arises from a "virtual charge" and does not allow conclusions about the actual elementary charge moved during any of the involved transitions. A small undershoot after the decay of I_{conf} was sometimes observed, particularly when I_{conf} was large (i.e., in the presence of DCB_i; see, e.g., Fig. 4 *A*). This current could reflect some electrogenic redistribution of Na-Ca exchangers after I_{conf}. Although we do not know the exact basis for this undershoot, we can exclude a significant effect on our analysis because it is slower than I_{conf} and because it has a small size.

In a simple consecutive transporter without additional states (e.g., states due to a narrow access channel or "ion well"), the transition giving rise to I_{conf} would correspond to the Ca^{2+} translocating step moving a fractional negative charge. The inward direction of I_{conf} is inconsistent with the existence of a deep and narrow access channel for Ca^{2+} on the inside, because movement of Ca^{2+} into an empty channel would generate an outward current. However, based on our data we cannot rule out the possibility that a conformational change induced by Ca^{2+}_i binding is followed by a rapid movement of extracellular Na^+ into an extracellular narrow access channel giving rise to I_{conf}. Similar narrow access channels have been proposed to exist in the rod outer segment Na-Ca-K exchanger (Lagnado et al., 1988) and the Na^+-K^+ ATPase (Gadsby et al. 1993; Rakowski, 1993; Hilgemann, 1994).

With each of these possible schemes a voltage dependence of the steady-state distribution of Na-Ca exchanger states may be expected. However, in our previous studies we were not able to reveal a significant voltage dependence of I_{conf} (Niggli and Lederer, 1991b,c). There are several reasons for this. First, the current I_{conf} is relatively small (average peak currents in the absence of DCB_i are around 80 pA with whole-cell membrane noise around 20 pA peak to peak; see Fig. 3 *A*). Secondly, the light-sensitive caged compound present in the cell is gradually photolyzed by repeated flash experiments giving rise to a decline of the responses from flash to flash. Thirdly, the flash intensity may vary from flash to flash by up to several percent.

Photoconsumption of DM-nitrophen

One of the most important determinants of the flash-to-flash variability of the Ca^{2+} concentration jumps is the photoconsumption of the caging compound within the cell. Every flash is supposed to photolyze a fraction of the compound present in the cytosol. Photoconsumption is thus expected to give rise to an exponentially decreasing response if there were no supply of additional caged compound through the pipette. It is important to note that the epiillumination arrangement we use keeps photolysis within the patch pipette to a minimum because only the pipette tip is illuminated. There is thus a small supply of unphotolyzed DM-nitrophen during the experiment. However, despite the fact that we are using dye loading through the patch pipette and flashes are only applied every 30 s (see Fig. 2 *A*), we still see a gradual decrease in the signal indicating that photoconsumption exceeds the supply by dialysis. Pressure injection of a small volume of pipette filling solution into the cell restores the original signal amplitude, confirming that the decay is due to the expected photolysis and not to rundown of the current-carrying mechanism (not shown). To reveal small changes of the conformation current I_{conf} we developed a protocol designed to correct for the photoconsumption (Fig. 2 *B*). Flashes were applied at various test voltages (◇) and each test flash was bracketed by two controls at −40 mV (◆). The correction for photoconsumption was finally performed by normaliza-

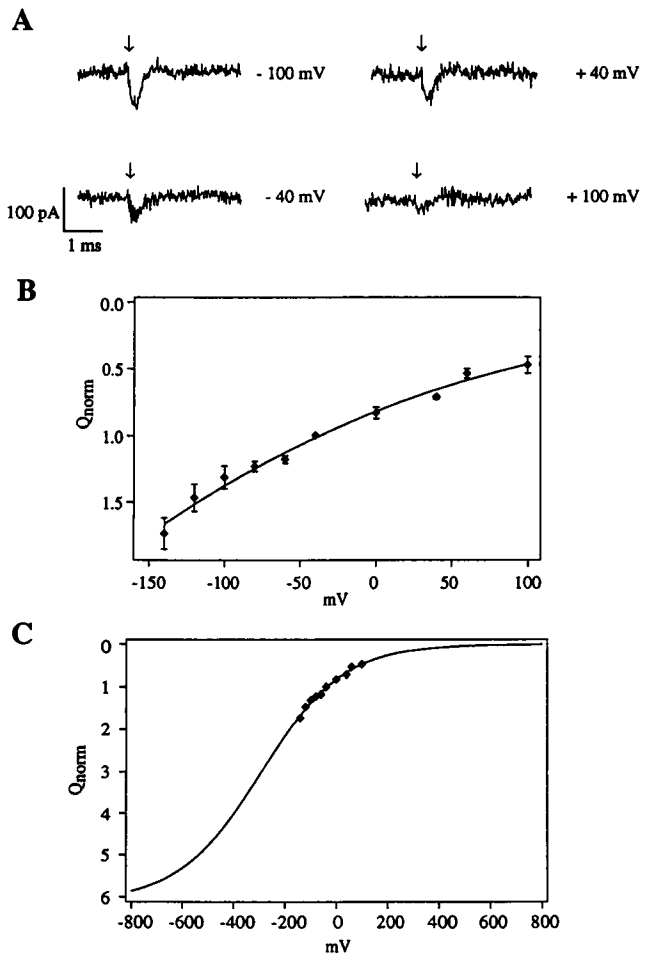

FIGURE 3 Voltage dependence of I_{conf} under control conditions. (*A*) Original records of I_{conf} recorded at membrane potentials between −100 mV and +100 mV. The flash was triggered at the arrow (↓). The effect of photoconsumption of DM-nitrophen precludes the direct quantitative comparison of the current records without normalization. (*B*) The voltage dependence of the charge movement during I_{conf} was normalized using the protocol presented in Fig. 2. The symbols indicate mean values from n = 3–8 data points (two control and one test flash for each data point; the error bars represent SE). The continuous line was calculated with a nonlinear least-squares fitting routine and represents the Boltzmann distribution of our two-state model. Q_{max}, 6.11; V_h, −296 mV; k, 159 mV; χ^2, 0.021. (*C*) Using the parameters obtained from the fitting routine, the predicted distribution was calculated beyond the experimentally accessible voltage range. Most data points recorded experimentally are clustered in the range of the curve corresponding to the states with the Ca^{2+}-binding sites facing the outside.

tion to a value determined by linear interpolation between two adjacent control values.

Distribution of exchanger states in control conditions

Fig. 3 *A* shows conformational currents (I_{conf}) before normalization recorded at voltages between +100 mV and −100 mV when an energy of 230 Ws was discharged through the flash lamp (↓). After normalization for photobleaching the peak inward current and the transported charge were consistently larger at more negative potentials. Data from 37 flashes to various test potentials and 42 control flashes at −40 mV were pooled to produce Fig. 3 *B*. Each data point represents the mean charge moved at a given voltage (± SE, n = 3–8) normalized with respect to the controls performed at a membrane potential of −40 mV. The continuous line shows the least-squares fit of a Boltzmann distribution describing the two-state model introduced in Fig. 1 *B*. In Fig. 3 *C* the fit of the model was extended beyond the voltage range accessible experimentally. The recorded data points appear to be clustered in one range of the Boltzmann plot suggesting that under our control conditions most of the exchangers were not available for intracellular Ca^{2+} binding but resided in states with the Ca^{2+} binding sites facing the outside of the cell. From the Boltzmann function the "virtual" charge of the two-state model resulting from the two real charges can be deduced. We found this virtual charge to correspond to +0.16 elementary charges. From the extrapolated fit it was predicted that the Na-Ca exchanger molecules would be evenly distributed between inside and outside states at a V_h of −296 mV.

Distribution of exchanger states in the presence of intracellular DCB

Depending on the exact molecular mechanism of the Na-Ca exchange the voltage dependence of the distribution may be influenced by ions and compounds interacting with the protein in various ways. It is important to note that, in the absence of an extracellular inhibitor for the Na-Ca exchange, I_{conf} is always "contaminated" by I_{NaCa}. Therefore, it is not straightforward to detect small or even moderate effects of extracellular inhibitors on I_{conf}. All ionic inhibitors (like Ni^{2+}, La^{3+}) cannot be applied intracellularly in our experiments, because they would displace Ca^{2+} from the EDTA-based caging compound DM-nitrophen. Therefore, we used the organic amiloride analog and Na-Ca exchange inhibitor DCB to investigate the effects of inhibitors on I_{conf}. As in a previous study (Niggli and Lederer, 1991b) DCB_i induced an about 5.7-fold increase of I_{conf} at −40 mV (compare Figs. 3 *A* and 4 *A*). In addition, we found that the voltage dependence of I_{conf} was changed dramatically by intracellular DCB (Fig. 4 *B*). Under control conditions, I_{conf} was more voltage sensitive at negative voltages, whereas in the presence of DCB I_{conf} was more voltage sensitive in the positive voltage range. Analysis of the data revealed that under control conditions the voltage dependence showed a decreasing slope at more positive voltages, but in the presence of intracellular DCB the slope increased with depolarization (compare Figs. 3 *B* and 4 *B*). Fitting the simple two-state model to the data suggests that, contrary to control conditions, in the presence of DCB_i most exchanger molecules occupied a state with the Ca^{2+}-binding site facing the inside of the cell. This redistribution of charge also resulted in a shift of the voltage dependence toward more positive potentials with V_h now at +172 mV. In summary the effects of DCB_i on I_{conf} match those predicted for an inhibitor interfering with either binding or transloca-

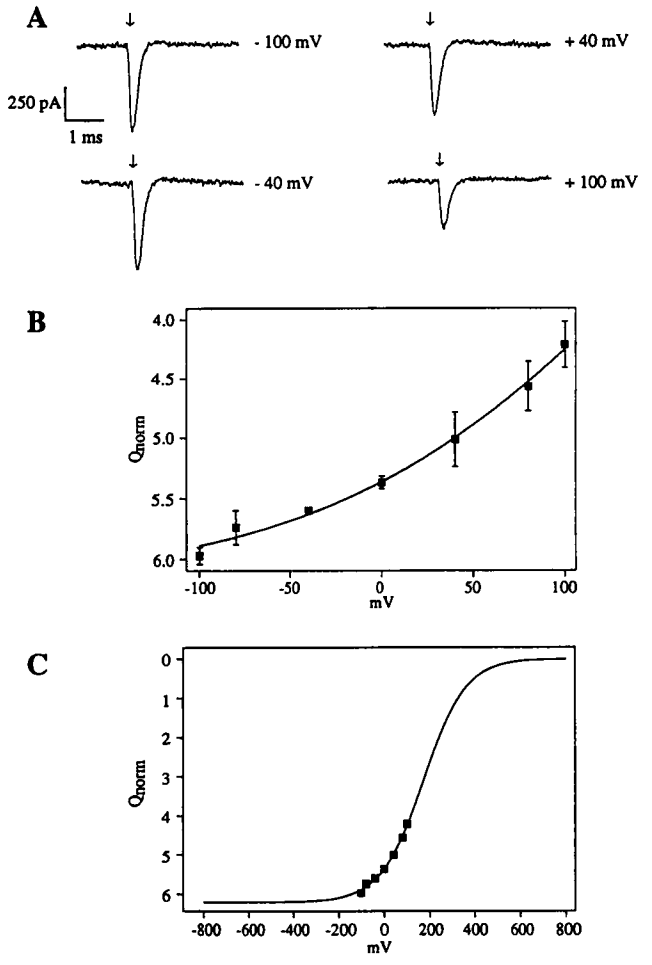

FIGURE 4 Voltage dependence of I_{conf} in the presence of intracellular DCB. (*A*) shows raw data (before normalization) recorded between −100 mV and +100 mV. After normalization the effect of voltage is apparently much smaller in the presence of DCB than under control conditions, especially in the negative voltage range. The charge/voltage relationship compiled from pooled data. (*B*) reveals an increase of I_{conf} of ~5.7-fold at −40 mV. In addition, the slope of the voltage dependence has changed dramatically and is now increasing at more positive potentials. Mean ± SE, n = 3–9 tests per data point. Q_{max}, 6.21; V_h, +172 mV; k, 93 mV; χ^2, 0.017. (*C*) Most data points recorded in the presence of intracellular DCB are now clustered in a range of the Boltzmann corresponding to the states with the Ca^{2+} binding sites facing the inside.

tion of intracellular Na^+ in a consecutive transporter and thus resulting in "trapping" of exchanger molecules in the inside configuration.

Voltage dependence after prolonged experiments

In a few cells we were able to perform a large number of flashes. In the presence of extracellular Ni^{2+}, an inhibitor of the Na-Ca exchange, these attempts usually resulted in a gradual shortening of the cell until the cell eventually was torn off the pipette by the shortening force. In the presence of intracellular DCB we observed a significant increase in the voltage dependence during these prolonged experiments. We have analyzed this phenomenon in more detail. To determine the voltage dependence rapidly, test voltages of +100 mV and −100 mV were used alternately, again normalized with respect to I_{conf} measured at −40 mV (Fig. 5 *B*). The difference between the two data points at −100 mV and +100 mV provides a rough estimate for the voltage dependence. The increasing divergence of the data points suggested that the voltage dependence became more pronounced during the experiment. Fig. 5 *C* shows a comparison of data points obtained early in an experiment (●) and later in the same experiment (◆). It is apparent that during the prolonged experiment Ca^{2+} binding sites are moved outward and that the voltage dependence is shifted along the voltage axis by about 200 mV (V_h from +251 to +59 mV). Because only a few data points clustered in one range of the Boltzmann distribution were available, we could not determine whether the apparent change of the slope (i.e., the "virtual" charge) was significant. A possible interpretation of this finding is that at the beginning most exchangers resided in states with the Ca^{2+} binding sites facing the inside of the cell and thus were in a range of the Boltzmann distribution where the voltage dependence is small. However, later the distribution of exchanger states was shifted toward the "center" of the Boltzmann, where the slope is greater. Although data recorded toward the end of prolonged experiments were not included in the other results presented in this study, we are aware of the fact that this phenomenon introduces additional noise to our data. At present, we do not know the exact cause for this progressive shift of charge during prolonged experiments but it may result from slow changes of intracellular resting Ca^{2+}. A gradual rise of resting Ca^{2+} is expected to occur during these experiments. With the Na-Ca exchange blocked by Ni^{2+} the cell behaves as a closed system with respect to Ca^{2+}, but the flashes repeatedly destroy some of the mobile Ca^{2+} buffer DM-nitrophen (see Fig. 5 *D* and Discussion). In the consecutive model a slow rise of $[Ca^{2+}]_i$ would gradually move $[Ca^{2+}]_i$-receptive exchangers into states facing the outside.

DISCUSSION

In voltage-clamped cardiac myocytes a transient inward current (I_{conf}) lasting about 2 ms can be recorded after a rapid intracellular Ca^{2+} concentration jump (Niggli and Lederer, 1991b). In previous studies no current reversal and no significant voltage dependence were found for I_{conf} between −100 and +100 mV. This observation excludes the possibility that the current arises from the movement of ions through a Ca^{2+}-activated channel. We therefore concluded that the current was most likely associated with a charge movement corresponding to Ca^{2+}-dependent gating of proteins. Although several channels are known to exhibit Ca^{2+}-dependent gating (or at least Ca^{2+}-dependent shifts between conformational states) the large amount of charge moved during I_{conf} was clearly inconsistent with the known low density of these channel proteins. However, the dramatic effect of DCB on I_{conf} suggested that the current may originate from Na-Ca exchanger molecules. DCB, an organic inhibitor of

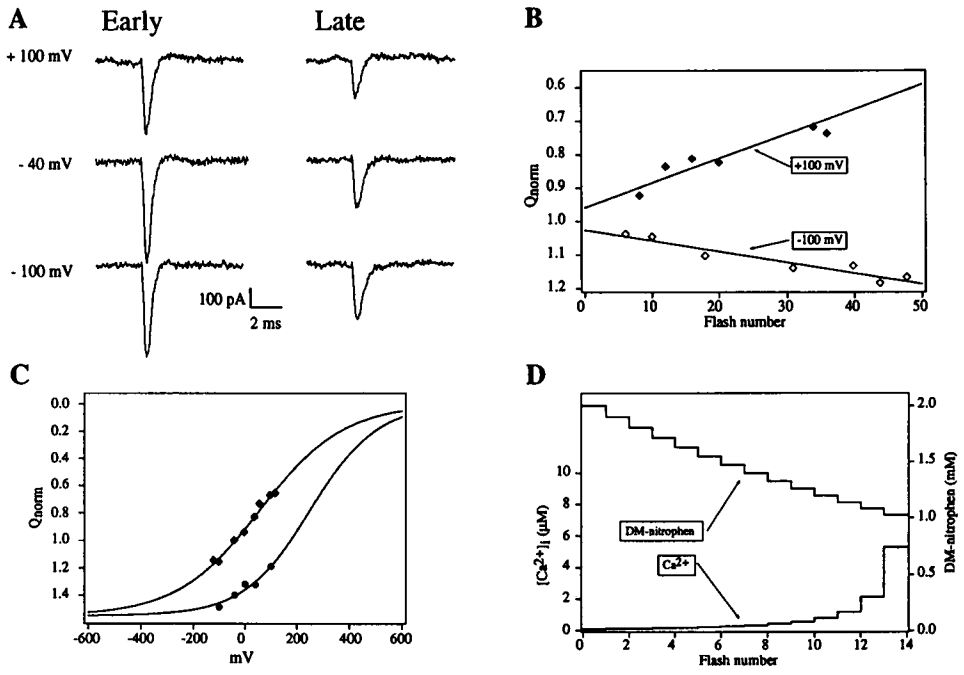

FIGURE 5 Voltage dependence of I_{conf} during prolonged experiments. (*A*) Original traces recorded from a single cell early and late in the experiment at voltages between −100 mV and +100 mV. (*B*) The difference between the normalized charge movement at +100 mV and −100 mV was used as a rough estimate for the steepness of the voltage dependence. In the course of the experiment, this difference became progressively larger, consistent with an outward shift of exchanger states with the Ca^{2+} binding sites facing the inside of the cell. To generate (*C*) the voltage dependence of I_{conf} was investigated in a single cell in the presence of intracellular DCB. Data points recorded at the beginning (●) and toward the end (♦) of this prolonged experiment were analyzed separately by fitting a Boltzmann distribution to each data set normalized for Q_{conf} at −40 mV toward the end of the experiment and with Q_{max} held constant. The fitting parameters for the early data points were V_h, 251 mV; k, 127 mV; χ^2, 0.003 (n = 5); and V_h, 59 mV; k, 160 mV; χ^2, 0.004 (n = 8) for the later data points. (*D*) Model calculations of a closed system containing 2 mM DM-nitrophen, 200 μM cellular Ca^{2+} buffers and Ca^{2+}. With each simulated flash, 5% of the remaining DM-nitrophen were "photolyzed". The predicted resting Ca^{2+} increased slowly at the beginning but more rapidly after several flashes. The SR and mitochondria were not included in these calculations. In the real experiments they served as additional buffers enabling many repeated flashes.

the Na-Ca exchange, is known to exhibit a number of nonspecific inhibitory side effects on different channels (Bielefeld et al., 1986). The stimulatory effect on I_{conf} we observed, however, was only consistent with a specific effect of DCB on the Na-Ca exchange cycle and not with nonspecific inhibitory actions on other current carrying systems. Based on these findings we associated I_{conf} with a partial reaction of the Na-Ca exchange cycle that moves charge after intracellular Ca^{2+} binding.

Voltage-dependent distribution of $[Ca^{2+}]_i$-receptive Na-Ca exchanger states

A number of features are expected for an Na-Ca exchange conformational current induced by a sudden Ca^{2+} concentration jump. Obviously, all predicted features are based on assumptions derived from models that are still controversial. In any case, for transporters moving charge across the electrical field of the membrane, voltage dependence of some partial reactions is expected. Voltage-dependence of membrane-crossing partial reactions results in a voltage-dependent steady-state distribution of exchanger states. Provided I_{conf} corresponds to a partial reaction moving ions across the membrane, the charge movement during I_{conf} would reflect the number of Na-Ca exchanger states with Ca^{2+} binding sites facing the inside of the cell membrane and therefore I_{conf} should also depend on voltage. This predicted voltage dependence may by small, however, because only one reaction step moves positive charge across the membrane, whereas the second step may represent the movement of negative charge. When cycling of the exchanger is inhibited by Ni^{2+}, these two transitions operate in parallel. Given that variations of membrane voltage would have opposite effects on these two transitions governing the inside versus outside distribution, changes of the four membrane-crossing rate constants may partially compensate each other and the voltage dependence may be shallow.

After adopting an experimental protocol that takes into account photoconsumption of the caging compound we were indeed able to reveal a weak voltage dependence of I_{conf}. The charge moved during I_{conf} increased at more negative potentials, suggesting that overall a small "virtual" positive charge governs the distribution of Ca^{2+}_i-receptive exchanger states. Fitting a simplified two-state model (Ca^{2+}_i-receptive E* versus other states E** lumped together) to the data suggested that under control conditions most exchanger molecules were not Ca^{2+}_i receptive, especially at positive membrane potentials.

In addition to the observed voltage-dependence of the steady-state charge distribution an effect of voltage on the current relaxation of I_{conf} may also be expected. Unfortunately, the rate at which we can increase intracellular Ca^{2+} is limited by the relatively long duration of the light flash (about 350 μs full width at half-maximal amplitude, overall duration about 2 ms). Because the Ca^{2+} release from photolyzed DM-nitrophen has been determined to be much faster ($\tau \approx 30$ μs; Vergara and Escobar, 1993) the observed current relaxation of I_{conf} most likely reflects the time-course of the flash and not the rate of the process underlying the current.

Intracellular DCB increases the fraction of exchangers in $[Ca^{2+}]_i$-receptive states

In earlier studies, we found that 100 μM extracellular DCB inhibited I_{NaCa} by about 90% without noticeably affecting I_{conf}. When applied from the inside DCB did not significantly block cycling of the Na-Ca exchange (i.e., inward I_{NaCa}), but I_{conf} was increased about sixfold. The absence of a blocking effect from the inside is in contrast to observations made in giant patches (Hilgemann et al., 1992b). Our finding was interpreted in the following way. In the presence of K^+, DCB acts as a competitive inhibitor of Na^+ binding for the exchanger in vesicles (Slaughter et al., 1988). Because I_{NaCa} activated by photorelease of Ca^{2+} only reflects $[Ca^{2+}]_i$-induced changes in the "forward" (i.e., Ca^{2+} extrusion) direction of Na-Ca exchange activity, extracellular DCB inhibits I_{NaCa} by interfering with extracellular Na^+-binding required for forward cycling (see Fig. 1 *A*). In contrast, when applied from the inside of the cell, the activation of cycling in the forward direction (i.e., inward I_{NaCa}) may not be markedly affected because no intracellular Na^+-binding is necessary for the forward mode. Although DCB_i has little effect on the forward mode, it is still expected to change the equilibrium distribution (i.e., immediately before the Ca^{2+} concentration jump) of Na-Ca exchanger states by preventing the loss of $[Ca^{2+}]_i$-receptive exchangers via the Na^+ translocating step. This inhibition is predicted to result in a shift of exchanger states to favor those with the inside facing ion binding sites and thus could account for the observed increase of I_{conf}.

In the present study the effect of intracellular DCB was also dramatic. Not only did the amount of charge increase about 5.7-fold at -40 mV, we also observed a change of the voltage dependence. Whereas under control conditions the voltage dependence showed a decreasing slope at more positive potentials, the opposite was observed in the presence of intracellular DCB; the charge/voltage relationship became steeper at positive potentials. Analyzing the data with the same two-state model we reached the conclusion that intracellular DCB increased the fraction of Ca^{2+}_i-receptive states until at -100 mV almost all exchanger molecules were residing in states with the Ca^{2+}-binding sites facing the inside. The Q_{max} values obtained from unconstrained nonlinear least-squares fits of the two-state model to both data sets (i.e., in the absence and presence of DCB_i) were similar (6.11 and 6.21, respectively), confirming that both sets of data reflect the same fundamental mechanism although under different conditions. However, when the fitting routine was constrained to keep Q_{max} and the slope k fixed, the fit became very poor (χ^2: 0.457 compared with 0.017), indicating that the change of the slope in the presence of DCB_i is significant. As outlined above, variable and even negative slopes are possible for the steady-state distribution of an exchanger transporting opposite charges across the membrane in parallel. The actual slope would depend on the prevailing ion binding and translocation rates and thus also on the presence of inhibitors like DCB. In conclusion, the effects of intracellular DCB are consistent with the notion that the compound interferes with a partial molecular reaction moving exchanger molecules to the outside.

The voltage dependence increases during prolonged experiments

In the presence of the extracellular Na-Ca exchange inhibitor Ni^{2+} it was observed, particularly during prolonged experiments, that the normalized charge moved at -100 mV and at $+100$ mV exhibited a gradually increasing voltage dependence, consistent with a progressive outward shift of Ca^{2+}_i-receptive exchanger states despite the presence of intracellular DCB. Thus, early in the experiment most exchangers occupied a range of the Boltzmann distribution where the voltage-dependence is shallow. However, in the course of the experiment the distribution was gradually shifted along the Boltzmann curve to the range with a steeper slope. This observation can be explained by the following mechanism. Because the cell had no potent means to extrude Ca^{2+} rapidly in the presence of Na-Ca exchange inhibitors, the myocyte was essentially a closed system with respect to Ca^{2+}. With every flash we photolyzed a certain fraction of the mobile Ca^{2+} buffer DM-nitrophen while the amount of Ca^{2+} within the cell remained roughly constant. Thus, we could expect a progressive elevation of "resting" $[Ca^{2+}]_i$, i.e. the $[Ca^{2+}]_i$ immediately before the flash was applied, a prediction that was routinely confirmed by the observation of progressive cell shortening in the course of prolonged experiments. This change of resting $[Ca^{2+}]_i$ could favor the movement of Ca^{2+}_i binding sites to the outside via the Ca^{2+}-translocating step and may be responsible for the observed shift of charge distribution. This interpretation would also imply that the DCB effect can be reversed by an increase of $[Ca^{2+}]_i$.

Similarly, changes of the concentration of extracellular substrates would be expected to shift the distribution of states. However, preliminary experiments revealed that extracellular substrates had little influence on the charge distribution in the presence of high extracellular concentrations of Ni^{2+} and intracellular DCB (Niggli and Lipp, 1993a). At present, we cannot yet rule out an unexpected effect of Ni^{2+} on the distribution of exchangers under these conditions (Niggli and Lipp, unpublished observations), but this result

could mean that the exchanger is able to equilibrate between the inside and outside states in the absence of substrate, similar to a two-step simultaneous mechanism. Alternatively, this could indicate that the charge movement reflects a conformational change of the Na-Ca exchanger other than an ion translocating step.

In conclusion, we have found the Ca^{2+}-induced charge movement of the Na-Ca exchange to be weakly voltage dependent. The voltage dependence was markedly shifted along the voltage axis by intracellular DCB, and indirect evidence suggests that it may also depend on intracellular resting Ca^{2+}.

We would like to thank Dr. J.A.S. McGuigan for his valuable comments on the manuscript; and D. deLimoges, P. Frei, J. Burkhalter, and H. U. Schweizer for their skillful help with electronics and with mechanical hardware. This work was supported by Swiss National Science Foundation grants to E. N. (31–28545.90 and 3100–037417.93).

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