Rapid Charge Translocation by the Cardiac Na^+-Ca^{2+} Exchanger after a Ca^{2+} Concentration Jump

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ABSTRACT The kinetics of Na^+-Ca^{2+} exchange current after a cytoplasmic Ca^{2+} concentration jump (achieved by photolysis of DM-nitrophen) was measured in excised giant membrane patches from guinea pig or rat heart. Increasing the cytoplasmic Ca^{2+} concentration from 0.5 μM to 100 μM in the presence of 100 mM extracellular Na^+ elicits an inward current that rises with a time constant $\tau_1 < 50$ μs and decays to a plateau with a time constant $\tau_2 = 0.65 \pm 0.18$ ms ($n = 101$) at 21°C. These current signals are suppressed by Ni^{2+} and dichlorobenzamil. No stationary current, but a transient inward current that rises with $\tau_1 < 50$ μs and decays with $\tau_2 = 0.28 \pm 0.06$ ms ($n = 53$, $T = 21$°C) is observed if the Ca^{2+} concentration jump is performed under conditions that promote Ca^{2+}-Ca^{2+} exchange (i.e., no extracellular Na^+, 5 mM extracellular Ca^{2+}). The transient and stationary inward current is not observed in the absence of extracellular Ca^{2+} and Na^+. The application of α-chymotrypsin reveals the influence of the cytoplasmic regulatory Ca^{2+} binding site on Ca^{2+}-Ca^{2+} and forward Na^+-Ca^{2+} exchange and shows that this site regulates both the transient and stationary current. The temperature dependence of the stationary current exhibits an activation energy of 70 kJ/mol for temperatures between 21°C and 38°C, and 138 kJ/mol between 10°C and 21°C. For the decay time constant an activation energy of 70 kJ/mol is observed in the Na^+-Ca^{2+} and the Ca^{2+}-Ca^{2+} exchange mode between 13°C and 35°C. The data indicate that partial reactions of the Na^+-Ca^{2+} exchanger associated with Ca^{2+} binding and translocation are very fast at 35°C, with relaxation time constants of about 6700 s^{-1} in the forward Na^+-Ca^{2+} exchange and about 12,500 s^{-1} in the Ca^{2+}-Ca^{2+} exchange mode and that net negative charge is moved during Ca^{2+} translocation. According to model calculations, the turnover number, however, has to be at least 2–4 times smaller than the decay rate of the transient current, and Na^+ inward translocation appears to be slower than Ca^{2+} outward movement.

INTRODUCTION

The cardiac Na^+-Ca^{2+} exchanger is an electrogenic transport protein of the sarcolemma that is driven by the electrochemical potential of the Na^+ gradient and plays an important role for the Ca^{2+} homeostasis of the cell. Under normal physiological conditions, it countertransports three extracellular Na^+ ions for one intracellular Ca^{2+} ion (Reeves and Hale, 1984; Ehara et al., 1989; Crespo et al., 1990). Depending on the electrochemical potential, a reverse transport mode, where three intracellular Na^+ are exchanged for one extracellular Ca^{2+}, can also be observed (Baker et al., 1969; Reeves and Sutko, 1983; Kimura et al., 1986). Experiments on the reverse mode of the Na^+-Ca^{2+} exchanger have revealed the existence of a regulatory Ca^{2+} binding site on the intracellular side, which is different from the Ca^{2+} transport binding site (DiPolo, 1979; DiPolo and Beaugé, 1986). It has been characterized by electrophysiological studies (Kimura et al., 1986; Hilgemann and Cash, 1990; Hilgemann et al., 1992a), and its position in the amino acid sequence of the protein has been identified (Levitsky et al., 1994; Matsuoka et al., 1995).

The transport mechanism and kinetics of the Na^+-Ca^{2+} exchanger are controversial. A consecutive transport has been suggested for Na^+ and Ca^{2+} translocation (Khananshvili, 1990; Hilgemann et al., 1991; Li and Kimura, 1991; Matusoka and Hilgemann, 1992), but very little is known about the individual reaction steps. Published turnover numbers for the transport cycle vary from 100 s^{-1} (Powell et al., 1993) to the order of 1000 s^{-1} (Cheon and Reeves, 1988; Hilgemann et al., 1991; Niggli and Lederer, 1991). Possible approaches for investigating the kinetics of an electrogenic transport protein like the Na^+-Ca^{2+} exchanger are fast perturbations of the steady state by voltage steps or concentration jumps. The induced relaxation of the system to a new steady state can give valuable information on the underlying kinetics. A useful tool for investigation of the fast kinetics of the Na^+-Ca^{2+} exchanger is DM-nitrophen (caged Ca^{2+}), an EDTA-based Ca^{2+} chelator, which releases Ca^{2+} with a time constant of $\tau < 30$ μs (Ellis-Davies et al., 1996) upon photolysis by UV light. Under conditions where other Ca^{2+} activated processes are blocked, it should be possible to measure the fast activation of Na^+-Ca^{2+} exchange current by photolysis of caged Ca^{2+}. This method has already been employed in combination with the whole-cell patch-clamp technique in ventricular myocytes (Niggli and Lederer, 1991; Niggli and Lipp, 1994; Powell et al., 1993) and with sarcolemmal vesicles from lobster muscle adsorbed to a black lipid membrane (Eisenrauch et al., 1995). Powell et al. reported a relatively slow ($\tau > 5$ ms) activation of the Na^+-Ca^{2+} exchange current by an intracellular Ca^{2+} concentration jump, whereas Niggli et al. observed a fast activation (~1 ms). In their experiments the stationary current

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was preceded by a transient signal, which was interpreted as a redistribution of the exchanger molecules to a new stationary state.

Interpretation of the data obtained after a concentration jump of intracellular Ca^{2+} under whole-cell conditions is complicated because Ca^{2+} acts as a trigger for a number of processes inside a muscle cell. The aim of this study was to establish a less complicated situation. Therefore the laser-induced photolysis of DM-nitrophen was combined with the giant excised patch-clamp technique (Hilgemann, 1989), offering the advantages of high clamp speed and complete control of ionic conditions on both sides of the isolated membrane patch, together with a sufficiently large number of exchanger molecules to generate measurable currents.

This article analyzes the time course of the current signal obtained after a Ca^{2+} concentration jump on the cytoplasmic side. Experiments under various ionic conditions establish its association with the activity of the Na^+-Ca^{2+} exchanger and allow some conclusions on the mechanism of Na^+-Ca^{2+} exchange.

Some of the results presented here have been published in abstract form (Kappl and Hartung, 1996).

MATERIALS AND METHODS

Preparation of cells

Single ventricular myocytes from adult rat and guinea pig were prepared using a procedure similar to that of Isenberg and Klöckner (1982) and Yazawa et al. (1990). The isolated cells were stored at 4°C in a nominally Ca^{2+}-free, K^+-rich buffer (in mM: 70 KOH, 50 glutamic acid, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4 with KOH), which promotes formation of large membrane blebs (Hilgemann, 1989), and were used for experiments within 2 to 48 h.

Solutions

A list of the solutions used for the experiments is given in Table 1. All concentrations are given in mM. The pH of all solutions was adjusted to 7.1. The abbreviations P1. . . DMN5 in the second line of the table are used for reference throughout the article. DM-nitrophen was obtained from Calbiochem Biochemicals (Bad Soden, Germany) in solid form (>95% purity) in 5-mg vials. The contents of one vial were dissolved in 14 ml of (in mM) 97 LiCl, 3 LiOH, 20 TEA-Cl, 20 CsCl, 10 HEPES (pH 7.1 with HCl). Free Ca^{2+} was monitored by a Ca^{2+}-sensitive electrode (see below) and titrated to 0.5 μM with $CaCl_2$, while the pH was kept at 7.1 by the addition of HCl. Under these conditions, DM-nitrophen is loaded by approximately 99% with Ca^{2+}, and the true DM-nitrophen concentration can be calculated from the amount of $CaCl_2$ added during titration, giving values of 0.42–0.48 mM (solution DMN1). In some experiments the DM-nitrophen concentration was increased by 50 μM in the above solution, resulting in a free Ca^{2+} concentration of $\leq$100 nm (solution DMN2). For control experiments, 1 mM Ca^{2+} (solution DMN3) or 1 mM EGTA and no Ca^{2+} (solution DMN4) were added.

2,4-Dichlorobenzamil (DCB) was obtained from Molecular Probes Europe BV (Leiden, The Netherlands). Stock solutions (10 mM) of DCB in dimethyl sulfoxide were used to prepare solution DMN5 with 200 μM DCB. Ca^{2+} activity of all solutions was measured using a Ca^{2+}-sensitive electrode (Kwik-Tip Tipca; World Precision Instruments, Sarasota, FL), which was calibrated using CALBUF-1 calcium buffers (World Precision Instruments).

Patch-clamp technique

Na^+-Ca^{2+} exchange current was measured using the giant excised patch-clamp technique (Hilgemann, 1989). Pipettes were pulled on a two-stage puller (Narishige PP-83, Tokyo, Japan) from Drummond N-51A glass (Drummond Scientific, Broomall, PA) and fire polished to diameters of 15–22 μm. Pipettes were dipped into α-tocopherol to improve seal formation and filled with the desired solution. Pipette resistance ranged from 130 to 190 kΩ.

Cells were placed in a small petri dish mounted on the stage of an inverted microscope. Gigaseals (1–5 GΩ) were formed between the pipette and a membrane bleb. By quickly retracting the pipette, an excised patch was established, with the former cytoplasmic side of the cell membrane facing the bath solution. The pipette was placed in front of the optical fiber at a distance of about 100 μm. Then negative pressure was applied to the suction line and nearly all of the solution was removed, leaving only a small remainder of approximately 50 μl, which was held back by surface tension and the surrounding experimental chamber. This chamber was developed in collaboration with Thomas Friedrich and Georg Nagel (Frankfurt) to combine rigid temperature control with the possibility to

TABLE 1 List of solutions used for the experiments

	Pipette solutions				Bath solutions				DMN solutions				
	P1	P2	P3	P4	B1	B2	B3	B4	DMN1	DMN2	DMN3	DMN4	DMN5
NaCl	75	97	—	—	—	—	75	75	—	—	—	—	—
NaOH	25	3	—	—	—	—	25	25	—	—	—	—	—
LiCl	—	—	96	94	96	97	—	—	97	97	97	97	97
LiOH	—	—	4	6	4	3	—	—	3	3	3	3	3
KCl	—	1	—	1	—	—	—	—	—	—	—	—	—
EGTA	10	—	—	1	1	1	10	10	—	—	—	1	—
DMN	—	—	—	—	—	—	—	—	0.45	0.455	0.45	0.45	0.45
$CaCl_2$	—	—	5	—	—	1.2	—	—	0.44	0.44	1	0.44	0.44
$NiCl_2$	—	5	—	—	—	—	—	—	—	—	—	—	—
CsCl	20	20	20	20	20	20	20	20	20	20	20	20	20
TEA-Cl	20	20	20	20	20	20	20	20	20	20	20	20	20
MgATP	—	—	—	—	—	—	—	0.5	—	—	—	—	—
$MgCl_2$	2	—	—	—	—	—	—	2	—	—	—	—	—
Verapamil	0.02	0.02	0.02	0.02	—	—	—	—	—	—	—	—	—
DCB	—	—	—	—	—	—	—	—	—	—	—	—	0.2
HEPES	10	10	10	10	10	10	10	10	10	10	10	10	10

All concentration in mM. pH adjusted to 7.1 with HCl.

photolyze caged compounds (for details see Friedrich et al., 1996). The small sample volume could be quickly exchanged (≤1 s) by solution flow from the inlet tube, which was connected to eight different solution lines that were used to activate the steady-state response of the Na^+-Ca^{2+} exchanger by superfusion or to inject the caged Ca^{2+} solution. The solution lines were all surrounded by a water jacket, and the solution temperature was measured continuously with a thermocouple, which was located near the pipette tip.

For concentration jump experiments, the sample volume was flushed by injection of 100–200 μl of the DM-nitrophen-containing solution. A XeCl excimer laser with 308-nm wavelength and 10-ns pulse duration was used to photolyze the DM-nitrophen. The attenuated laser output was coupled into an optical silica fiber (Lot Oriel UV/VIS, 400 μm core diameter; Lot Oriel, Darmstadt, Germany), yielding a pulse energy of 0.6 mJ at the end of the fiber (corresponding to 480 mJ/cm^2). A single flash photolyzed approximately 30% of the DM-nitrophen within the illuminated volume, creating a saturating (>100 μM) cytoplasmic Ca^{2+} concentration for Na^+-Ca^{2+} exchange activity.

Laser flashes sometimes caused abrupt deterioration of the seal/membrane resistance. Therefore, 5-mV pulses were applied before and after every flash to measure seal/membrane resistance; recordings exhibiting a decrease in seal resistance were discarded.

Data acquisition

A digitizer board with the pClamp 6.0.2 software package (Axon Instruments, Foster City, CA) was used for data recording, control of the solution valves, and triggering of the laser. The filtered output of the Axopatch 200A patch clamp amplifier (Axon Instruments) was sampled at a rate of 90 kHz. The effective recording bandwidth under experimental conditions was checked with a spectrum analyzer (R9211C FFT signal analyzer; Advantest Corp., Tokyo, Japan) and was between 3 and 23 kHz, depending on filter settings. The capacitive feedback mode of the amplifier was employed, and the reset of the input circuit (lasting 50 μs) was activated 22 μs before the laser was triggered, to cancel the flash-induced positive transient that could otherwise be observed (cf. Fig. 2). For continuous monitoring of the membrane current, the output of the patch-clamp amplifier was filtered at 50 Hz and digitized by a second computer at a sampling rate of 100 Hz.

Exponential fits of the current signals were made with pClamp 6.0.2 software, and the data were modeled with a four-state exchange cycle model and using Scientist software (MicroMath Scientific Software, Salt Lake City, UT).

RESULTS

Na^+-Ca^{2+} exchange current after a Ca^{2+} concentration jump

If not indicated otherwise, the results reported in this and the following sections were obtained from guinea pig heart muscle cells at 22°C and 0 mV membrane potential, and patches were treated with α-chymotrypsin (1 mg/ml) for 30 s.

In the experiments described in this section, the pipette (extracellular) solution contained 100 mM Na^+ (solution P1). Stationary Na^+-Ca^{2+} exchange current was always activated by changing bath (cytoplasmic) solution from zero free Ca^{2+} (1 mM EGTA) to free Ca^{2+} concentrations of 50 or 200 μM Ca^{2+} before the photolytic Ca^{2+} concentration jumps, to verify correct patch configuration (no vesicles). Fig. 1 *A* shows an example for the current response to a Ca^{2+} concentration jump from 0.5 μM to >100 μM, generated by flash photolysis of solution DMN1. A fast transient inward current is observed, which precedes the stationary inward Na^+-Ca^{2+} exchange current. This transient current rises with a time constant of τ_1 = 0.06 ms and decays to the plateau phase with a time constant of τ_2 = 0.54 ms. The amplitude of the stationary current induced by the photolytic release of Ca^{2+} is identical to that measured in an earlier part of the experiment by superfusion with a saturating intracellular Ca^{2+} concentration.

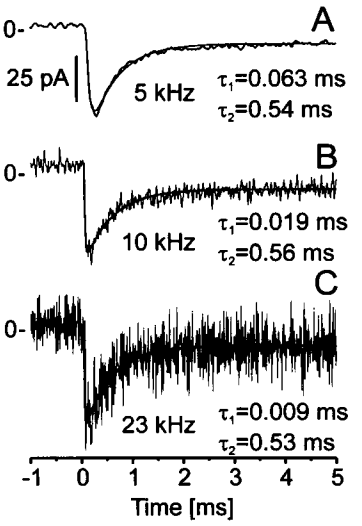

FIGURE 1 Current response to a Ca^{2+} concentration jump, recorded at a bandwidth of 5, 10, and 23 kHz for traces *A*, *B*, and *C*, respectively. The moment of the laser flash is taken as the origin of the time axis. The solid lines are fits of the data with two exponential functions plus a constant. All data are from the same guinea pig membrane patch, which has been treated with α-chymotrypsin (1 mg/ml) for 30 s. The temperature is 22°C and the membrane potential is clamped to 0 mV.

The data in Fig. 1, *B* and *C*, demonstrate that the rise time τ_1 of the transient current is limited by the recording bandwidth. These recordings are from the same membrane patch as in Fig. 1 *A* under identical conditions but at different settings of the low pass filter. With increasing bandwidth, the measured rise time τ_1 becomes shorter, whereas the decay time τ_2 remains unchanged. The experimental time resolution for τ_1 is in fact lower than suggested by the fitted time constant, because within the first 50 to 100 μs after the laser flash, the signal is dominated by the laser-induced artefact. A slight improvement can be achieved by triggering the 50-μs lasting reset of the patch-clamp amplifier 22 μs before the laser trigger, as shown in Fig. 2, but the first 50 μs after the flash still cannot be resolved.

In summary, the photolytic release of cytoplasmic Ca^{2+} in the presence of 100 mM Na^+ activates a fast transient inward current with a time constant $\tau_1 < 50$ μs that decays to the stationary inward Na^+-Ca^{2+} exchange current with a time constant of τ_2 = 0.65 ± 0.18 ms (mean ± SD, n = 101) at 21°C.

When the laser energy is reduced by a factor of 2 with an optical filter, photolysis of caged Ca^{2+} still produces saturating conditions for the stationary Na^+-Ca^{2+} exchange current. The time constants of the transient current also

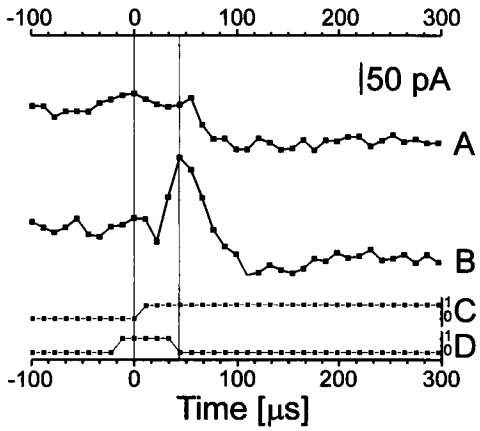

FIGURE 2 Recordings of the current response to a Ca^{2+} concentration jump, demonstrating the time resolution of the experimental setup and the effect of the reset of the amplifier, which is used to cancel the laser-induced artefact. Both current traces (*A*, *B*) are recorded at a bandwidth of 23 kHz from the same patch. In trace *A*, the reset function of the amplifier is triggered 22 μs before the laser flash. In trace *B*, the reset function is disabled. Beneath the current traces the timing of the laser flash (*C*) and the "Data not valid" output of the patch clamp amplifier during the reset (*D*) are shown.

remain unchanged, indicating that the applied Ca^{2+} concentration is also saturating for the reaction step associated with the current transient. The results are not changed when $MgCl_2$ is omitted from the pipette solution. Treatment of the patches with α-chymotrypsin does not change the time constants of the transient signal, but avoids an influence of the regulatory Ca^{2+} binding site and maximizes signal amplitudes (see below). There is no significant difference in the time constants between recordings from rat or guinea pig membrane patches, but patches from guinea pigs on average yield two- to threefold higher Na^+-Ca^{2+} exchange current amplitudes.

Large variations of the ratio of stationary to transient signal and variation in the time constants, which were observed in preliminary experiments (Kappl and Hartung, 1996), could be greatly reduced by treatment with α-chymotrypsin and strict temperature control of the solutions.

Control experiments

In a first series of experiments, the effect of DM-nitrophen on the stationary Na^+-Ca^{2+} exchange current was tested. A solution containing 1 mM DM-nitrophen loaded by 85% with Ca^{2+} is prepared. A fraction of this solution is placed in a quartz cuvette and exposed to 50 laser flashes to completely photolyze the DM-nitrophen. The stationary inward Na^+-Ca^{2+} exchange current is activated by the application of 50 μM Ca^{2+} on the cytoplasmic side in the presence of 100 mM extracellular Na^+. Incubation of the membrane patch for 1 min with solutions containing photolyzed or unphotolyzed DM-nitrophen does not alter the amplitude of the stationary Na^+-Ca^{2+} exchange current.

In a second series of experiments, the effect of the laser flash and the photolysis of DM-nitrophen were investigated. Fig. 3 shows current recordings from the same membrane patch under different experimental conditions. The pipette solution contains 100 mM Na^+ and no Ca^{2+} (solution P1), and the patch has not been treated with α-chymotrypsin. Trace A demonstrates the effect of a laser flash in the absence of caged Ca^{2+} (solution B1). Only a small artefact is visible. Trace B shows the current response after photolysis of caged Ca^{2+} (solution DMN1), raising free Ca^{2+} from 0.5 μM to more than 100 μM, a level that fully activates the stationary exchange current. The plateau phase is preceded by a transient current, which is clearly distinct from the laser artefact observed in trace A. Trace C is a continuation of trace B, showing the current response to a second laser flash, which is triggered 10 s later, during the plateau phase of trace B. The further increase in free cytoplasmic Ca^{2+} does not induce any change, as is expected in the case of a fully activated exchanger. Trace D was recorded in the presence of DM-nitrophen, which is not loaded with Ca^{2+} (1 mM EGTA present, solution DMN4). The photolysis of the chelator itself does not produce a current signal. Trace E shows the current in the presence of DM-nitrophen, which is overloaded with Ca^{2+} (0.5 mM DM-nitrophen, 1 mM $CaCl_2$, solution DMN3). Under these conditions, the Na^+-Ca^{2+} exchanger is already saturated with free Ca^{2+} before the flash, and the photolytic release of Ca^{2+} does not induce a transient signal, nor does it alter the stationary Na^+-Ca^{2+} exchange current.

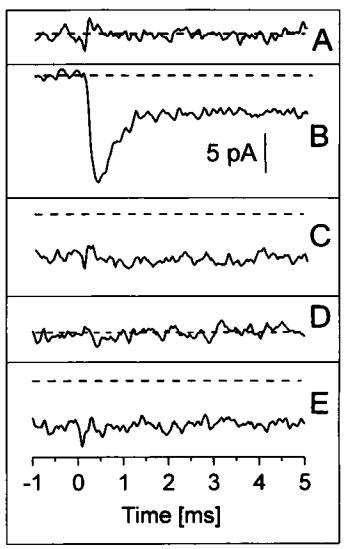

FIGURE 3 Current recordings from the same membrane patch under various control conditions (no α-chymotrypsin treatment). The moment of the laser flash is taken as the origin of the time axis. The dotted horizontal lines mark the zero current baseline for each trace. (*A*) Laser flash in the absence of DM-nitrophen. (*B*) Photolysis of caged Ca^{2+}, raising free Ca^{2+} from 0.5 μM to >100 μM. (*C*) A second laser flash is applied during the plateau phase of trace *B*. (*D*) Photolysis of DM-nitrophen not loaded with Ca^{2+}. (*E*) Photolysis of DM-nitrophen that has been overloaded with Ca^{2+} (0.5 mM DM-nitrophen, 1 mM $CaCl_2$).

Regulatory effect of intracellular Ca^{2+}

The Na^+-Ca^{2+} exchanger is regulated by intracellular free Ca^{2+} via a high-affinity Ca^{2+} binding domain, which is separate from the transport binding site (DiPolo and Beaugé, 1986; Hilgemann, 1990; Levitsky et al., 1994; Matsuoka et al., 1995). Removal of Ca^{2+} from this site leads to a reversible inactivation. Treatment with α-chymotrypsin removes the inactivating effect of low intracellular Ca^{2+}, leaving the exchanger in a highly activated state (Hilgemann, 1990). Because of the much higher Ca^{2+} affinity of the regulatory domain, this regulatory effect of intracellular Ca^{2+} on the native Na^+-Ca^{2+} exchanger could so far only be studied under reverse-mode conditions. In the following it is demonstrated that the application of Ca^{2+} concentration jumps also allows the investigation of this phenomenon for the forward running Na^+-Ca^{2+} exchanger.

When the membrane patch is incubated in a solution with a low free Ca^{2+} concentration (approximately 0.1 μM, solution DMN2), the signal seems unchanged in its time course, but relatively small on the fast time scale of Fig. 4 *A*. On the slow time scale of Fig. 4 *B*, the fast transient is invisible and a biphasic increase in the Na^+-Ca^{2+} exchange current can be observed. This biphasic behavior results from a combination of the fast activation of the exchange current shown in Fig. 4 *A*, followed by a slow increase in the Na^+-Ca^{2+} exchange current with a time constant of 0.5 to 1 s. After the application of α-chymotrypsin (1 mg/ml) for 40 s to deregulate the exchanger, a Ca^{2+} concentration jump under otherwise identical conditions caused the immediate activation of the maximum Na^+-Ca^{2+} exchange current, resulting in a much higher amplitude of the transient current and a stable plateau phase, as can be seen in Fig. 4, *C* and *D*.

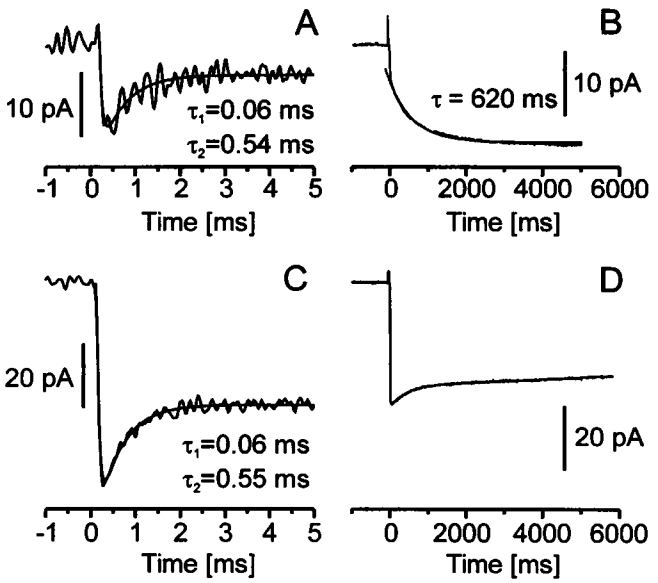

FIGURE 4 Current signals after a Ca^{2+} concentration jump, plotted at two different time resolutions, recorded before (*A*, *B*) and after (*C*, *D*) treatment of the patch with α-chymotrypsin (1 mg/ml) for 40 s. The preflash Ca^{2+} concentration is less than 100 nM (solution DMN2). At high temporal resolution, the fast transient signal followed by the plateau phase is visible (*A*). At lower temporal resolution (*B*), a slow biphasic increase of Na^+-Ca^{2+} exchange current after the concentration jump is observed, which is a combination of the fast activation observed in (*A*) and a slow removal of inactivation. The positive transient visible in (*B*) and (*D*) is the current response to a +10 mV test pulse to monitor seal resistance, applied 40 ms before the laser flash. After treatment with α-chymotrypsin, full activation of the Na^+-Ca^{2+} exchange current immediately after the concentration jump is observed (*C*, *D*).

Activation of Ca^{2+}-Ca^{2+} exchange by photolysis of caged Ca^{2+}

It is known that the Na^+-Ca^{2+} exchanger may perform electroneutral Ca^{2+}-Ca^{2+} exchange (Bartschat and Lindenmayer, 1980; Slaughter et al., 1983). To investigate whether the Ca^{2+}-Ca^{2+} exchange cycle contains electrogenic reaction steps, extracellular Na^+ was replaced by Li^+. With 5 mM $CaCl_2$ in the pipette (solution P3), stationary outward Na^+-Ca^{2+} exchange current can be elicited by the replacement of Li^+ in the bath solution with 100 mM Na^+ to demonstrate correct patch configuration. The amplitudes of stationary forward and reverse-mode Na^+-Ca^{2+} exchange current are of comparable size under saturating conditions (Matsuoka and Hilgemann, 1992). Therefore, the reverse Na^+-Ca^{2+} exchange current can be used as an indicator for relative patch size and correct seal formation.

Fig. 5 *A* shows an example for current response to the photolysis of caged Ca^{2+} in the absence of extracellular Na^+ and the presence of 5 mM extracellular Ca^{2+}. The patch has not been treated with α-chymotrypsin and the temperature is held at 22°C. The Ca^{2+} concentration jump from 0.1 μM to >100 μM induces an inward transient current but no stationary current. This transient rises with a time constant τ_1 = 0.05 ms (recording bandwidth 5 kHz) and decays with a time constant of 0.33 ms.

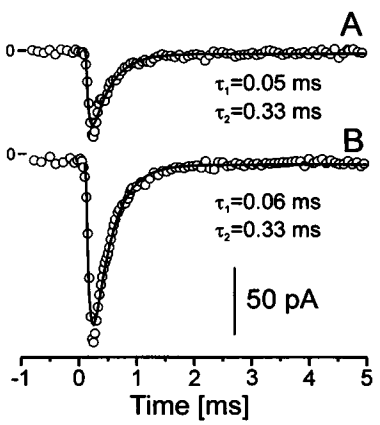

FIGURE 5 Transient current signal observed after photolysis of caged Ca^{2+} in the absence of extracellular Na^+ before (*A*) and after (*B*) treatment of the membrane patch with α-chymotrypsin (1 mg/ml, 40 s). Free Ca^{2+} is elevated from 0.1 μM to >100 μM. The solid lines are biexponential fits with time constants τ_1 and τ_2 as indicated. The temperature is 22°C and the recording bandwidth is 5 kHz.

The time constant τ_1 for the rise is again limited by the time resolution of the measurements. For the decay of the current transient, a time constant $\tau_2 = 0.28 \pm 0.06$ ms (mean $\pm$ SD, $n = 53$) at 22°C is obtained. The average amount of total charge moved during the current transient is 15 ± 4 fC.

The idea that the transient current observed under these conditions indeed originates from the Na^+-Ca^{2+} exchanger is supported by the data in Fig. 5 *B*. Treatment of the patch with α-chymotrypsin (1 mg/ml, 40 s) leads to an increase in the amplitude of the transient current without changing the time constants, showing that this current too is regulated by the cytoplasmic high-affinity binding site.

Photolysis of caged Ca^{2+} in the absence of extracellular Na^+ and Ca^{2+}

To further investigate the substrate dependence of the transient current, experiments without extracellular Na^+ and Ca^{2+} were carried out. The pipette solution was free of Na^+ and Ca^{2+} (solution P4). Under these conditions, the Na^+-Ca^{2+} exchanger is inactive and no stationary exchange current can be elicited in either direction. To control a correct patch configuration, the cytoplasmic application of 0.5 mM MgATP in the presence of 100 mM cytoplasmic Na^+ is used to activate the stationary outward Na^+,K^+-ATPase pump current.

To validate this method, experiments are performed with a pipette solution containing 100 mM Na^+ and 1 mM KCl (solution P1 with 1 mM KCl added). Under these conditions, a direct comparison of Na^+,K^+-ATPase and Na^+-Ca^{2+} exchange current for the same patch is possible. Stationary Na^+-Ca^{2+} exchange current is elicited by cytoplasmic application of 200 μM Ca^{2+} (solution B2). Although no strict linear correlation between maximum stationary currents is found in these experiments, in all patches with levels of stationary Na^+,K^+-ATPase current greater than 5 pA, photolytic release of Ca^{2+} elicits readily detectable transient and stationary Na^+-Ca^{2+} exchange current ($n = 7$).

Photolysis of caged Ca^{2+} in the absence of extracellular Na^+ and Ca^{2+} does not produce a transient or stationary current, although Na^+,K^+-ATPase current amplitudes between 10 pA and 29 pA are obtained from these patches ($n = 6$). Fig. 6 shows the current response after a Ca^{2+} concentration jump, recorded from the patch with the largest level of stationary Na^+-K^+-ATPase current (29 pA).

Block by $NiCl_2$

Ni^{2+} is known to block Na^+-Ca^{2+} exchange reversibly from both sides of the membrane (Kimura et al., 1987). A straightforward approach to testing the effect of Ni^{2+} on the current response after a Ca^{2+} concentration jump would be to photolyze caged Ca^{2+} in the presence and absence of intracellular Ni^{2+} in a single experiment. Unfortunately,

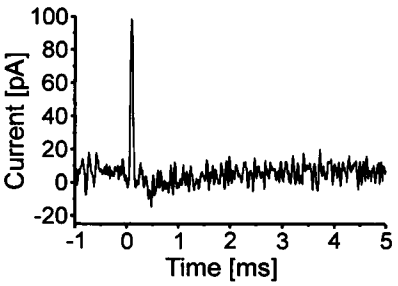

FIGURE 6 Photolysis of caged Ca^{2+} in the absence of extracellular Na^+ and Ca^{2+}, raising the cytoplasmic Ca^{2+} concentration from 0.5 μM to 100 μM. A pronounced outward directed laser artefact is visible, as was sometimes observed before in experiments with patches with a very large membrane area. No significant transient inward current is elicited, although the stationary Na^+/K^+ ATPase current from this patch reached 29 pA (cf. Fig. 7). The temperature was 25°C and the patch had been treated with α-chymotrypsin (1 mg/ml) for 30 s.

DM-nitrophen, as an EDTA-based chelator, binds Ni^{2+} with higher affinity than it does Ca^{2+}, making a Ca^{2+} concentration jump in the presence of intracellular Ni^{2+} impossible. Therefore one must compare the results obtained from different membrane patches with and without extracellular Ni^{2+} under otherwise identical experimental conditions.

As described before, the current generated by the Na^+,K^+-ATPase was used as a control for correct patch configuration and relative patch size. Experiments are carried out comparing patches from the same cell preparation and with similar levels of stationary Na^+,K^+-ATPase pump current. Fig. 7 contrasts current recordings from different patches with the same amplitudes of stationary Na^+,K^+-ATPase current. Trace A is obtained under Ni^{2+}-free conditions (pipette solution as P2, but without $NiCl_2$), trace B in the presence of 5 mM extracellular Ni^{2+} (pipette solution

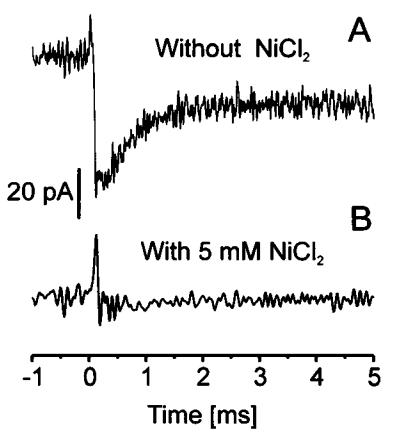

FIGURE 7 Current response to a Ca^{2+} concentration jump in the absence (*A*) and presence (*B*) of 5 mM extracellular $NiCl_2$. Recordings are from different patches with comparable levels of stationary Na^+,K^+ ATPase current (12 pA and 11 pA for traces *A* and *B*, respectively; see text for details).

P2). Both peak and plateau current are lacking in the presence of extracellular Ni^{2+}.

In summary, no significant transient or stationary current can be observed with Ni^{2+} present in the pipette in patches with stationary Na^+,K^+-ATPase currents between 5 and 20 pA (n = 13).

Block by 2,4-dichlorobenzamil

Na^+-Ca^{2+} exchange is inhibited by 2,4-dichlorobenzamil (DCB), an amiloride derivative, at low concentrations (IC_{50} = 12 μM) (Kleyman and Cragoe, 1988) from the intracellular side. DCB failed, however, to block stationary Na^+-Ca^{2+} exchange current in ventricular muscle cells from the intracellular side (Niggli and Lederer, 1991).

Preliminary measurements showed that DCB can indeed block Na^+-Ca^{2+} exchange current in membrane patches, that treatment of the patches with α-chymotrypsin before the addition of DCB does not show any influence on the block of the stationary Na^+-Ca^{2+} exchange current by DCB, and that for a complete block, concentrations above 100 μM are necessary.

These experiments are somewhat complicated by the fact that DCB causes abrupt breakdown of seal resistance, limiting the time left for current measurements after the addition of DCB to less than 1 min.

Therefore a special protocol is used. A series of laser flashes is delivered while the bath is continuously superfused with a solution containing DCB (0.2 mM) and DM-nitrophen (solution DMN5). The pipette solution contains 100 mM Na^+ (solution P1). Trace A of Fig. 8 shows the current response to a photolytic Ca^{2+} concentration jump in the absence of DCB, immediately (1 s) before the flow of solution DMN5 is started. Traces B and C are recorded 3 s and 6 s after the beginning of superfusion with solution DMN5. The data demonstrate that transient and stationary current components are blocked in the same way. The change in seal resistance between trace A and C is less than 2%. That the observed reduction of the current response to the concentration jump not is due to the washout of the released Ca^{2+} by the superfusion was shown in a set of control experiments during superfusion with a solution containing DM-nitrophen without DCB.

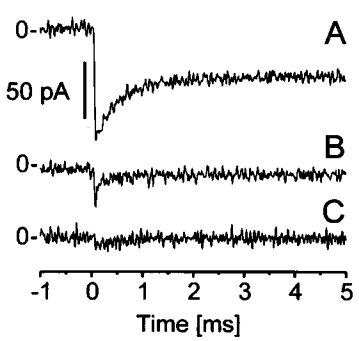

FIGURE 8 Block of transient and stationary current components by 2,4-dichlorobenzamil. Trace *A* is recorded immediately before the perfusion with the DCB containing solution is started. Traces *B* and *C* show the response to a laser flash 3 s and 6 s after the perfusion with a solution containing DM-nitrophen and 0.2 mM DCB is started. Both the transient and the stationary current are blocked by DCB. The temperature for all recordings is 21°C.

In all experiments, both the stationary and transient current responses to the Ca^{2+} concentration jump, which are clearly visible in the control before addition of DCB, are abolished in the presence of 0.2 mM DCB (n = 7).

Temperature dependence of stationary and transient Na^+-Ca^{2+} exchange current

For measurements of the temperature dependence of the stationary inward Na^+-Ca^{2+} exchange, the pipette solution contained 100 mM Na^+ (solution P1). Fig. 9 summarizes the data from 10 membrane patches. Exchange current is activated either by superfusion of the bath with the Ca^{2+} containing solution B2 (solid symbols) or by photolysis of caged Ca^{2+} (open symbols). Between 21°C and 38°C, the data can be fitted with an Arrhenius equation, using an activation energy of 70 ± 4 kJ mol^{-1} (solid line). Below 21°C, the slope is steeper, with an apparent activation energy of 138 ± 8 kJ mol^{-1} (broken line).

In Fig. 10 the temperature dependence of the rate $1/\tau_2$ for the decay of the transient current after photolysis of solution DMN1 is plotted between 13°C and 38°C. For experiments with extracellular Na^+, solution P1 is used, whereas for the experiments without Na^+ the pipette is filled with solution P3.

The rate of decay is significantly faster for Ca^{2+}-Ca^{2+} exchange compared to Na^+-Ca^{2+} exchange conditions at all temperatures, but in both cases an activation energy of ~70

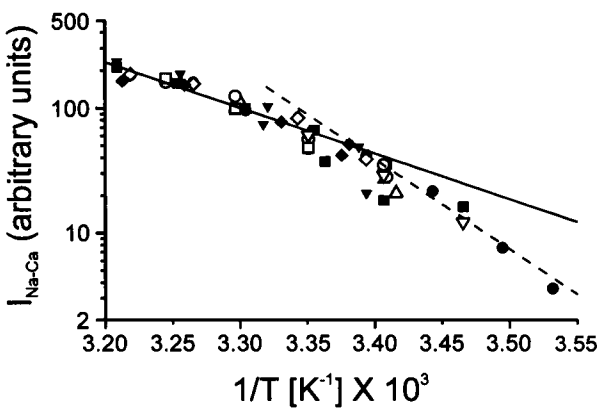

FIGURE 9 Temperature dependence of stationary Na^+-Ca^{2+} exchange current. Stationary Na^+-Ca^{2+} exchange current is activated at different temperatures by the application of intracellular Ca^{2+} via solution changes (■, ●, ◆, ▲, ▼) or by photolysis of caged Ca^{2+} (□, ○, ◇, △, ▽). Data from 10 different membrane patches are shown. Current amplitudes are shown in arbitrary units to make results from different patches comparable. The solid line is an Arrhenius fit of the data between 21°C and 38°C, giving an activation energy of 70 ± 4 kJ mol^{-1}. For the temperature range between 10°C and 21°°C, a much steeper dependence is observed, yielding an activation energy of 138 ± 8 kJ mol^{-1} (broken line).

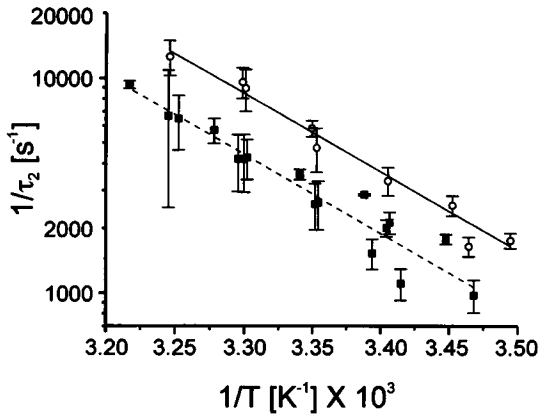

FIGURE 10 Temperature dependence of the rate constant $1/\tau_2$ for the decay of the transient current. Data from 28 different patches and 270 laser flashes are summarized in this plot. Open circles indicate data from experiments without extracellular Na^+ (5 mM Ca^{2+}). The solid line is an Arrhenius fit of these data, with an activation energy of 70 ± 6 kJ mol^{-1} (mean ± SD). Filled squares mark data obtained in the presence of 100 mM extracellular Na^+. The broken line is an Arrhenius fit of these data, with an acitvation energy of 70 ± 4 kJ mol^{-1}.

kJ mol^{-1} is derived by fitting the data with an Arrhenius equation. At 35°C, the rate of decay $1/\tau_2$ reaches a value of 6700 s^{-1} in the presence and 12,500 s^{-1} in the absence of extracellular Na^+.

In accordance with the idea that τ_1 is determined by the time resolution of the recording circuit, the values obtained for τ_1 are independent of the temperature between 13°C and 38°C.

DISCUSSION

In the previous sections, experiments are described which show that Ca^{2+} concentration jumps on the cytoplasmic side of excised heart muscle membrane patches elicit an inward current within a few tens of microseconds, which rises to a peak and decays to a plateau. As discussed in the following section, this current can be attributed to the Na^+-Ca^{2+} exchanger.

Photolabile Ca^{2+} chelators were previously used to study properties of the Na^+-Ca^{2+} exchange current with the patch-clamp technique under whole-cell conditions (Niggli and Lederer, 1991; Powell et al., 1993; Niggli and Lipp, 1994). Although there is general agreement that Ca^{2+} concentration jumps generate an inward current, there are considerable differences in detail between the studies of Niggli and co-workers and of Powell et al., as well as all of those previous studies and ours.

Linkage between the Ca^{2+}-induced current and the Na^+-Ca^{2+} exchanger

The experiments were performed under conditions that are considered to be standard to isolate Na^+-Ca^{2+} exchange currents from other membrane currents in heart muscle (e.g., Kimura et al., 1986; Hilgemann, 1989), and a variety of control experiments indicate that the transient current signal is not due to photochemical reactions of the DM-nitrophen. It nevertheless seems appropriate to discuss other sources for the current signal, because the rapid and large Ca^{2+} concentration jump may lead to charge translocation by a variety of mechanisms. Increasing the cytoplasmic Ca^{2+} concentration from 0.5 μM to 100 μM may change the surface potential of the plasma membrane and may lead to a capacitive current flow. Furthermore, screening of negative charges on the cytoplasmic side of the membrane by Ca^{2+} has the same effect on ion channels as a depolarization and may induce conformational changes in voltage-dependent channels ("gating currents"), even in the presence of blockers and in the absence of permeable ions. In both cases, however, the direction of current flow is expected to be outward (for gating currents see Bean and Rios, 1989), whereas the observed current flow is inward. Furthermore, the addition of Ni^{2+} on the extracellular side and of DCB on the cytoplasmic side as well as the removal of extracellular Na^+ or Ca^{2+}, which should not abolish the effect of Ca^{2+} on the surface potential, effectively suppress the Ca^{2+}-generated current signal. Furthermore, reduction of preflash Ca^{2+} below 0.1 μM markedly reduces the current amplitude.

Another possibility to be considered is current flow through Ca^{2+} channels or ion channels activated directly by cytoplasmic Ca^{2+} or indirectly via the surface potential. This may be a residual current through blocked (inactivated) Na^+, Ca^{2+}, and K^+ channels. In addition, Ca^{2+}-activated cation channels and chloride channels that were not specifically blocked were observed in heart muscle cells (Ehara et al., 1988). The long mean open time of Ca^{2+}-activated cation channels (~100 ms; Ehara et al., 1988) and the single-channel conductance of 10 to 30 pS are, however, not compatible with the decay time constant (<1 ms) of the current transient and the signal-to-noise ratio of small current signals. The mean open time of Ca^{2+}-activated Cl^- channels in heart muscle is unknown, but in other cells it is about 100 ms (Koumi et al., 1994). Thus it seems unlikely that Ca^{2+}-activated Cl^- channels are involved in the generation of the current signal. Residual currents through K^+, Ca^{2+}, and Na^+ channels are not likely because there is no K^+ present in most experiments, currents through Ca^{2+} channels should reverse under Ca^{2+}-Ca^{2+} exchange compared to Na^+-Ca^{2+} exchange, and with identical Na^+ and Li^+ concentrations on both sides of the membrane there is practically no driving force for currents through Na^+ channels.

In conclusion, it seems unlikely that ion channels contribute to the observed current signal. On the other hand, the current observed after a Ca^{2+} concentration jump shows properties which indicate that it is due to the Na^+-Ca^{2+} exchanger—it depends on extracellular substrate (Na^+, Ca^{2+}), and it is blocked by extracellular Ni^{2+} and cytoplasmic DCB, both known blockers of Na^+-Ca^{2+} exchange. Furthermore, preincubation with a Ca^{2+} concentration of

0.1 μM reversibly reduces the amplitudes of both the transient signal and the stationary Na^+-Ca^{2+} exchange current. Treatment with α-chymotrypsin irreversibly abolishes this inactivating effect of low cytoplasmic Ca^{2+}. This behavior demonstrates that both stationary inward Na^+-Ca^{2+} exchange current and transient current are regulated by the cytoplasmic high-affinity Ca^{2+} binding site of the exchanger.

Kinetic properties of the current signal and limitations of the recording system

As shown above, the laser flash generates an initial artefact that lasts for about 50 μs. Although its size can be reduced by shielding the electrodes and using petri dishes with a bottom made of glass, it could not be abolished completely. Thus, the initial artefact is suppressed by using the reset function of the patch-clamp amplifier, which short-circuits the input of the amplifier for about 50 μs. This efficiently eliminates the initial artefact, but part of the current signal generated by the exchanger is probably lost. After the end of the reset, the current signal rises with a time constant that is determined by the bandwidth of the recording system (maximally 23 kHz, corresponding to 7 μs). As the photolysis of caged Ca^{2+} starts 30 μs before the reset function is terminated, we assume that the rise of the current signal is mainly determined by the recording circuit and only partially by the release of Ca^{2+}. The maximum of the peak current may be reached during the reset phase of the amplifier. This has little effect on the amplitude of the peak current as long as the decay is not too fast. At higher temperatures, however, this may become a problem. So far, the data are compatible with the assumption that binding of Ca^{2+} is very rapid ($k_{on} \geq 10^8\ M^{-1}\ s^{-1}$) and that it may be described by its equilibrium constant. The peak current decays in an exponential manner with a time constant of about 0.5 ms at 25°C if Na^+-Ca^{2+} exchange is promoted, and about twice as fast in the Ca^{2+}-Ca^{2+} exchange mode. Time constants of this magnitude are easily resolved, but at temperatures above 35°C, the decay time constant becomes so small that it is in the range of the time resolution of the recording circuit. The significance of the decay time will be discussed below.

Comparison with previous Ca^{2+} concentration jump experiments

Photolabile Ca^{2+} chelators were used previously in combination with the whole-cell configuration of the patch-clamp technique to investigate the properties of Na^+-Ca^{2+} exchange current in heart muscle cells (Niggli and Lederer, 1991, 1993; Niggli and Lipp, 1994; Powell et al., 1993). These studies agree with the results presented here in so far as a Ca^{2+} concentration jump on the cytoplasmic side elicits an inward current in the presence of extracellular sodium. But in detail there are considerable differences between these studies and ours. Powell et al. report that an intracellular Ca^{2+} concentration jump generates an inward current that rises exponentially to a plateau with a time constant of 5 ms at 0 mV and 26°C. As a transient current is not observed, Powell et al. concluded that the Ca^{2+} translocation step is electroneutral. One explanation for the lack of a transient response may be the small Ca^{2+} concentration jump ($\Delta Ca^{2+} \approx 2.5\ \mu M$) applied by these authors. Under these conditions, Ca^{2+} binding may be rate limiting.

In contrast, Niggli and co-workers observed an initial inward peak current lasting for about 2 ms, which was followed by a stationary current. The peak current could be distinguished from the stationary current at 20°C but not at 35°C. Its time course could not be determined because the duration of the light flash was 0.4 ms. This peak current, which was called a conformational current by Niggli and Lederer, looks similar to the initial current observed here. But the pharmacological properties are very different because it is resistant to 4–8 mM extracellular Ni^{2+} and is increased sixfold by 3,4-DCB (1 mM) on the cytoplasmic side. The application of 1 mM intracellular DCB increased the transient current but did not block the stationary Na^+-Ca^{2+} exchange current. In contrast, the transient and stationary currents in excised membrane patches are abolished by 5 mM extracellular Ni^{2+} or 0.2 mM cytoplasmic 2,4-DCB, as shown above. The major experimental difference between previous studies and the one presented here is that previous studies were performed in the whole-cell mode, raising questions about the control of intracellular Ca^{2+} and a possible interference from intracellular Ca^{2+} stores (i.e., Ca^{2+}-induced Ca^{2+} release).

Hilgemann et al. (1991) performed slow Na^+ and Ca^{2+} concentration jumps on the cytoplasmic side by doing solution switches. Current transients were observed in the reverse Na^+-Ca^{2+} exchange mode and in the Na^+-Na^+ exchange mode after a cytoplasmic Na^+ jump. In the forward Na^+-Ca^{2+} exchange mode or in the Ca^{2+}-Ca^{2+} exchange mode, a cytoplasmic Ca^{2+} jump elicited a very small or no transient current signal. The observed charge movements were abolished by intracellular DCB with $K_1 < 10\ \mu M$. It was concluded that no or very little charge translocation is correlated with Ca^{2+} translocation. One possible explanation for the lack of a Ca^{2+}-dependent signal is that the application of Ca^{2+} by solution switches is too slow.

Implications of the relaxation current for the transport mechanism of the Na^+-Ca^{2+} exchange

The observation of inward peak and plateau current in the Na^+-Ca^{2+} exchange mode and of inward peak current in the Ca^{2+}-Ca^{2+} exchange mode sheds some light on the transport mechanism of this transport protein. Before the results are discussed in terms of a specific model, some qualitative conclusions are drawn.

The very short time to peak of the current (<100 μs) indicates that the release of Ca^{2+} from DM-nitrophen is

fairly rapid, as described by Ellis-Davies et al. (1996), and that early steps of the reaction cycle of the exchanger are electrogenic. One possibility is that Ca^{2+} binding is very fast and is followed by a slower electrogenic step. Alternatively, one might assume that Ca^{2+} binding itself is slow and electrogenic and is followed by a fast reaction step. In principle, we favor the first possibility, because it is easier to reconcile with the different time constants of the decay observed for the Na^+-Ca^{2+} exchange and Ca^{2+}-Ca^{2+} exchange mode. The rapid decay (τ_2 = 0.66 ms at 21°C) of the peak current to the steady state and the brief peak current in the Ca^{2+}-Ca^{2+} exchange mode demonstrate that at least some reaction steps of the Na^+-Ca^{2+} exchange are very fast, but it does not imply that the reaction cycle is as short as the relaxation time constant under the same conditions. For example, it may be envisioned that the exchanger performs a fast electrogenic reaction step after the concentration jump, and that this is followed by a slow electrogenic or nonelectrogenic reaction that does not contribute significantly to the relaxation time but determines the turnover time.

We observe a peak (transient) and a plateau inward current in the Na^+-Ca^{2+} exchange mode and peak inward current in the Ca^{2+}-Ca^{2+} exchange mode. This is most easily reconciled with the assumption that the peak current in both modes reflects the movement of negative charge in the outward direction during outward Ca^{2+} translocation, whereas net positive charge is moved inward in the stationary state, in accordance with the known stoichiometry of the Na^+-Ca^{2+} exchange. Simultaneous and consecutive Na^+ and Ca^{2+} translocation mechanisms have been suggested (Blaustein and Santiago, 1977; Ledvora and Hegyvary, 1983; Khananshvili, 1990; Hilgemann et al., 1991; Li and Kimura, 1991; Matsuoka and Hilgemann, 1992). Simple (one-step) simultaneous models are not at all compatible with the observation of a peak in the current signal, as in this case the Na^+-Ca^{2+} exchange current rises from zero to the stationary state with the time constant of Ca^{2+} binding. Within the framework of a consecutive transport model one expects that at saturating external Na^+ or Ca^{2+} concentrations and substrate concentrations far below saturation on the cytoplasmic side, the exchanger is ready to bind Ca^{2+} on the cytoplasmic side and to translocate it outward (e.g., Hilgemann et al., 1991). Current transients are expected after the rapid application of Ca^{2+} on the cytoplasmic side if the redistribution of carrier sites involves electrogenic steps (Powell et al., 1993; Hilgemann et al., 1991). But no transient is expected if the substrate concentrations on both sides are such that binding sites are oriented to the extracellular side. In the absence of extracellular Na^+ and Ca^{2+} and a preflash cytoplasmic Ca^{2+} concentration of 0.5 μM, no transient current is observed. This indicates that under these conditions binding sites are oriented to the extracellular side.

The possibility cannot be totally excluded that the initial peak is related to the inward movement of Na^+. However, this would imply that Ca^{2+} translocation is nonelectrogenic and extremely fast, occurring completely within 40 μs (during the reset time of the amplifier), and a nonelectrogenic, rate-limiting step must follow the movement of Na^+. Furthermore, the observation of an inward peak in the Ca^{2+}-Ca^{2+} exchange mode is not easily explained. One would have to assume that Ca^{2+} translocation is nonelectrogenic in the Na^+-Ca^{2+} exchange mode but electrogenic in the Ca^{2+}-Ca^{2+} exchange mode.

In the following the current signal is discussed in terms of a four-step reaction cycle (e.g., Niggli and Lederer, 1991; Powell et al., 1993), as shown in Fig. 11. This model has been used to determine which combinations of rate constants are possible and which fraction of charge may be assigned to each ion translocating step. The two ion translocating steps ($E_1 \rightarrow E_2$ and $E_3 \rightarrow E_4$) are interconnected by ion exchange reactions on the extracellular and the cytoplasmic side of the membrane ($E_2 \rightarrow E_3$ and $E_4 \rightarrow E_1$). Steps $E_1 \rightarrow E_2$ and $E_3 \rightarrow E_4$ are considered to be irreversible in the forward Na^+-Ca^{2+} exchange mode because there is no intracellular Na^+ and extracellular Ca^{2+}. It is assumed that the ion exchange reactions are fast (in equilibrium) at saturating ion concentrations and nonelectrogenic. Thus the Na^+ and Ca^{2+} exchange at the external and at the internal binding site have been lumped into a single step. A fast binding reaction of Ca^{2+} on the cytoplasmic side is implied by the time to peak after the release of Ca^{2+}, which is less than 100 μs. No experimental data on the dissociation rates of Na^+ and Ca^{2+} and on the association rate of Na^+ are available. The effect of possible slow ion exchange rates is discussed below. It should be pointed out, however, that the monoexponential relaxation is compatible with fast ion exchange reactions (Läuger, 1991).

In agreement with the 3 Na^+:1 Ca^{2+} stoichiometry, one net positive charge is translocated per cycle. In individual reaction steps, however, different amounts of charge may be translocated. The fraction of charge translocated in the $E_1 \rightarrow E_2$ transition is denoted by q, and therefore $(1 - q)$ elementary charges must be moved per exchanger during

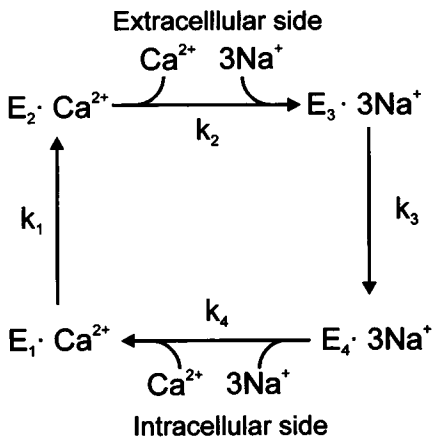

FIGURE 11 Scheme of four-state model used for fitting and discussion of the experimental data (see text for details).

Na^+ translocation. It will be seen below that the shape of the current signal and the results obtained by other investigators impose certain restrictions on the amount of charge placed on individual reaction steps. Other conditions are as follows. Initially all exchangers are in state E_4, which is susceptible to Ca^{2+} binding. All rate constants are first-order or pseudo-first-order rate constants.

Investigations conducted by Hilgemann et al. (1991) and Niggli and Lederer (1991) show that Na^+ translocation is a major electrogenic step. Thus $q > 0.5$ seems not to be a reasonable choice. With $q < 0.2$, very slow Na^+ translocation and low turnover numbers ($<50\ s^{-1}$) are necessary to reproduce the peak/plateau ratio of the current signal. Therefore, $q = 0.4$ is used for the simulations. Fig. 12 shows a fit using the four-step model, with $q = 0.4$ to an average current signal. Symbols mark data generated by the equation $I = I_S - I_P \cdot \exp(-t/\tau_2)$, to calculate a curve exhibiting the characteristics of the mean of the measured current signals at 21°C with $\tau_2 = 0.66 \pm 0.18$ and a peak/plateau ratio $I_P/I_S = 3.6 \pm 0.8$ ($n = 101$). The rise of the current to the transient peak, which is too fast to be resolved in the experiments, is assumed to be instantaneous for the model calculations. The solid line is a differential equation fit with the four-state model. The reaction rates k_1 and k_3 obtained are 1310 and 165 s^{-1}, respectively, and the values for k_2 and k_4 each reached the upper limit of 20,000 s^{-1} (given in the fitting constraints) during the fit procedure. For a temperature of 35°C, the mean values of the experimental data are 0.11 ms for τ_2 and 1.95 for the peak/plateau ratio. The corresponding rate constants obtained by the four-state model fit are 5260 and 1540 s^{-1} for k_1 and k_3, and the values for k_2 and k_4 again reached the upper limit of 20,000 s^{-1}. A fit of equal quality can be obtained by exchanging the values of k_2 and k_3. This would imply that Na^+ binding is electrogenic, as suggested by Hilgemann et al. (1991), and slow compared to the Na^+ translocation step. For values of $q > 0.5$, it is possible to find a set of parameters with Na^+ translocation faster than Ca^{2+} translocation (i.e., $k_1 < k_3 < k_2, k_4$) that produces a transient signal followed by a stationary current. But the peak/plateau ratio under these conditions is always smaller than 1.5, and therefore a reasonable fit of the data is not possible. A set of parameters that makes k_4 the rate-limiting step does not allow any reasonable fit of the data.

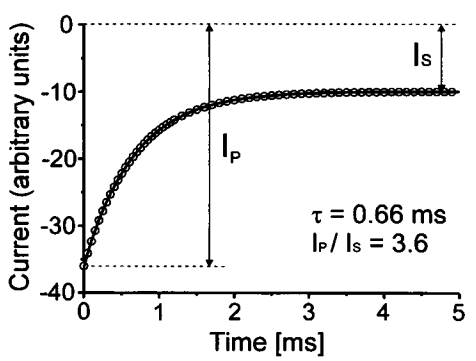

FIGURE 12 Fit of an average current signal with $\tau_2 = 0.66$ and a peak/plateau ratio of 3.6, using the four-state model. Open circles are calculated values corresponding to a mean data set at 21°C, and the solid line is the fit curve (see text for details).

Therefore, the current transient must mainly arise from the Ca^{2+} translocation step, which has to be followed by a rate-limiting Na^+ binding or translocation step, and the relaxation rate $1/\tau_2$ is mainly determined by the rate of Ca^{2+} translocation k_1, whereas the turnover is determined by the rate of Na^+ translocation k_3 or Na^+ binding k_2. That Na^+ translocation is rate limiting has been deduced from the current/voltage relation of the stationary exchange current (Niggli and Lederer, 1991).

No estimate for the value of q can be derived from the fitting procedure, as fitting is equally possible for a wide range of values for q, but the choice of q strongly influences the turnover number calculated from the fit parameters. This is demonstrated by the equation $k_t = q \cdot k_1 \cdot I_s/I_p$, which is derived from the equations $I_p = k_1 \cdot q \cdot E_1^0 \cdot e^-$ and $I_s = k_t E_1^0 e^-$. E_1^0 corresponds to the total number of exchanger molecules, which is assumed to be in a state ready to bind Ca^{2+} before the Ca^{2+} concentration jump. Using the data obtained at 21°C, one gets $k_t = q \cdot 420\ s^{-1} = 168\ s^{-1}$ for $q = 0.4$. At a temperature of 35°C, the corresponding values are $k_t = q \cdot 4600\ s^{-1} = 1840\ s^{-1}$ for $q = 0.4$. Both values are in good agreement with the numbers obtained by the differential equation fits of the data with the four-state model.

Although the data presented here do not permit us to determine the various rate constants of the model, the above equation for k_t sets an upper limit for the turnover number of the forward Na^+-Ca^{2+} exchange, as $q \leq 1$ by definition and $I_s/I_p < 0.5$ for temperatures below 35°C. Therefore, high turnover numbers of $\sim 2000\ s^{-1}$ at physiological temperatures (37°C) are certainly possible, as has been suggested by others (Cheon and Reeves, 1988; Hilgemann et al., 1991; Niggli and Lederer, 1991). A lower limit for the turnover rate k_t of Na^+-Ca^{2+} exchange cannot be derived from our data. Independently of the precise turnover rate, one can conclude that Ca^{2+} translocation is very fast and must be followed by a slower rate-limiting step, which is probably Na^+ translocation.

Under conditions that promote Ca^{2+}-Ca^{2+} exchange (i.e., no extracellular Na^+ and 5 mM extracellular Ca^{2+}), a Ca^{2+} concentration jump generates a transient inward current that decays to zero with an exponential time course. The observation of transient inward charge movement in the Ca^{2+}-Ca^{2+} exchange mode strongly supports the idea that the Ca^{2+}-loaded exchanger bears negative charge, and it is easier to reconcile with a consecutive transport model than with a simultaneous one.

The transient current seen in the Ca^{2+}-Ca^{2+} exchange mode looks very similar to the peak current observed in the Na^+-Ca^{2+} exchange mode, but its relaxation time constant is lower by a factor of 2 at all temperatures. The peak current is nearly identical in both transport modes. This may be fortuitous, but it could indicate that initial conditions are

very similar in both modes, i.e., nearly all exchangers are in state E_4, and that the occupancy of states in the stationary state is also comparable. Within the framework of the four-step model, the exchanger shuttles between states E_1 and E_2 in the Ca^{2+}-Ca^{2+} exchange mode. The relaxation time constant is then determined by $1/(k_1 + k_{-1})$. If it is assumed that the relaxation rate constant observed in the Na^+-Ca^{2+} exchange mode equals k_1, then it follows that $k_{-1} \approx k_1$. The turnover in the Ca^{2+}-Ca^{2+} exchange mode is given by $k_t = 1/(1/k_1 + 1/k_{-1})$ and may be faster than in the Na^+-Ca^{2+} exchange mode. This, however, is in contradiction to measurements of stationary isotope fluxes, which indicate that forward Na^+-Ca^{2+} exchange is faster than Ca^{2+}-Ca^{2+} exchange (Khananshvili, 1991; Khananshvili et al., 1995). At present, we have no explanation for this discrepancy. One possibility is that the simple four-step model is not sufficient to describe the stationary transport. Another possibility is that isotope exchange and electrical currents are not identical. This may happen, for example, if the transport sites are connected via narrow channels to the medium outside the exchanger protein. Such narrow ion channels ("single-file channels"), which show discrepancies between isotope fluxes and electrical currents, are described by Hodgkin and Keynes (1955).

Inactivation of exchange current at low intracellular Ca^{2+}

At low Ca^{2+} concentration on the cytoplasmic side (<100 nM), the Na^+-Ca^{2+} exchanger enters a state of inactivation that is controlled by a Ca^{2+}-binding site that is not identical to the transport site (DiPolo and Beaugé, 1986; Kimura et al., 1986). The properties of this regulatory site were extensively studied in giant excised patches of guinea pig by Hilgemann et al. (1992a) using the reverse Na^+-Ca^{2+} exchange mode. Studies in the forward Na^+-Ca^{2+} exchange mode were not possible so far with the wild-type Na^+-Ca^{2+} exchanger, because Ca^{2+} binds to the regulatory site with higher affinity, and Ca^{2+} concentration jumps performed by solution switches were not fast enough to distinguish between both sites. Matsuoka et al. (1995) used mutants of the Na^+-Ca^{2+} exchanger expressed in *Xenopus* oocytes to investigate the regulatory influence of cytoplasmic Ca^{2+} on the forward Na^+-Ca^{2+} exchange current. For these mutants, the Ca^{2+} affinity of the regulatory binding site was lower than that of the transport binding site. Under these conditions, they could demonstrate that inward Na^+-Ca^{2+} exchange current is also regulated by the regulatory binding site.

In our experiments, where intracellular Ca^{2+} is changed very quickly, the regulatory effect also becomes visible in case of the forward Na^+-Ca^{2+} and the Ca^{2+}-Ca^{2+} exchange modes of the native Na^+-Ca^{2+} exchanger. This is possible because of the different activation kinetics for exchanger molecules with and without an occupied regulatory binding site. If the regulatory binding site is occupied by Ca^{2+} (free $Ca^{2+} \approx 0.5$ μM) or if the exchanger molecules are deregulated by treatment with α-chymotrypsin, a Ca^{2+} concentration jump activates the Na^+-Ca^{2+} exchanger on a time scale of <1 ms. In the case of a lower free Ca^{2+} concentration (<100 nM), a substantial fraction of the exchanger molecules will have an unoccupied regulatory Ca^{2+}-binding site and will therefore be inactivated. This fraction shows much slower activation kinetics in response to a sudden rise in intracellular free Ca^{2+}, with a time constant for the recovery from inactivation of ~0.6 s at room temperature.

The slow response of the regulatory binding site may have some physiological relevance, because during the excitation-contraction cycle, the Ca^{2+} concentration oscillates between 120 and 1100 nM (Beuckelmann and Wier, 1988), which is on the order of the dissociation constant of the regulatory binding site (Hilgemann et al., 1992a; Levitsky et al., 1994). Furthermore, the time constant of recovery is in the range of one contraction cycle.

Temperature dependence of Na^+-Ca^{2+} and Ca^{2+}-Ca^{2+} exchange

For the stationary inward Na^+-Ca^{2+} exchange current, an activation energy of 70 ± 4 kJ/mol is derived from the Arrhenius plot for temperatures between 21°C and 38°C. This corresponds to a Q_{10} value of 2.6 for T = 21°C, which is similar to the values observed by others in whole cells (Kimura et al., 1987; Niggli and Lederer, 1993) or giant excised patches (Hilgemann et al., 1992b) from guinea pig. For temperatures below 21°C, we obtained a much higher activation energy of 138 ± 8 kJ/mol (Q_{10} = 6.7). Breaks in the Arrhenius plot of the temperature dependence of Na^+-Ca^{2+} exchange in the range of 22°C have been observed before by Bersohn et al. (1991) for sarcolemmal vesicles from rabbit and by Khananshvili et al. (1995) in calf cardiac sarcolemmal vesicles.

In the Arrhenius plot of the decay time constant, no discontinuity is visible for either Na^+-Ca^{2+} or Ca^{2+}-Ca^{2+} exchange, but because of the larger scatter of these data, the existence of such a break cannot be ruled out completely. On the other hand, the absence of such a discontinuity would not be surprising, if it is assumed that the reaction steps associated with the transient current signal are not rate limiting for the turnover of Na^+-Ca^{2+} exchange, as discussed above.

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