Activation of Na-Ca Exchange Current by Photolysis of "Caged Calcium"

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ABSTRACT Intracellular photorelease of Ca^{2+} from "caged calcium" (DM-nitrophen) was used to investigate the Ca^{2+}-activated currents in ventricular myocytes isolated from guinea pig hearts. The patch-clamp technique was applied in the whole-cell configuration to measure membrane current and to dialyze the cytosol with a pipette solution containing the caged compound. In the presence of inhibitors for Ca^{2+}, K^+, and Na^+ channels, concentration jumps of $[Ca^{2+}]_i$ induced a rapidly activating inward Na-Ca exchange current which then decayed slowly ($\tau \approx 500$ ms). The initial peak of the inward current and the time-course of current decay were voltage-dependent, and no reversal of the current direction was found between −100 and +100 mV. The observed shallow voltage dependence can be described in terms of the movement of an apparently fractional elementary charge ($+0.44\,e^-$) across an energy barrier located symmetrically in the electrical field of the membrane. The currents were dependent on extracellular Na^+ with a half-maximal activation at 73 mM and a Hill coefficient of 2.8. No change of membrane conductance was activated by the Ca^{2+} concentration jump when extracellular Na^+ was completely replaced by Li^+ or *N*-methyl-D-glucamine (NMG) or when the Na-Ca exchange was inhibited by extracellular Ni^{2+}, La^{3+}, or dichlorobenzamil (DCB). The velocity of relengthening after a twitch induced by photorelease of Ca^{2+} was only reduced drastically when both the sarcoplasmic reticulum and the Na-Ca exchange were inhibited suggesting that all other Ca^{2+} removing mechanisms have a low transport capacity under these conditions. In conclusion, we have used a novel approach to study Na-Ca exchange activity with photolysis of "caged" calcium. We found that in guinea pig heart muscle cells the Na-Ca exchange is a potent mechanism for Ca^{2+} extrusion, is weakly voltage-dependent (118 mV for *e*-fold change) and can be studied without contamination with other Ca^{2+}-activated currents.

INTRODUCTION

The Na-Ca exchange is a unique countertransport mechanism that uses the electrochemical Na^+ gradient across the cell membrane to perform uphill transport of Ca^{2+} ions (1–3). In cardiac myocytes, the Na-Ca exchange is the most important system removing the Ca^{2+} ions which enter the cell with the Ca^{2+} current to initiate contraction. The Na-Ca exchange has also obtained recent attention because it may be involved in rapid events during excitation-contraction coupling in cardiac muscle (5–7) and because the primary structure of several cardiac Na-Ca exchangers has been deduced from the cDNA sequence (canine (8), human (9), bovine (10)).

For the cardiac exchange, a stoichiometry of 3 Na^+: 1 Ca^{2+} is well established and results in net charge translocation during ion transport (for review see Ref. 11, but Ref. 12 for the stoichiometry in rod outer segments). Depending on the electrochemical gradients for Na^+ and Ca^{2+}, the Na-Ca exchange can also move Ca^{2+} ions into the cell ("backward" mode). Recently, the electrogenicity has been used experimentally to measure the activity (or changes in activity) of the Na-Ca exchange in single cells from different tissues (e.g., Refs. 13–17). Because no specific pharmacological blocker is available for the Na-Ca exchange, the exchange current (I_{NaCa}) has usually been defined as a *cis* Ca^{2+}-activated *trans* Na^+-dependent difference current (or vice versa). Since $[Ca^{2+}]_o$ and $[Na^+]_o$ as well as $[Na^+]_i$ are not expected to change significantly during intracellular Ca^{2+} signaling, the activation of the Na-Ca exchange by $[Ca^{2+}]_i$ is the most relevant mechanism for the cellular function of this transporter (but see Refs. 5 and 6). Experimental analysis of $[Ca^{2+}]_i$-induced activation of the Na-Ca exchange has been difficult in cardiac myocytes, because changes in $[Ca^{2+}]_i$ may not selectively activate the Na-Ca exchange, but are also expected to influence other Ca^{2+}-dependent currents like the Ca^{2+}-activated nonselective cation channels (18, 19). Consequently, identification of Na-Ca exchange activity as the whole-cell current I_{NaCa} under conditions of changing $[Ca^{2+}]_i$ is not straightforward.

Recently developed photolabile biologically inactive molecules ("caged compounds") allow rapid concentration jumps of the biologically active substance by photolysis with an intense flash of ultraviolet light. In our experiments we have used DM-nitrophen ("caged calcium") to increase $[Ca^{2+}]_i$ transiently (20). This technique promised to be a convenient and reliable tool to investigate the $[Ca^{2+}]_i$-activated currents in ventricular myocytes isolated from guinea pigs under a variety of experimental conditions including different membrane potentials and the absence and presence of extracellular substrates or inhibitors of the Na-Ca exchange. One goal of the present study was thus to characterize the membrane currents activated by photorelease of Ca^{2+} and to examine whether contamination of the measured currents by activation of $[Ca^{2+}]_i$-dependent currents other than the Na-Ca exchange was significant.

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Surprisingly, we only observed Ca^{2+}-induced current transients consistent with the activation of the Na-Ca exchange. No currents related to the calcium-sensitive nonselective cation channels were recorded. Thus, photorelease of $[Ca^{2+}]_i$ turned out to be a useful approach to investigate the cellular function of the Na-Ca exchange without contamination from other Ca^{2+}-activated currents under a variety of experimental conditions. Furthermore, the rapid concentration jumps of $[Ca^{2+}]_i$ ($t_{½}$ less than 30 μs) may also allow the investigation of the molecular function of the Na-Ca exchanger (21, 22). Part of the present work has been presented in abstract form to the Swiss Society of Physiology (23).

METHODS

System overview

The set-up consisted of a vibration-isolation table (Kinetic Systems, Roslindale, MA) with a Faraday-cage containing the experimental chamber mounted on the stage of an inverted microscope (Nikon, Inc., Garden City, NY) and of racks holding the electronics for data acquisition and experiment control. The chamber was equipped with an electronically controlled perfusion system and with a Peltier element for cooling and heating. The flash-photolysis unit was enclosed in another small Faraday cage located under the vibration isolation table. The individual experimental systems are described in more detail below.

Voltage-clamp and cell-length measurements

Whole-cell voltage-clamp currents were recorded with a Dagan 8900 patch clamp amplifier (Dagan Corp., Minneapolis, MN) using the giga-seal technique. Microelectrodes were pulled to 1–2 MΩ from borosilicate glass (WPI, New Haven, CT). Timing, voltage-clamp protocols and simultaneous data acquisition at appropriate sampling rates were controlled by an 80386 CPU-based computer (Dell Computer Corp., Austin, TX) running pClamp software (Axon Instruments, Burlingame, CA). In addition, up to eight signal channels could be digitized with a PCM recorder (Neurodata Inc., New York, NY) and stored on videotape.

The cell image was recorded on videotape with a CCD camera (Cohu Inc., San Diego, CA). Cell length was measured on-line or off-line from the video signal using a video dimension-analyzer (Crescent Electronics, Sandy, UT).

Flash-photolysis

A flash-unit which discharges electrical energy up to 230 J through a Xenon short-arc flash-lamp was used to produce a pulse of light of ~400-μs duration (Chadwick-Helmuth, El Monte, CA). The light produced was collected with an ellipsoidal mirror (Opti-Forms, Temecula, CA) and a portion of the spectrum (330–390 nm) was reflected by a dichroic mirror (Acton Research, Acton, MA) onto the input aperture of a liquid light guide (Oriel, Stratford, CT). The output aperture of the liquid light-guide was imaged onto the cell in an epi-illumination arrangement using a plano-convex quartz condenser lens and the microscope objective (Neofluar, 63x Oel, N.A. 1.25; Zeiss, Thornwood, NY). Wavelength selection was enhanced between the condenser lens and the preparation by reflecting the ultraviolet light off two additional dichroic mirrors centered around 350 nm (Omega Optical, Brattleboro, VT). Contamination of the voltage-clamp records by electromagnetic interference (EMI) resulting from the capacitor discharge was carefully minimized. We could reduce artifacts below the whole-cell noise in most cases using a combination of techniques. The flash-unit was enclosed in a Faraday cage. A liquid light guide was used to direct the flash, while a fiber-optic connection communicated the trigger signals to the flash unit. During the capacitor discharge, the power supply was disconnected briefly (100 ms) from the power line by custom-built electronics in order to suppress voltage transients mediated by the power cords.

Isolation of cells

Single ventricular myocytes from guinea pigs were isolated by an enzymatic procedure (24). Briefly, heparinized (300 units) guinea pigs were anesthetized with 25 mg of pentobarbital sodium. The hearts were quickly removed and mounted on a Langendorff perfusion system. Enzymatic digestion was performed by retrogradely perfusing the heart with 50 ml of a solution containing 25 mg of collagenase B (Boehringer-Mannheim, Indianapolis, IN) and 5 mg of protease (Sigma, St. Louis, MO). After perfusion with the enzymes, the ventricles were chopped into small pieces and gently agitated in a solution containing 200 μM Ca^{2+}. Cells were harvested from the supernatant and transferred into the experimental solution in the chamber immediately before the experiment.

Solutions

The electrode filling solution had the following composition (in millimolar): Cs-gluconate 120; 4-(hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20; TEA-Cl, 20; Na_4-DM-nitrophen, 2; $CaCl_2$, 0.5; K-ATP 5; pH adjusted to 7.2 with CsOH. Reduced glutathione (GSH, 2 mM) was added to the filling solution to prevent side-effects of the photolytic byproducts (25). In this solution, DM-nitrophen is only partially loaded with Ca^{2+}. In order to maintain the cells close to a physiological $[Ca^{2+}]_i$ during loading of the caged compound, the cells were continuously stimulated to produce twitches at 1 Hz. With an apparent K_d of 5 nM for Ca^{2+} (in the absence of Mg^{2+}) DM-nitrophen does not represent a significant intracellular Ca^{2+} buffer, because it is almost completely loaded with Ca^{2+} at physiological $[Ca^{2+}]_i$. The liquid junction potential of the Cs-gluconate filling solution was not corrected in the results presented (≈13 mV). Cells were superfused with a solution of the following composition (in millimolar): NaCl, 145; K, 4; $CaCl_2$, 1; CsCl, 1; $BaCl_2$, 0.5; HEPES, 10; glucose, 10; pH was adjusted to 7.4 with NaOH. $NiCl_2$, 8; verapamil, 0.01; ryanodine, 0.01 were added for some experiments. Unless indicated otherwise, the experiments were carried out at room temperature (20–23°C).

RESULTS

Membrane current and twitch contraction induced by concentration jumps of $[Ca^{2+}]_i$

When cells dialyzed with a pipette filling-solution containing Ca^{2+} and the light-sensitive Ca^{2+}-buffer DM-nitrophen are exposed to a flash of ultraviolet light (duration ≈400 μs), a concentration jump of intracellular Ca^{2+} is expected to occur. DM-nitrophen has an apparent K_d of 5 nM for Ca^{2+}, whereas the photolytic products of DM-nitrophen exhibit a K_d of 3 mM (measured at pH 7 and in the absence of Mg^{2+} (20)). This 10^6-fold decrease of Ca^{2+} affinity is followed by a rapid release of Ca^{2+} ions with a $t_{½}$ of ≈30 μs (26, 27). The Ca^{2+} ions photoreleased intracellularly affect all Ca^{2+}-sensitive membrane currents. In addition, an isolated cardiac myocyte shortens as a consequence of the activation of the contractile proteins by photoreleased Ca^{2+}. Fig. 1 *A* shows the whole-cell membrane current and the cell-length signal measured during such an experiment in a cell which was dialyzed with the DM-nitrophen containing filling solution for 10 min. During the loading period Ca^{2+} currents were elicited at 1 Hz by changing the membrane potential from −40 to 0 mV for 300 ms. This protocol enabled us to continuously examine the low access resistance and the Ca^{2+}

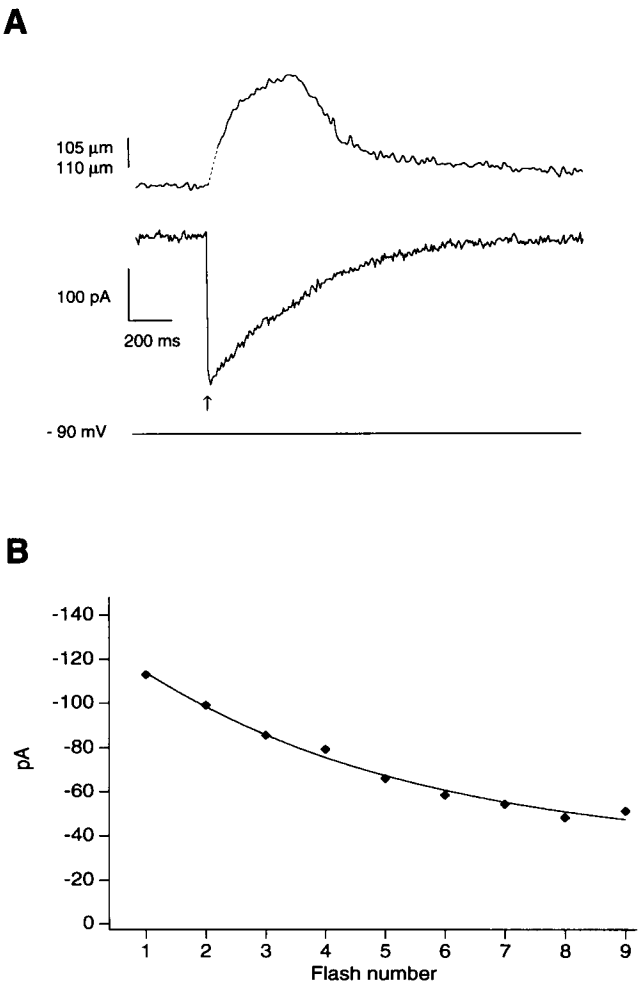

FIGURE 1 Inward current and cell shortening induced by flash photolysis of caged Ca^{2+}. (*A*) Cell length, membrane current, and voltage are shown as recorded from a single myocyte when a flash of intense ultraviolet light was applied. The cell was dialyzed with a pipette filling solution containing DM-nitrophen and Ca^{2+}. At the arrow a flash was triggered, resulting in a concentration jump of $[Ca^{2+}]_i$ in the cytosol of the myocyte. Immediately after the flash, the cell started to shorten and an inward current was fully activated. The inward current then slowly decayed, presumably because the Na-Ca exchange and the Ca^{2+} pump of the SR removed the Ca^{2+} ions from the cytosol (as indicated by relengthening of the cell). (*B*) The plot shows peak inward currents obtained in one myocyte by repeatedly applying 230-J flashes at a holding potential of −40 mV. As expected for photoconsumption of the light-sensitive caged compound, the decreasing peak inward currents can be fitted by a monoexponential function (*continuous line*).

current kept the $[Ca^{2+}]_i$ at physiological concentrations during the process of loading with DM-nitrophen. Once the flash experiments started, the cells were usually held at 0 mV to elevate resting Ca^{2+} slightly. This maneuver ensures complete loading of DM-nitrophen with Ca^{2+}. Under these conditions, photolysis of DM-nitrophen results in a stepwise increase of $[Ca^{2+}]_i$ and rebinding of photoreleased Ca^{2+} to unphotolyzed DM-nitrophen is minimized. When Ca^{2+} induced currents were activated at voltages other than 0 mV, the membrane potential was changed 400 ms prior to the flash and 10 μM verapamil was present to prevent changes of $[Ca^{2+}]_i$ before the flash was applied. During the record shown in Fig. 1 *A*, the membrane potential was kept unchanged at −90 mV. At the arrow, a flash of light was produced by discharging an energy of 230 J through the xenon flash lamp. Immediately after the flash, mechanical shortening of the myocyte and an inward membrane current were activated. The current subsequently decayed slowly, presumably because the Na-Ca exchange and the Ca^{2+} pump of the SR removed Ca^{2+} from the cytosol, as indicated by relengthening of the cell. In control experiments where DM-nitrophen was replaced by EGTA, no current was observed after the flash. Additional control experiments in the absence of Ca^{2+} were performed to verify that photolytic by-products of DM-nitrophen do not change the membrane current. The inward current activated by photolysis of caged Ca^{2+} is consistent with the electrogenic Na-Ca exchange as the current source. Because the Na-Ca exchange removes one Ca^{2+} ion from the cytosol in exchange for 3 Na^+ ions it carries net positive charge into the cell and thus produces inward current. But other Ca^{2+}-activated changes of the membrane conductance could contribute to the observed current at −90 mV as well (e.g., the Ca^{2+}-activated nonselective cation channels, see below and Ref. 28).

Usually, between 10 to 20 photolytic flashes (at 230 J) could be applied in one myocyte, separated at least by the time required to recharge the capacitors (30 s). Repeated flash illumination of a single cell loaded with DM-nitrophen resulted in a gradual decrease of the peak current response. Fig. 1 *B* shows the measured peak currents elicited by repeatedly discharging flashes (230 J each) at 30-s intervals. The decay can be fitted with a monoexponential function as expected for photoconsumption of the light-sensitive Ca^{2+} buffer DM-nitrophen. The analysis of the photoconversion suggests that with our optical system each flash photolyzes at least ≈13% of the DM-nitrophen present in the cell (not including any additional supply of unexposed DM-nitrophen through the pipette tip between subsequent flashes). Pressure-injecting pipette filling solution into the cell after photobleaching restored the large currents suggesting that the decrease is not due to run-down of the Na-Ca exchange. Currently, we are not yet able to measure the $[Ca^{2+}]_i$ immediately after the flash. However, the resting cell length as well as the observation of vigorous unloaded shortening after photorelease of Ca^{2+} indicate that, at a membrane potential of 0 mV, resting $[Ca^{2+}]_i$ was slightly above the physiological concentration (≈100 nM) and that $[Ca^{2+}]_i$ after the flash was considerably more elevated than during a Ca^{2+} transient induced by a voltage-clamp depolarization.

Identification of membrane currents activated by photolysis of caged Ca^{2+}: voltage dependence

In order to further characterize and identify the current induced by the $[Ca^{2+}]_i$-jump a number of different approaches were chosen. First, the voltage dependence of the currents and of the transport rate was investigated under experimental conditions designed to make the Na-Ca exchange the most

important system removing Ca^{2+} ions from the cytosol. In order to pharmacologically eliminate interference by the SR with the photolytically generated Ca^{2+} transient, cells were preincubated in 10 μM ryanodine which was also added to the superfusion solution. Verapamil 10 μM was used to avoid voltage-dependent Ca^{2+} influx through L-type Ca^{2+} channels preceding the flash. Although the Na-Ca exchange current has a reversal potential E_{NaCa} (29, 30) and can produce outward current at potentials positive to E_{NaCa} and inward current at more negative potentials, the difference current induced by an increase of $[Ca^{2+}]_i$ is expected to be always inwards at any given potential. If the rate-limiting partial reaction of the Na-Ca exchange cycle or a partial reaction closely associated with the rate-limiting step move charge, the current induced by $[Ca^{2+}]_i$ jumps is expected to depend on voltage (31). However, Ca^{2+}-activated cation channels seem to have fairly similar permeabilities for Na^+, K^+, and other small monovalent cations. Ca^{2+}-activated difference currents through these channels would exhibit a reversal potential around 0 mV (19). The results presented in Fig. 2 show observations we made when investigating effects of voltage on $[Ca^{2+}]_i$-induced currents. Comparison of the records in Fig. 2 *A* reveals that the amplitude of the peak inward current immediately after the flash had a tendency to increase with more negative potentials. The peak inward current was −90 pA at +60 mV and increased to −260 pA when the flash was triggered at −40 mV. Since the peak of the inward current reflects the turnover rate of the Na-Ca exchange, removal of photoreleased Ca^{2+} ions from the cytosol is expected to be slower at more positive voltages if the Na-Ca exchange is the dominant system removing $[Ca^{2+}]_i$ (32). Consistent with this notion, the time-course of current decay was indeed slowed down at more positive voltages. At +60 mV, the time-course of the current decay could be fitted by a single exponential with a τ of 614 ms, whereas at −40 mV the τ was 302 ms. This finding suggests that after elimination of the SR function a voltage-dependent transport system became the most important process extruding $[Ca^{2+}]_i$, most likely the Na-Ca exchange. This idea was confirmed when the Na-Ca exchange was also eliminated (see below). Pooled data from 106 measurements performed in 26 different cells show the voltage dependence for the current immediately after the flash normalized for the peak current at −40 mV (Fig. 2 *B*.) In the entire voltage range examined (−100 to +100 mV) reversal of the current direction was not observed suggesting that contamination by a Ca^{2+}-activated membrane conductance other than I_{NaCa} was small or lacking. A hyperpolarization of 118 mV was required to increase the peak-current *e*-fold (e.g., from +50 to −68 mV). This voltage dependence can be modeled as a rate-limiting movement of charge through the electrical field of the membrane. Eyring rate theory can be used to predict the voltage dependence of a chemical transition moving charge across a symmetrical energy barrier in an electrical field (33). Such a model is described by Eq. 1, where I denotes current, I_0 current at zero mV, E membrane potential, and z the amount of charge moved. R, F, and T have their usual meaning.

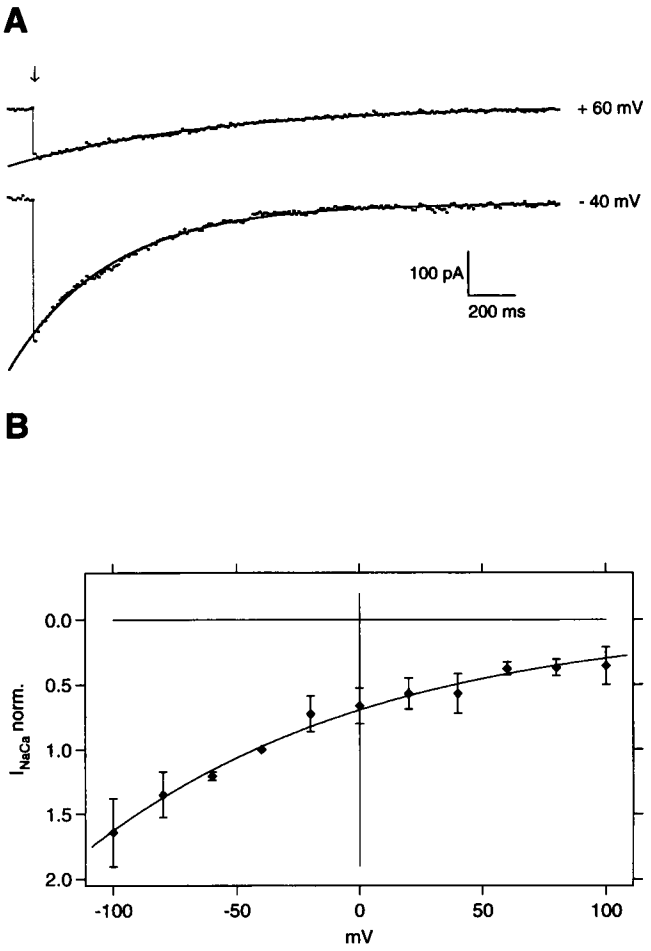

FIGURE 2 Voltage dependence of inward current induced by photolysis of caged Ca^{2+}. (*A*) The upper current trace shows the current recorded with the membrane potential clamped at +60 mV, the lower trace was measured at −40 mV. Flashes were triggered at the arrow. The continuous lines represent the fits of monoexponential functions to the data with τ of 614 and 302 ms, respectively. Ryanodine 10 μM and verapamil 10 μM was present in all tests of voltage dependence. (*B*) Summary figure illustrating the voltage dependence of peak inward current. Data are pooled from 26 cells and represent 106 flash experiments. Currents are normalized with respect to the current elicited at −40 mV. For each voltage the mean of n = 5–18 flashes (mean ± SE) are shown. The continuous line represents a nonlinear least-squares fit of a rate-theory model to the observed voltage dependence (for details see text).

$$I = I_0 \exp(zFE/2RT) \quad (1)$$

This equation predicts an *e*-fold change in current for every 51 mV if one elementary charge is moved (z = 1) with the rate-limiting transition. The continuous line in Fig. 2 *B* represents a nonlinear least-squares fit of this equation to the data. The voltage dependence obtained by fitting this equation to our data corresponds to an apparently fractional charge of +0.44 elementary charges moving into the cell with the rate-limiting step for cycling. In order to estimate the statistical confidence limits for the apparent charge, the model and the data were linearized by taking logarithms on both sides of equation 1. A linear fit to the data produced an

identical slope (+0.44 charges) with 95% confidence limits corresponding to +0.40 and +0.48 apparent charges, respectively. Other studies have reported a more pronounced voltage dependence for I_{NaCa} activated by intracellular Ca^{2+} (e.g., Refs. 29 and 32). Part of this difference may be explained by the inevitable changes of resting Ca^{2+} at different test potentials. The resting Ca^{2+} immediately before a spontaneous Ca^{2+} release or before a Ca^{2+} concentration jump induced by a flash will depend on voltage (because the Na-Ca exchange itself moves Ca^{2+} into the cell at more positive potentials). This experimental difficulty tends to make I_{NaCa} smaller at more positive voltages but larger at more negative voltages, thus apparently increasing the voltage dependence of I_{NaCa} in a manner which depends on the duration the cell was maintained at the test potential. When using flash photolysis of caged Ca^{2+} we employed a voltage protocol which kept the time at the test potential relatively short (≈400 ms) in order to avoid changes in resting Ca^{2+} before the flash. A steeper voltage dependence for I_{NaCa} was also described when I_{NaCa} was activated by rapid superfusion of extracellular Na^+ (34). Since the extracellular Na^+ has a pronounced effect on the voltage dependence of I_{NaCa} under some conditions (35) peak exchange currents during these experiments may also be affected by the changing Na^+ during activation of the Na-Ca exchange.

Identification of membrane currents activated by photolysis of caged Ca^{2+}: effect of inhibitors for the Na-Ca exchange

Although no specific inhibitor for the Na-Ca exchange is available, a number of ions and compounds are known to inhibit the transporter. In particular, the application of extracellular Ni^{2+} ions was shown to selectively inhibit the Na-Ca exchange under conditions where all other ionic currents usually affected by Ni^{2+} were already eliminated by more specific blockers (15). On the other hand, Ni^{2+} ions do not appear to block a $[Sr^{2+}]_i$-induced membrane conductance, which may be produced by the Ca^{2+}-activated nonselective cation channels (28). Nickel therefore appeared to be a suitable tool to separate I_{NaCa} from nonspecific currents. This approach however only works provided $[Ca^{2+}]_i$ transients can still be elicited despite the presence of Ni^{2+}. Flash photolysis of caged Ca^{2+} enabled us to perform this type of experiment. The records presented in Fig. 3 *A* show the surprising finding that in the presence of 8 mM Ni^{2+} no change in membrane current could be detected at a holding potential of −40 mV. Note that under these conditions at a higher sampling rate a conformation current (I_{conf}) could be seen, presumably reflecting a molecular rearrangement of the exchanger molecules. Successful photorelease of calcium under these conditions was still demonstrated by the twitch contraction of the cell. The observation that Ni^{2+} eliminated the current activated by the intracellular Ca^{2+} jump suggests, that the current was exclusively carried by the Na-Ca exchange (I_{NaCa}) and that the contribution of other potentially contaminating Ca^{2+}-activated currents was negligible. This

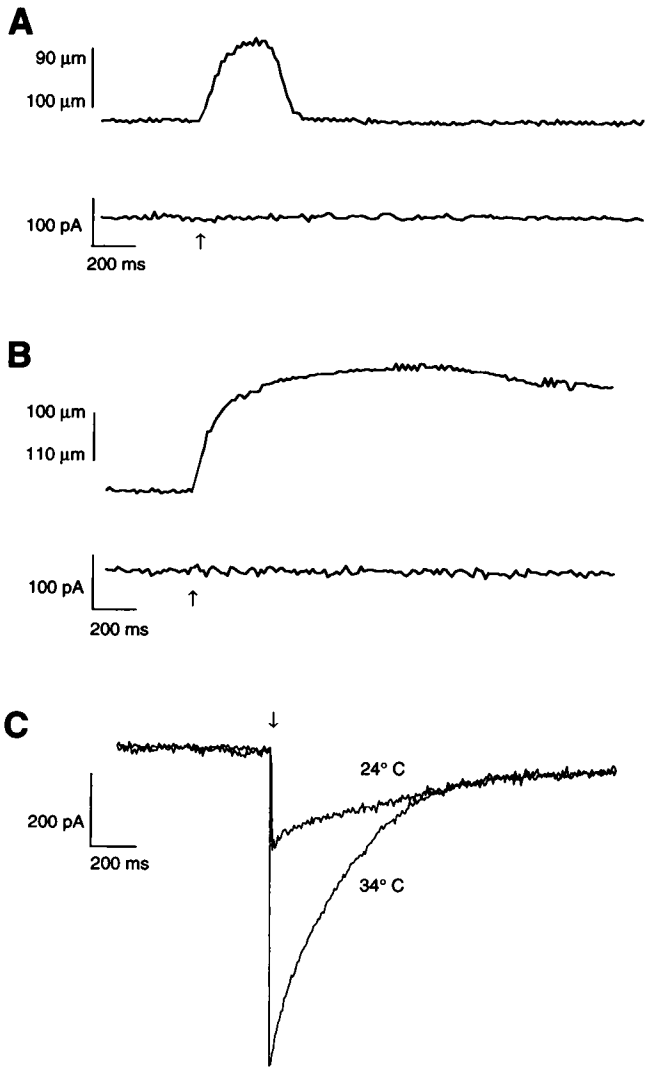

FIGURE 3 Effect of inhibitors and temperature on I_{NaCa}. (*A*) In the presence of 8 mM extracellular Ni^{2+} no currents were elicited when the cell preloaded with caged Ca^{2+} was flashed (at the *arrow*, V_h = −40 mV). Successful release of $[Ca^{2+}]_i$ was still demonstrated by mechanical shortening of the cell. Relengthening appears not to be dramatically slowed despite the inhibition of Na-Ca exchange. (*B*) When the cells were preexposed to ryanodine (10 μM), the addition of Ni^{2+} greatly slowed relengthening after the concentration jump of $[Ca^{2+}]_i$. V_h was −40 mV. (*C*) Changing the temperature of the superfusion solution remarkably affected the inward current activated by photorelease of $[Ca^{2+}]_i$. The currents shown were recorded at 24 and 34°C, respectively. Cooling resulted in a 3.3-fold reduction of the peak current in the experiment shown. The records were sampled at a rate of 133 Hz. This accounts for the absence of I_{conf} in *A* and *B*.

interpretation is supported by identical results which were obtained between +100 and −100 mV in zero $[Na^+]_o$ (replaced by Li^+ or *N*-methyl-D-glucamine, NMG) and in the presence of La^{3+} (4 mM), dichlorobenzamil (DCB; 100 μM), or quinacrine (1 mM) in the extracellular solution. These observations indicate that, under the present experimental conditions, $[Ca^{2+}]_i$ had no influence (did not activate or inhibit) on any membrane conductance which could not be blocked by Ni^{2+} or the other inhibitors. Occasionally, we

noticed a step-like change in the whole-cell current closely resembling opening and closing of an ion channel. The observation of single channel events on whole-cell current records is a rather unusual finding and suggests that the conductance of this channel has to be fairly high. Indeed, a linear regression fitted to the current/voltage relationship of single channel currents revealed a conductance of 305 pS with a reversal potential near +7 mV. This channel is likely to be identical with the large conductance Ca^{2+}-activated ion channel described in cultured guinea pig atrial cells (14), where it can be observed frequently under conditions of elevated $[Ca^{2+}]_i$. Because the single-channel conductance of this channel is very high, we can reliably exclude a current contribution to I_{NaCa} by inspection of our current records. The physiological role of this large channel is still unclear, but the channel is not related to the Ca^{2+}-activated nonselective cation channel mentioned earlier, which has a much lower single-channel conductance of 14.8 pS (19). Taken together, the findings suggest that the currents we observed reliably represent I_{NaCa} and thus the activity of the Na-Ca exchange. Please also note that relengthening of the ventricular myocyte after the twitch contraction was fairly rapid despite the absence of Na-Ca exchange, indicating that the SR can compensate for the eliminated transport capacity of the Na-Ca exchange and take up the Ca^{2+} photoreleased after the flash (Fig. 3 *A*). Interestingly, when the function of the SR was also inhibited by the continued presence of extracellular ryanodine (10 μM), relengthening of the cell was greatly slowed (Fig. 3 *B*). About 30 s were required for complete reextension, suggesting that ryanodine and Ni^{2+} together eliminate the most important cellular systems able to reduce $[Ca^{2+}]_i$. Similar observations have been described when the relaxation from rapid cooling contractions was measured in the presence of caffeine in a Na^+-free solution (36).

Temperature dependence of Na-Ca exchange current

Another characteristic of active ion transport systems is the relatively high temperature dependence. In guinea pig myocytes, the outward Na-Ca exchange current activated by extracellular Ca^{2+} (representing Ca^{2+} influx) has a reported Q_{10} of 3.6 (15). In native and reconstituted vesicles from different species, the Q_{10} for Ca^{2+} uptake varied between 2 and 6 in mammalian tissue (37). In our experiments, the inward current activated by photorelease of intracellular Ca^{2+} decreased by 4.0 ($\pm$ 0.27 mean $\pm$ SE, $n = 4$) when the temperature was reduced from 34 to 24°C, again suggesting that the current we observed was generated by a transporter and represents I_{NaCa} (see Fig. 3 *C*).

Dependence of Na-Ca exchange current on $[Na^+]_o$

The inwardly directed electrochemical Na^+ gradient provides the energy required for Ca^{2+} removal by the Na-Ca exchange. In addition, extracellular Na^+ is the countersubstrate for removal of intracellular Ca^{2+}. Both properties of $[Na^+]_o$ result in a complex Na^+ dependence of I_{NaCa}. We used a rapid superfusion device ($t_{1/2} < 1$ s) to investigate the effects of varying Na^+ concentration on I_{NaCa}. Fig. 4 *A* shows exchange currents activated at a holding potential of 0 mV in the presence of 145, 72.5, and 0 mM Na^+, respectively. Replacing 50% Na^+ with Li^+ resulted in a marked reduction of the peak I_{NaCa}. In addition, the time-course of the current decay was slowed considerably. At zero Na^+ no change in membrane was observed after the Ca^{2+} concentration jump. Normalized peak exchange currents were used to fit a Hill equation to the data. The current was activated half-maximally at 73 mM Na^+ with a slope of 2.8, suggesting a high degree of cooperativity for extracellular Na^+ on I_{NaCa}.

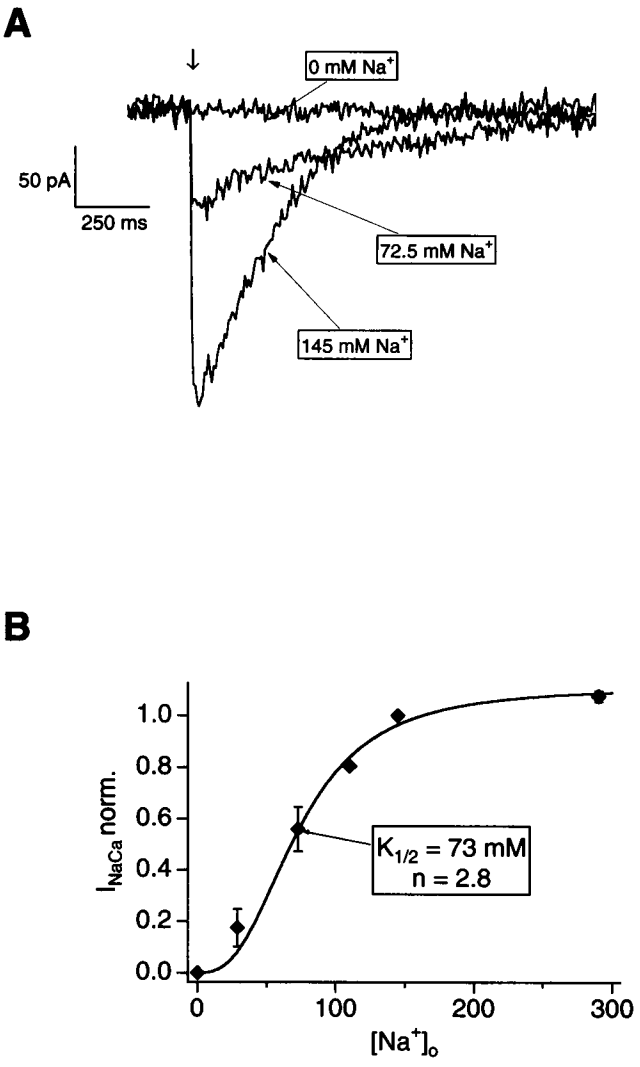

FIGURE 4 I_{NaCa} activated by photorelease of Ca^{2+} depends on extracellular Na^+. (*A*) Shows membrane currents recorded at 0 mV after Ca^{2+} concentration jumps ($\downarrow$) at various Na^+ concentrations. Extracellular Na^+ was changed briefly with a rapid superfusion system. Peak I_{NaCa} was smaller at lower Na^+ concentrations while the time-course of current decay was slowed down. No change of membrane current is observed in zero Na^+. (*B*) A Hill equation was fitted to the peak currents normalized for 145 mM Na^+. Half-maximal activation was observed at 73 mM Na^+, the slope was 2.8.

A similar slope (2.9) and Na^+ sensitivity (87 mM) was obtained in a study in which exchange activity was determined as extracellular Na^+-dependent membrane current in Ca^{2+} loaded cardiac myocytes (15).

DISCUSSION

Electrogenicity of Na-Ca exchange

In the past a variety of experimental approaches has been used to measure the activity of the Na-Ca exchange including tracer-flux measurements in intact (2, 3) and vesicle preparations (37, 38). After the 3 Na^+:1 Ca^{2+} stoichiometry and the electrogenicity of the exchange has been established, investigators have also associated membrane currents with the activity of the exchanger (for review, see Ref. 11). The introduction of the giga-seal voltage-clamp technique (39) has allowed membrane current measurements in single cells while controlling the ionic composition of the intracellular environment. This technique has enabled the investigation of membrane currents activated by *cis* Ca^{2+} but depending on *trans* Na^+ (or activated by *cis* Na^+ and depending on *trans* Ca^{2+}) in a variety of preparations, most notably in rod outer segments (12, 13) and in cardiac myocytes (14, 15, 32, 35, 40, 41). Such currents have been associated with the electrogenic Na-Ca exchange and have been used to investigate various properties of this transporter. In particular, the voltage dependence and the steady-state kinetic activation of the exchange current (I_{NaCa}) by extracellular and intracellular Na^+ and Ca^{2+} ions have been explored with this technique (15, 42).

Unfortunately, some results obtained in isolated intact cells may suffer from problems arising from inadequate control of intracellular ion concentrations, particularly under conditions of substantial ionic flux across the membrane (43). The transport mechanisms of the cell (including the Na-Ca exchange) may be able to generate significant concentration gradients between the pipette-filling solution and the actual concentration in the cytosol, especially in the subsarcolemmal space where movement of ions may be restricted (6). This phenomenon may result in an apparent saturation of the Na-Ca exchange at high turnover rates, because the flux of ions from the bulk cytosol into this space may become rate-limiting for cycling of the Na-Ca exchange.

Certain problems possibly associated with changes of subsarcolemmal ion concentration can be avoided with rapid photolytic release of intracellular Ca^{2+}. Immediately after the flash, depletion of subsarcolemmal Ca^{2+} ions by the Na-Ca exchanger molecules starting to turn over rapidly is not expected. Since the dissociation of Ca^{2+} from the photolytic products of DM-nitrophen is very fast ($\tau \approx 30$ μs (27)) the photochemical dark reaction is essentially complete immediately after the flash (which has a duration of $\approx$400 μs). Therefore, delivery of Ca^{2+} ions to the exchanger is unlikely to be limited by Ca^{2+} dissociation from photolyzed DM-nitrophen, a complication which may be relevant when EGTA is used to buffer Ca^{2+}. But concentration gradients in the subsarcolemmal space may build up later after the flash when Na-Ca exchange turnover results in subsarcolemmal concentration changes of Ca^{2+}. Using this method, we found no indication of a saturation of peak I_{NaCa} at negative potentials. In several other studies inward Na-Ca exchange currents had a tendency to saturate at moderately negative voltages (41, 42), a phenomenon which may thus be related to subsarcolemmal concentration gradients and not necessarily represent a molecular feature of the Na-Ca exchanger transport cycle. Saturation at extreme potentials has been interpreted to result from a voltage-independent partial reaction of the exchanger cycle becoming rate-limiting at high turnover rates. But, as outlined above, processes not directly linked to the reaction cycle may become rate-limiting as the result of concentration changes. But our results can also not exclude the existence of genuine Na-Ca exchange saturation at more extreme potentials, because the voltage-range in which saturation will appear depends on the actual reaction rates (which are unknown at present) and may well be beyond the range of membrane potentials accessible experimentally.

Voltage dependence of Na-Ca exchange

$[Ca^{2+}]_i$-induced difference currents with a comparably shallow voltage dependence have also been found by other investigators under slightly different conditions and have been linked to the electrogenic Na-Ca exchange (78 mV for an *e*-fold change (29), 77 mV (32)). The voltage dependence of cardiac Na-Ca exchange currents in heart has frequently been less steep than expected for a full elementary charge moving across the membrane electrical field with single rate-limiting transition. In several models of the Na-Ca exchange (e.g., Ref. 44) a parameter (r) has been introduced to accommodate this observation. The parameter r has been interpreted to represent the position of an energy-barrier located asymmetrically in the electric field of the cell membrane. According to this model Na-Ca exchange inward currents depend on voltage as

$$I_{NaCa,i} = a \exp[-(1 - r)EF/RT], \quad (2)$$

where a is a scaling factor determining the magnitude of the current. E, F, R, and T have their usual meanings. Using this model, r values between 0.67 and 0.78 have been found to reasonably well describe the voltage dependence of I_{NaCa} (15, 32, 45, and this study). If an asymmetrically located energy-barrier of a single reaction step were the only mechanism underlying the shallow voltage dependence of inward currents (i.e., $r > 0.5$), then the currents moving charge in the outward direction should be much more voltage-dependent since according to the model they depend on voltage as

$$I_{NaCa,o} = a \exp(rEF/RT), \quad (3)$$

However, experimental results do not appear to support this concept. Outward currents generated by the Na-Ca exchange do not have a steeper voltage dependence than the corresponding inward currents. Exchange currents in the out-

ward direction were best fitted with r values around 0.36 (15, 42). However, according to this model the value of r should not depend on transport direction. If anything, only the voltage dependence should be different for the "forward" and "backward" mode, yielding the same value for r. Based on the data on voltage dependence of I_{NaCa} in heart cells available at present, it seems not justified to postulate a priori an asymmetrically located energy barrier as the only molecular mechanism to explain the shallow voltage dependence.

It appears to be implausible, that exactly one elementary charge moves across the entire electrical field with the rate-limiting step of an electrogenic transporter (31, 33). There are several reasons why this is unlikely. 1) The transported ion may not move through the entire field in one single step. It is possible that the ion has to pass through a fraction of the field even before it can bind to a binding site on the transporter (narrow access channel type of transporter (46)). In this case, the molecular transition actually transposing the binding site across the membrane only crosses the remaining fraction of the electrical field. 2) Together with the ion translocation step, the transporter undergoes a molecular conformational change which is likely to include movement of charged residues and submolecular dipoles within the electrical field. The rearrangement of charges during the conformational transition contributes to the charge moved with the ion translocation step. Both processes result in an apparently fractional (i.e., not integer multiple of) elementary charge moving with this transition. Taking these ideas into account, we represented the rate-limiting (and voltage-dependent) step for cycling with a simplified Eyring rate-theory model assuming an energy-barrier symmetrically located within the membrane electrical field (which would be equivalent to $r = 0.5$). The molecular transition responsible for the voltage dependence was allowed to move an apparently fractional charge during this transition. The voltage dependence of inward I_{NaCa} would thus be described by Eq. 1. Using this model, the results indicated that +0.44 charges move inwards with the rate-limiting step. In order to satisfy the 3:1 stoichiometry, one full elementary charge has to move during each complete turnover of the molecule. This suggests that the remainder of the charge has to move through the electrical field in a separate step. When a simple consecutive model without occluded intermediate states and without voltage-dependent ion binding is assumed for ion transport by the Na-Ca exchanger, these considerations indicate that the unloaded transporter has a charge of −2.56, while the charge is +0.44 on the Na-Ca exchanger with three Na^+ ions bound and −0.56 when one Ca^{2+} ion is bound (but see Ref. 35). These calculations are also consistent with the observation of a pre-steady state inward current transient immediately after a Ca^{2+} concentration jump (I_{conf}) (21). While these conclusions are model-dependent, the shallow voltage dependence of the Na-Ca exchange may, in reality, be the result of both the movement of apparently fractional charge and the presence of an asymmetrically located energy barrier. Yet detailed information about the function of the Na-Ca exchanger on the molecular level is required to more clearly define the precise molecular conformational changes responsible for the observed voltage dependence.

Other currents activated by $[Ca^{2+}]_i$

Based on the voltage dependence and the absence of reversal the currents that we measured (Fig. 2) are consistent with electrogenic Na-Ca exchange currents. However, another potential problem has to be considered which is associated with the interpretation of membrane currents when intracellular Ca^{2+} concentrations are changed. This complication is related to additional effects of $[Ca^{2+}]_i$ on the membrane conductance. Ca^{2+}-activated nonselective cation channels have been found in numerous preparations (18, 47) including guinea pig heart cells (19). Activation of these channels may contaminate I_{NaCa} measurements (28). Although the absence of a reversal of the current direction in our records excludes a major contribution from a channel reversing around 0 mV, a minor contamination (smaller than I_{NaCa} at the most positive potential) cannot be excluded based on the voltage dependence alone.

Addition of Ni^{2+} to the superfusion solution has been used to inhibit I_{NaCa} (15, 41) and to define I_{NaCa} as a Ni^{2+}-sensitive difference current. Unfortunately, in most experimental settings Ni^{2+} ions do not only block the Na-Ca exchange but also suppress the Ca^{2+} transient during the voltage-clamp depolarizations (which activate I_{NaCa}). Thus, Ni^{2+} would tend to block both, the I_{NaCa} *and* any contaminating $[Ca^{2+}]_i$-induced membrane conductance change. In consequence, the Ni^{2+}-sensitive difference current may still be contaminated by nonspecific membrane currents, as outlined above. Flash photolysis of caged Ca^{2+} is a technique which allows one to produce intracellular Ca^{2+} concentration jumps independently of membrane voltage and despite the presence of extracellular blockers for Ca^{2+} channels and the Na-Ca exchange. We have used this new approach in combination with extracellular Ni^{2+} to probe the presence of any nonspecific Ca^{2+}-induced membrane conductance. However, in the presence of extracellular Ni^{2+} we found no indication of a current change over the time-course of the Ca^{2+} transient and in the voltage range tested (−100 to +100 mV). Successful photorelease of Ca^{2+} under these conditions could still be demonstrated as $[Ca^{2+}]_i$-induced shortening of the cardiac myocytes. Identical results were observed when no Ni^{2+} ions were added, but when extracellular Na^+ was replaced with Li^+ or NMG. We do not know, at present, why no current other than I_{NaCa} was activated by flash photolysis of caged Ca^{2+}, but the reportedly rapid run-down of the nonspecific cation channels may be a possible explanation (19). The large-conductance channel (305 pS) we occasionally observed is not identical with the nonspecific cation channels, although it is also activated by elevated $[Ca^{2+}]_i$.

Because flash photolysis of caged Ca^{2+} allows changes of $[Ca^{2+}]_i$ independently of membrane potential and membrane conductance for Ca^{2+} ions and also enables reliable and rapidly repeatable determinations of the activity of the

Na-Ca exchange, it turned out to be an ideal method to study cellular regulatory modulation of the Na-Ca exchange as well as pharmacological actions of chemicals on the transporter. Due to the rapid concentration jump, it may also be possible to obtain transient state kinetic information and thus insight into the molecular function of the exchanger molecules (21, 22).

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