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Na^+/Ca^{2+} EXCHANGE CURRENT IN VENTRICULAR MYOCYTES OF FISH HEART: CONTRIBUTION TO SARCOLEMMAL Ca^{2+} INFLUX

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Summary

Influx of extracellular Ca^{2+} plays a major role in the activation of contraction in fish cardiac cells. The relative contributions of Na^+/Ca^{2+} exchange and L-type Ca^{2+} channels to Ca^{2+} influx are, however, unknown. Using a physiological action potential as the command pulse in voltage-clamped heart cells, we examined sarcolemmal Ca^{2+} influx through Na^+/Ca^{2+} exchange and L-type Ca^{2+} channels in crucian carp (*Carassius carassius* L.) ventricular myocytes. When other cation conductances were blocked, a Ni^{2+}-sensitive current with the characteristic voltage- and time-dependent properties of the Na^+/Ca^{2+} exchange current could be distinguished. At the maximum overshoot voltage of the ventricular action potential (+40 mV; $[Na^+]_i$=10 mmol l^{-1}), the density of the Na^+/Ca^{2+} exchange current was 2.99±0.27 pA pF^{-1} for warm-acclimated fish (23 °C) and 2.38±0.42 pA pF^{-1} for cold-acclimated fish (4 °C) (means ± S.E.M., *N*=5–6; not significantly different, *P*=0.26). The relative contributions of the Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channels to Ca^{2+} influx were estimated using two partly different methods. Integration of the Ni^{2+}-sensitive Na^+/Ca^{2+} exchange current and the verapamil- and Cd^{2+}-sensitive L-type Ca^{2+} current suggests that, during the action potential, approximately one-third of the activating Ca^{2+} comes through Na^+/Ca^{2+} exchange and approximately two-thirds through L-type Ca^{2+} channels. An alternative method of analysis, using the inward tail current as a measure of the total sarcolemmal Ca^{2+} flux from which the Ni^{2+}-sensitive Na^+/Ca^{2+} exchange current was subtracted to obtain the Ca^{2+} influx through the channels, suggests that L-type Ca^{2+} channels and Na^+/Ca^{2+} exchange are almost equally important in the activation of contraction. Furthermore, the time course of cell shortening is not adequately explained by sarcolemmal Ca^{2+} influx through the channels alone, but is well approximated by the sum of Ca^{2+} influx through the channels and the exchanger. The present results indicate that reverse Na^+/Ca^{2+} exchange in crucian carp ventricular myocytes has sufficient capacity to trigger contraction and suggest that the exchange current makes a significant contribution to contractile Ca^{2+} during the physiological action potential. The relative significance of channels and exchanger molecules in sarcolemmal Ca^{2+} entry into crucian carp ventricular myocytes was unaffected by thermal acclimation when determined at 22 °C.

Key words: Na^+/Ca^{2+} exchange, L-type Ca^{2+} current, fish, heart, cardiac myocyte, action potential, excitation–contraction coupling, thermal acclimation, activation of contraction, cell shortening, *Carassius carassius*, carp.

Introduction

The sarcoplasmic reticulum of fish cardiac cells is relatively poorly developed and obviously a minor factor in contractile activation in most fish species (Santer, 1985; Driedzic and Gesser, 1988; Vornanen, 1989, 1996b; Tibbits et al., 1991; Keen et al., 1994; Shiels and Farrell, 1997; Aho and Vornanen, 1998). The sparsity of sarcoplasmic reticulum means that activation of cardiac myofibrils necessitates an ample sarcolemmal Ca^{2+} influx during the action potential. Although L-type Ca^{2+} channels can mediate a relatively large Ca^{2+} entry into fish ventricular cells (Vornanen, 1997, 1998), the force of contraction in fish heart is not completely abolished by 100 μmol l^{-1} Cd^{2+} (Vornanen, 1996b), a Ca^{2+} channel blocker. These findings suggest that, in addition to L-type Ca^{2+} channels, Na^+/Ca^{2+} exchange may also make a significant contribution to contractile Ca^{2+} entry in fish cardiac myocytes. The Na^+/Ca^{2+} exchange process is determined by the electrochemical gradients for Na^+ and Ca^{2+} across the sarcolemma, so that the exchanger is sensitive to the membrane potential. At the normal resting potential and during a major part of the action potential, the Na^+/Ca^{2+} exchanger extrudes Ca^{2+} from the cell (forward mode), while during the upstroke of the cardiac action potential, the exchanger may reverse direction and bring Ca^{2+} into the cell (reverse mode) during the overshoot (Mullins, 1979). Thus, the Na^+/Ca^{2+} exchange system is not only the major Ca^{2+} efflux pathway but can, at least in principle, also mediate some of the trans-sarcolemmal

Ca^{2+} influx. The function and significance of Na^+/Ca^{2+} exchange in the contraction of cardiac muscle has not been examined previously in any teleost species. The present study was therefore designed to measure trans-sarcolemmal Ca^{2+} influx through Na^+/Ca^{2+} exchange in fish cardiac myocytes. The properties of Na^+/Ca^{2+} exchange currents were characterized and the relative contributions of sarcolemmal Ca^{2+} channels and the Na^+/Ca^{2+} exchanger to contractile Ca^{2+} during the action potential were estimated using voltage-clamp experiments on fish ventricular cells.

Materials and methods

Animals

Crucian carp *Carassius carassius* L. were captured in August and September 1997 from local ponds located approximately 5 km from the campus of the University of Joensuu (Finland). In the laboratory, the fish were kept in 500 l metal tanks filled with aerated and circulating tap (ground) water. The fish were randomly divided into two groups that were acclimated to either 4 or 23 °C for more than 4 weeks. The warm-acclimated (WA) fish were fed daily with commercial fish food (Ewos; Turku, Finland). In nature, crucian carp stop feeding after adaptation to low ambient temperature (Penttinen and Holopainen, 1992) and refuse food in the laboratory when kept at 4 °C. Accordingly, cold-acclimated (CA) fish were not fed. The mass of the fish varied between 20 and 60 g, with a mean body mass of 34.4±3.9 g (means ± S.E.M., N=12). Animals of both sexes were used.

Solutions

The nominally Ca^{2+}-free low-Na^+ solution used for cell isolation contained (mmol l^{-1}): NaCl, 100; KCl, 10; KH_2PO_4, 1.2; $MgSO_4$, 4; taurine, 50; glucose, 20; and Hepes, 10, pH 6.9 (KOH). Physiological saline, used as the extracellular solution when recording action potentials, contained (mmol l^{-1}): NaCl, 150; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 2.0; glucose, 10; and Hepes, 10, pH 7.6. This solution was modified for current recordings by replacing KCl with equimolar CsCl. The pipette-filling solution for action potential recordings contained (mmol l^{-1}): KCl, 140; $MgCl_2$, 1; Na_2ATP, 5; and Hepes, 10, pH 7.2 (adjusted with KOH). For current recordings, the pipette-filling solution contained (mmol l^{-1}): CsCl, 140, $MgCl_2$, 1; Na_2ATP, 5; Na_2GTP, 0.03; and Hepes, 10, pH 7.2 (adjusted with CsOH).

Cell isolation

The procedure used for isolation of fish ventricular cells has been previously described in detail (Vornanen, 1997, 1998). In brief, the heart was retrogradely perfused first with nominally Ca^{2+}-free saline for 10 min and then with the same solution with added proteolytic enzymes (collagenase IA, 1.5 mg ml^{-1}; trypsin IX, 0.75 mg ml^{-1}; both from Sigma) and fatty-acid-free bovine serum albumin (0.75 mg ml^{-1}) for 30–40 min. The cells were suspended in the Ca^{2+}-free saline by trituration through the opening of a Pasteur pipette and stored in low-Na^+ solution at room temperature for less than 6 h.

Patch-clamp experiments

The experiments were initially designed to characterize the properties of the Na^+/Ca^{2+} exchange current and the relative contributions of the L-type Ca^{2+} current and the exchanger to sarcolemmal Ca^{2+} entry in fish cardiac myocytes. The information about the effects of thermal acclimation on these phenomena was regarded as secondary. Consequently, all experiments were conducted at room temperature (22±2 °C), which is close to the physiological body temperature of the WA fish but considerably higher than the normal body temperature of the CA fish. Also, the action potential waveform used for voltage-clamping is representative for the heart of WA fish (Vornanen, 1996b), but much shorter than the action potential of the CA fish at their body temperature.

Contamination of current recordings by unwanted conductances was minimized by the ionic composition of the intra- and extracellular solutions and by using selective blockers. K^+-free intra- and extracellular saline solutions were used to block K^+ currents. Omission of K^+ from the solutions also inhibits the Na^+/K^+ pump. Tetrodotoxin (TTX, 1 μmol l^{-1}) is an effective blocker of fast Na^+ channels in fish cardiac cells (Vornanen, 1997, 1998) and was included in the external solution in all experiments. When needed, fast and complete blockade of L-type Ca^{2+} channels was achieved by adding 10 μmol l^{-1} verapamil together with 25 μmol l^{-1} $CdCl_2$. This concentration of $CdCl_2$ does not inhibit the Na^+/Ca^{2+} exchange current (Hobai et al., 1997). In some experiments, 10 μmol l^{-1} nifedipine was used instead of verapamil and Cd^{2+}, with identical results. Blockade of both Ca^{2+} channels and Na^+/Ca^{2+} exchange was achieved by including 2 mmol l^{-1} $NiCl_2$ with verapamil and $CdCl_2$ (or nifedipine). Although the contribution of the sarcoplasmic reticulum to contractile activation seems to be negligible in crucian carp ventricular cells, 5 μmol l^{-1} ryanodine was included in the extracellular saline solution to impair any possible sarcoplasmic reticulum function. Intracellular [Ca^{2+}] was allowed to change freely in the absence of any Ca^{2+} buffers. Ca^{2+} contamination from the water (0.7 μmol l^{-1}) and from reagents is buffered by free ATP so that the concentration of free Ca^{2+} in the pipette solution, determined using Fura-2, was approximately 0.1 μmol l^{-1}. Ca^{2+} transients could, in principle, lead to contamination of the recorded current by a Ca^{2+}-activated Cl^- current (Zygmunt, 1994). The use of 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS; 100 μmol l^{-1}), a blocker of Ca^{2+}-activated Cl^- channels (Zygmunt, 1994), had no effect on current recordings under the present experimental conditions (not shown), suggesting that there was no interference from Cl^- currents.

Cells were allowed to attach to the glass bottom of a small chamber (0.5 ml) and superfused with physiological saline at 2 ml min^{-1}. Membrane potential and currents were recorded using standard whole-cell voltage-clamp techniques (Hamill et al., 1981). Patch pipettes were pulled from borosilicate glass (Modulohm A/S, Denmark) and had a resistance of approximately 2 MΩ. All recordings were made using an Axopatch 1D amplifier (Axon Instruments) equipped with a CV-4 1/100 headstage. Junction potentials were zeroed before

formation of the seal. The pipette capacitance (4–5 pF) was compensated for after formation of a gigaohm seal. The patch was ruptured by delivering a short voltage pulse (zap) to the cell, and capacitative transients were eliminated by iteratively adjusting the series resistance and cell capacitance compensation circuits. The cell capacitance was read directly from the Axopatch 1D amplifier. There was no leakage correction. Membrane potentials and currents were filtered at 10 kHz and 2 kHz, respectively, and were sampled at 5 and 1 kHz with an analog-to-digital converter (TL-1 DMA, Axon Instruments).

The Ca^{2+} current and the Na^+/Ca^{2+} exchange current were elicited using both action-potential and square-pulse voltage waveforms delivered at a frequency of 0.1 Hz. A representative action potential was recorded from a ventricular myocyte of WA crucian carp at 22 °C and used in all experiments as the stimulus waveform to activate the Ca^{2+} current and the Na^+/Ca^{2+} exchange current. The resting potential, and therefore the holding potential, was −70 mV. The overshoot of the action potential was +40 mV, and the duration of the plateau (0 mV) was 276 ms. The square pulses were elicited from the holding potential of −70 mV to +10 mV for 500 ms.

In some experiments, shortening of the cell was measured in addition to current. To record shortening, the cell was observed using a video camera attached to the side port of a microscope (Nikon Eclipse 200), and the amplitude of cell motion was traced using a video edge detector (Crescent Electronics). Unattached fish cardiac myocytes, because of their long and narrow shape, do not usually shorten precisely in the direction of their longitudinal axis. Consequently, the amplitude of shortening is given in arbitrary units rather than as a percentage change in cell length. Both voltage-clamp and contraction recordings were stored on the hard disk of a computer using pClamp 6.02 software. Analysis of recordings was conducted off-line using the Clampfit program of the pClamp software package.

The contribution of the Ca^{2+} current and the Na^+/Ca^{2+} exchange current to total cellular $[Ca^{2+}]$ was calculated from the transferred charges and cell volume, as described previously (Vornanen, 1996a, 1997). Charge transfer was determined in two somewhat different ways. The analysis of charge transfer is described in detail in the Results section, so only a brief outline is given here. In method 1, the L-type Ca^{2+} current was first measured as the verapamil- and Cd^{2+}-sensitive current, and the Na^+/Ca^{2+} exchange current was then measured as the Ni^{2+}-sensitive current in the presence of Ca^{2+} channel blockers. Total Ca^{2+} influx was calculated as the sum of the two blocker-sensitive currents. In method 2, the total Ca^{2+} influx was measured by integrating the inward tail current during the repolarizing phase of the action potential or after repolarization of the square voltage step. This estimation is based on the assumption that, in the presence of sarcoplasmic reticulum blockade, Ca^{2+} efflux occurs solely through forward Na^+/Ca^{2+} exchange, which is the sum of Ca^{2+} influx through L-type Ca^{2+} channels and reverse Na^+/Ca^{2+} exchange. The Ca^{2+} current was calculated as the difference between the maximum inward current and the steady-state current at the end of the depolarizing voltage pulse. Subtraction of Ca^{2+} influx through the channels from the total Ca^{2+} influx gave the value for Ca^{2+} influx through the exchanger.

When using the action-potential clamp, method 2 was modified so that the total Ca^{2+} influx (efflux) was obtained by integrating the inward current during the action potential repolarization from +10 mV (the mean reversal potential of the exchanger) to the end of the action potential (−70 mV). Ca^{2+} influx through the exchanger was calculated as the integral of the Ni^{2+}-sensitive current (method 1), and Ca^{2+} influx through the channels was calculated as the difference between total Ca^{2+} influx and Ca^{2+} influx through the exchanger. After integration of the currents, the change in total cellular Ca^{2+} concentration ($[Ca^{2+}]_{tot}$, μmol l^{-1}) was obtained from the equation:

$$\Delta[Ca^{2+}]_{tot} = \int I_{Ca/Ex}(t)dt \times (z \times F \times V)^{-1}, \quad (1)$$

where z is the equivalent charge movement per ion of Ca^{2+} entry (z=2 for I_{Ca} and z=1 for I_{Ex}, assuming a stoichiometry of $3Na^+$:$1Ca^{2+}$), F is the Faraday constant with a value of 96 500 A s^{-1} mol^{-1}, $\int I_{Ca/Ex}$ is the time integral of Ca^{2+} current or Na^+/Ca^{2+} exchange (either outward current or inward tail current), t is time and V is the Ca^{2+}-accessible cell volume. The latter was assumed to be equal to the myofibrillar space, which is located immediately beneath the sarcolemma and constitutes approximately 40 % of the whole-cell volume in crucian carp ventricular myocytes (Vornanen, 1997). The cell volume was derived from the measured cell capacitance using a specific membrane capacitance of 1.59 μF cm^{-2} and a surface-to-volume ratio of 1.15 μm^{-1}. The two transforming factors are experimentally determined values for crucian carp ventricular myocytes (Vornanen, 1997).

Intracellular free Ca^{2+} concentration during the activation of the reverse Na^+/Ca^{2+} exchange was calculated from the experimentally determined reversal potential of the exchanger ($E_{Na/Ca}$). Since the reversal potentials of the exchanger and Na^+ (E_{Na}) were known, the Ca^{2+} reversal potential (E_{Ca}) was obtained from the equation:

$$E_{Na/Ca} = 3E_{Na} - 2E_{Ca}. \quad (2)$$

Intracellular free Ca^{2+} concentration was then calculated from the Nernst equation.

Current–voltage relationships for Na^+/Ca^{2+} exchange were fitted by a single-exponential equation:

$$I_{Ex} = a e^{bE}, \quad (3)$$

where I_{Ex} is the density of exchanger current, b is a variable describing its voltage-dependence, a is a scaling factor that determines the magnitude of the current, and E is the membrane potential.

Statistics

Where possible, data are presented as means ±1 S.E.M., and

statistical comparisons were made using a two-tailed Student's *t*-test. Differences were considered significant at $P<0.05$.

Results

Characterization of Na^+/Ca^{2+} exchange current

When stepped to voltages positive to the reversal potential of the exchanger, Ca^{2+} influx through the Na^+/Ca^{2+} exchanger will occur until a new equilibrium at an elevated intracellular Ca^{2+} concentration is attained (Hume and Uehara, 1986). If the recorded current is carried by the Na^+/Ca^{2+} exchanger, therefore, the outward current should decay with time, and the integrals of outward and inward exchange currents should increase with the duration of the depolarizing clamp step. Furthermore, the time-dependent current should be abolished by Ni^{2+}, a blocker of Na^+/Ca^{2+} exchange (Kimura et al., 1987; Iwamoto and Shigekawa, 1998). Fig. 1 shows that increasing the duration of the square-wave pulse (−80 to +40 mV) from 50 to 500 ms induced a slowly decaying outward current and enhanced the inward tail current upon repolarization. Approximately 70 % of this current was blocked by 2 mmol l^{-1} $NiCl_2$, leaving a small time-independent current component (Fig. 1B). The integrals of the Ni^{2+}-sensitive outward current and the Ni^{2+}-sensitive inward tail current matched, suggesting that the Na^+/Ca^{2+} exchange current was recorded. Similar results were obtained from four WA and four CA cells.

If the recorded current is mediated by Na^+/Ca^{2+} exchange, its voltage-dependence should show outward rectification with a roughly exponential course (Kimura et al., 1987; Ehara et al., 1989). The voltage-dependence of the Na^+/Ca^{2+} exchange current was initially examined using a voltage-ramp protocol (Fig. 2). Ca^{2+} influx through Na^+/Ca^{2+} exchange was activated by a depolarizing clamp step (200 ms) from −80 to +40 mV, and a subsequent voltage ramp from +40 to −120 mV (at 800 mV s^{-1}) was used to evaluate the voltage-dependence of the Na^+/Ca^{2+} exchange current. The ramp was followed by a voltage step back to the holding level (−80 mV). It was again evident that a major part (approximately 70 %) of both the outward and inward currents is sensitive to 2 mmol l^{-1} $NiCl_2$ (Fig. 2B). The outward current shows a steep increase with increasing voltage (outward rectification), while the inward current reached its maximum at −60 mV and then declined at more negative voltages, probably because of the depletion of subsarcolemmal Ca^{2+} in the vicinity of the Na^+/Ca^{2+} exchanger (no Ca^{2+} buffering). The Ni^{2+}-sensitive current reversed its direction at 9.73±0.86 mV (N=6) for WA fish and at 12.8±3.03 mV (N=6) for CA fish (Fig. 2D). The reversal potential of the exchanger (+10 mV) gives a Ca^{2+} reversal potential of 98 mV ($[Na^+]_i$=10 mmol l^{-1}; $[Na^+]_e$=150 mmol l^{-1}; $[Ca^{2+}]_e$=2 mmol l^{-1}), which suggests that depolarization to +40 mV for 200 ms increases the intracellular free Ca^{2+} concentration to 0.85 µmol l^{-1}. This amount of Ca^{2+}, if it were in a steady state with the myofibrils, would produce approximately half the maximal contraction force in the crucian carp ventricle (Vornanen, 1996). The Ni^{2+}-resistant current showed linear voltage-dependence and crossed the voltage axis close to 0 mV; this current is largely explained by a leakage conductance through a 2 GΩ seal (Fig. 2C). Similar recordings were obtained from six WA and six CA cells (Fig. 2D). The density of the Na^+/Ca^{2+} exchange current was similar in WA and CA fish, reaching a value of approximately 2 pA pF^{-1} at the overshoot voltage of the action potential (+40 mV).

The voltage-dependence of the Na^+/Ca^{2+} exchange current was also studied using 200 ms voltage steps from −80 mV to different membrane potentials between −40 and +70 mV

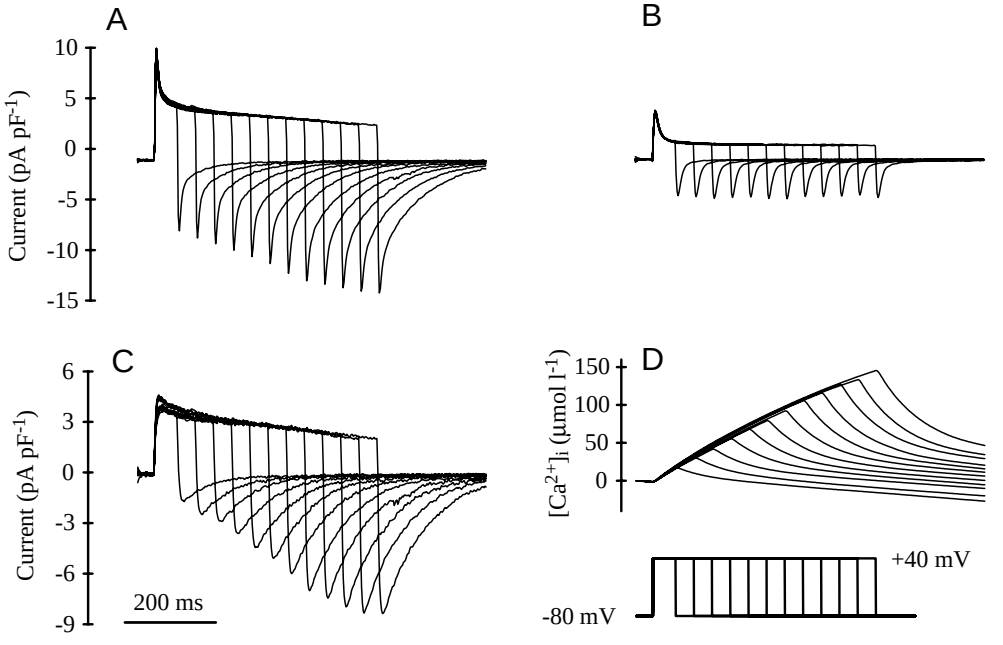

Fig. 1. Effects of the duration of membrane depolarization on the Ni^{2+}-sensitive current in a ventricular myocyte of crucian carp heart. (A,B) Membrane currents in the presence of Ca^{2+} channel blockers (10 µmol l^{-1} verapamil + 25 µmol l^{-1} Cd^{2+}) (A) and in the presence of both Ca^{2+} channel blockers and Ni^{2+} (10 µmol l^{-1} verapamil + 25 µmol l^{-1} Cd^{2+} + 2 mmol l^{-1} Ni^{2+}) (B). The Ni^{2+}-sensitive difference currents are indicated in C, and the calculated rises in intracellular Ca^{2+} concentration and their decay due to reverse and forward Na^+/Ca^{2+} exchange, respectively, were obtained by integration of the Ni^{2+}-sensitive currents and are shown in D. The applied voltage protocol is shown in D. The recordings are from a myocyte (18.4 pF) from a cold-acclimated fish at 22 °C.

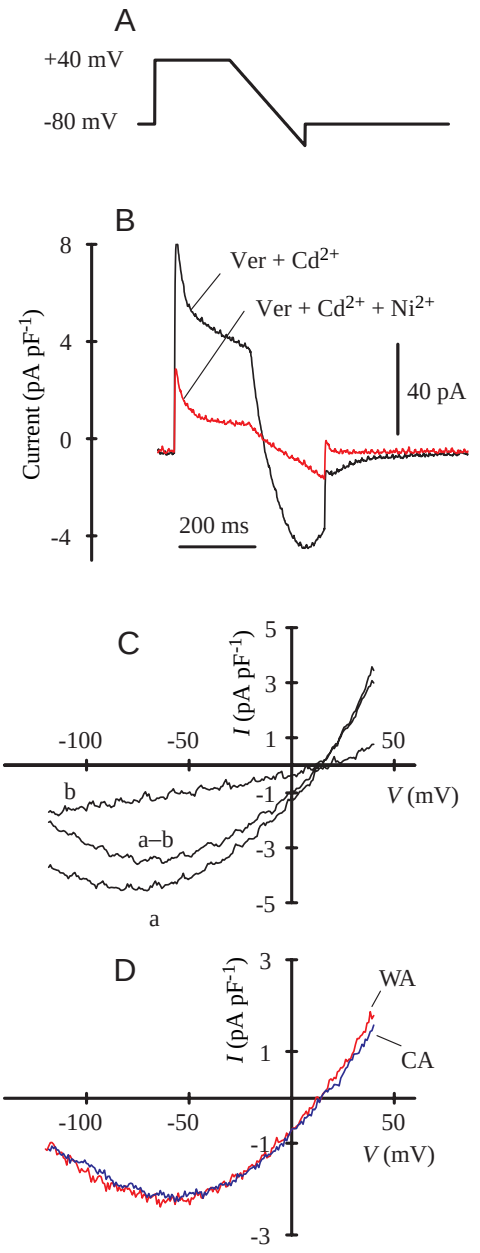

Fig. 2. Voltage-dependence of the Ni^{2+}-sensitive current in crucian carp ventricular myocytes. The voltage-clamp waveform is shown in A. Original current recordings in the presence of Ca^{2+} channel blockers (10 μmol l^{-1} verapamil + 25 μmol l^{-1} Cd^{2+}) and in the presence of both Ca^{2+} channel blockers and Ni^{2+} (10 μmol l^{-1} verapamil + 25 μmol l^{-1} Cd^{2+} + 2 mmol l^{-1} Ni^{2+}) are shown in B. The voltage-dependence of the membrane current in the absence (a) and in the presence (b) of Ni^{2+} and the voltage-dependence of the Ni^{2+}-sensitive difference current (a minus b), for the voltage ramp from –120 to +40 mV, are shown in C. The recordings are for a myocyte (22.9 pF) from a warm-acclimated fish at 22 °C. Mean values for warm-acclimated (WA) and cold-acclimated (CA) fish (N=6 for both acclimation groups) are shown in D. I, current; V, voltage; Ver, verapamil.

(Fig. 3). At –40, –30 and –20 mV, very little Ca^{2+} influx occurred and, consequently, the accompanying inward current was also negligible. With increasing depolarization, both Ni^{2+}-sensitive outward and inward exchange currents increased strongly without any significant difference between acclimation groups. The current–voltage relationships were well fitted by a single-exponential equation with a mean correlation coefficient (r) of 0.97±0.01. Thus, when other membrane conductances were blocked, the time- and voltage-dependent properties and Ni^{2+}-sensitivity of the remaining membrane current identified it as a Na^+/Ca^{2+} exchange current. Furthermore, this current is able to trigger contraction when sarcolemmal Ca^{2+} channels and sarcoplasmic reticulum Ca^{2+} release are blocked (Fig. 3B). The amplitude of cell shortening shows very similar voltage-dependence to that of the Ni^{2+}-sensitive current components, indicating that Ca^{2+} fluxes through the Na^+/Ca^{2+} exchanger can activate contraction and subsequent relaxation. The ability to trigger contraction is in agreement with the intracellular free Ca^{2+} concentration derived from the reversal potential of the exchanger. Similar results were obtained from three other cells (two WA and one CA).

Relative contributions of the Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channels to sarcolemmal Ca^{2+} entry

After confirming that the Na^+/Ca^{2+} exchange current can be measured in these cells and that the Ca^{2+} fluxes through the exchanger can mediate contraction, we attempted to quantify the Ca^{2+} fluxes through Na^+/Ca^{2+} exchange and L-type Ca^{2+} channels during a cardiac action potential. The procedure for distinguishing the Ca^{2+} current and the Na^+/Ca^{2+} exchange current as blocker-sensitive currents (method 1) is shown in Fig. 4. First, membrane current was recorded in the absence of channel and exchanger blockers (Fig. 4B). Square pulses from –70 to +10 mV elicited a large and rapid inward current that inactivated quickly during the pulse. Initial depolarization by an action-potential clamp rises to +40 mV, reducing the electrochemical driving force for Ca^{2+} influx through channels and activating more overlapping outward current through the exchanger; the rapid surge of inward current during the action-potential clamp therefore remains small in comparison with the current recorded during the square-wave pulse. The inward current surge was followed by a small outward current and a slower secondary inward deflection during the plateau phase of the action potential. Addition of Ca^{2+} channel blockers (10 μmol l^{-1} verapamil + 25 μmol l^{-1} Cd^{2+}) abolished fast inward currents and shifted the current recordings in the outward direction. The verapamil- and Cd^{2+}-sensitive difference currents are shown in Fig. 4D (I_{Ca}). During a square pulse, the rapidly inactivating inward current was much bigger and the maintained inward current was slightly smaller than during the action-potential clamp. In the next phase, 2 mmol l^{-1} $NiCl_2$ was added to block the Na^+/Ca^{2+} exchange current (Fig. 4C). Addition of $NiCl_2$ caused an inward shift of the current recordings during both the square-wave and action-potential clamps and strongly inhibited inward tail currents associated with repolarization. The Ni^{2+}-sensitive currents are shown in Fig. 4D (I_{Ex}). The voltage-dependence

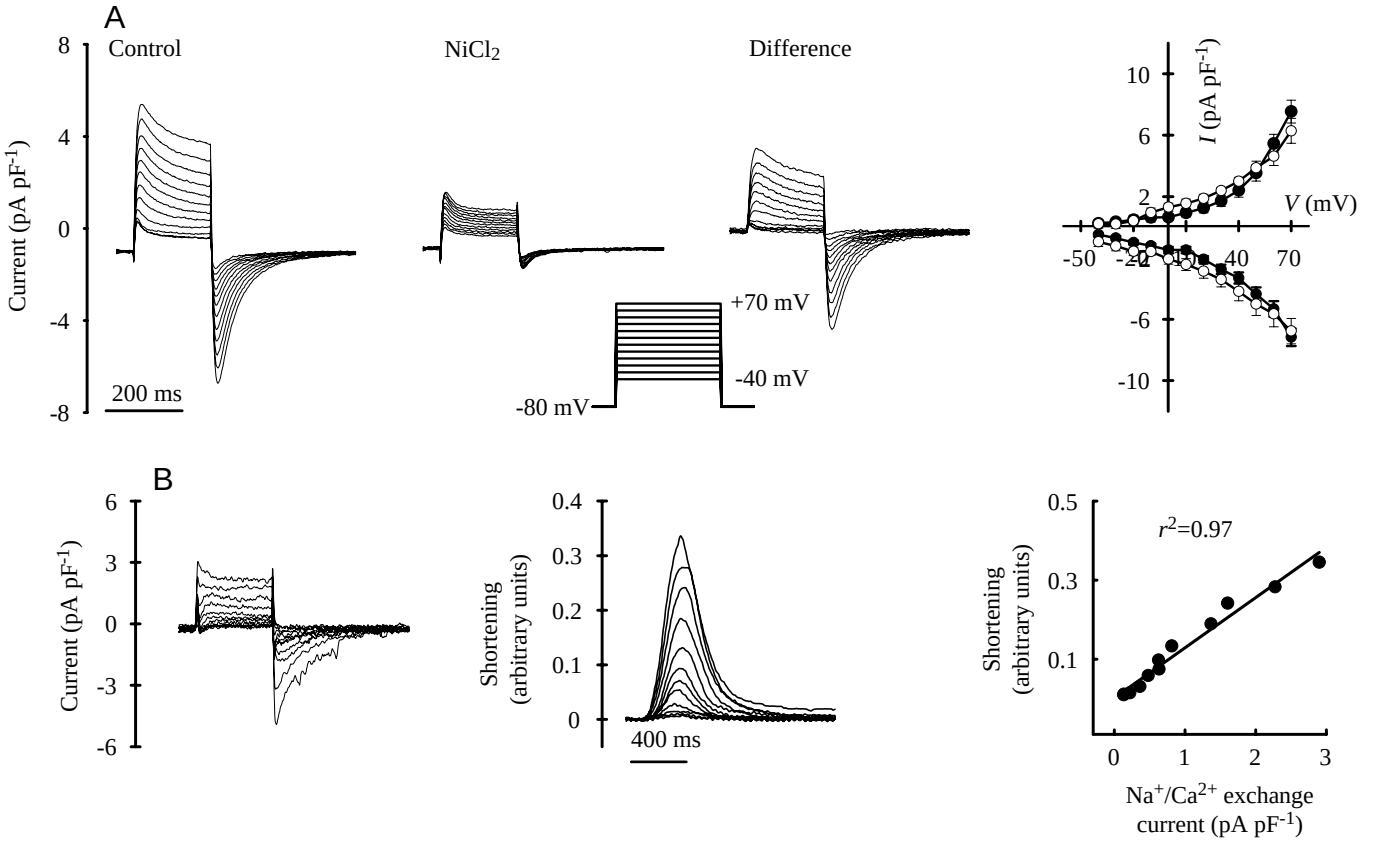

Fig. 3. Voltage-dependence of Ni^{2+}-sensitive outward and inward currents upon step changes in membrane potential. Currents were activated by square-wave pulses (200 ms) from –80 mV to between –40 and +70 mV, as shown by the voltage protocol in the middle of the figure. The upper row of recordings (A) shows superimposed recordings of membrane current in the presence of Ca^{2+} channel blockers (10 μmol l^{-1} verapamil + 25 μmol l^{-1} Cd^{2+}; control), in the presence of both Ca^{2+} channel blockers and Ni^{2+} (10 μmol l^{-1} verapamil + 25 μmol l^{-1} Cd^{2+} + 2 mmol l^{-1} Ni^{2+}; $NiCl_2$) and the Ni^{2+}-sensitive difference current (Difference). The recordings are for a myocyte (22.6 pF) from a warm-acclimated fish at 22 °C. Mean (± 1 S.E.M.) values for warm- (open circles; *N*=6) and cold-acclimated (filled circles; *N*=5) fish are shown on the right. The lower row of recordings (B) shows the Ni^{2+}-sensitive difference current and the shortening of the myocyte from another cell (20.7 pF, cold-acclimated fish). The Ni^{2+}-sensitive outward current and the amplitude of shortening are closely correlated (y=0.127x+0.02, r^2=0.97, P<0.001).

of the Ni^{2+}-sensitive current during the action potential clamp is similar to that of the currents recorded during step and ramp pulses (Figs 2, 3): outward rectification during the initial depolarization, reversal of the current at +10 mV and maximum inward current at –60 mV (Fig. 4E). It is notable that, during the action potential, the Na^+/Ca^{2+} exchange current turns from outward to inward at a membrane potential of approximately +10 mV, indicating the removal of intracellular Ca^{2+} through the Na^+/Ca^{2+} exchanger during the latter half of the action potential.

The above experimental procedure was performed on eight WA and eight CA cells, and the mean values for the integrals of the Ca^{2+} current and Na^+/Ca^{2+} exchange current are shown in Table 1. The charge transfer through the Na^+/Ca^{2+} exchange (outward current) during the action potential was 29.8 % of the charge transfer of Ca^{2+} channels (inward current) for CA fish and 21.9 % (*P*=0.32) for WA fish. When these data are transformed to give the increment of intracellular [Ca^{2+}], the contributions of Ca^{2+} channels are 64.5±4.9 and 63.5±3.4 % and the contributions of the Na^+/Ca^{2+} exchange are 35.5±4.9 and 36.5±3.4 % for WA and CA fish, respectively (Table 1). Thus, according to the blocker method, approximately two-thirds of the sarcolemmal Ca^{2+} influx comes through the L-type Ca^{2+} channels and one-third through the Na^+/Ca^{2+} exchanger in crucian carp ventricular myocytes.

It is possible that the Na^+/Ca^{2+} exchange current is smaller in the presence of an intact Ca^{2+} current than it is after blockade of the Ca^{2+} channels. This would lead to an overestimation of both the exchange current and the Ca^{2+} current by method 1, although the relative sizes of the Ca^{2+} current and the Na^+/Ca^{2+} exchange current would not necessarily be altered. To alleviate this problem, an alternative method was used. Method 2 is based on the assumption that, in the absence of a functional sarcoplasmic reticulum, Na^+/Ca^{2+} exchange alone is responsible for relaxation. Accordingly, the total sarcolemmal Ca^{2+} influx through Ca^{2+} channels and the reverse Na^+/Ca^{2+} exchange should be equal to the Ca^{2+} efflux by forward exchange. The total sarcolemma Ca^{2+} efflux (=influx) was

Table 1. *Contribution of L-type Ca^{2+} current and Na^+/Ca^{2+} exchange current to total cellular [Ca^{2+}] in the ventricular myocytes of cold- and warm-acclimated crucian carp heart*

	Cell capacitance (pF)	Cell volume (μm^3)	$\int I_{Ca}$ ($\mu mol\ l^{-1}$)		$\int I_{Ex}$ ($\mu mol\ l^{-1}$)		$\int I_{Ca} + \int I_{Ex}$ or $\int I_{it}$ ($\mu mol\ l^{-1}$)		Fraction contributed by I_{Ex} (%)	
			AP clamp	Square wave	AP clamp	Square wave	AP clamp	Square wave	AP clamp	Square wave
Method 1										
WA	25.9±3.1	1417±167	141±31	158±35	62±3[a]	41±6	204±31	198±33	35.5±4.9	25.6±5.6
CA	22.2±1.5	1214±80	117±8	116±9	69±10[b]	47±8	186±14	164±13	36.5±3.4	27.9±4.3
Method 2										
WA	25.9±3.1	1417±167	84±18	83±15	64±5[a]	20±8[c]	148±20	103±9	47.4±4.9	22.8±10.2[c]
CA	22.2±1.5	1214±80	74±15	70±9	70±10[b]	65±24[c]	144±14	135±24	49.8±6.8	35.6±10.6[c]

Sarcolemmal Ca^{2+} influx was determined by the two methods described in detail in the text.

All values are means ± S.E.M. of eight cells for both acclimation groups.

Cell volume was calculated from the whole-cell capacitance assuming a specific membrane capacitance of 1.59 $\mu F\ cm^{-2}$ and a surface-to-volume ratio of 1.15 μm^{-1} for the fish myocytes (Vornanen, 1996a, 1997).

Ca^{2+} entry was calculated from the transferred charges and cell volume; Ca^{2+} influx was assumed to distribute evenly in the subsarcolemmal myofibrillar space which occupies 40 % of the whole-cell volume.

$\int I_{Ca}$ ($\mu mol\ l^{-1}$), integrated Ca^{2+} influx through L-type Ca^{2+} channels; $\int I_{Ex}$ ($\mu mol\ l^{-1}$), integrated Ca^{2+} influx through reverse Na^+/Ca^{2+} exchange; $\int I_{it}$, integrated Ca^{2+} efflux through forward Na^+/Ca^{2+} exchange during action potential (AP) repolarization or inward tail current after the repolarising clamp step to −70 mV (= the total sarcolemmal Ca^{2+} influx).

[a, b]Two successive analyses of the same recordings.

[c]In those cells in which the calculated Na^+/Ca^{2+} exchange current was inwards (Ca^{2+} efflux) during depolarization, the contribution of Na^+/Ca^{2+} exchange was set to be zero (not negative).

There were no statistically significant differences between acclimation groups (*t*-test, $P<0.05$).

CA, cold-acclimated; WA, warm-acclimated.

determined as the integral of the inward tail current. For the square-pulse clamp, Ca^{2+} current was estimated as an inactivating Ca^{2+} current, which should be representative of the total Ca^{2+} current because the Ca^{2+} window current is small at +10 mV (Vornanen, 1997). The exchange current was then obtained by subtraction.

During action-potential clamp, inactivation of the Ca^{2+} current is more complex and cannot be measured using the above method. Furthermore, there is no clearly distinguishable inward tail current. The reversal potential of the exchanger under the present experimental conditions is close to +10 mV, and forward Na^+/Ca^{2+} exchange can be therefore calculated from the integral of the inward current at voltages more negative than the $E_{Na/Ca}$. Reverse Na^+/Ca^{2+} exchange was measured as in method 1, and Ca^{2+} influx through the channels was calculated as the difference between the total Ca^{2+} influx and the influx through the exchanger (Fig. 5). The results of this analysis are shown in Table 1.

When using a square-wave pulse, the cells could be divided in two different groups. In five (three WA and two CA) out of 16 cells, the integral of the Ca^{2+} current was larger that the integral of the inward tail current, suggesting that all the activating Ca^{2+} was coming through the channels and that the exchanger was in forward mode during the depolarization. This was to be expected, because the depolarization came close to the reversal potential of the exchanger. In the remaining 11 cells, the Ca^{2+} current could explain only part of the sarcolemmal Ca^{2+} influx. When all 16 cells were included, the subtraction indicated that the exchanger was responsible for 22.8±10.2 % of the Ca^{2+} influx in WA fish and 35.6±10.6 % and CA fish. During action-potential clamp, the exchanger contributed 47.4±4.9 and 49.8±6.8 %, respectively, of the total sarcolemmal Ca^{2+} influx. These high values are not unexpected, since the action potential overshoot rises far above the reversal potential of the exchanger, where the Ca^{2+} channel current is relatively small.

Integration of the Ca^{2+} current and the Na^+/Ca^{2+} exchange current indicates that Ca^{2+} influx through the channels and the exchanger has a different time course during action-potential clamp and square-pulse clamp. If the electrophysiological analysis of Ca^{2+} influx approximates correctly the true sarcolemmal Ca^{2+} influx, the time course of cell shortening should reveal whether only Ca^{2+} channels are contributing or whether both entry routes are involved. Cell contraction is faster during action-potential clamp than during a square-wave pulse in the presence of Ca^{2+} channel blockers, which accords with the calculated Ca^{2+} influx through the exchanger. More importantly, the time course of contraction in the absence of blockers cannot be adequately explained by Ca^{2+} influx through the channels alone, but is very similar to the sum of Ca^{2+} influxes through the channels and the exchanger (Fig. 6). This strongly suggests that Na^+/Ca^{2+} exchange is not only operative during Ca^{2+} channel blockade but also contributes to contractile Ca^{2+} in the presence of an intact Ca^{2+} current during the physiological action potential. In two cells, the amplitude of cell shortening in the presence of Ca^{2+} channel blockers

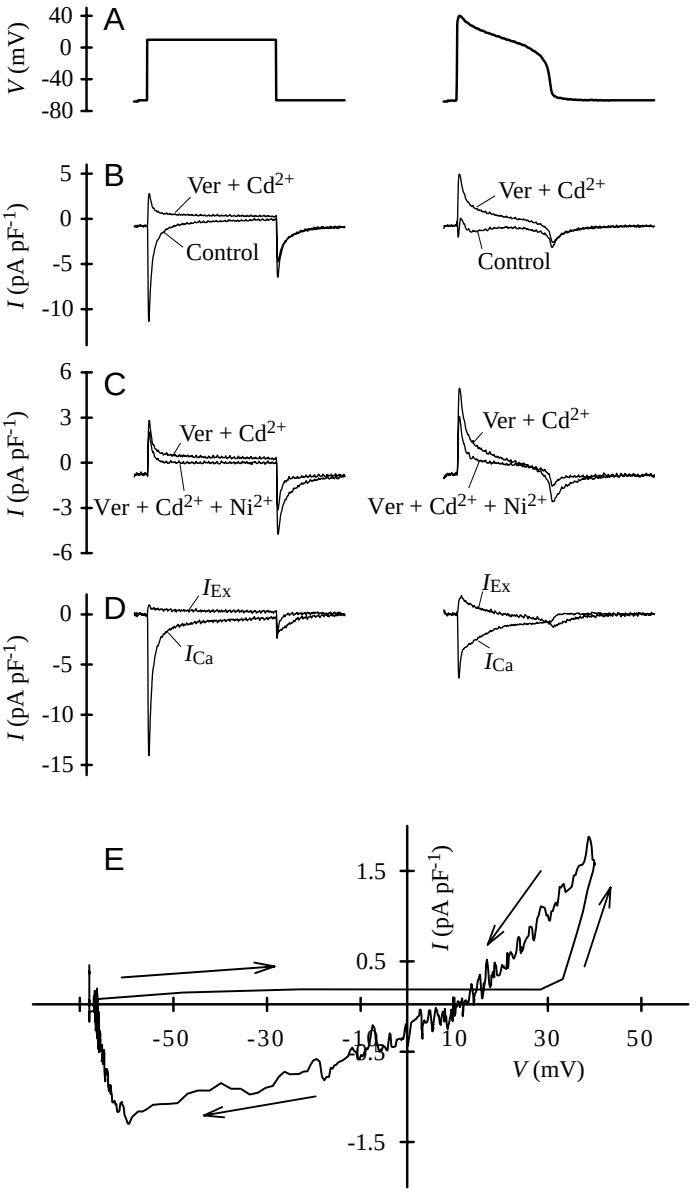

Fig. 4. Determination of sarcolemmal Ca^{2+} influx through L-type Ca^{2+} channels and by Na^+/Ca^{2+} exchange in crucian carp ventricular myocytes (method 1). Myocytes were voltage-clamped by square-wave pulses (from −70 to +10 mV for 500 ms) and action potential waveforms (A) at the rate of 0.1 Hz. The L-type Ca^{2+} current was determined as the verapamil (10 µmol l^{-1}) + Cd^{2+} (25 µmol l^{-1}) -sensitive component (B,D) and the Na^+/Ca^{2+} exchange current as the Ni^{2+}-sensitive current component (C,D). The verapamil- and Cd^{2+}-sensitive difference current (I_{Ca}) and Ni^{2+} (2 mmol l^{-1}) -sensitive difference current (I_{Ex}) are shown in D. The voltage-dependence of the Ni^{2+}-sensitive current during an action potential clamp is shown in E. The arrows indicate the direction of voltage change during the action-potential clamp. All recordings are for a ventricular myocyte (24.5 pF) from a cold-acclimated crucian carp at 22 °C. *I*, current; *V*, voltage; Ver, verapamil.

during action-potential clamp was 57.9 and 43.1 % of the control value, and the calculated Ca^{2+} influx through the exchanger was correspondingly 59.6 and 50.0 % of the total Ca^{2+} influx determined using method 1.

Discussion

Density of the Na^+/Ca^{2+} exchange current

The Na^+/Ca^{2+} exchanger is the major Ca^{2+} efflux pathway in cardiac muscle cells and is therefore intimately involved in relaxation from contraction, especially in preparations in which the sarcoplasmic reticulum is relatively poorly developed (Hume and Uehara, 1986). In mammalian cardiac cells, Ca^{2+} influx through Na^+/Ca^{2+} exchange can function as a trigger for more extensive Ca^{2+} release from the sarcoplasmic reticulum (Vornanen et al., 1994; Levi et al., 1994), whereas in cardiac myocytes of lower vertebrates, which rely on extracellular Ca^{2+} for contractile activation, the exchanger may provide a significant pathway for trans-sarcolemmal Ca^{2+} entry (Mullins, 1979; Fan et al., 1996). Accordingly, the density of the Na^+/Ca^{2+} exchange current should be relatively low in cardiac tissues, in which the sarcoplasmic reticulum plays a dominant role in cardiac Ca^{2+} regulation, and relatively high in the hearts of lower vertebrates with a rudimentary sarcoplasmic reticulum.

The available literature on the Na^+/Ca^{2+} exchanger lends some support to this assumption (Table 2). In ventricular myocytes of neonatal rabbit heart, in which the sarcoplasmic reticulum is poorly developed, the density of the Na^+/Ca^{2+} exchange current is much higher than in the cardiac cells of adult rabbit heart, which have a fully matured sarcoplasmic reticulum (Artman et al., 1995). A particularly high density of the Na^+/Ca^{2+} exchange current has been reported for frog ventricular myocytes (Fan et al., 1996), in which the sarcoplasmic reticulum is considered to be even sparser than in heart cells of teleost fish (Santer, 1985). Although the results of different studies are not directly comparable because of the varying experimental conditions, the density of the Na^+/Ca^{2+} exchange current in crucian carp ventricular myocytes seems to be lower than that in frog ventricular cells and similar to that in neonatal rabbit heart, but higher than in adult rabbit heart (Table 2).

Thus, the present results on fish heart conform to the assumed rule of an inverse relationship between the abundance of sarcoplasmic reticulum and the density of the Na^+/Ca^{2+} exchange current. It is notable that the high densities of frog (Fan et al., 1996) and fish (present study) Na^+/Ca^{2+} exchange currents were measured at significantly lower experimental temperatures than the respective values in the mammalian preparation (Table 2). Thus, in spite of low body temperature, relatively high densities of the Na^+/Ca^{2+} exchange current are present in these poikilothermic animals. This could be due either to a higher density of Na^+/Ca^{2+} exchange molecules per unit of sarcolemmal surface or to a different temperature-dependence of the exchanger. In fact, the temperature-dependence of the trout cardiac Na^+/Ca^{2+} exchanger is much weaker (Q_{10}=1.2) than that of mammalian preparations (Q_{10}>2) (Tibbits et al., 1992). The difference in temperature-dependence between mammals and fishes seems to be due to

Table 2. *Density of the Na^+/Ca^{2+} exchange current (I_{Ex}) in ventricular myocytes of different vertebrate species*

Species	I_{Ex} (pA pF^{-1})	Membrane potential (mV)	Temperature (°C)	$[Na^+]_i$ (mmol l^{-1})	Reference
Adult rabbit[1]	0.8±0.25	+50	35–36	10	Artman et al. (1995)
Newborn rabbit[1]	3.0±0.13	+50	35–36	10	Artman et al. (1995)
Frog (*Rana pipiens*)	8	+20	Room temperature	20	Fan et al. (1996)
Crucian carp[2]					
WA	2.99±0.27	+40	22±2	10	Present study
CA	2.38±0.42				

[1]Values are means ± S.E.M., N=3 for adult rabbits; N=24 for newborn rabbits.
[2]Values are means ± S.E.M. from Fig. 3A; N=5 for cold-acclimated (CA) fish and N=6 for warm-acclimated (WA) fish.
$[Na^+]_i$, total concentration of Na^+ in the pipette solution.
There were no statistically significant differences between acclimation groups (P=0.26).

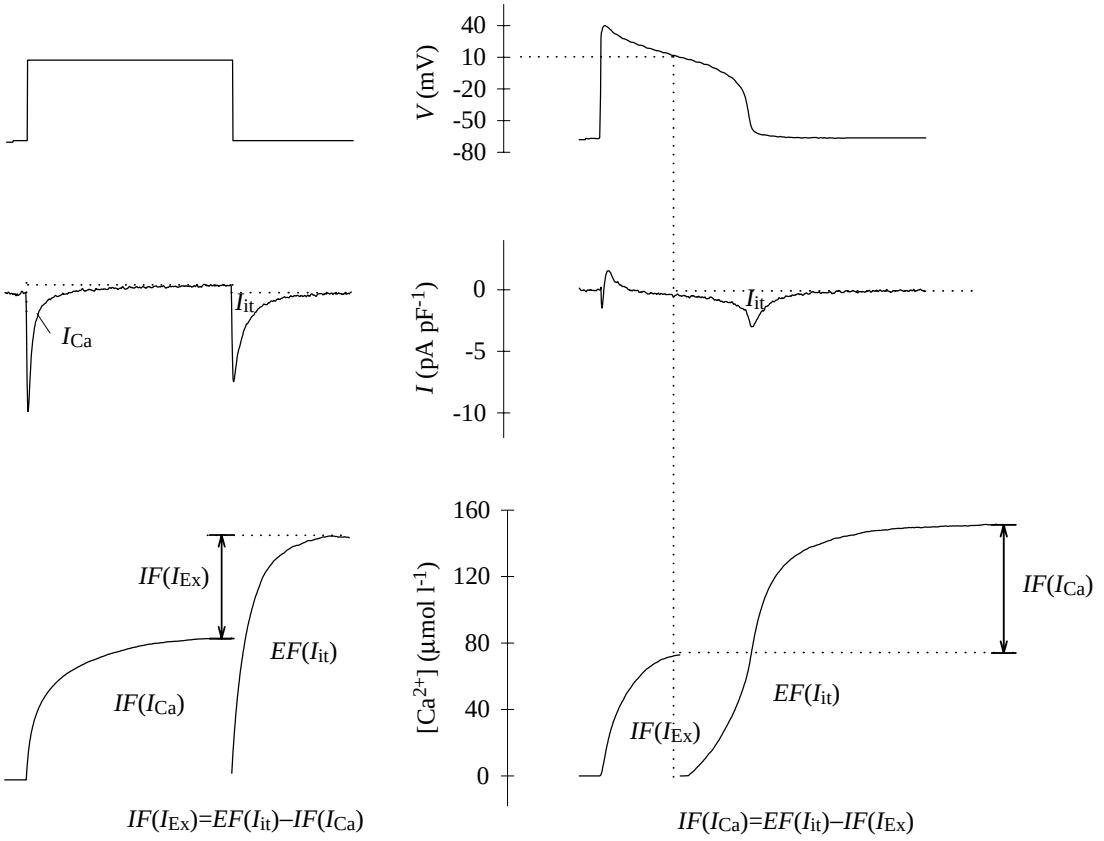

Fig. 5. Estimation of sarcolemmal Ca^{2+} influx from the inward tail current (I_{it}) (method 2). Sarcoplasmic reticulum Ca^{2+} uptake was inhibited by 5 µmol l^{-1} ryanodine, so that the total sarcolemmal Ca^{2+} influx through Ca^{2+} channels and reverse Na^+/Ca^{2+} exchange should be equal to sarcolemmal Ca^{2+} efflux through forward Na^+/Ca^{2+} exchange, as seen in the slowly inactivating inward tail current. The total Ca^{2+} efflux/influx, $EF(I_{it})$, during a square-wave pulse (left) was estimated from the integral of the inward tail current (I_{it}) after the repolarizing voltage step to −70 mV. Influx of Ca^{2+} through Ca^{2+} channels, $IF(I_{Ca})$, was determined by integrating the inactivating Ca^{2+} current (I_{Ca}), and Ca^{2+} influx through Na^+/Ca^{2+} exchange, $IF(I_{Ex})$, was then obtained by subtraction: $IF(I_{Ex})=EF(I_{it})–IF(I_{Ca})$. During action-potential clamp (right), the total Ca^{2+} efflux/influx was calculated as the integral of the inward current from +10 mV (the assumed reversal potential of the exchanger) to the end of the action potential (I_{it}). Influx of Ca^{2+} through Na^+/Ca^{2+} exchange was obtained from the Ni^{2+}-sensitive current determined using method 1 (see Fig. 4), and Ca^{2+} influx through Ca^{2+} channels was obtained by subtraction: $IF(I_{Ca})=EF(I_{it})–IF(I_{Ex})$. The results are expressed as increments in intracellular Ca^{2+} concentration which were calculated from the charges carried (two charges per Ca^{2+} for I_{Ca} and one charge per Ca^{2+} for I_{Ex} and I_{it}) as described in Materials and methods. All recordings are for a myocyte (20.7 pF) from a warm-acclimated fish at 22 °C. I, current; V, voltage.

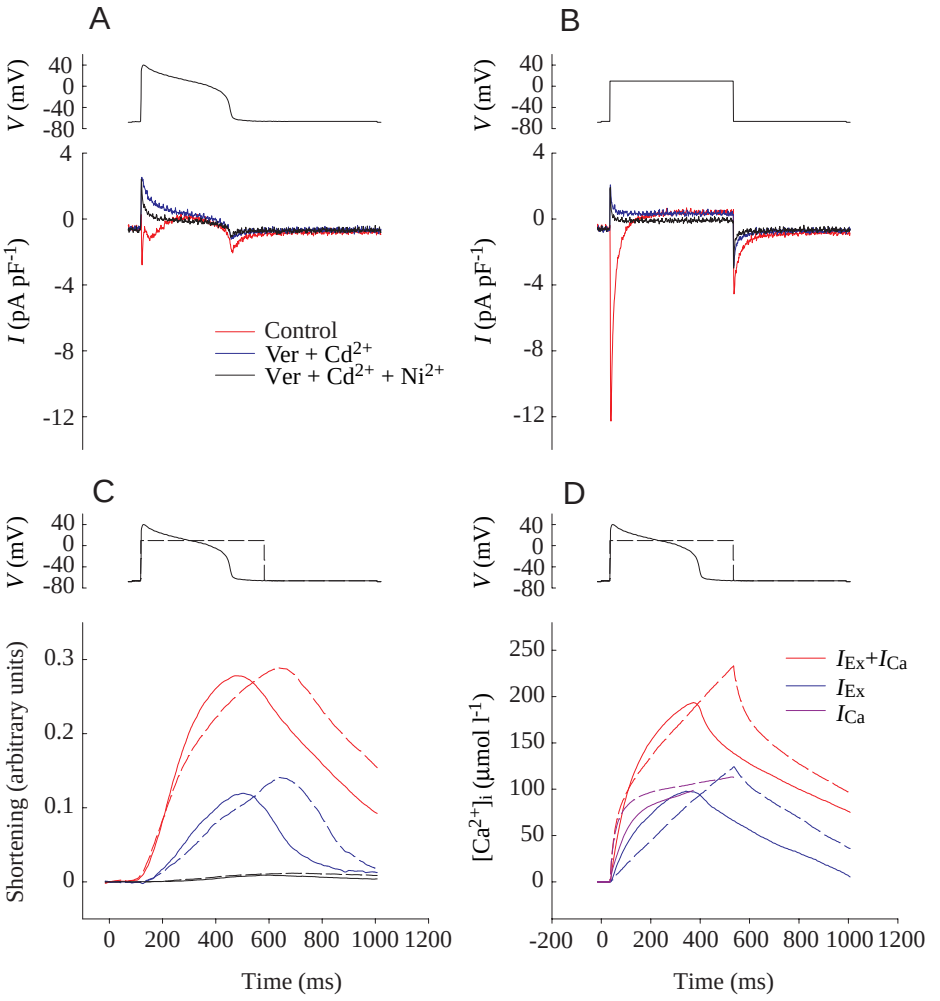

Fig. 6. Comparison of the time course of cell shortening with the calculated changes in intracellular Ca^{2+} concentration due to sarcolemmal Ca^{2+} fluxes through L-type Ca^{2+} channels and Na^+/Ca^{2+} exchange. Current recordings in the absence (control) and in the presence of Ca^{2+} channel blockers (verapamil + Cd^{2+}), and in the presence of both Ca^{2+} channel and Na^+/Ca^{2+} exchange blockers (verapamil + Cd^{2+} + Ni^{2+}) are shown in the upper half of the figure; (A) action-potential clamp and (B) square-wave clamp. Time courses of cell shortening (C) and the calculated intracellular Ca^{2+} transients (D) from the same cell are shown in the lower part of the figure. Sarcolemmal Ca^{2+} influx was calculated using method 1. Sarcolemmal Ca^{2+} efflux was obtained from the inward tail currents using method 2. The continuous line is for the action-potential clamp, and the dashed line is for the square-wave clamp. All recordings are for a myocyte (24.4 pF) from a cold-acclimated fish at 22 °C. *I*, current; *V*, voltage; Ver, verapamil.

differences in the primary structure of the Na^+/Ca^{2+} exchange molecule rather than to its lipid environment (Tibbits et al., 1992). Furthermore, direct comparison of the Na^+/Ca^{2+} exchange current densities between mammals and other vertebrates might underestimate the significance of Na^+/Ca^{2+} exchange in the hearts of poikilothermic animals. Heart cells of frogs and fishes are long and narrow in comparison with mammalian cardiac cells and therefore possess 4–5 times more sarcolemmal surface area per unit cell volume. Accordingly, the same density of the exchanger current in fish and frog cardiac cells can induce much larger changes in the intracellular Ca^{2+} concentration than would occur in cardiac cells of adult mammals.

Relative contributions of L-type Ca^{2+} current and Na^+/Ca^{2+} exchange to sarcolemmal Ca^{2+} influx during the action potential

The most straightforward way of measuring the Ca^{2+} current and the Na^+/Ca^{2+} exchange current during action-potential clamp is the method of subtraction, i.e. the measurement of Ca^{2+} fluxes through the channels and the exchanger as blocker-sensitive currents. In the present work, fairly complete block of L-type Ca^{2+} current was obtained by verapamil and Cd^{2+}, whereas the Na^+/Ca^{2+} exchange current was distinguished as a Ni^{2+}-sensitive current. Although Ni^{2+} is a nonspecific blocker, all major ionic currents other than Na^+/Ca^{2+} exchange should already have been abolished before the administration of Ni^{2+}. We used Ni^{2+} at a concentration of 2 mmol l^{-1}, which should completely inhibit the cardiac isoform of the Na^+/Ca^{2+} exchanger (Ehara et al., 1989; Iwamoto and Shigekawa, 1998). In crucian carp ventricular myocytes, 2 mmol l^{-1} Ni^{2+} abolished 70±2 % (*N*=18) of the total membrane current when other cation conductances had already been blocked (see, for example, Figs 1, 2). At a concentration of 2 mmol l^{-1}, Ni^{2+} caused a mean inhibition of cell shortening of 89±4 % (*N*=6) and of the inward tail current of 86±2 % (*N*=12), suggesting that almost 90 % of the exchanger activity was blocked.

Both the Ca^{2+} current and the Na^+/Ca^{2+} exchange current are regulated by intracellular Ca^{2+} concentration. The rate of Ca^{2+} current inactivation is largely dependent on intracellular free [Ca^{2+}], and the function of the exchanger is determined by the electrochemical gradient for Ca^{2+}. The rationale for not buffering intracellular Ca^{2+} was to allow the negative feedback of intracellular Ca^{2+} to regulate the function of channels as well as the exchanger. By this means, we could demonstrate the reversal of the Na^+/Ca^{2+} exchange current during the action

potential and we could correlate the Na^+/Ca^{2+} exchange current with the amplitude of contraction. The current–voltage relationship of the exchanger under these conditions does not represent pure voltage-dependence of the Na^+/Ca^{2+} exchange but rather a 'physiological' voltage-dependence which includes modification of the exchange current by changes in intracellular $[Ca^{2+}]$.

The integrals of the L-type Ca^{2+} current for crucian carp ventricular myocytes in the present study, when using method 1, are 52–90 % higher than reported in our previous studies (Vornanen, 1997, 1998). This could be partly due to the concentration of intracellular Mg^{2+}, which was much higher in earlier studies (total $[Mg^{2+}]$ 6 mmol l^{-1}, free $[Mg^{2+}]$ 1.28 mmol l^{-1}) than in the present study (total $[Mg^{2+}]$ 1 mmol l^{-1}, free $[Mg^{2+}]$ 0.02 mmol l^{-1}). Intracellular Mg^{2+} modulates L-type Ca^{2+} current so that a reduction in internal free $[Mg^{2+}]$ increases the amplitude of the Ca^{2+} current (White and Hartzell, 1988; Yamaoka and Seyama, 1998; Howarth and Levi, 1998). The physiological level of the internal free Mg^{2+} concentration of fish cardiac cells is not known, but if it is similar to that in mammalian cardiac cells (0.4–1.0 mmol l^{-1}; Buri et al., 1993), the amplitude of the L-type Ca^{2+}current may have been overestimated because of the relatively low Mg^{2+} concentration in the pipette.

Another source of error is inherent to the method used: it is possible that reverse Na^+/Ca^{2+} exchange is more strongly inhibited by the physiological Ca^{2+} transient when Ca^{2+} channels are functional than in the presence of Ca^{2+} channel blockers. Consequently, part of the outward shift of the current after the addition of Ca^{2+} channel blockers could be due to the activation of reverse Na^+/Ca^{2+} exchange, which would cause an overestimation of Ca^{2+} current. In fact, when the Ca^{2+} current was determined as the difference between the peak current and the current at the end of pulse, the Ca^{2+} current integrals in the present study came close to the values in our previous determinations. For the same reason, the Na^+/Ca^{2+} exchange current may also be overestimated, especially during the square-wave pulse when the Ca^{2+} current is large. The results obtained using method 1 should therefore be regarded as a maximum estimate of sarcolemmal Ca^{2+} influx; this method will give realistic values for the capacity of the exchanger in the absence of an intact Ca^{2+} current, but it may overestimate the total sarcolemmal Ca^{2+} influx by overestimating both the Ca^{2+} current and Na^+/Ca^{2+} exchange during the physiological Ca^{2+} transient.

The alternative method of analysis (method 2), using the inward tail current as a measure of the total unidirectional sarcolemmal Ca^{2+} flux, gives lower values for the total sarcolemmal Ca^{2+} influx. This should be considered, however, as a minimum estimate, since the inward tail current was not always completely inactivated during the acquisition period of the current (see Fig. 6), and it is likely that part of the cytosolic Ca^{2+} efflux occurs through the sarcolemmal Ca^{2+} pump (Choi and Eisner, 1999) or into mitochondria. The estimates of the total sarcolemmal Ca^{2+} influx using the two methods (action-potential clamp) differed by 38 % for WA fish and by 29 % for CA fish, suggesting that our estimates are not far from the real Ca^{2+} influx. Furthermore, the methods give similar values (approximately 2:1) for the relative significance of Ca^{2+} channels and Na^+/Ca^{2+} exchange in sarcolemmal Ca^{2+} influx when using square-wave voltage pulses. However, when action-potential clamp is used, method 2 gives somewhat higher values (approximately 1:1) for the relative importance of the exchanger compared with method 1 (approximately 2:1).

The results of the two methods were combined in the analysis of cell shortening by calculating the sarcolemmal Ca^{2+} influx through Ca^{2+} channels and the reverse Na^+/Ca^{2+} exchange using method 1 and estimating the Ca^{2+} efflux through the forward Na^+/Ca^{2+} exchange using method 2. It is notable that the 'Ca^{2+} transient' obtained by combining the two methods of analysis explains the time course of cell shortening very well. Thus, the two methods of electrophysiological analysis as well as measurements of cell shortening show that Na^+/Ca^{2+} exchange has a capacity adequate to provide significant amounts of activator Ca^{2+} in crucian carp ventricular myocytes. Furthermore, the present results lend strong support to the hypothesis that, during the physiological contraction, a significant part of the activator Ca^{2+} (from one-third to half) comes from reverse Na^+/Ca^{2+} exchange in crucian carp ventricular myocytes.

The total absence of a ryanodine-sensitive component of contraction in the crucian carp ventricle (Vornanen, 1996) indicates that the activation of contraction is exclusively dependent on sarcolemmal Ca^{2+} influx in this fish species. The total sarcolemmal Ca^{2+} influx of 144–148 μmol l^{-1}, as estimated using method 2, is approximately one-third larger than the total Ca^{2+}-handling capacity of the sarcoplasmic reticulum and sarcolemma of the mammalian ventricular cells (approximately 100 μmol l^{-1}) (Delbridge et al., 1996; Terracciano and Macleod, 1997). This is not unexpected since, under basal conditions, the crucian carp heart is working close to its contractile limit. Maximum inotropic stimulation, e.g. by treatment with isoprenaline or by cooling, increases the force of contraction by only approximately 60 % in crucian carp ventricle (Vornanen, 1989), whereas corresponding increases in mammalian and rainbow trout heart are 200–300 %. This property of the crucian carp ventricle is also evident in the density of sarcolemmal Ca^{2+} current which, under basal conditions, is double that of the rainbow trout ventricle. In the presence of maximal β-adrenergic activation, the density of the Ca^{2+} current is almost the same in the two fish species (Vornanen, 1998).

In the literature, there are some estimates for sarcolemmal Ca^{2+} influx through Ca^{2+} channels and Na^+/Ca^{2+} exchange in mammalian hearts. A comparison of the peak densities of the Na^+/Ca^{2+} exchange current and the Ca^{2+} current suggests that, in guinea-pig ventricular myocytes, a reverse Na^+/Ca^{2+} exchange can provide less than 30 % of the Ca^{2+} influx contributed by L-type Ca^{2+} channels (approximately 20 % of the total sarcolemmal Ca^{2+} influx) (Grantham and Cannell, 1996). In rabbit ventricular myocytes, net Ca^{2+} entry *via* reverse Na^+/Ca^{2+} exchange was calculated to be only 10 % of

the amount mediated by L-type Ca^{2+} channels at a membrane potential of +30 mV (Litwin et al., 1998). The present results, using a physiological action potential as the voltage waveform in fish cardiac myocytes, suggest that the relative contribution of Na^+/Ca^{2+} exchange to sarcolemmal Ca^{2+} influx is significantly larger in fish ventricular cells than in mammalian cardiac cells.

Effects of thermal acclimation

The same experimental conditions were used for recording membrane currents in ventricular myocytes from WA and CA crucian carp. This allows a direct comparison between acclimation groups and could also reveal putative temperature-induced changes in the intrinsic properties of the Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channels. Direct effects of low ambient temperature on action potential duration and on the function of Ca^{2+} channels and the Na^+/Ca^{2+} exchanger are, however, lacking. The similarity of the Na^+/Ca^{2+} exchange current and the L-type Ca^{2+} current in WA and CA fish hearts suggests that thermal acclimation has little effect on the density and intrinsic properties of these sarcolemmal proteins or, alternatively, that temperature-induced changes cancel each other out, with little net effect on current densities. It is, however, impossible to say whether the relative contributions of Ca^{2+} channels and the Na^+/Ca^{2+} exchanger to sarcolemmal Ca^{2+} influx would also hold at the physiological body temperature of the CA fish.

A low environmental temperature will probably reduce the peak amplitudes of the Na^+/Ca^{2+} exchange current and the Ca^{2+} current, whereas prolongation of the action potential will allow more time for sarcolemmal Ca^{2+} influx through these pathways. The very weak temperature-dependence of the fish Na^+/Ca^{2+} exchanger (Tibbits et al., 1992) suggests that the small temperature-dependent decrease in the amplitude of the Na^+/Ca^{2+} exchange current would be more than compensated for by prolongation of the action potential. It should be remembered, however, that the plateau height of the action potential is a very significant determinant of the function of the Na^+/Ca^{2+} exchanger and that any temperature-dependent changes in action potential plateau height would have immediate effects on the Na^+/Ca^{2+} exchange current.

The temperature-dependence of the Ca^{2+} current in fish heart is not known, but a temperature-related decrease in current amplitude could be compensated for by slower inactivation of individual Ca^{2+} channels and prolonged depolarization. An acute temperature decrease from 20 to 1 °C increases the force of contraction by 60 % in crucian carp heart (Vornanen, 1989), suggesting that sarcolemmal Ca^{2+} influx is increased rather than compromised at low ambient temperature. Whether the relative contributions of the Na^+/Ca^{2+} exchanger and Ca^{2+} channels to contractile Ca^{2+} change upon thermal acclimation or following acute temperature changes remains to be shown by direct analysis of sarcolemmal Ca^{2+} influx at different experimental temperatures.

In conclusion, the present results indicate that ventricular myocytes of fish heart have a relatively high density of Na^+/Ca^{2+} exchange current, which is able to activate contraction in the presence of sarcolemmal Ca^{2+} channel and sarcoplasmic reticulum Ca^{2+}-release channel inhibitors. Sarcolemmal Ca^{2+} entry through the Na^+/Ca^{2+} exchanger also contributes to contractile Ca^{2+} in the presence of an intact Ca^{2+} current. It is estimated that, during a physiological action potential, 33–50 % of the total trans-sarcolemmal Ca^{2+} influx occurs through the exchanger. Estimates of the total sarcolemmal Ca^{2+} influx suggest that L-type Ca^{2+} channels and the Na^+/Ca^{2+} exchanger in crucian carp ventricular myocytes have sufficient capacity to activate contraction without a contribution from the sarcoplasmic reticulum.

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