The Effects of Sodium Removal on the Two Types of Calcium Currents in Single Frog Atrial Cells

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Abstract. The effects of sodium removal on the two types of calcium currents were studied in enzymatically dispersed frog (*Rana esculenta*) atrial myocytes with the single pipette patch-clamp technique. Reduction of calcium currents was recorded when NaCl was replaced either by TEACl, LiCl, TrisCl, cholineCl or by mannitol. An involvement of the Na-Ca exchange mechanism could be ruled out since the decrease was also observed after replacing external Ca with Ba. The slight shift of the apparent reversal potential recorded in our study suggests that the inward flow of Na ions through calcium channels does not contribute significantly to the L-type calcium current. Once again, the slight negative shift of the steady-state inactivation curve of the L-type calcium current cannot explain this decrease while no shift was recorded for the T-type calcium current. Even if a TTX-resistant Na current was recorded from a few cells this current cannot explain the decrease of calcium currents which was always observed upon sodium removal. To date we have no explanation for this effect.

Key words: Ca current — Frog heart — Single cells — Na ions

Introduction

In the past, a variety of effects of external sodium reduction have been reported on the calcium current in multicellular cardiac preparations (for review see Coraboeuf 1980). For example, in mammalian cat ventricular muscle, calcium current was unaffected by lowering the sodium concentration to 10% of the normal level (New and Trautwein 1972), whereas in bovine heart calcium current was decreased by 33% upon reducing the sodium concentration from 149.3 to 72.3 mmol/l (Reuter and Scholz 1977). In frog atrial fibres, calcium current has been reported to be both sensitive (Rougier et al. 1969; Lenfant et al. 1972; Mentrard et al. 1984) and insensitive (Benninger et al. 1976; Horackova and Vassort 1979) to changes in

external sodium. Some investigators concluded that both Na and Ca contribute to the cardiac slow inward current (Rougier et al. 1969; Reuter and Scholz 1977) or that two different types of slow channels may coexist in the membrane, one for Na ions and another for Ca ions (Chesnais et al. 1975), while an involvement of the Na-Ca exchange mechanism was assumed by others (Tarr 1971). Improvements in methodology, with the use of single cells which allow the application of the suction pipette methods of Hamill et al. (1981), have produced results which indicate that the Ca channel is considerably more selective for divalent cations than previously thought. According to Tsien et al. (1987b), a practical consequence of this selectivity is that at physiological Ca concentrations the calcium current is unaffected by the presence or absence of Na. Nevertheless, it has been shown recently that reduction of extracellular Na decreases the Ca current in guinea-pig ventricular cells (Orkand et al. 1991) despite the high selectivity of Ca channels in this preparation (Lee and Tsien 1984). Also such a high selectivity for Ca ions was reported in bull-frog (*Rana catesbeiana*) atrial cells (Campbell et al. 1988b). The present work, performed with the whole-cell patch-clamp technique, demonstrates a significant reduction in the magnitude of the two types of calcium currents recorded in atrial cells from the frog (Rana esculenta) after sodium withdrawal.

Materials and Methods

The experiments were performed at 20–23 °C on frog (*Rana esculenta*) isolated atrial cells using the standard whole-cell, tight-seal, voltage-clamp technique (Hamill et al. 1981). Normal Ringer solution contained (mmol/l): NaCl 110; KCl 2.5; $CaCl_2$ 1.8; $MgCl_2$ 2; HEPES/NaOH 10; glucose 10; pH 7.4. Patch pipettes (2 to 5 MΩ) were filled with (mmol/l) : NaCl 12; CsAspartate 130; CsCl 20; NaH_2PO_4 1; $MgCl_2$ 1; EGTA/CsOH 2; HEPES/CsOH 10; pH 7.2. Cells were enzymatically dispersed as previously described by Bonvallet (1987). Briefly, frogs were killed by decapitation and the heart rapidly removed and placed in normal Ringer solution. After dissection, all the auricular tissue was bathed in nominally Ca-free Ringer (composition as normal Ringer but without $CaCl_2$) for 30 min at room temperature, then transferred to 600 U/ml collagenase (Sigma type I) + 1.5 U/ml protease (Sigma type XIV) for 60 min at 25 ± 0.5 °C. Subsequently, the tissue was placed in Ca-free Ringer and stored at 5 °C for at least 12 hours before starting the experiment. The tissue was gently shaken in the superfusing chamber containing Ca-free Ringer. The cells were left to settle down for 15 min and then superfusion was started with normal Ringer solution. After a gigaseal was obtained, the bathing solution was changed to a Cs-Ringer solution, further referred to as control solution (composition as normal Ringer solution except for KCl and the buffer which were replaced by 2.5 mmol/l CsCl and 10 mmol/l HEPES/TRIS, respectively), containing 10^{-6} mol/l tetrodotoxin (TTX; Sankyo, Japan) in order to separate Ca currents from K and Na currents, and the patch was ruptured to allow a whole-cell voltage-clamp. All modifications concerning the composition of the control solution will be indicated in the text. The series resistance was usually 5–15 MΩ, 50–80% of which could be compensated. The frequency of stimulation was set at 0.1 Hz. A patch-clamp RK300 amplifier (Biologic, Meylan, France) was used to monitor cell currents. Voltage commands and simultaneous signal recordings used

pCLAMP software (Axon Instr., Burlingame, CA, USA) and a microcomputer. All traces shown were filtered with a 5-pole Bessel filter and were not corrected for leakage current or capacitive transients. Ca currents amplitude was measured as the difference between peak inward current and the zero current; when the currents became outward the amplitude was measured at the point corresponding to the peak of the last measurable inward current. The low-threshold and high-threshold calcium currents (Bonvallet and Rougier 1989) will be referred to the text as the T-type (or T-component) and the L-type (or L-component) respectively, according to the nomenclature proposed by Nowycky et al. (1985). Data are expressed as means ± S.E.M.. The number of cells is indicated by "n=" in brackets.

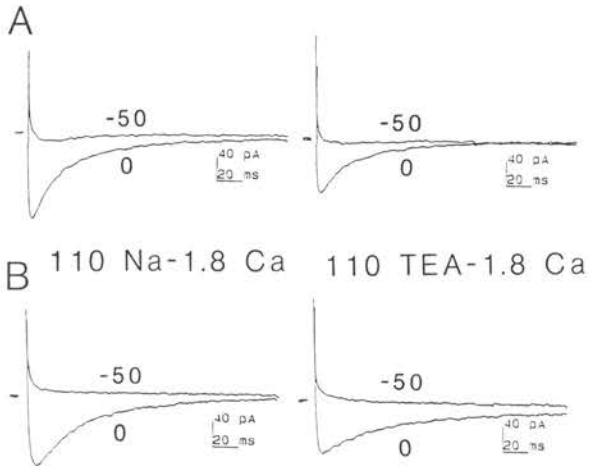

Figure 1. Effect of Na removal on the calcium currents. Step depolarizations are indicated at each trace. HP=−100mV. Control: left column; Na-free: right column. A) cell showing the two types of calcium currents. B) cell showing only the L-type of calcium current. Note the absence of inward current for the depolarizing pulse at −50mV.

Results

Figure 1*A* shows the effects of sodium removal on the two types of Ca currents elicited at −50 mV (for T-component) and 0 mV (for the L-component), both from a holding potential HP=−100 mV. In control solution (Fig. 1*A* ; left panel) the amplitude of the T-component and L-component was 16 pA and 122 pA, respectively. A 6 min exposure to Na-free external solution (TetraethylammoniumCl=TEACl substitute; right panel) caused a marked reduction in the magnitude of the two currents. In this medium, the amplitude was 8 pA and 78 pA for the T-component and L-component, respectively. The inactivation time courses of the T-component and

L-component were not significantly modified in Na-free external solution: indeed, the inactivation time constants ($= \tau_{inac}$) of the T-component and L-component were about 43 ms and 28 ms respectively in control solution, and 40 ms and 30 ms respectively in Na-free solution. No change in the leakage or background current was observed upon modifying $[Na]_o$. Similar results were obtained with four other cells. The average decrease in the magnitude of the T-component at −50 mV was 58.50 ± 8.43% (n=4) and 39.12 ± 9.91% (n=4) for the L-component at 0 mV. These effects appeared and reversed rapidly (between 30–60 s; results not shown).

Owing to the fact that the T-component was not recorded in all atrial cells (see Bonvallet and Rougier 1989), the above experiments were performed on cells showing only one type of current, the L-component. The results are shown in Fig. 1*B*. After about 4 min in Na-free external solution (TEACl substitute; right panel), the magnitude of the L-component (at 0 mV) was reduced by about 27% as compared to the control (left panel). In this cell the inactivation time course was slowed down in Na-free medium (τ_{inac} = 38.65 ms in control; τ_{inac} = 53.24 ms in Na-free medium).

At first sight, it seemed as if this decrease in the amplitude of the L-type Ca current, upon reducing external sodium, could be attributed either to (*i*), an increase in Ca-dependent inactivation mechanism due to an intracellular calcium rise in Na-free, Ca-containing solution as a consequence of the membrane Na-Ca exchange operating in the "reverse mode" (even though the presence of EGTA in the pipette was not in favour of this hypothesis); or to (*ii*) an outward current generated by Na-Ca exchange. To test these possibilities, we performed two kinds of experiments with 1.8 mmol/l Ba in place of Ca in the external solution since Ba ions do not replace Ca ions either in the Ca-mediated processes involved in the inactivation of the L-type calcium current (Argibay et al. 1988) or in the Na-Ca exchanger (Kimura et al. 1987; Campbell et al. 1988a).

Figure 2 shows that the decrease in the amplitude of the two components of the current was still observed when Ba ions served as the charge carriers. Indeed, on a cell showing the two current components (Fig. 2*A*), sodium removal (TEACl substitute) caused, after 3 min, a reduction by about 67.5% (from −20 pA to −6.5 pA) in the magnitude of the T-component at −50 mV, and by about 62% (from −162 pA to −62 pA) for the L-component at 0 mV. Fig. 2*B* shows a cell which was held at HP=−50 mV in order to inactivate the T-component (note the absence of the inward current for the depolarizing pulse at −45 mV; see Bonvallet and Rougier 1989). Once again, sodium removal caused after about 2 min a reduction by about 44% (from −468 pA to −260 pA) in the amplitude of the L-component recorded at 5 mV. The average decrease in the magnitude of the T-component at −50 mV was 47.16 ± 15.47% (n=6) and 60.75 ± 15.72% (n=6) for the L-component at 0 mV. In Ba, as in Ca, these effects were reversible (results not shown).

Owing to the above results, it was important to see whether, in our experi-

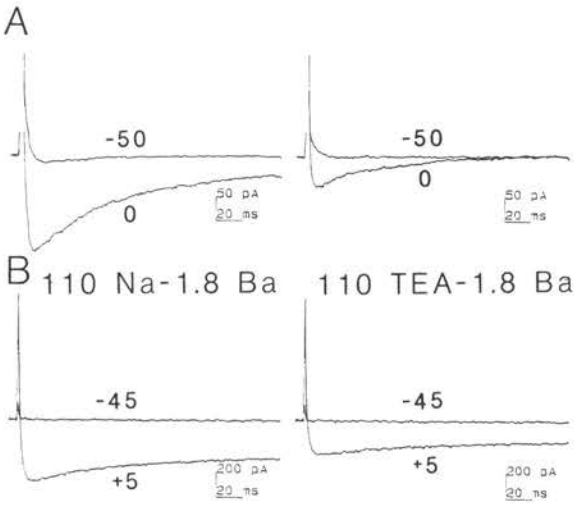

Figure 2. Effect of Na removal on the barium currents. Step depolarizations are indicated at each trace. Control: left column; Na-free: right column. A) cell showing the two types of barium currents; HP=−100 mV. B) cell showing only the L-type of barium current; HP=−50 mV. Note the absence of inward current for the depolarizing pulse at −45 mV.

mental conditions, Ba could replace Ca in the Na-Ca exchanger and whether the latter one could generate an outward current or "creep currents" (Hume and Uehara 1986a, b) upon external sodium removal. This hypothesis was tested in the following way. The cell was washed in the control solution containing 1.8 mmol/l Ba plus 40 μmol/l Ni and 20 μmol/l Cd in order to block the two components of calcium current (Bonvallet and Rougier 1989; also see Fig. 6). After 12 min in this medium (an interval sufficient for a complete diffusion of the pipette solution into the cell interior), currents were elicited by step depolarizations from HP=−100 mV to membrane potentials varying between −60 to +60 mV (Fig. 3*A*, 1). Then the external sodium concentration was reduced (TEACl substitute), and 10 min later the same pulse protocol was applied (Fig. 3*A*, 2). Figure 3*A*, 3 shows the digitally subtracted difference between currents elicited in 0% Na and in 100% Na. The difference current (delta I), which is the mean current measured during the last 50 ms of the depolarizing pulses, is plotted as a function of potential in Fig. 3*B*. It is clear that the reduction of $[Na]_o$ did not evoke an outward current. Identical results were obtained in two other cells. Therefore, in our experimental conditions it seems that Ba ions cannot replace Ca ions in the Na-Ca exchange, and that an involvement of Na-Ca exchange is not a likely explanation for the observed reduction of the T and L currents upon sodium removal.

Figure 4*A* shows current-voltage relationships of the L-component elicited from

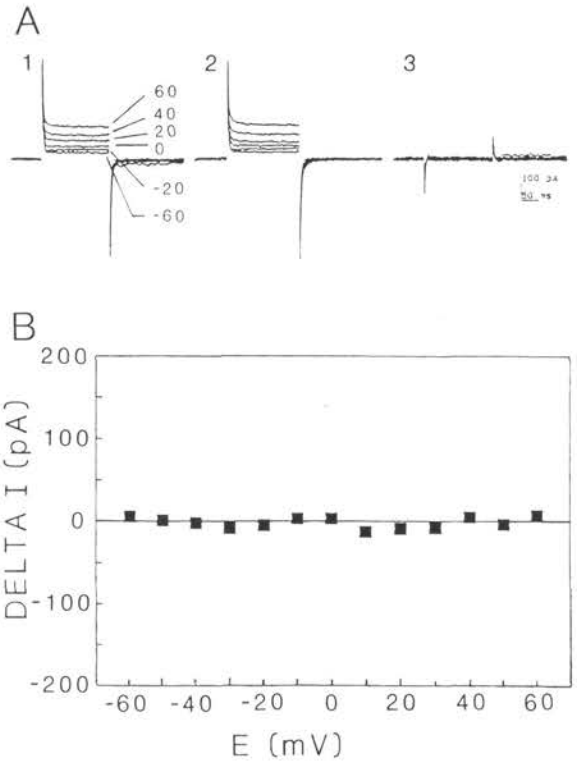

Figure 3. A) Effect of Na removal on the current recorded in 1.8 mmol/l Ba plus 40 μmol/l Ni and 20 μmol/l Cd. HP=−100 mv. Step depolarizations are indicated only at the upper left traces. 1) Control: 110 mmol/l Na. 2) After 12 min in 0 mmol/l Na (TEACl substitute). 3) Digitally subtracted difference between traces 2 and traces 1. B) Plot of difference current (Delta I) versus test potential in the same cell as A(1–3). See text for more explanation.

a HP=−50 mV in 110 mmol/l Na, 1.8 mmol/l Ba and after about 10 min in Na-free (TEACl substitute), 1.8 mmol/l Ba. Each point corresponds to the average obtained for 4 cells, each cell being superfused in control solution and then in Na-free solution. Changing the external sodium concentration induced a decrease of the L-component at all potentials tested. The apparent reversal potential was slightly shifted (≈3–4 mV) in the negative direction.

Since the decrease of T and L currents was always observed when sodium was replaced by tetraethylammonium, we could not rule out an inhibitory effect of this ammonium derivative on the calcium channels. To test this possibility, experiments

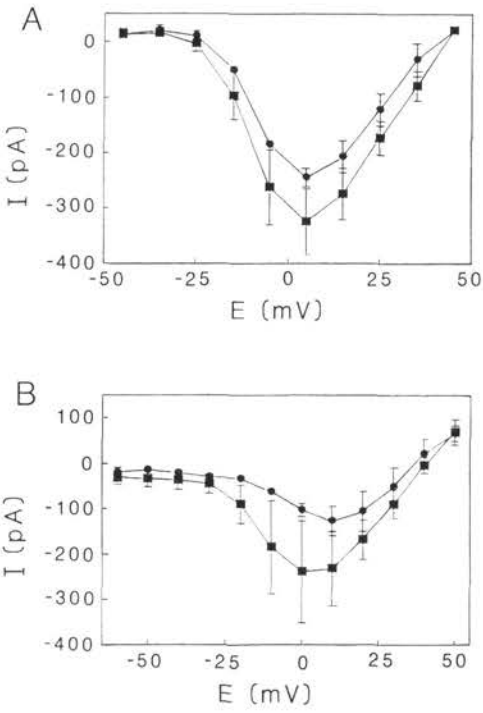

Figure 4. Average peak current-voltage relationships recorded in 110 mmol/l Na (▪) and 0 mmol/l Na (•). $[Ba]_o$=1.8 mmol/l. Data points are means ± S.E.M.. A) HP=−50 mV. TEACl substitute. (n=4). B) HP=−100 mV. LiCl substitute. (n=3). Note the reduction in the amplitude of currents and the slight shift of the apparent reversal potential in the negative direction.

were performed with external sodium replaced by lithium. Figure 4*B* shows the current-voltage relationships of T- and L-components in 110 mmol/l Na, 1.8 mmol/l Ba and after about 5 min in 110 mmol/l Li, 1.8 mmol/l Ba. Each point is the mean of 3 cells; each cell was superfused in control solution and then in Na-free solution. Substituting Li ions for Na ions induced qualitatively the same effects as previously, i.e. a slight shift of the apparent reversal potential towards more negative potentials and a decrease in the amplitude of the two current components. However, the L-component seemed more depressed between −25 mV and +10 mV than for higher potentials. The decrease of the two current components was similar (not shown) when sodium was replaced by Tris (n=1), mannitol (n=1) or by choline (n=1).

Figure 5 shows the effects of sodium removal on the inactivation curves of the two current components. Inactivation characteristics of T- and L-components were studied by a classical double-pulse protocol (Bonvallet and Rougier 1989). Briefly, for the T-component, a 200 ms pre-pulse to eight different potentials ranging from −110 to −35 mV was followed by a 297 ms test pulse to −40 mV. Pre-pulse and test pulse were separated by a 3 ms return to −100 mV holding potential. The

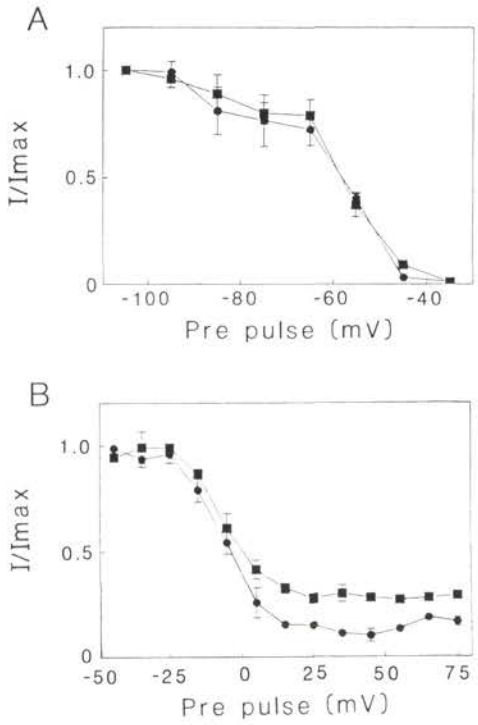

Figure 5. Effect of Na removal on the inactivation curves of the two types of barium currents. $[Ba]_o$=1.8 mmol/l. (▪)=110 mmol/l Na, (•)=110 mmol/l Li. Data points are means ± S.E.M.. A) Inactivation curve of T-component (n=4). B) Inactivation curve of L-component (n=4). See text for more explanation.

noninactivated fraction of the T-component during the test pulse, normalized to the maximal current obtained in the absence of a prepulse, has been plotted against the amplitude of the pre-pulse. For the L-component, the HP and the test pulse were set at −50 and +10 mV, respectively, and the duration of the pulses was the same as before. Substituting Li ions for Na ions (in 1.8 mmol/l Ba) was without significant effect on the steady-state inactivation of the T-component (Fig. 5*A*) and slightly shifted the steady-state inactivation of the L-component (Fig. 5*B*) towards more negative potentials, but more inactivation was obtained with pre-pulses to high positive potentials. Identical results were obtained when exchanging NaCl by TEACl (results not shown).

The reduction in magnitude of the two current components in Na-free solution may indicate the presence of a contaminating inward current through a TTX-insensitive sodium channel. This possibility was tested by using 40 μmol/l Ni and 20 μmol/l Cd to block the two components of calcium current (Bonvallet and Rougier 1989). Figure 6*A* shows membrane currents elicited by step depolarizations from HP=−100 mV to membrane potentials varying between −50 and +60 mV

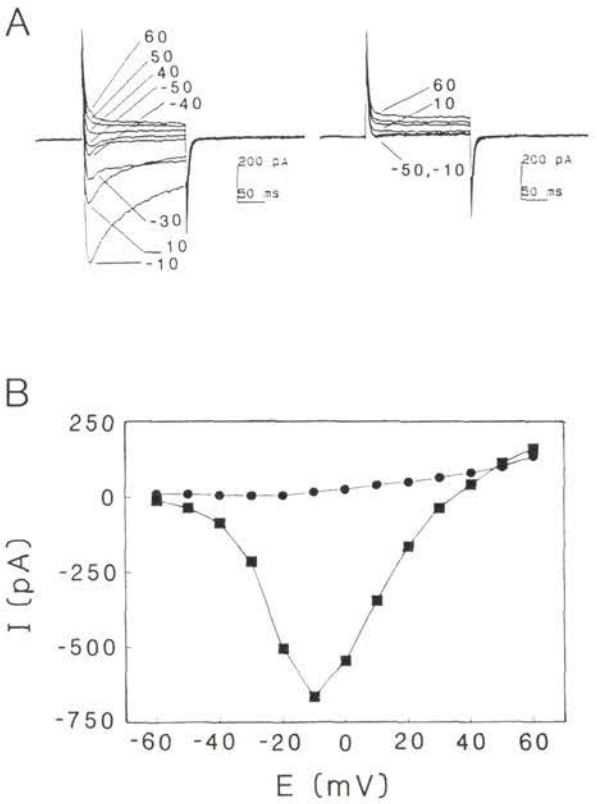

Figure 6. Effect of inorganic calcium channel blockers Ni and Cd on the two types of barium current. HP=−100 mV. $[Ba]_o$=1.8 mmol/l. Step depolarizations are indicated at each trace. A) Current traces obtained for various voltages in control (left panel) and then after addition of 40 μmol/l Ni and 20 μmol/l Cd (right panel). B) Current-voltage relationships measured in control (▪) and in the presence of blockers (•). Note that currents are completely blocked by Ni and Cd.

in 110 mmol/l Na, 1.8 mmol/l Ba (Fig. 6A, left panel). Two minutes after the addition of Ni and Cd ions into the control solution (Fig. 6*A*, right panel), inward currents were completely blocked. The corresponding current-voltage relationships are illustrated in Fig. 6*B*. In few cells, the same protocol gave different results. Such a behaviour is shown in Fig. 7. Figure 7*A* (left panel) shows that an inward current was still recorded in a solution containing 110 mmol/l Na, 1.8 mmol/l Ba plus 40 μmol/l Ni and 20 μmol/l Cd. This current disappeared when sodium ions were replaced by choline (Fig. 7*A*, right panel). The current-voltage relationships (Fig 7*B*) demonstrate clearly the presence of an inward current between about −20

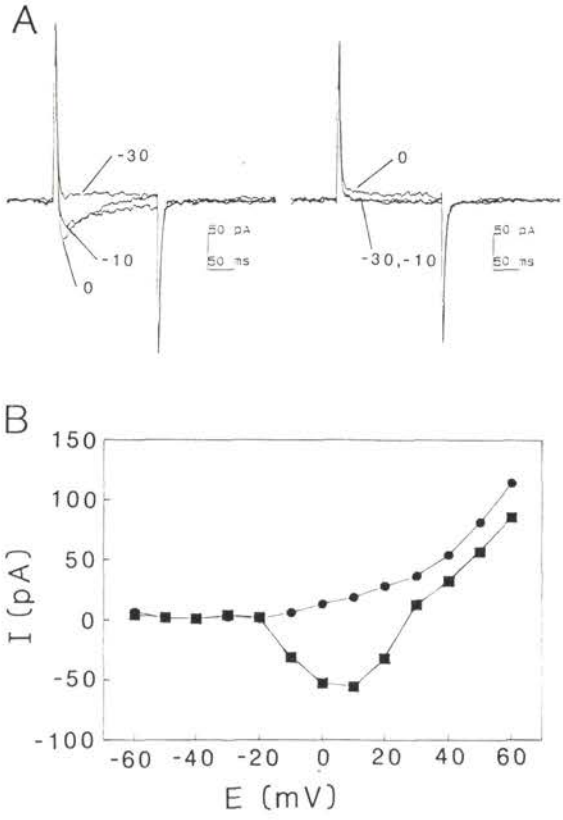

Figure 7. Effect of Na removal on the current recorded in the presence of inorganic calcium channel blockers Ni and Cd. HP=−100 mV. $[Ba]_o$=1.8 mmol/l. Step depolarizations are indicated at each trace. A) Current traces obtained for various voltages in 40 μmol/l Ni and 20 μmol/l Cd (left panel) and then after the suppression of Na (choline substitute; right panel). B) Current-voltage relationships measured in the presence of Ni and Cd (▪) and after Na withdrawal (•). Note the disappearance of the inward current when sodium is removed. See text for more explanation.

mV and +20, +30 mV and its disappearance with sodium being removed.

Discussion

The present study shows that sodium removal causes a marked reduction of the two types of calcium currents in single atrial cells from the frog (Fig. 1). Indeed, the decrease of the L-component observed at 0 mV (Fig. 1*A*) is unlikely to be due to a contamination of the L-component by the T-component since this effect is

also recorded on cells showing the L-type current only (Fig. 1*B*). The reduction in amplitude of the T- and L-component is not consistent with an increase in Ca-dependent inactivation mechanism due to an intracellular calcium rises in Na-free, Ca-containing solution as a consequence of inhibition of membrane Na-Ca exchange (Mitchell et al. 1983) or to an outward current generated by the Na-Ca exchange operating in the "reverse mode" (Hume and Uehara 1986a,b) since (*i*) inactivation of the T-component seems purely voltage-dependent in heart cells (Tsien et al. 1987a; Bonvallet 1989; Bonvallet and Rougier 1989; Charnet et al. 1991), and (*ii*) Ba ions do not replace Ca ions in the Na-Ca exchanger (Fig. 3; Kimura et al. 1987; Campbell et al. 1988b) while a decrease in amplitude of the two components of current is still recorded in Na-free, Ba containing solution (Fig. 2). Moreover, an inhibitory effect of TEA on calcium channels (Lee and Tsien 1984) can be ruled out since the reduction of calcium currents was also recorded when NaCl was replaced either by LiCl (Fig. 4*B*), TrisCl, cholineCl or by mannitol (results not shown). It has been suggested that the marked reduction of L-type Ca current, observed upon external sodium concentration being reduced, is due to a high sodium permeability of calcium channels in isolated rabbit sino-atrial node cells (Porciatti and DiFrancesco 1990) whereas in rat and guinea-pig ventricular cells, a shift in the voltage dependence of the inactivation process has been evoked (Orkand et al. 1991). According to Orkand et al. (1991), the shift of the inactivation curve is due to a specific interaction between Na ions and the Ca channel rather than to a general surface charge effect. The slight shift of the apparent reversal potential recorded in our study (see Fig. 4) suggests that an inward flow of Na ions through the L-type calcium channels does not contribute significantly to the L-component. This suggestion is consistent with the results of Campbell et al. (1988b) concerning the calcium channel selectivity in bull-frog (*Rana catesbeiana*) atrial myocytes. Concerning the shift of the steady-state inactivation of the L-component (Fig. 5*B*) recorded in our study, it is certainly too slight to explain the reduction in the amplitude of this current. In most cases, 40 μmol/l Ni and 20 μmol/l Cd completely blocked the two components of the current (Fig. 6) however in a few cells a persistent inward current was still recorded in these conditions and this current disappeared when sodium was removed. Such a result is consistent with the existence of a sodium current which is not blocked by 1 μmol/l TTX. Even if a TTX-insensitive Na current was already reported in a few frog auricular trabeculae by Tarr (1971), it seems impossible that the TTX-resistant current recorded in our conditions can explain all of the decrease of the two components of calcium current upon sodium removal since (*i*) the T-component is activated in a voltage range more negative than those of the TTX-resistant current; (*ii*) this TTX-resistant current was observed only in a few cells whereas the decrease of calcium currents was always observed upon sodium removal.

In ventricular cells of the guinea pig, it has been shown (Sato et al. 1985) that

when the Ca current was recorded in Na-free (Tris substitute) external solution, the current was decreased by 71% in the presence of a weakly acidic (pH 6.8) pipette solution whereas it was decreased by only 37% in normal Na in the presence of a more acidic (pH 5) pipette solution. This effect was attributed to a more extensive intracellular acidification caused by a depressed extrusion of proton via Na-H exchange mechanism in Na-free solution rather than to an increased sensitivity of the calcium channel to protons. In our experimental conditions the reductions in amplitude of the T- and L-components are not consistent with an intracellular acidification since such a reduction is also observed when Na is replaced by Li. Indeed, Li is the only monovalent cation used in this study that can substitute for Na in the Na-H antiporter (Lazdunski et al. 1985).

Even if none of the effects tested in this study can alone explain the decrease of calcium currents in Na-free solution we cannot exclude that the combination of some of these effects can explain it. An alternative explanation could be that Na ions play a facilitating role on calcium currents by modifying the movement of Ca ions through the channels as proposed for starfish egg cell membrane by Hagiwara et al. (1975). The hypothesis concerning the presence of an external binding site for Na ions which could modulate some parameters of the calcium channels (elementary current, probability of opening,...) is currently being investigated.

Acknowledgements. The authors gratefully appreciate Jocelyne Barbéry for doing the artwork.

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Final version accepted October 20, 1991