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The control of the contraction of myocytes from guinea-pig heart by the resting membrane potential

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1 The influence of different holding potentials (−120 to −70 mV) on the contraction of enzymatically dispersed myocytes from guinea-pig hearts was evaluated. Contractions were elicited by repetitive depolarizations to 0 mV at 0.5 Hz.

2 While ineffective at 140 and 5 mmol l^{-1} $[Na^+]_o$ and pipette Na^+, respectively, depolarization of the resting membrane with the holding potential increased myocyte shortening at reduced Na^+ gradients ($[Na^+]_o$ 70 or $[Na^+]_i$ 10–15 mmol l^{-1}). Elevated intracellular Na^+ after Na^+-pump inhibition with ouabain 1–10 μmol l^{-1} was similarly effective with regard to the inotropic response to different holding potentials.

3 At −70 mV holding potential, reduction of $[Na^+]_o$ from 140 to 70 mmol l^{-1} increased myocyte shortening and induced an inwardly directed component of the holding current which peaked at -44 ± 10 pA and declined thereafter in parallel with the inotropic effect. The relation of this inward current to $[Ca^{2+}]_i$ was confirmed by experiments at high Ca^{2+} buffer capacity where $[Na^+]_o$ reduction induced a Ni^{2+}-insensitive, outwardly directed component (36 ± 15 pA) of the holding current. The observed inward current is suggested to reflect the extrusion of $[Ca^{2+}]_i$ in exchange for $[Na^+]_o$ as a counter-regulatory mechanism which limits the increase of $[Ca^{2+}]_i$.

4 The interventions which increased the strength of the contraction also enhanced the transient tail current after repolarization, suggesting its close relation to $[Ca^{2+}]_i$. This finding confirmed the pattern found with cell shortening.

5 It is concluded that under certain conditions, voltage-dependent and Na^+-dependent Na^+–Ca^{2+} exchange during the interval between the contractions is relevant to the diastolic concentration of $[Ca^{2+}]_i$ which in turn determines the accumulation of Ca^{2+} in the sarcoplasmic reticulum and the magnitude of the subsequent contraction.

Keywords: Resting potential; sodium-calcium exchange; sodium; guinea-pig heart; myocytes

Introduction

While depolarization of the cell membrane with high K^+ concentrations leads to contracture (e.g., Niedergerke, 1956), moderate changes of K^+ concentration preserve the physiological contraction cycle. Negative inotropic effects (Reiter *et al.*, 1971), presumably related to Na^+-pump activation and alterations of the action potential, and transient increases of contraction (Kavaler *et al.*, 1972), however, indicate the diversity of K^+ actions. The reversal from a negative to a positive inotropic effect of K^+ after reduction of extracellular Na^+ in guinea-pig papillary muscle (Ebner & Siegl, 1986) and the role of K^+ in the inotropic response to intracellular Na^+ (Ebner *et al.*, 1986) could suggest a control of the contraction by the resting membrane potential via Na^+–Ca^{2+} exchange in a way which resembles the effect of high K^+ concentrations in frog atrial trabeculae on the Na^+ withdrawal contracture (Chapman & Tunstall, 1980) or the Na^+-dependence of the K^+-induced contracture (Chapman & Tunstall, 1981). The influence of high K^+ concentrations on Ca^{2+} fluxes (Busselen, 1982) or intracellular Ca^{2+} (Sheu *et al.*, 1986) is in line with the contracture experiments. However, the membrane potentials at those high, contracture-inducing K^+ concentrations which under physiological conditions only occur during the action potential and a possible role of voltage-dependent Ca^{2+} release from the sarcoplasmic reticulum (cf. Reiter, 1988) weaken the arguments in support of a common basis of the effects of different resting membrane potentials and of high K^+ concentrations. Moreover, some observations preclude a direct comparison of the effects of the resting membrane potential and of K^+. Firstly, an effect of K^+ different from its influence on the membrane potential cannot be excluded *a priori* (Cervetto *et al.*, 1989). Secondly, with respect to the resting membrane potentials of −100 to −70 mV associated with the K^+ effect on the regular contraction, reports on the electrical control of mechanical restitution seem to be at variance (Beeler & Reuter, 1970; Lipsius *et al.*, 1982). Potentials negative to −70 mV did not alter or only slightly altered the contraction after a priming event although the Na^+ withdrawal contracture of frog atrial trabeculae depended on the membrane potential (Chapman & Rodrigo, 1986). At presence, therefore, the question regarding a conceivable effect of the resting membrane potential on the regular contraction by interfering with the sarcoplasmic Ca^{2+} concentration and its precise mechanism seems to be unsettled.

Since the first description of Na^+–Ca^{2+} exchange (mammalian cardiac muscle; Reuter & Seitz, 1968; squid giant axons; Baker *et al.*, 1969; Blaustein & Hodgkin, 1969), the basic parameters of the exchange have been evaluated (for reviews see Eisner & Lederer, 1985; Sheu & Blaustein, 1986; Hilgemann, 1988; Wier, 1990), the features of which comprise: (1) the role of intra- and extracellular Na^+ and Ca^{2+} (Reuter & Seitz, 1968; Glitsch *et al.*, 1970; Miura & Kimura, 1989); (2) the stoichiometry of the exchange of 1 Ca^{2+} for n Na^+ ions (consistently $n > 2$; $n = 4$, Mullins & Brinley, 1975; $n = 3$, Reeves & Hale, 1984), and (3) the voltage-dependence (Ca^{2+} efflux from squid axon; Mullins & Brinley, 1975; $[Na^+]_i$-dependent accumulation of $[Ca^{2+}]_i$ in cardiac vesicles; Philipson & Nishimoto, 1980; Reeves & Sutko, 1980). These experimental details are condensed in equation 1 which couples the Na^+ and Ca^{2+} gradients to yield (Blaustein & Hodgkin, 1969; Mullins, 1976; Mullins, 1977) $2(V-E_{Ca}) = n(V - E_{Na})$ (1) and $[Ca^{2+}]_i = [Ca^{2+}]_o([Na^+]_i[Na^+]_o^{-1})^n \exp -[(2-n)(V\,F)(R\,T)^{-1}]$ (2); whereby V, E_{Ca}, and E_{Na} are membrane potential, and Ca^{2+} and Na^+ equilibrium potential, respectively; n is the number of Na^+ ions exchanged per

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Ca^{2+} ion; $[Ca^{2+}]_{i,o}$ and $[Na^+]_{i,o}$ are intra- and extracellular concentrations of the respective ions; F, R, T are Faraday number, gas constant, and absolute temperature, respectively.

With respect to the features of Na^+–Ca^{2+} exchange, depolarization of the cell membrane on excitation and changes of the resting membrane potential could both interfere with Na^+–Ca^{2+} exchange and alter the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$). Its accumulation in the sarcoplasmic reticulum in the interval between the beats could therefore depend on the membrane potential. Although the reports on K^+ and voltage effects appear to be conflicting, an influence of Na^+–Ca^{2+} exchange during diastole on the subsequent contraction may be overlooked when the effectiveness of a voltage change on $[Ca^{2+}]_i$ disappears with an inappropriate ratio of $[Na^+]_i$:$[Na^+]_o$; according to equation 2, depolarization of the membrane from -120 to -70 mV increases $[Ca^{2+}]_i$ from 0.6 to 3.9 nmol l^{-1} at a ratio of $[Na^+]_i$:$[Na^+]_o$ = 5:140, whereas this value rises from 16 to 105 nmol l^{-1} at 15:140; n was 3, $[Ca^{2+}]_o$ 1.2 mmol l^{-1}. Irrespective of the factors which in addition to Na^+–Ca^{2+} exchange modulate $[Ca^{2+}]_i$ and lead to actually higher concentrations than predicted by equation 2, the relation of voltage to $[Ca^{2+}]_i$, and consequently the relation of resting membrane potential to contraction, should vary with the ratio of $[Na^+]_i$:$[Na^+]_o$. Isolated myocytes were therefore studied under conditions of whole-cell voltage clamp with adequate control of the intracellular ionic milieu.

Methods

Cell preparation

Ventricular myocytes from the hearts of adult guinea-pigs (250–300 g) were isolated enzymatically. The procedure of Bendukidze *et al.* (1985) was modified as follows. The heart was retrogradely perfused at 36°C with a pressure of 6.8 kPa. The solutions were vigorously gassed with 5% CO_2 in O_2; pH was 7.0. Perfusion with 30–50 ml of a nominally Ca^{2+}-free solution at a flow rate of approximately 10 ml min^{-1} was followed by 30–40 ml of pronase solution. The ventricle was then minced with scissors, pronase solution containing 0.5 mmol l^{-1} $CaCl_2$ was added, and the tissue fragments were incubated for 5 min at 37°C. After filtration the myocytes were spun down at 37 *g*, 2–3 min, resuspended in modified Krebs-Henseleit solution and kept for use at room temperature.

On average, one third of the cells recovered were rod-shaped with clear cross-striation. Cell length was 107 ± 2 μm, diameter 20 ± 0.5 μm ($n = 101$) and estimated height 7.5 μm, yielding a cell surface of 6.24×10^3 μm^2. This value compares with 6.99×10^3 μm^2 calculated from membrane capacity (69.9 ± 3.4 pF, $n = 43$) assuming a specific capacity of 1 μF cm^{-2}. Determined with low-ohmic patch-clamp pipettes, the resting membrane potential was -93.3 ± 0.42 ($n = 99$), -74.0 ± 0.8 ($n = 42$), and -60.3 ± 1.49 ($n = 15$) mV at 2.4, 5.9, and 9.6 mmol l^{-1} K^+, respectively. In the voltage-clamp mode at 2.4 mmol l^{-1} K^+ the holding current was -658 ± 25 pA at -120 mV holding potential and 211 ± 17 pA at -70 mV in 20 cells.

Experimental set-up and procedure

The cell suspension was placed in a thermostated perfusion chamber (volume 0.5 ml) mounted on an inverse microscope (IM 35, Zeiss, Oberkochen, West Germany). After the cells had attached to the bottom, the bath was perfused at a flow rate of 2–3 ml min^{-1} with pre-warmed modified Krebs-Henseleit solution continuously gassed with 5% CO_2 in O_2. The temperature in the bath (35–36°C) was continuously monitored.

Voltage-clamp experiments were performed in the whole-cell clamp configuration (Hamil *et al.*, 1981). The pipettes were made from haematocrit glass in a two-step procedure. After fire-polished they were filled with pre-filtered solution (see below). D.c. resistance ranged from 1.5 for 4 MΩ. An Ag-AgCl electrode connected via an agar bridge (3% agar-agar in modified Krebs-Henseleit medium) to the bath solution served as a reference. The pipette was mounted on a micromanipulator (Narishige, Japan) and connected to a patch-clamp amplifier (List LM/EPC7) for control of voltage and monitoring current. After giga-seal formation cell capacity was compensated before the experiment. Series resistance was compensated until 'ringing' occurred (usually at 30–50% compensation). The signals were filtered with a 3 kHz, 3-pole Bessel filter.

Cell shortening was recorded simultaneously with a photometer attached to the microscope (MPM01K, Zeiss, Oberkochen, West Germany). A window was positioned in the light beam on the cell end or on the cell itself and the change of transmission, either an increase or decrease with cell shortening depending on the position of the window, was monitored. The contraction signal was filtered with a cut-off frequency of 100 Hz.

The voltage, current, and contraction signals were displayed on an oscilloscope and a pen recorder. Simultaneously, the signals were recorded and stored on diskette by a personal computer (IBM AT02) equipped with an AD board (Labmaster, Scientific Solutions, Solon, USA) at a sampling rate per channel of 1.33 and 1.1 kHz in the experiments with change of $[Na^+]_o$, respectively.

From a holding potential of -120 mV the cell was depolarized to 0 mV for 100 ms at a stimulus rate of 0.5 Hz. Following stabilization of the signals the holding potential was set to -70 mV and the cell was depolarized to 0 mV as before. While the voltage control with fast voltage-clamp steps may not have been ideal, this is unlikely to have influenced the principal results of the experiments. In some experiments perfusion was changed to a solution of different $[Na^+]_o$ at constant holding potential and otherwise identical experimental conditions. Exchange of solution in the dead space of the apparatus required approximately 1 min, but depending on the flow rate, $[Na^+]_o$ concentration in the vicinity of a cell could be changed within 15–30 s as monitored by the fast Na^+ inward current.

Data analysis and statistics

The active shortening was evaluated from the difference between baseline and peak shortening of the signals stored on diskette. Since contraction data could not be normalized sufficiently fast during the experiment in terms of μm shortening, the actual photometer output is given instead. It is representative for the shortening of an individual cell. Inwardly directed tail currents after repolarization from 0 to -70 or -120 mV were integrated numerically (Simpson rule) after subtraction of the holding current. Arithmetic means ± s.e.mean or the data of individual experiments are shown. Statistical significance was assessed with the two-tailed t test. Significance was assumed at $P < 0.05$.

Solutions

The concentrations are in mmol l^{-1} if not stated otherwise. pH was measured after gassing with 5% CO_2 in O_2 except the pipette solution. Modified Krebs-Henseleit solution: NaCl 115, $NaHCO_3$ 24.9, KCl 1.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.2, glucose 10, pH 7.4; at reduced $[Na^+]_o$ concentration 70 mmol l^{-1} NaCl was replaced by an equimolar amount of choline chloride; pipette solution: KCl 140, NaCl 5–15, $MgCl_2$ 2, Tris-ATP 3, HEPES 10, EGTA (if not stated otherwise) 0.02; adjusted to pH 7.2 with KOH; assuming a total of 3 μmol Ca^{2+} l^{-1} (Bendukidze *et al.*, 1985) on the basis of the log binding constants of Fabiato (1988) (H_1EGTA 9.353, H_2EGTA 8.794, Ca_1EGTA 10.593, Ca_2EGTA 5.33, Mg_1EGTA 5.21, Mg_2EGTA 3.37, Ca_1ATP 3.936, Ca_2ATP 1.735, Mg_1ATP 4.422, Mg_2ATP 2.841) non-complexed Ca^{2+}

is 57 nmol l^{-1} in the pipette solution at 35°C as calculated by solving a set of 10 non-linear equations (Newton-Raphson method) with Ca^{2+}, Mg^{2+}, and H^+ competing for EGTA and Ca^{2+} and Mg^{2+} for ATP according to

$$\left(I_{0,i} - \sum_{j=1}^{j=2}\sum_{k=1}^{k=n} I_{i,j}L_k\right) \times \left(L_{0,k} - \sum_{j=1}^{j=2}\sum_{i=1}^{i=m} I_{i,j}L_k\right) - K_{i,k,1}I_{i,1}L_k = 0$$

and

$$\left(I_{0,i} - \sum_{j=1}^{j=2}\sum_{k=1}^{k=n} I_{i,j}L_k\right)I_{i,1}L_k - K_{i,k,2}I_{i,2}L_k = 0;$$

where I_i, L_k, and $K_{i,k,1,2}$ are ion i ligand k, and binding constant K, respectively, and the subscript $_0$ denotes the total ion or ligand concentration; nominally Ca^{2+}-free solution: NaCl 100, $NaHCO_3$ 10, KCl 10, KH_2PO_4 1.2, $MgSO_4$ 5, glucose 20, taurine 50, pH 7.0; pronase solution: NaCl 80, $NaHCO_3$ 10, KCl 30, KH_2PO_4 1.2, $MgSO_4$ 5, glucose 20, taurine 50, $CaCl_2$ 0.04, pronase E 100 mg l^{-1}, essentially fat free bovine serum albumin 1 g l^{-1}, pH 7.0.

Materials

Pronase E from *Streptomyces griseus* 7.4 DMC-u mg^{-1}, taurine, Dowex G50WX8, and ouabain were obtained from Serva, Heidelberg, West Germany. Essentially fat free bovine serum albumin, EGTA (ethylene glycol *bis*-(β-aminoethylether)N,N,N',N'-tetraacetic acid), and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) were obtained from Sigma (München, West Germany). Tris-ATP was prepared from the disodium salt after passage over Dowex AG 50WX8 and adjustment with Tris(trishydroxymethyl-aminomethane) to pH 7.5. ATP, Tris and all other chemicals were purchased from Merck (Darmstadt).

Results

The effect of the holding potential (V_{hold}) on contraction depends on extracellular Na^+ ($[Na^+]_o$)

While the change of the holding potential from −120 to −70 mV did not alter the contraction elicited by repetitive depolarizations to 0 mV at 140 mmol l^{-1} $[Na^+]_o$ and 5 mmol l^{-1} pipette Na^+, depolarization with the same settings of the holding potential nearly doubled myocyte shortening when $[Na^+]_o$ was reduced to 70 mmol l^{-1} (Figure 1). The effect of depolarization to −70 mV was reversible on hyperpolarization to −120 mV, although some run down of contraction was usually found. When the holding potential was gradually changed to −120, −100 and −70 mV the shortening of myocytes followed in a stepwise manner. Relative to the value at −70 mV, V_{hold} the normalised shortening was 0.7 ± 0.05 ($n = 5$) at −100 mV and 0.58 ± 0.03 ($n = 13$) at −120 mV, 15 s after alteration of the holding potential.

The recordings of a single experiment illustrate the effect of depolarization of the membrane to less negative values of the holding potential (Figure 2). Figure 2a demonstrates the shortening of the cell subsequent to the voltage protocol in Figure 2c. The current traces in Figure 2b indicate the reversal of the holding current from the inward to the outward direction and some increase of the tail current after repolarization when the holding potential was set from −120 to −70 mV. When the membrane was clamped to 0 mV the flow of currents seemed to be hardly affected by the preceding V_{hold} although at higher resolution the inactivation phase of the initial inward current appeared to be accelerated and, in some cells, the subsequent current was slightly shifted to the inward direction.

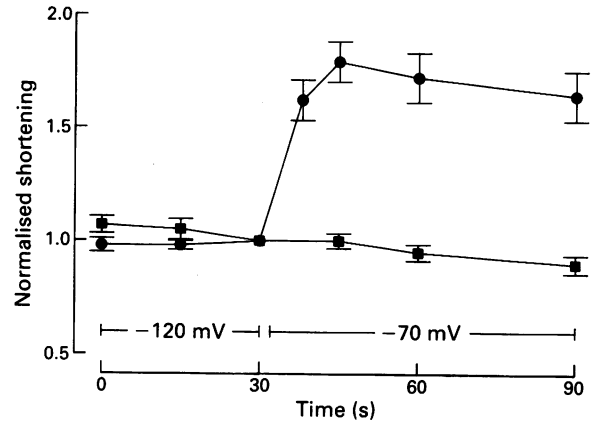

Figure 1 The effect of the holding potential on myocyte shortening depends on extracellular Na^+ ($[Na^+]_o$). The time courses of normalised shortening of 11 and 13 cells at 140 (■) and 70 (●) mmol l^{-1} $[Na^+]_o$ are shown when the holding potential is altered from −120 to −70 mV. The contractions were elicited by depolarization to 0 mV at a stimulus rate of 0.5 Hz. Na^+ concentration in the pipette was 5 mmol l^{-1}. Abscissa scale: time in s; ordinate scale normalised shortening, i.e., peak shortening relative to its value before V_{hold} was changed. Arithmetic means are presented with s.e.mean shown by vertical bars.

The influence of $[Na^+]_o$ on the holding current

The interference from several voltage-dependent processes precludes the isolation of a Na^+–Ca^{2+} exchange current in the preceding experiments with different holding potentials. The relevance of the exchange to the holding current was therefore tested with change of $[Na^+]_o$ at −70 mV holding potential in regularly depolarized cells. The individual recordings of clamp protocol, current, and myocyte shortening of

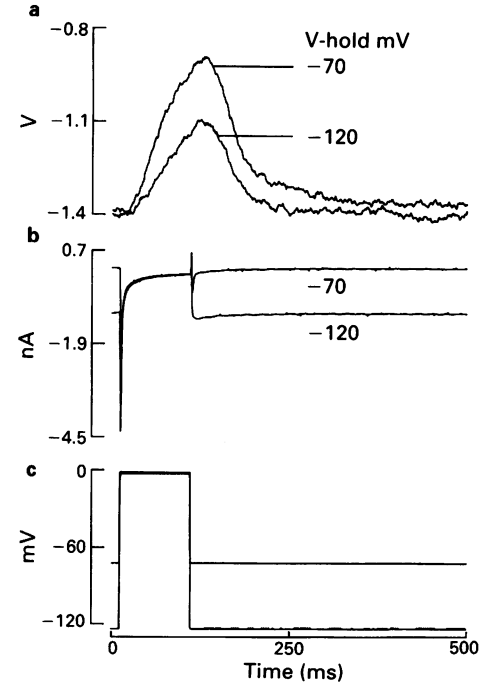

Figure 2 The influence of the holding potential on myocyte shortening and membrane currents at reduced $[Na^+]_o$. At 70 mmol l^{-1} $[Na^+]_o$, 5 mmol l^{-1} pipette Na^+, the effect of depolarization from a holding potential of −120 to −70 mV on the shortening (a) and membrane currents (b) is illustrated with data from a single myocyte. The digitized recordings were taken immediately before and 16 s after change of the holding potential. The myocyte was regularly stimulated at 0.5 Hz by depolarization to 0 mV; see (c) for clamp protocol. Ordinates: (a) photometer output in V, (b) membrane currents in nA; (c) clamp voltage in mV; common abscissae: time in ms.

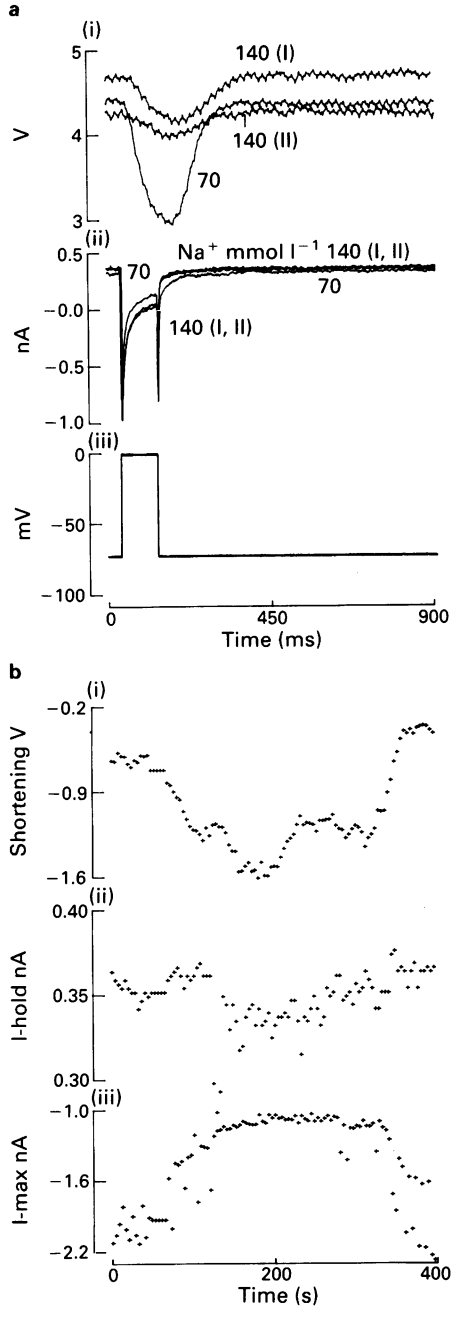

Figure 3 The influence of $[Na^+]_o$ reduction on the membrane currents and myocyte shortening at low intracellular Ca^{2+} buffer capacity: (a) individual recordings; (b) the time courses of some current parameters and of cell shortening. At low intracellular Ca^{2+} buffer capacity (20 μmol l^{-1} EGTA) superfusion was changed from 140 to 70 and back to 140 mmol l^{-1} $[Na^+]_o$; $Na^+_{pipette}$ was 5 mmol l^{-1}. Stimulation frequency was 0.5 Hz, temperature 35°C, V_{hold} −70 mV. (a) The digitized recordings of clamp potential (mV, (iii)), membrane current (nA, (ii)), and cell shortening (photometer output in V, (i) were taken before (140I), during (70), and after (140II) $[Na^+]_o$ reduction; for better resolution the maximal inward current was cut off; common abscissa scale: time in ms. (b) From recordings corresponding to (a) and of the same cell the maximal inward current to monitor $[Na^+]_o$ (averaged data from peak ± 1.8 ms, in nA, (iii)), the holding current (averaged data from 660 to 715 ms after repolarization, in nA, (ii)), and the cell shortening (peak-background value of photometer output, in V, (i) were evaluated and their time courses were followed during the change of $[Na^+]_o$; common abscissa scale: time in s. $[Na^+]_o$ was altered at $t = 0$.

Figure 3a before, during, and after superfusion with reduced $[Na^+]_o$ illustrate this effect at low $[Ca^{2+}]_i$ buffer capacity. The development of the positive inotropic effect of $[Na^+]_o$ reduction was paralleled by an outward shift of the current during depolarization while the holding current was slightly but reversibly shifted to the inward direction. For closer inspection the time courses of the maximal shortening and of the holding current were evaluated (Figure 3b). To monitor the actual Na^+ concentration in the vicinity of the cell we chose the maximal inward current flowing on depolarization as an indirect measure of $[Na^+]_o$, although the sampling rate of 1.1 kHz and the incomplete control of fast voltage changes in large cells preclude its precise evaluation. Nevertheless the change of $[Na^+]_o$ became clearly visible in its time course as exemplified in Figure 3b (lower panel). With the reduction of $[Na^+]_o$ the shortening of the myocyte increased and, beyond a maximum, approached a somewhat lower level of contractility (Figure 3b, upper panel). When the onset of Na^+ reduction became detectable in the shortening, the holding current (middle panel) was at first slightly and barely visibly shifted to the outward direction before an inward current of approximately 20 pA in this cell superimposed with the further development of the inotropic effect. This inward current faded to a variable extent in the different cells in parallel with shortening. After $[Na^+]_o$ had been reset to 140 mmol l^{-1}, the holding current returned to its previous value. In a total of 5 cells $[Na^+]_o$ reduction increased the shortening by 1.94 ± 0.33 and induced an inwardly directed component of the holding current of −44 ± 10 pA.

The relation between the inotropic effect of $[Na^+]_o$ reduction and an inwardly directed component of the holding current suggests a role of $[Ca^{2+}]_i$ in it. This idea was confirmed by clamping $[Ca^{2+}]_i$ with high buffer capacity of the pipette solution (EGTA 10 mmol l^{-1}). When under these conditions $[Na^+]_o$ was reduced from 140 to 70 mmol l^{-1} (Figure 4a) the membrane currents during depolarization to 0 mV were altered in a similar manner to that occurring in low Ca^{2+} buffer capacity, although the outward shift was particularly prominent in this cell. By contrast, the holding current was now differently affected. Reduction of $[Na^+]_o$ as monitored by the maximal inward current induced an outwardly directed component of the holding current which clearly dissociated from the overall outward drift (Figure 4b). The outward current related to $[Na^+]_o$ reduction was 36 ± 15 pA in 4 cells. To test its susceptibility to Ni^{2+}, the experiment with high Ca^{2+} buffer capacity was repeated while the cells were constantly superfused with 1 mmol l^{-1} Ni^{2+}, a concentration which substantially reduces the Na^+–Ca^{2+} current (Kimura *et al.*, 1987). Under otherwise identical conditions, $[Na^+]_o$ reduction induced an outward current of similar magnitude (58 ± 13 pA, $n = 3$). After prolonged superfusion with Ni^{2+}, however, this $[Na^+]_o$ effect increased progressively in repeated tests.

The effect of the holding potential (V_{hold}) on contraction depends on intracellular Na^+ ($[Na^+]_i$)

The role of $[Na^+]_i$ in the effect of different holding potentials was tested at 140 mmol l^{-1} $[Na^+]_o$ by raising Na^+ concentrations in the pipette or alternatively by inhibition of the Na^+ pump with ouabain. When, in the absence of ouabain, pipette Na^+ was 15 mmol l^{-1} a few minutes after the giga seal was formed, cell shortening increased reversibly with depolarization from V_{hold} = −120 to −70 mV (Figure 5). Qualitatively at least, the changes of contractile and current parameters correspond to the situation at reduced $[Na^+]_o$. The result shown in Figure 5 was confirmed in two other cells. In further experiments with 6 cells, pipette Na^+ was 10 mmol l^{-1}. Change of the holding potential from −120 to −70 mV increased the shortening relative to its value at −120 mV by 1.66 ± 0.25.

At low access resistance the pipette solution could entirely determine the intracellular milieu. The development of inotropic effects in myocytes in the whole-cell-clamp configuration after the addition of a cardioactive steroid to the superfusion medium (e.g., Berlin *et al.*, 1989), however, suggests that intracellular Na^+ is incompletely controlled in large cells. Super-

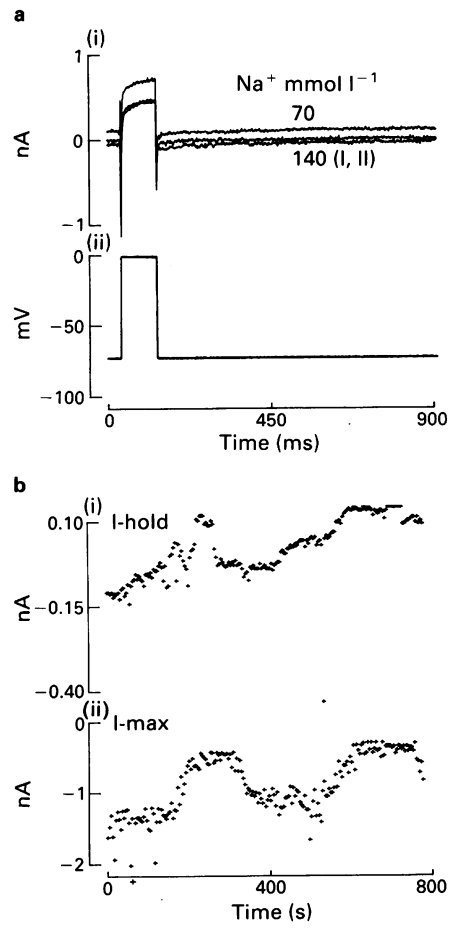

Figure 4 The influence of $[Na^+]_o$ reduction on membrane currents at high intracellular Ca^{2+} buffer capacity: (a) individual recordings; (b) the time courses of some parameters. At high intracellular Ca^{2+} buffer capacity (10 mmol l^{-1} EGTA) superfusion was changed twice from 140 to 70 and back to 140 mmol l^{-1} $[Na^+]_o$; $Na^+_{pipette}$ was 5 mmol l^{-1}. Stimulation frequency was 0.5 Hz, temperature 35°C, V_{hold} −70 mV. (a) The digitized recordings of clamp protocol (mV, (ii)) and membrane current (nA, (i)) were taken before (140I), during (70), and after (140II) the first $[Na^+]_o$ reduction; for better resolution the maximal inward current was cut off; common abscissa scale: time in ms. (b) From recordings corresponding to (a) and of the same cell the maximal inward current (in nA, (ii)) as an indirect measure of $[Na^+]_o$ concentration in the vicinity of the cell and averaged values of the holding current (from 660 to 715 ms after repolarization in nA, (i)) were evaluated and their time courses were followed during the change of $[Na^+]_o$; common abscissa scale: time in s. $[Na^+]_o$ was altered at t = 110 s.

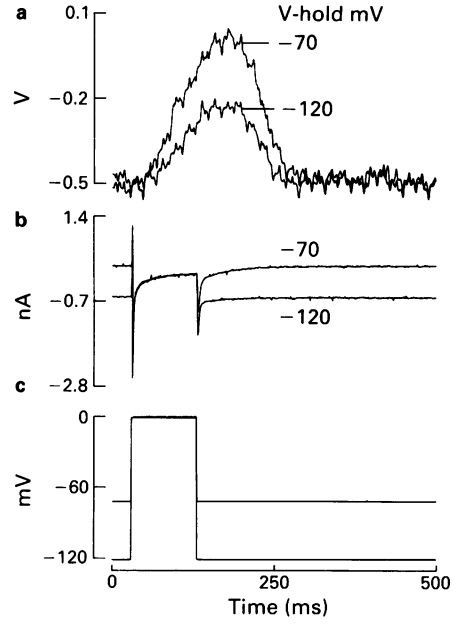

Figure 5 The influence of the holding potential on myocyte shortening and membrane currents at elevated intracellular Na^+. At 140 mmol l^{-1} $[Na^+]_o$, 15 mmol l^{-1} pipette Na^+, the effect of a change of the holding potential from −120 to −70 mV on the shortening (a) and membrane currents (b) of a single myocyte is shown. The digitized recordings were taken immediately before and 16 s after change of the holding potential. The myocyte was regularly stimulated by depolarization to 0 mV; see (c) for clamp protocol. Ordinates: (a) photometer output in V, (b) membrane currents in nA, (c) clamp voltage in mV; common abscissa scale: time in ms.

fusion of myocytes with ouabain therefore could also increase $[Na^+]_i$ in view of the effect of Na^+-pump inhibition in multicellular preparations and raise the inotropic effectiveness of different holding potentials. In resting guinea-pig papillary muscle under comparable conditions (K^+ 2.4, Ca^{2+} 1.2 mmol l^{-1}) and under the influence of 0.3 μmol l^{-1} ouabain $[Na^+]_i$ rose from 8 to 14, and to 20 mmol l^{-1} at 3.2 mmol l^{-1} Ca^{2+}, respectively (Ebner *et al.*, 1986). Corresponding to the situation in multicellular preparations in the isolated myocytes, too, a considerable inotropic effect developed at −120 mV V_{hold} (Figure 6a). Since in this experiment the window was positioned on the cell, light transmission reduced with contraction. In accordance with the development of the inotropic effect the currents both during excitation and diastole changed; alterations of $[Ca^{2+}]_i$-dependent K^+ conductivity (Siegelbaum & Tsien, 1980) and of the current generated by the Na^+ pump (Daut & Rüdel, 1981) could be relevant to this observation. After the maximum inotropic effect of ouabain had developed, contracture ensued with reduced active shortening; compare baseline and active shortening in upper panels of Figure 6a and b. When under these conditions the holding potential was changed from −120 to −70 mV, this resulted in 4 cells (as exemplified in Figure 6b), showing enhanced contracture (see the shift of the baseline in the upper panel), reduced active shortening, after-contractions associated with transient inward currents, shift of the current to the outward direction when the cell was clamped to 0 mV, and in large tail currents on repolarization. These effects were at least partially reversible on hyperpolarization (Figure 6b) with the exception of the current at 0 mV clamp potential. Before the signs of Ca^{2+} overload appeared and as long as contraction was regular, fractional shortening increased on depolarization by factors of 2.1 and 2 at 10 μmol l^{-1} ouabain in 2 cells and by 1.5 and 1.44 at 1 μmol l^{-1} ouabain in 2 additional cells (see Figure 6c).

The tail current on repolarization in relation to contraction

The inwardly directed tail current on repolarization has been shown to reflect the intracellular Ca^{2+} load (Fedida *et al.*, 1987; Shimoni & Giles, 1987; Mitchell *et al.*, 1987) subsequent to an activation of Na^+–Ca^{2+} exchange and possibly of non-selective cation channels (Colquhoun *et al.*, 1981). Accordingly, different magnitudes of the tail current and of its integral as a measure of total charge flow could be expected with the interventions of the preceding experiments. Increased pipette Na^+ and reduced $[Na^+]_o$ actually influenced the integral of charge similarly (Figure 7). Depolarization of the resting membrane to −70 mV augmented this effect. Ouabain also potentiated the flow of charge and, like the situation with different pipette Na^+, V_{hold} was a relevant factor with higher effectiveness under conditions of a reduced Na^+ gradient.

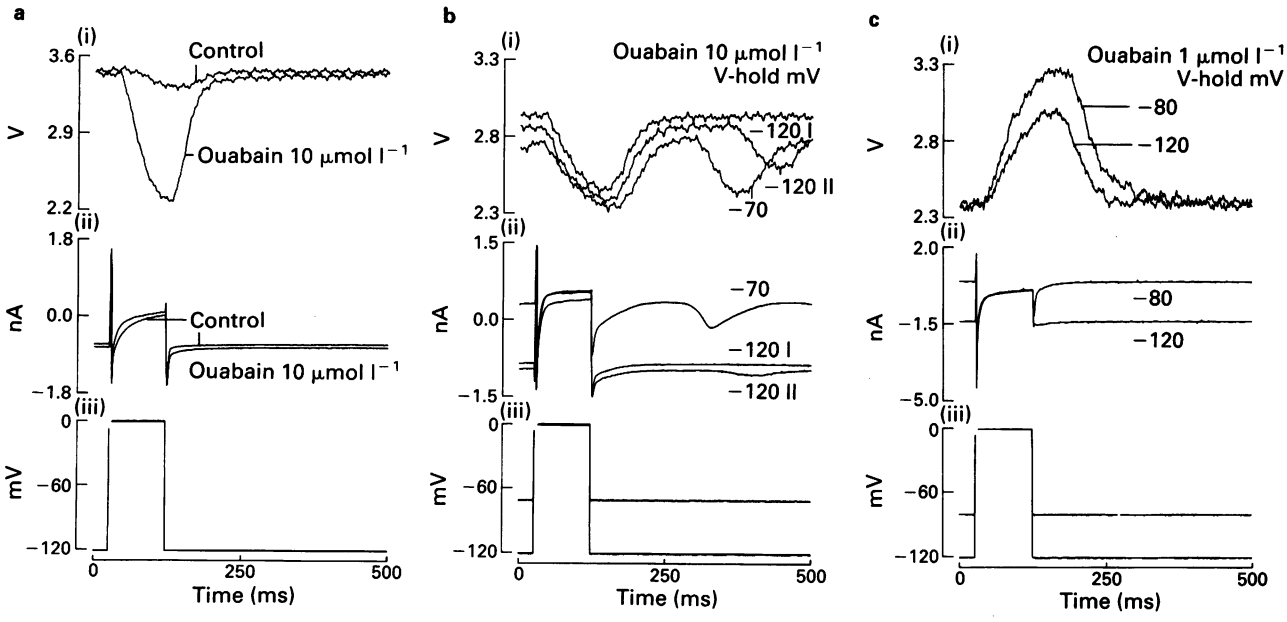

Figure 6 The influence of the holding potential on myocyte shortening and membrane currents in the presence of ouabain. The ouabain effect at constant holding potential (a); the effect of different holding potentials at 10 (b) and 1 (c) μmol l^{-1} ouabain. The shortening (upper panel) and membrane currents (middle panel) of a single myocyte subsequent to the voltage protocol of the lower panel are demonstrated with digitized data. In (a) at constant holding potential the signals were recorded before and 3 min after superfusion had been switched to 10 μmol l^{-1} ouabain; 3.5 min later the holding potential was changed from −120 to −70 and back to −120 mV after additional 1.5 min. The data of (b) were sampled immediately before (−120 I) and 80 s (−70) after the holding potential was altered while the traces (−120 II) were recorded 90 s after changing back to −120 mV. (a and b) are derived from the same cell. In a different myocyte (c) 11 min after superfusion with 1 μmol l^{-1} ouabain the holding potential was altered from −120 to −80 and back to −120 mV 1 min later. Immediately before and 80 s after resetting the holding potential from −80 to −120 mV the traces of (c) were recorded. The cells were regularly stimulated by depolarization to 0 mV at 0.5 Hz. $[Na^+]_o$ was 140, pipette Na^+ 5 mmol l^{-1}. Ordinates: (i) photometer output in V, (ii) membrane currents in nA, (iii) clamp voltage in mV; common abscissae: time in ms.

Although the tendency of an increased effectiveness of depolarization could be observed under all conditions of a reduced Na^+ gradient, this was statistically significant only at 70 mmol l^{-1} $[Na^+]_o$ ($P < 0.05$) and in the presence of ouabain ($P < 0.01$).

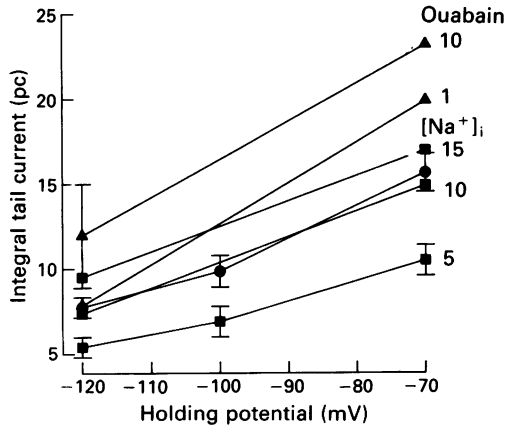

Figure 7 The charge carried with the tail current after repolarization. Influence of holding potential, intra- and extracellular Na^+, and ouabain. The tail current after repolarization from 0 to the respective V_{hold} of −120, −100, or −70 mV as indicated on the abscissa scale was integrated numerically to obtain the exchange of charge in pC (ordinate scale). At 140 mmol l^{-1} $[Na^+]_o$ (■) the number of cells was 18 (at −100 mV, $n = 7$), 6, and 3 at 5, 10, and 15 mmol l^{-1} pipette Na^+. At reduced $[Na^+]_o$ (70 mmol l^{-1}, pipette Na^+ 5 mmol l^{-1}; ●) the number of cells was 7 ($n = 5$ at −100 mV). In the experiments with ouabain (▲) two groups with 4 cells each were evaluated after the effect had stabilized. $[Na^+]_o$ was 140; pipette Na^+ 5 and 10 mmol l^{-1} in the experiments with 1 and 10 μmol l^{-1} ouabain, respectively. In the graph the Na^+ concentrations in the pipette ($[Na^+]_i$) are given in mmol l^{-1}, ouabain concentrations are in μmol l^{-1}. Arithmetic means and some s.e.mean (vertical bars) are shown).

Discussion

Depolarization of the resting membrane in the diastolic interval between the stimuli has been shown to interfere with the strength of the regular contraction in this paper, i.e., the resting membrane potential is a factor in the regulation of contraction. This effect of membrane potentials negative to −70 mV on contraction differs from the potential dependence of mechanical restitution (Beeler & Reuter, 1970; Lipsius *et al.*, 1982) where the relation of tension to membrane potential was S-shaped with saturation at potentials negative to −70 mV, while tension declined at more positive potentials. The relevance of the Na^+ gradient to our experiments, however, suggests that it plays a special role in this discrepancy. Moreover, decrease in a^i_{Na} (13.0 and 8.5 mmol l^{-1} at −110 and −80 mV, respectively, in sheep Purkinje fibres; January & Fozzard, 1984) with depolarization could contribute to the difference between the effects of the resting membrane potential in single cells and in multicellular preparations where $[Na^+]_i$ is not controlled. Decrease of $[Na^+]_i$ would evidently compensate for a positive inotropic effect of different origin.

Our results with regard to the contraction and the tail current as indirect measures of Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_i$) indicate that depolarization increases the availability of Ca^{2+} in the cell. The role of the holding potential in contraction is supported by the increase of $[Ca^{2+}]_i$ found when, after a conditioning pulse to 60 mV, the holding potential was set to less negative values ($Na^+_{pipette}$ 15 mmol l^{-1}; Beuckelmann & Wier, 1989). Our experiments agree with the features of equation 2 which defines the effectiveness of a potential change on $[Ca^{2+}]_i$ relative to the Na^+ gradient. Therefore, and after considering the conceivable contribution of Na^+–Ca^{2+} exchange to the holding current (see below), Na^+–Ca^{2+} exchange may be inferred as the basis of the potential effect on contraction. Secondary to the effect of the holding potential on cytosolic Ca^{2+}, the accumulation of

Ca^{2+} in the sarcoplasmic reticulum must have been altered. The comparably slow process of mechanical restitution which indicates the filling of the sarcoplasmic reticulum with Ca^{2+} during diastole in periods of time between 0.5 and 1 s is consistent with the influence of the resting membrane potential in this process (Hoffman *et al.*, 1956; Edman & Johannson, 1976; Lipsius *et al.*, 1982). Relative to the role of Na^+–Ca^{2+} exchange, the activation of ion channels by depolarization from −120 to −70 mV appears to be irrelevant to the present findings since potentials step negative to −60 mV do not activate the Ca^{2+} channels of guinea-pig myocardial cells (Josephson *et al.*, 1984). Moreover, the driving force of passive Na^+ and Ca^{2+} influx would even be reduced with depolarization. The difference between the effects at −120 as compared with −100 mV V_{hold} on contraction (see Results) likewise indicates that ion channels did not measurably interfere with the effect of depolarization on contraction.

The relation to the concentration of $[Ca^{2+}]_i$ and its reversal potential determine the role of Na^+–Ca^{2+} exchange in cellular Ca^{2+} homeostasis. The low K_M value of 0.6 μmol l^{-1} $[Ca^{2+}]_i$ for $[Na^+]_o$–$[Ca^{2+}]_i$ exchange in guinea-pig ventricular cells as compared with 20.7 mmol l^{-1} $[Na^+]_i$ and 0.14 mmol l^{-1} $[Ca^{2+}]_o$ in the absence of external $[Na^+]_o$ for $[Na^+]_i$–$[Ca^{2+}]_o$ exchange (Miura & Kimura, 1989) and its high capacity as opposed to Ca^{2+}-ATPase (for review see Carafoli, 1988) support the relevance of the exchanger to Ca^{2+} extrusion at the $[Ca^+]_i$ concentration which prevails during diastole (in nmol l^{-1}): 272 (sheep ventricular muscle, Ca^{2+}-sensitive microelectrodes; Sheu & Fozzard, 1982) 100 and 137–177 (guinea-pig and rat myocytes, Quin-2; Powell *et al.*, 1984; Sheu *et al.*, 1986). In stimulated preparations diastolic $[Ca^{2+}]_i$ was 290 nmol l^{-1} in canine cardiac Purkinje fibres (aequorin; Wier & Hess, 1984) or 75 nmol l^{-1} in rat myocytes (Fura-2; Berlin *et al.*, 1989). In addition to the K_M value, the reversal potential of Na^+–Ca^{2+} exchange is essential to an assessment of its mode of action under special experimental conditions. The consistency of theoretical with observed reversal potentials of Ni^{2+}-sensitive currents in guinea-pig ventricular cells (Ehara *et al.*, 1989) validates the data of Figure 8 which were calculated from equation 1 assuming an exchange of 3 Na^+ for 1 Ca^{2+}. Its features are: increase of $[Na^+]_i$, or equivalent reduction of $[Na^+]_o$, and a shift of the reversal potential to negative potentials while $[Ca^{2+}]_i$ has the opposite effect; since low concentrations of $[Ca^{2+}]_i$ are especially effective, Na^+–Ca^+ exchange seems to be particularly sensitive to any change of its parameters during rest. Accordingly, at fixed membrane potential (see horizontal lines at −120 and −70 mV in Figure 8) and at 0.1–0.3 μmol l^{-1} $[Ca^{2+}]_i$ the driving force of Ca^{2+} extrusion ($V_m - E_r$) declines with increase of $[Na^+]_i$ and reduction of $[Na^+]_o$, respectively, or, dependent on membrane potential and $[Ca^{2+}]_i$, the mode of the exchanger even reverses. Depolarization from −120 to −70 mV reduces Ca^{2+} extrusion depending on the Na^+ gradient or leads to transient influx of Ca^{2+} at high $[Na^+]_i$ (low $[Na^+]_o$). Both mechanisms will increase $[Ca^{2+}]_i$ and shift the reversal potential to less negative values while establishing the new equilibrium. Since the driving force of the Ca^{2+} leak current also diminishes on depolarization, the potential effect on $[Ca^{2+}]_i$ via Na^+–Ca^{2+} exchange would be partially compensated for.

To consider the effects of $[Na^+]_o$ reduction with respect to Na^+–Ca^{2+} exchange it may be inferred that, as long as $[Ca^{2+}]_i$ is unaltered, the reduced driving force and competition by Na^+ (Reeves & Sutko, 1983) induce an outward current. Since the outward current of Figure 4 was insensitive to Ni^{2+}, participation of the exchanger is unlikely and its origin remains obscure. When on the other hand $[Ca^{2+}]_i$ increases and shifts the reversal potential towards positive potentials, a transiently reduced driving force subsequent to the reduction of $[Na^+]_o$ would be compensated for. Its magnitude could even increase in spite of the low Na^+ gradient depending on the extent of the increase of $[Ca^{2+}]_i$ (see Figure 8). A slowly developing inward current, related to the inotropic effect and susceptible to EGTA (Figures 3 and 4), which is consistent with this interpretation, was actually found. This inward current may therefore represent a counter-regulatory mechanism which limits the increase of $[Ca^{2+}]_i$. At first glance our finding appears to be incompatible with the transient inward current related to mechanical relaxation and induced by the rapid increase of $[Na^+]_o$ from 0 to 145 mmol l^{-1} in the presence of 10 mmol l^{-1} caffeine (−40 mV holding potential; Bridge *et al.*, 1990). However, different initial $[Ca^{2+}]_i$ concentrations resolve this apparent contradiction. The finding of Bridge *et al.* (1990) reflects the acute activation of Ca^{2+} extrusion by $[Na^+]_o$ from a level of high $[Ca^{2+}]_i$ where the reversal potential is less sensitive to any change of $[Ca^{2+}]_i$. In consequence, a higher driving force for Ca^{2+} extrusion is also less rapidly balanced by the $[Ca^{2+}]_i$ reduction. By contrast, diastolic $[Ca^{2+}]_i$ is low in our experiment. In view of the high sensitivity of the reversal potential to low $[Ca^{2+}]_i$ the activation of an outward current could be obscured and over-compensated by the inward current that is induced subsequent to an even small rise in diastolic $[Ca^{2+}]_i$. Our observation therefore supports the contribution of Na^+–Ca^{2+} exchange to the holding current whereas in view of a threshold concentration of 0.3 μmol l^{-1} $[Ca^{2+}]_i$ required for their activation (Ehara *et al.*, 1988) $[Ca^{2+}]_i$-activated non-selective cation channels do not seem to be involved.

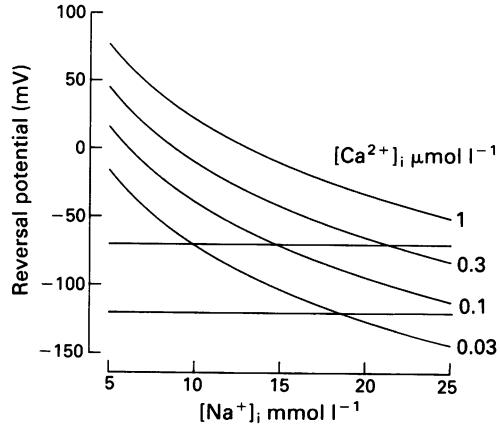

Figure 8 The reversal potential of Na^+–Ca^{2+} exchange in dependence on Na^+ and Ca^{2+} gradients. From equation 1 reversal potentials were calculated under variation of $[Na^+]_i$ and $[Ca^{2+}]_i$. Extracellular concentration of Ca^{2+} and Na^+ was 1.2 and 140 mmol l^{-1}, respectively; temperature was 35°C. The horizontal lines indicate the position of holding potentials (−70 and −120 mV). Changes of the same relative magnitude of intra- and extracellular Na^+ concentration are equivalent; e.g., increase of $[Na^+]_i$ from 5 to 10 mmol l^{-1} has the same effect as reduction of $[Na^+]_o$ from 140 to 70 mmol l^{-1}. Abscissa scale: intracellular Na^+ concentration in mmol l^{-1}, ordinate scale: reversal potential in mV.

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