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REGULATION OF UNLOADED CELL SHORTENING BY SARCOLEMMAL SODIUM–CALCIUM EXCHANGE IN ISOLATED RAT VENTRICULAR MYOCYTES

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SUMMARY

1. Regulation of unloaded cell shortening and relaxation by sarcolemmal Na^+–Ca^{2+} exchange was investigated in rat ventricular myocytes. Contraction of single cells at 22 ± 1 °C was measured simultaneously with membrane current and voltage using the whole-cell voltage clamp technique in combination with a video edge-detection device.

2. The extent of mechanical activation (cell shortening amplitude) was strongly dependent on diastolic membrane potential over the voltage range −140 to −50 mV. This voltage sensitivity of contraction was abolished completely when a recently described inhibitory peptide of the cardiac Na^+–Ca^{2+} exchanger (XIP, 2×10^{-5} M) was present in the recording pipette, demonstrating that in rat ventricular cells Na^+–Ca^{2+} exchange is modulated by diastolic membrane potential.

3. Possible influences of Na^+–Ca^{2+} exchange on contraction were studied from a holding potential of −80 mV. Depolarizations (−50 to + 60 mV) resulted in a bell-shaped shortening–voltage (*S–V*) relationship. These contractions were suppressed completely by either Cd^{2+} (10^{-4} M) or verapamil (10^{-5} M), but remained unchanged during superfusion with tetrodotoxin (TTX, $1{\cdot}5 \times 10^{-5}$ M), when $[Na^+]_o$ was reduced from 140 to 10 mM by substitution with either Li^+ or Cs^+ ions or when pipette Na^+ was varied between 8 and 13 mM. XIP (2×10^{-5} M) increased the magnitude and duration of twitch contractions, but had no effect on the shape of the *S–V* relationship. Thus, the Ca^{2+} current but not the Na^+ current or Ca^{2+} influx due to reversed Na^+–Ca^{2+} exchange can release Ca^{2+} from the sarcoplasmic reticulum (SR) under these experimental conditions.

4. The effect of the rate of repolarization on cell shortening was studied under voltage clamp by applying ramp waveforms immediately following the depolarizations which activated contraction. Although slowing of the rate of repolarization had no effect on the first contraction following a train of conditioning depolarizations, a positive inotropic effect developed thereafter.

5. Caffeine (10 mM) was applied to determine whether Na^+–Ca^{2+} exchange and/or Ca^{2+} sequestration/buffering by the sarcoplasmic reticulum were primarily

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responsible for these inotropic effects. In the presence of caffeine the positive inotropic effect developed fully during the first test depolarization. Changes in the rate of repolarization had much less effect on shortening in cells dialysed intracellularly with XIP (2×10^{-5} M). In combination, these results suggest that the changes in the inotropic effects resulting from changes in rate of repolarization may be due to altered loading and release of Ca^{2+} from the SR.

6. We conclude that the dependence of Na^+–Ca^{2+} exchange activity on the diastolic membrane potential and the waveform (e.g. rate) of repolarization are very important parameters in modulating contractility in rat ventricle; and that release of Ca^{2+} from intracellular stores depends primarily on Ca^{2+} influx through sarcolemmal Ca^{2+} channels.

INTRODUCTION

Many of the important steps in excitation–contraction coupling on mammalian ventricle are now understood in some detail (Fabiato, 1983, 1985; Beuckelmann & Wier, 1988; cf. Wier, 1990; Gibbons & Zygmunt, 1992). It is known that Ca^{2+} influx through L-type Ca^{2+} channels initiates contraction by triggering Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR). This rise in intracellular Ca^{2+} allows the myofilaments to interact and force is developed. Relaxation results from sequestration of myoplasmic Ca^{2+} into the SR via an ATP-dependent Ca^{2+} pump, as well as extrusion of Ca^{2+} across the sarcolemma by Na^+–Ca^{2+} exchange (cf. Wier, 1990).

Although some of the evidence for this sequence of events has been obtained from rat ventricular trabeculae or from single myocytes from rat ventricle, even in this tissue a number of important phenomena remain incompletely understood. For example, there is doubt concerning whether L-type Ca^{2+} channels provide the sole source of Ca^{2+} influx or whether Na^+–Ca^{2+} exchange operating in the reverse mode (thereby mediating Ca^{2+} influx) is also involved (Eisner & Lederer, 1985; Terrar & White, 1989; Sham, Cleeman & Morad, 1992) in triggering Ca^{2+} release from the SR. Both indirect indications of Na^+–Ca^{2+} exchange activity (Schouten & ter Keurs, 1985; 1991) and direct measurements of the relevant transmembrane current and changes in intracellular Ca^{2+} (Cannell, Berlin & Lederer, 1987; Bers, Lederer & Berlin, 1990; Cleeman & Morad, 1991) have shown that in rat ventricle the Na^+–Ca^{2+} exchanger extrudes Ca^{2+} during diastole. However, it has also been proposed that at diastolic potentials the Na^+–Ca^{2+} exchanger may be operating in reverse mode in rat heart so that it produces a Ca^{2+} influx (Kort & Lakatta, 1988; Shattock & Bers, 1989). In addition, Leblanc & Hume (1990) have reported that the influx of Na^+ through TTX-sensitive channels may reduce the electrochemical gradient for Na^+, reverse Na^+–Ca^{2+} exchange and cause Ca^{2+} influx. This suggestion and the validity of these experimental findings have been questioned (Sham *et al.* 1992; cf. Callewaert, 1992; Carmeliet, 1992).

One of the limitations of previous experiments which have attempted to determine the physiological role of the Na^+–Ca^{2+} exchanger, (either as a source of Ca^{2+} influx for excitation contraction coupling or as an extrusion mechanism for Ca^{2+}), is the lack of a selective blocker for this process (Kaczorowski, Slaughter,

King & Garcia, 1989). Non-selective agents, e.g. La^{3+}, Ni^{2+}, Co^{2+}, or amiloride, have often been used under circumstances where rapid changes in the electrochemical gradient for Na^+ cannot be made. Recently, however, Philipson and his colleagues (Nicholl, Longoni & Philipson, 1990) have isolated and cloned the integral membrane protein which is responsible for Na^+–Ca^{2+} exchange, and have utilized this information to synthesize a short peptide (20 amino acid) inhibitor, denoted XIP, for exchange inhibitor peptide (Li *et al.* 1991). XIP potently (K_D approximately 10^{-6} M) and quite selectively inhibits the Na^+–Ca^{2+} exchanger both in sarcolemmal vesicle preparations and when it is introduced into the cytosol of intact myocytes (Chin, Spitzer, Philipson & Bridge, 1993). In the experiments reported here we have used isolated rat ventricular myocytes together with whole-cell voltage clamp and cell length measurements in an attempt to define the functional role of the Na^+–Ca^{2+} exchanger at potentials near the normal resting or diastolic potential, and to learn more about the relative roles of the Ca^{2+} current and Na^+–Ca^{2+} exchange as triggers for excitation–contraction coupling at potentials corresponding to the plateau of the action potential. Our results provide direct evidence for the functional importance of Na^+–Ca^{2+} exchange at diastolic membrane potentials in rat ventricle, and suggest that the L-type Ca^{2+} current, as opposed to reversed Na^+–Ca^{2+} exchange, is the most important source of intracellular Ca^{2+} which triggers Ca^{2+}-induced Ca^{2+} release in rat ventricle.

METHODS

Cell isolation

The isolation method used to obtain single cells from rat ventricle was a modification of procedures used previously for enzymatic digestion of rabbit atrium and ventricle (Giles & Imaizumi, 1988). Briefly, male Sprague–Dawley rats (250–300 g) were killed by cervical dislocation under light ether anaethesia, and the heart was retrogradely perfused at 35 °C using a horizontal Langendorff apparatus. After 4–6 min perfusion with normal Tyrode solution containing collagenase (0·014 g/ml; Yakult Co. Ltd, Japan) and pronase (0·014 g/ml; Sigma Chemical Co., St Louis, MO, USA), the heart was cut down and the right ventricle was dissected off and minced into 10 ml of a low-Ca^{2+} Tyrode solution containing 0·2 mg/ml (each) collagenase and pronase, 10 mg/ml bovine serum albumin (Sigma; fatty acid free) with 50 μM free Ca^{2+}. The tissue segments were then agitated gently for 8–10 min at 35 °C, following which they were drawn off with a Pasteur pipette at 2 min intervals and placed into 4 ml aliquots of enzyme-free Tyrode solution containing 10 mg/ml albumin, and 100 μM Ca^{2+}. They remained in this solution until most were digested into single cells. The ventricular myocytes were stored in enzyme-free control Tyrode solution at room temperature (22–23 °C) until use.

Solutions

The control Tyrode solution used to superfuse the cells consisted of (mM): NaCl, 121; KCl, 5; $CaCl_2$, 1; sodium acetate, 2·8; $MgSO_4$, 1; glucose, 10; $NaHCO_3$, 24; Na_2HPO_4, 1·1. For experiments in which a local superfusion device was employed (see below) the Tyrode solution consisted of (mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; sodium acetate, 2·8; $MgSO_4$, 1; glucose, 10; Hepes ([4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)]), 10; pH was adjusted to 7·4 with NaOH. When indicated, external Na^+ concentration, $[Na^+]_o$, was reduced to 10 mM with equimolar Li^+ or Cs^+ substitution. The control pipette-filling solution contained (mM): potassium aspartate, 120; KCl, 30; $MgCl_2$, 1·2; Hepes, 5; Na_2ATP, 5; pH was adjusted to 7·1 with KOH. In a series of experiments in which internal $[Na^+]$ was either 8 or 13 mM, Na_2ATP was 4 mM, and $[Na^+]$ was increased to 13 mM by addition of 5 mM NaCl. In some experiments, a recently described peptide, XIP, which inhibits the cardiac sarcolemmal Na^+–Ca^{2+} exchanger (Li *et al.* 1991), was included in the pipette solution. XIP was generously supplied by Dr K. D. Philipson of the University of

California at Los Angeles. XIP was dissolved in distilled water (1 mM), and subsequently diluted to a final concentration of 2×10^{-5} M in the pipette solution. Verapamil, caffeine and tetrodotoxin (TTX) were obtained from Sigma Chemical Co. (St Louis, MO, USA). TTX was dissolved in sodium acetate buffer and diluted to a final concentration of $1{\cdot}5 \times 10^{-5}$ M immediately before use.

Electrophysiological procedures

All whole-cell voltage clamp/cell shortening experiments (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were conducted at 22 °C using a List EPC-7 amplifier (List Electronics, Darmstadt, Germany). Patch electrodes were fabricated from thin-walled borosilicate glass tubing (TW150; World Precision Instruments, Sarasota, FL, USA) and had DC resistances of 1·5–2·5 MΩ when they contained the normal filling solution. The use of potassium aspartate led to a 10 mV liquid junction potential (pipette solution negative), for which membrane potentials were subsequently corrected. Membrane potential and currents were filtered at 5 kHz, sampled at 1 kHz with a 12-bit A–D converter (DT2801A; Data Translation, Marlbourough, MA, USA) and stored in a microcomputer for later analyses.

In the majority of experiments, a bulk superfusion system was used in which cells were placed in a recording chamber (volume 250 μl) and superfused by gravity at a flow rate of 1–2 ml/min with Tyrode solution gassed with 95 % O_2 and 5 % CO_2. A series of electronically operated solenoid valves (Lee Company, Westbrook, CT, USA) was used to select different solutions. Some of the experiments required rapid solution changes which were made using a local superfusion device similar to that described by Shimoni, Clark & Giles (1992) in which a multi-barrelled pipette was placed 50–100 μm away from the cell. A complete solution change surrounding the cell was completed in approximately 500 ms using this device. During these experiments, the cells were superfused 'in parallel' using both the local superfusion device and bulk superfusion systems using Hepes-buffered solution gassed with 100 % O_2 to minimize the effects on solution pH of gas permeation through the semipermeable walls of the polyethylene tubes used to carry the solution from the reservoir to the superfusion pipette.

In some experiments, voltage clamp command potentials consisting of a rectangular step from −80 to + 20 mV, followed immediately by a linear ramp from +20 to −80 mV, were used. The ramp had a rate of change of −2, −0·4 or −0·2 V/s. These waveforms were generated on the D–A output of a DT2801A convertor board in a separate microcomputer, and filtered at 4 kHz before the voltage was applied to the command input of the patch clamp amplifier.

Cell contraction

Changes of cell length during the cardiac cycle were recorded with a video edge detection circuit (Steadman, Moore, Spitzer & Bridge, 1988), which monitored changes in position of either the right or left cell edge. Cell shortening signals were filtered at 30–50 Hz, and were digitized at 1 kHz simultaneously with either membrane potential or current. Records of cell shortening were also recorded on-line using a chart recorder (Gould Instruments, Cleveland, OH, USA). Plotted records of cell shortening were compensated for a video delay of 40 ms (cf. Spurgeon *et al.* 1990).

RESULTS

Unloaded cell shortening and holding potential

Figure 1 illustrates effects of changes in holding potential (V_h) on membrane currents and unloaded cell shortening elicited by a train of 100 ms voltage clamp steps to + 20 mV, applied at 0·2 Hz. The continuous chart recording in Fig. 1*A* shows peak membrane current (I_m) and cell shortening (PS) for a cell voltage clamped at three different holding potentials. These results show that the magnitude of peak shortening depended on V_h, and that after an abrupt change in V_h, changes in peak shortening occurred slowly, with at least 2 min being required to reach a new steady-state value. An example of the changes in time course and magnitude of membrane currents and cell shortening following an abrupt change in V_h is shown in Fig. 1*B*. Maximum cell shortening increased by about 10 % within a single diastolic interval (5 s) after V_h was changed from −80 to −60 mV. This

relatively small change was followed by a gradual and more extensive positive inotropic response; both the magnitude and duration of contraction were increased. These slow changes in contraction contrasted with the accompanying changes in background and outward membrane currents, which developed fully within the first diastolic interval following the change of V_h.

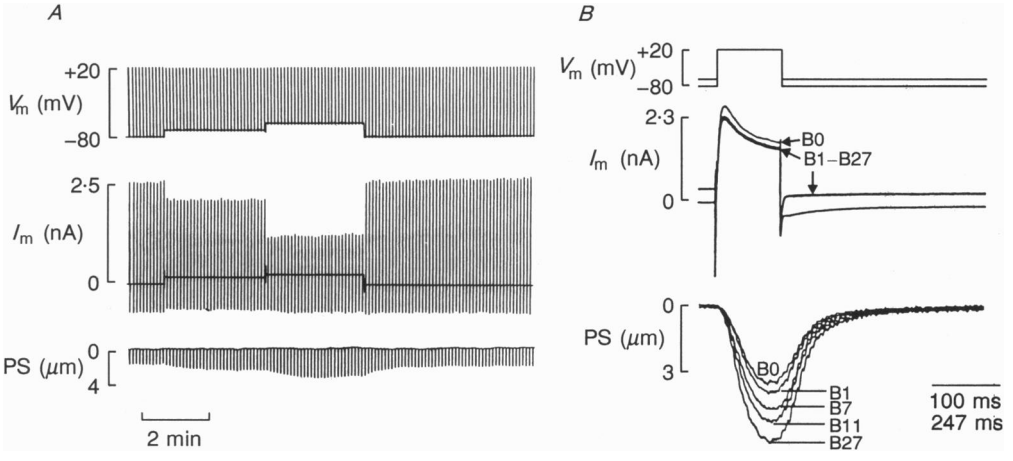

Fig. 1. Effects of holding potential on membrane current and unloaded cell shortening during repetitive voltage clamp depolarizations to a fixed test potential. *A*, continuous chart recording of membrane current (I_m) and peak cell shortening (PS), in response to 100 ms voltage clamp steps to + 20 mV from a series of different holding potentials (V_h). Note that changes in the magnitude of membrane current occurred within one diastolic interval (5 s) after an abrupt change in the holding potential, but changes in peak cell shortening required about 2 min to reach a new steady-state value. *B*, an example of changes in membrane currents (middle panel) and cell shortening (bottom panel) in a cell in which the holding potential (V_h) was abruptly changed from −80 to −60 mV. B0–B27 indicates contractions elicited before (B0), and 1, 7, 11 and 27 beats after depolarization of V_h to −60 mV. Time calibration represents 100 and 247 ms for current and cell shortening measurements, respectively.

The relationship between V_h and unloaded cell shortening is shown in more detail in Fig. 2. Graded changes in V_h over the range of membrane potentials from −140 to −50 mV resulted in significant increases in both amplitude and duration of twitch contractions elicited by voltage clamp steps to + 20 mV (Fig. 2*A*). Contractions at V_h positive to −50 mV could not be studied due to the appearance of spontaneous contractile waves and/or inactivation of the Ca^{2+} current (cf. London & Krueger, 1986). The decrease in peak outward membrane current during depolarization of V_h (Fig. 2*A*) is consistent with the steady-state voltage dependence of inactivation of the transient outward current in rat ventricular myocytes (Apkon & Nerbonne, 1991). Figure 2*B* summarizes the dependence of peak cell shortening on V_h in eight different myocytes. Note that there was a steep increase in peak shortening as V_h was depolarized from the normal resting potential of −80 mV.

To investigate whether the dependence of contraction on V_h was related to Na^+–Ca^{2+} exchange, the effects of a recently described inhibitory peptide of the

cardiac Na^+–Ca^{2+} exchanger (XIP; Li *et al.* 1991) were studied under identical conditions to those of Figs 1 and 2. Other blockers of Na^+–Ca^{2+} exchange such as La^{3+} ($1–2 \times 10^{-3}$ M) or Ni^{2+} (5×10^{-3} M) were not used as they also blocked the Ca^{2+} current at concentrations required for inhibition of Na^+–Ca^{2+} exchange activity (cf.

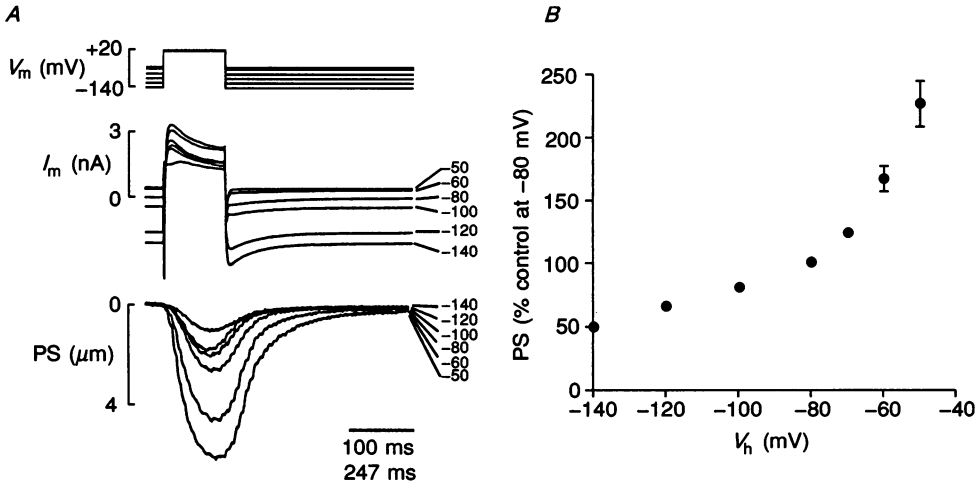

Fig. 2. Relationship between holding potential (V_h) and unloaded cell shortening. *A*, example of changes in cell shortening and membrane currents in response to graded changes in V_h between −140 and −50 mV. Two minutes were required for contractions to reach a steady state after a change in V_h. Note that outward membrane currents during the depolarizing step were partially inactivated at a V_h more positive than about −100 mV. The large inward holding currents at a V_h more negative than −100 mV were due to activation of inward rectifier K^+ current. Time calibration represents 100 and 247 ms for current and cell shortening, respectively. *B*, relationship between unloaded cell shortening and V_h ($n = 8$, mean ± S.E.M.). Peak shortening is expressed as a percentage of control shortening at $V_h = -80$ mV. Temperature = 22 ± 1 °C.

Beuckelmann & Wier, 1989). Figure 3 shows the effects of XIP on membrane currents and contraction under steady-state conditions (following trains of depolarizations) at selected holding potentials. At a V_h of −80 mV (Fig. 3*A*), dialysis of the cell for 10 min with a standard pipette solution containing 2×10^{-5} M XIP resulted in a marked increase in both the amplitude and duration of cell shortening elicited by membrane depolarizations to + 20 mV applied at 0·2 Hz. This inotropic effect occurred without significant changes in the transmembrane ionic currents.

Changes of V_h in the range from −120 to −60 mV in the presence of XIP had little or no effect on either the magnitude or duration of contraction (Fig. 3*B*). A maximum of only four different holding potentials could be studied in a given cell before the appearance of spontaneous contractile waves. Pooled data from five experiments are presented in Fig. 3*C*. The lack of sensitivity of peak shortening to changes in V_h in the presence of XIP suggests that the marked dependence of peak shortening on V_h observed in the control conditions is due primarily to diastolic Na^+–Ca^{2+} exchange.

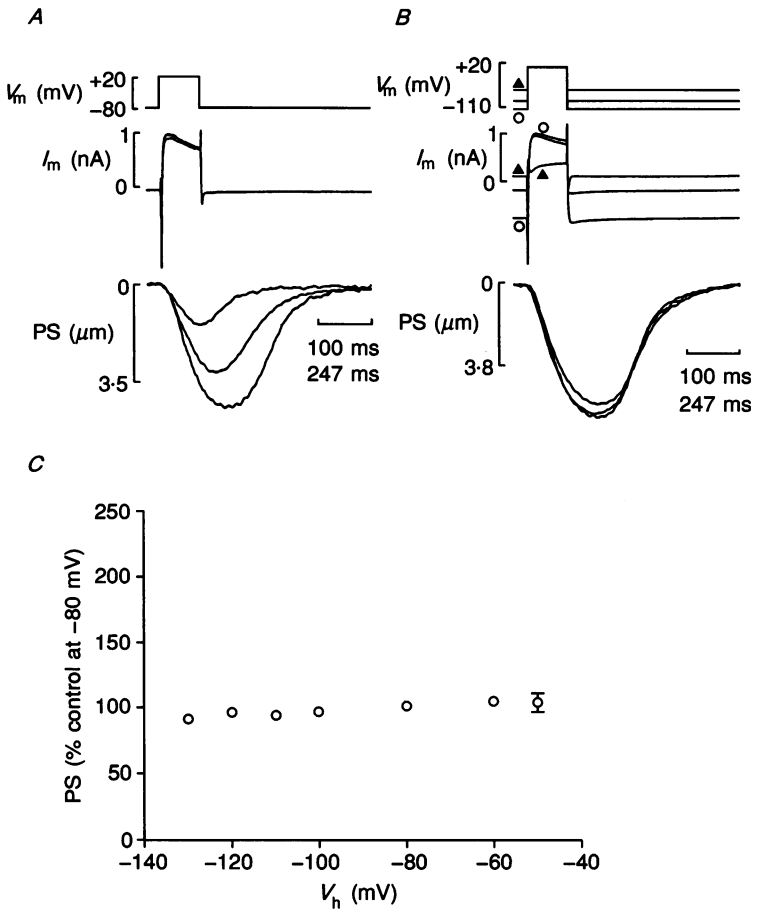

Fig. 3. Effect of Na^+–Ca^{2+} exchange inhibitory peptide (XIP) on holding potential dependence of unloaded cell shortening. *A*, time-dependent increase in the magnitude and duration of cell shortening following membrane rupture and onset of cell dialysis from a recording pipette containing 2×10^{-5} M XIP. Lower traces show contractions 0·5 (smallest), 2·5 and 10 (largest) min after establishment of whole-cell recording conditions. Contractions were elicited by 80 ms voltage clamp pulses from −80 to + 20 mV applied at 0·2 Hz. *B*, lack of sensitivity of contraction to changes in V_h in the presence of intracellular XIP. Both the peak and duration of contraction remained unchanged when V_h was changed from −80 to −110 and then to −60 mV (▲). *C*, summary of relationship between peak contraction and V_h in 5 cells dialysed internally with XIP. Peak shortening was normalized in each experiment to the corresponding control pulse at $V_h = -80$ mV.

Cell shortening–membrane potential relationship

Evidence for the involvement of reverse Na^+–Ca^{2+} exchange in the activation of contraction has been obtained previously in rat and guinea-pig isolated ventricular myocytes (cf. Horackova, 1989; Terrar & White, 1989; Callewaert, 1992). To examine the possible role of this process in contraction of rat ventricular myocytes, the dependence of cell shortening on membrane potential was studied. Figure 4 *A* illustrates an example of the dependence of cell shortening on the membrane

potential from a V_h of −80 mV, with 10 mM Na^+ in the pipette. In agreement with previous measurements in rat ventricular myocytes (Cannell *et al.* 1987; Cleeman & Morad, 1991), a bell-shaped shortening–voltage (*S–V*) relationship was obtained (Fig. 4; see also Fig. 6). The threshold for contraction was near −40 mV, and the

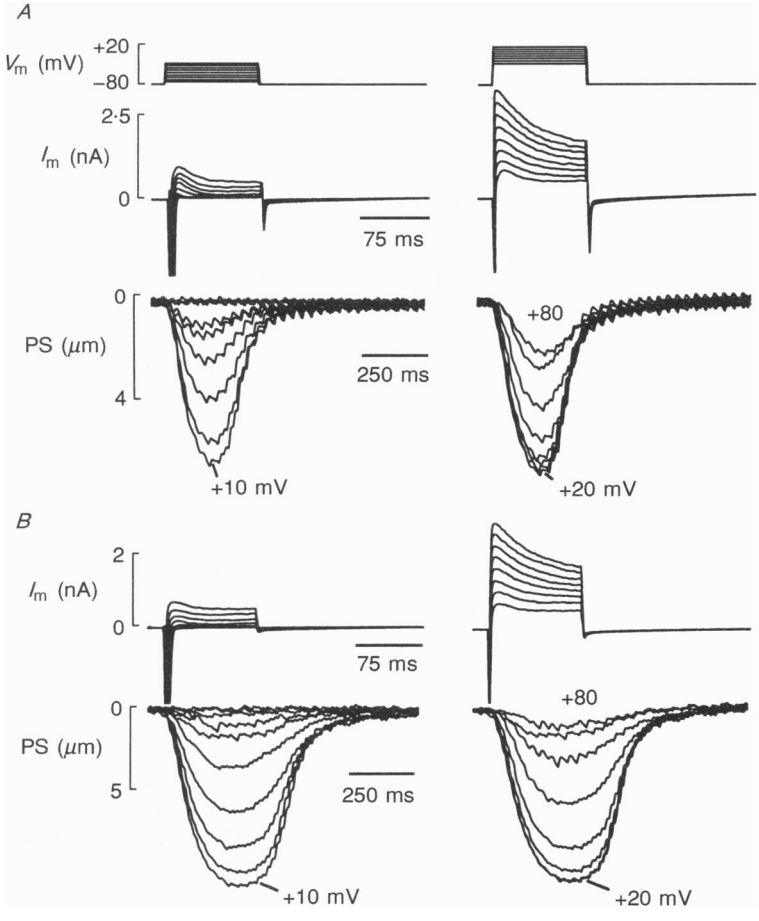

Fig. 4. Voltage dependence of contraction at a holding potential of −80 mV. *A*, unloaded cell shortening and membrane current elicited by a train of 100 ms voltage clamp pulses which increased in 10 mV increments from −70 to + 10 mV (left) and from + 20 to + 80 mV (right). Note the decrease in magnitude of cell shortening as the voltage clamp step was increased from + 20 to + 80 mV. Stimulation frequency was 0·2 Hz. Time calibration bars are 75 and 250 ms for all current and cell length recordings, respectively. *B*, voltage dependence of contraction in a cell dialysed intracellularly with 2×10^{-5} M XIP. The voltage protocol was identical to that described above. Stimulation frequency was 0·17 Hz.

maximum shortening occurred at about + 20 mV, i.e. cell shortening and the calcium current, I_{Ca}, had similar voltage dependence (Josephson, Sanchez-Chapula & Brown 1984; Callewaert, 1992). This relationship was not changed when the duration of the depolarization was increased from 70 to 150 ms (data not shown), or

by application of three to five conditioning depolarizations (–80 to + 20 mV lasting 100 ms) before each 'test' depolarization.

Figure 4 *B* shows the voltage dependence of contraction in a cell dialysed with 2×10^{-5} M XIP. Note that in this XIP-loaded myocyte the magnitude and duration

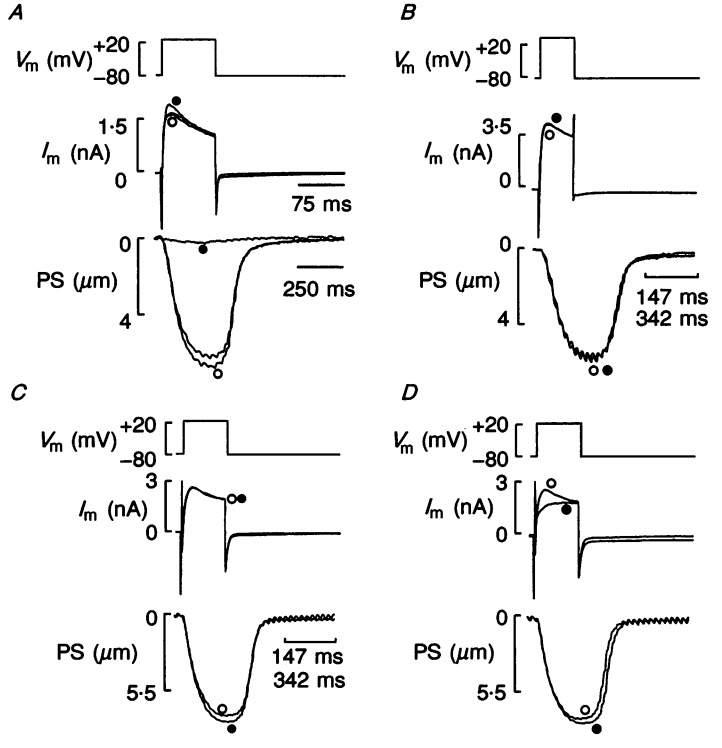

Fig. 5. Dependence of contraction on Na^+ and Ca^{2+} current. *A*, suppression of contraction by Ca^{2+} current blocker. Cd^{2+} (1×10^{-4} M) was applied using the local superfusion device approximately 500 ms after the control twitch (○) and was removed immediately following relaxation of the first test beat (●). Unmarked traces indicate wash-off recordings. *B*, lack of effect of TTX ($1{\cdot}5 \times 10^{-5}$ M) on twitch amplitude or duration. *C–D*, lack of effect of rapid $[Na^+]_o$ reduction on contraction. $[Na^+]_o$ was reduced to 10 mM by substitution with either Li^+ (*C*) or Cs^+ (*D*). Contractions were recorded 1–2 s after switching to low-$[Na^+]_o$ solution. Reduction of $[Na^+]_o$ with either Li^+ or Cs^+ slightly increased peak contraction. All test depolarizations were from –80 to + 20 mV for 80 or 100 ms, and were preceded in each case by 5–10 conditioning depolarizations to + 20 mV. Stimulation frequency, 0·2 Hz. Time calibration bars represent 75 and 250 ms in *A* and 147 and 342 ms in *B–D* for all current and cell length measurements, respectively.

of contraction were increased compared with control conditions (cf. Fig. 2 *A*), but there was no significant effect on the shape of the *S–V* relationship in this or any of eight other cells studied (Fig. 6 *A*).

To evaluate the importance of I_{Ca} in triggering contraction, Cd^{2+} or verapamil were applied in effective blocking doses. Activation of contraction at all test potentials (–70 to + 90 mV; Fig. 6 *A*) was suppressed completely within a single test

beat following blockade of L-type Ca^{2+} channels with 1×10^{-4} M Cd^{2+} (Fig. 5*A*). Similar results were obtained when cells were superfused with verapamil (1×10^{-5} M; Fig. 6*A*).

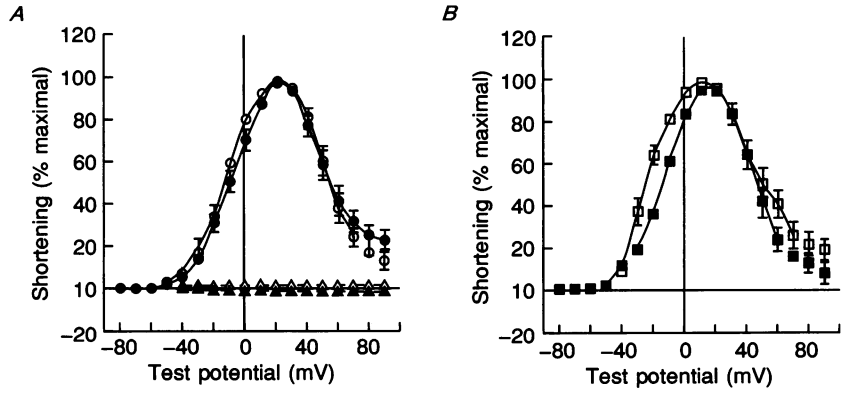

Fig. 6. *A*, summary of the voltage dependence of contraction in cells dialysed with control filling solution (●; $n = 12$), compared with data obtained using filling solution containing 2×10^{-5} M XIP (○; $n = 9$). Pipette $[Na^+]$ was 10 mM. ▲ and △, suppression of contraction with control pipette solution by Cd^{2+} (1×10^{-4} M) or verapamil (1×10^{-5} M), respectively. *B*, effect on the voltage dependence of contraction of changes in pipette $[Na^+]$. □, 13 mM $[Na^+]_i$ ($n = 7$); ■, 8 mM $[Na^+]_i$ ($n = 12$). Note the slight broadening of the *S–V* relationship obtained with increased pipette $[Na^+]$.

The dependence of contraction on the Na^+ current was investigated by altering the size of the Na^+ current with (i) tetrodotoxin (TTX) which, at a concentration of $1{\cdot}5 \times 10^{-5}$ M, blocked approximately 95 % of the Na^+ current, and (ii) by using rapid reduction of extracellular $[Na^+]$. Three to five conditioning pulses were applied before each test pulse to obtain a constant level of SR Ca^{2+} loading. Figure 5*B* shows that application of TTX had no significant effect on contractions elicited by depolarizations from a V_h of −80 to + 20 mV. Similar results were obtained when cells were superfused with Na^+-depleted Tyrode solution. In these experiments a rapid reduction of $[Na^+]_o$ from 140 to 10 mM using either Li^+ (Fig. 5*C*) or Cs^+ (Fig. 5*D*) as Na^+ substitutes had little immediate effect on the amplitude of contractions elicited by stepping the membrane potential from −80 to + 20 mV. The slight increase in contraction seen in Fig. 5*C* and *D* probably was due to reversal of Na^+–Ca^{2+} exchange during the 1–2 s exposure to low $[Na^+]_o$ which preceded the test pulse.

Contractions of multicellular and single-cell cardiac preparations have been shown to be strongly dependent on intracellular Na^+ activity (Eisner, 1990; Harrison, McCall & Boyett, 1992). The effects of $[Na^+]_i$ on voltage dependence of contraction were studied with a pipette $[Na^+]$ of 8 or 13 mM. These values of pipette $[Na^+]$ were based on recent measurements of intracellular sodium activity in rat ventricular cells which yielded 7–8 mM at rest; and 9–11 mM following 2 Hz stimulation (Harrison *et al.* 1992). To ensure adequate equilibration of the pipette solution with the myoplasm, measurements were made 5–10 min after establishment of whole-

cell recording conditions. Raising the pipette Na^+ concentration from 10 to 13 mM increased the magnitude and duration of contraction (data not shown), but resulted in only a slight broadening of the *S–V* relationship (Fig. 6*B*; □). Similarly,

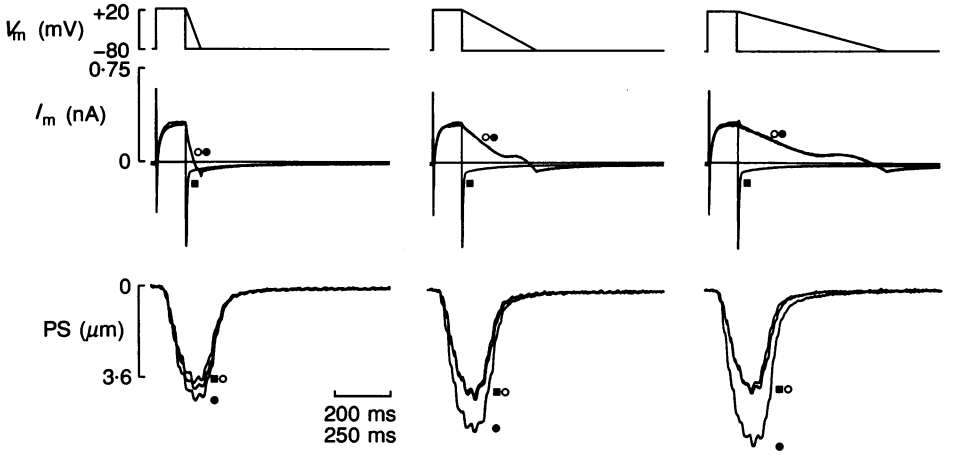

Fig. 7. Effect of changes in the rate of membrane repolarization on cell shortening. A linear, repolarizing voltage ramp was added to the rectangular voltage clamp step (top row). Rectangular depolarization pulses were from V_h of −80 mV to + 20 mV for 100 ms at 0·2 Hz. Ramps were 50 (left), 250 (middle) and 500 ms (right) in duration and immediately followed the + 20 mV step, giving repolarization rates of −2·0, −0·4 and −0·2 V/s, respectively. Membrane currents (middle row) and shortening traces (bottom row) corresponding to the final control step are indicated by the filled squares (■). Recordings corresponding to the first step + ramp are indicated by open circles (○), and the filled circles (●) indicate the response to the final (tenth) ramp pulse in the train. Note that the time-dependent inotropic effect was greater for smaller rates of repolarization, and that while membrane current changes developed fully within the first ramp (○), the inotropic effects required ten beats to reach steady state (●). Calibration bars represent 200 and 250 ms for all current and cell length recordings, respectively.

reduction of pipette Na^+ from 10 to 8 mM had little effect on the shape of the *S–V* relationship (Fig. 6*B*; ■).

These results show that the voltage dependence of contraction in isolated rat ventricular cells does not depend strongly on intracellular Na^+ concentration, the electrochemical gradient for Na^+ (Figs 5 and 6), entry of Na^+ into the cell through TTX-sensitive channels (Fig. 5*B*) or sarcolemmal Na^+–Ca^{2+} exchange activity (Figs 4*A* and 6*A*). The suppression of contraction by either Cd^{2+} or verapamil (Fig. 6*A*) shows that SR Ca^{2+} release, and hence unloaded cell shortening, is dependent almost exclusively on Ca^{2+}-induced Ca^{2+} release (Beuckelmann & Wier, 1988; Valdeolmillos, O'Neill, Smith & Eisner, 1989; Cleeman & Morad, 1991).

Relationship between cell shortening and rate of repolarization

The relationship between membrane voltage and cell shortening was investigated further by changing the rate of repolarization using voltage clamp commands having a linear ramp during repolarization (see Methods). In these protocols the cell

was voltage clamped with a 100 ms step from −80 to + 20 mV at 0·2 Hz until the amplitude of cell shortening reached a steady state; the command signal was then switched to one with a ramp waveform of repolarization.

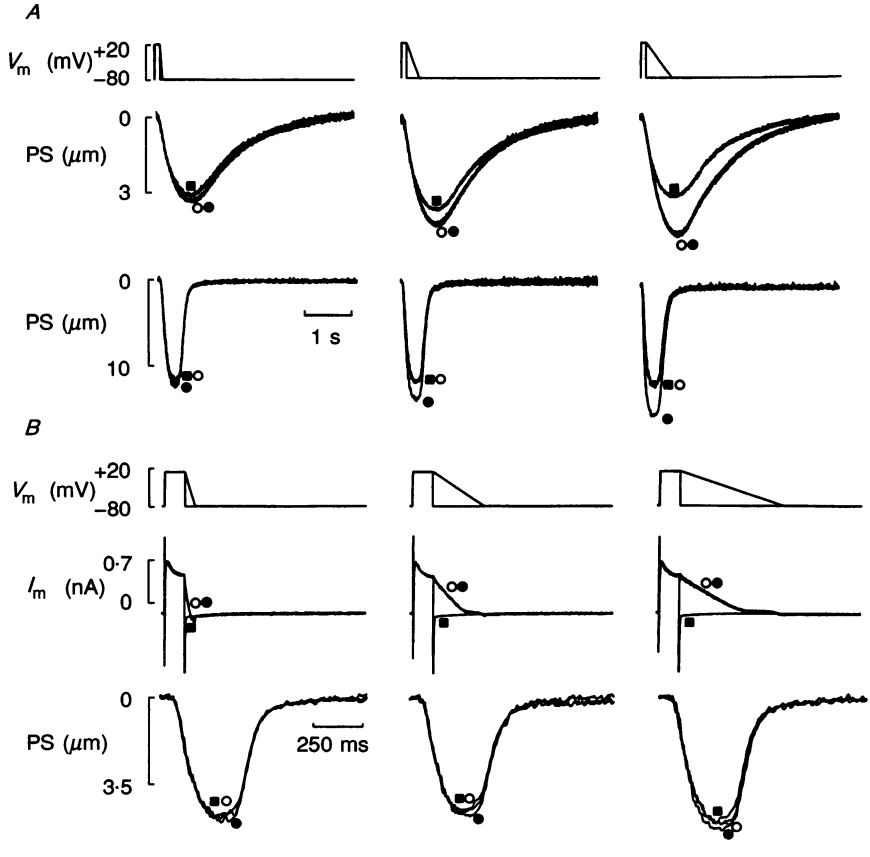

Fig. 8. *A*, effect of caffeine on changes in unloaded cell shortening in response to selected rates of repolarization. Upper panel, voltage clamp command waveforms. Stimulation frequency was 0·2 Hz. Middle panel, effect of 10 mM caffeine on the first (○) and sixth (●) contractions associated with ramp repolarizing waveforms. Contractions associated with the final control waveform are indicated by the filled square (■). Note that in contrast to the data shown in Fig. 7, inotropic effects developed fully within the first ramp waveform, when SR Ca^{2+} sequestration was inhibited with caffeine. Lower panel, reversal of inotropic effects following wash-off of caffeine. *B*, effects of ramp repolarizing waveforms in the presence of 2×10^{-5} M internal XIP. Voltage clamp protocols and corresponding membrane currents and contractions are given in the upper, middle and lower panels, respectively. Stimulation protocol and symbols are identical to those in *A*. Note that in the presence of XIP the positive inotropic response to slowing the rate of repolarization is absent.

The effects of a train of ten ramp voltage commands on membrane currents and accompanying contractions are shown in Fig. 7. The filled squares (■) in Fig. 7 denote control membrane current (middle row) and cell shortening (bottom row) produced by the final rectangular step. The open (○) and filled circles (●) denote currents and contractions produced by the first and tenth ramp voltage commands,

respectively. Note that the first ramp of the train produced no significant inotropic effect, but a positive inotropic effect developed with subsequent commands, and it required at least ten pulses to reach steady state. The extent of increase in cell shortening was greatest for ramps with the slowest rate of repolarization. Note also that changes in membrane current in response to the ramp repolarizations developed almost fully within the first ramp waveform. Similar observations were made in six other cells.

Caffeine has been shown to depress SR Ca^{2+} uptake and release (Bridge, 1986), and in its presence the time course of relaxation is very similar to that of the decline of $[Ca^{2+}]_i$ (Spurgeon *et al.* 1992), and is strongly dependent on sarcolemmal Na^+–Ca^{2+} exchange (Bridge, Spitzer & Ershler, 1988). Caffeine was used in an attempt to determine the contribution of the SR in the ramp-induced inotropy. Figure 8*A* shows the effect of caffeine (10 mM) on the inotropic responses to these slowed rates of repolarization. In the presence of caffeine the inotropic effects associated with ramp repolarization were still observed. However, they developed fully during the first ramp in contrast to the time-dependent changes recorded when caffeine was absent. These inotropic effects were also reversible within a single beat following the return to rectangular voltage clamp waveform (data not shown).

Figure 8*B* shows membrane currents and accompanying contractions in the presence of ramp repolarizations at selected rates in the presence of 2×10^{-5} M internal XIP. The voltage command signals and stimulation protocol were identical to those described in Fig. 7. As shown in Fig. 8*B*, when XIP was present in the recording pipette, cell shortening was much less sensitive to changes in the rate of repolarization. Membrane currents associated with ramp repolarization in XIP-dialysed cells were also similar in magnitude and time course to those observed in the absence of the peptide (Fig. 8*B*, middle panel). Similar results were obtained in two other cells.

DISCUSSION

Inhibitory peptide of Na^+–Ca^{2+} exchanger

Many of our conclusions regarding the contribution of sarcolemmal Na^+–Ca^{2+} exchange to contractility in rat ventricular myocytes depend on XIP being a relatively specific inhibitor for the Na^+–Ca^{2+} exchange. Previous data have shown that XIP blocks Na^+–Ca^{2+} exchange without significantly inhibiting Na^+–K^+-ATPase or Ca^{2+}-ATPase activities in sarcolemmal or SR vesicles prepared from canine hearts (Li *et al.* 1991). In addition, XIP blocks the ionic current attributed to Na^+–Ca^{2+} exchange in excised sarcolemmal patches (Hilgemann, Nicoll & Philipson, 1991) and in intact guinea-pig ventricular cells (Chin *et al.* 1993), where this peptide has no effect on L-type Ca^{2+} currents. Our data show that 2×10^{-5} M XIP had no significant effect on the background (Fig. 3) or outward K^+ currents (Fig. 4). In combination, these findings provide strong evidence that XIP is a relatively selective inhibitor for sarcolemmal Na^+–Ca^{2+} exchange activity in mammalian cardiac muscle.

Relationship between unloaded cell shortening and holding potential

Most previous studies on the voltage dependence of contraction in mammalian cardiac muscle, including those using single cells, have focused on changes in

contractility as a function of depolarization. Early sucrose-gap or two-microelectrode experiments showed that the relationship between V_h and twitch tension was very similar to the I–V relationship for the Ca^{2+} current, I_{Ca}, and that prepulses which inactivated I_{Ca} also reduced force development (cf. Trautwein, McDonald & Trepathi, 1975; Fozzard, 1977). More recent experiments (London & Krueger, 1986) on guinea-pig ventricular cells also demonstrated a decrease of cell shortening in response to depolarization of V_h from −50 to −20 mV, the voltage range in which I_{Ca} is inactivated.

The experiments in Figs 1–3 provide detailed information on the relationship between diastolic membrane potential and cell contraction. A depolarization to + 20 mV was chosen to ensure that a maximal Ca^{2+} influx via I_{Ca} would trigger Ca^{2+} release from the SR (Josephson *et al.* 1984; cf. Callewaert, 1992). Under conditions where the SR Ca^{2+} load was allowed to reach a steady state, contraction was strongly dependent on the diastolic potential (Figs 1 and 2). This voltage dependence was lost completely following dialysis of the cell with XIP (Fig. 3). This result is consistent with previous data from mammalian heart showing that ionic currents and intracellular Ca^{2+} transients attributed to Na^+–Ca^{2+} exchange have a strong dependence on membrane potential in this voltage range (Beuckelmann & Wier, 1989; Crespo, Grantham & Cannell, 1990; Bers *et al.* 1990).

In addition to decreasing the magnitude of contraction, hyperpolarization of V_h significantly shortened the duration of unloaded cell shortening elicited by short depolarizing stimuli (Fig. 2). In caffeine-treated rabbit ventricular muscle (Bridge, 1986) and guinea-pig ventricular myocytes (Bridge *et al.* 1988) the rate of relaxation varied steeply with membrane potential between −80 and 0 mV.

Relationship between unloaded cell shortening and depolarization

The relationship between contraction and the Ca^{2+} current has been studied extensively (cf. Fozzard, 1977; Fabiato, 1985; London & Kreuger, 1986; Beuckelmann & Wier, 1988; Cleeman & Morad, 1991). It is well known that control of twitch force or unloaded cell shortening depends strongly on Ca^{2+}-induced Ca^{2+} release in most mammalian species (Fabiato, 1983). However, recent studies have suggested that Ca^{2+} release from the SR may also be modulated by membrane potential (Cannell *et al.* 1987), Ca^{2+} entry through T-type Ca^{2+} channels, a Ca^{2+} 'window' current, or a Na^+ current-induced Ca^{2+} release (cf. Callewaert, 1992; Carmeliet, 1992).

Significant Ca^{2+} influx through T-type Ca^{2+} channels could be ruled out in our experiments since contraction was blocked by either Cd^{2+} or verapamil (Figs 4 and 7), both of which block Ca^{2+} entry through L-type, but not T-type, Ca^{2+} channels in mammalian heart (Bean, 1985). Entry of Ca^{2+} through non-inactivated Ca^{2+} channels is unlikely to have triggered cell shortening since the test potential (+ 20 mV) was much more positive than the voltage range in which the steady-state component of I_{Ca} has been demonstrated (Trautwein *et al.* 1975; Josephson *et al.* 1984).

Previous theoretical work has suggested that Na^+–Ca^{2+} exchange can modulate contraction at strongly depolarized potentials (cf. Noble, 1986) and this has been demonstrated in guinea-pig ventricular cells (Wier, 1990). Leblanc & Hume (1990) recently reported that a significant component of the transient rise of myoplasmic

Ca^{2+} during contraction in guinea-pig ventricular myocytes may be attributed to reversed Na^+–Ca^{2+} exchange. This contrasts with our results from rat ventricular cells (Fig. 6*A*) showing that the voltage dependence of unloaded cell shortening remained unaffected by intracellular dialysis with XIP and when extracellular Na^+ was rapidly reduced (Fig. 5*C* and *D*). These findings suggest that release of Ca^{2+} from the SR in rat ventricle can occur independently of Na^+–Ca^{2+} exchange activity.

Our results are also consistent with the recent data of Sham *et al.* (1992) showing that Ca^{2+} transients in fura-2-loaded rat ventricular cells remained unaffected when extracellular Na^+ was reduced rapidly, and when the sodium current was blocked with TTX. In our experiments, contractions were blocked completely by Cd^{2+} or verapamil (Figs 5 and 6*A*). However, their voltage dependence was not affected by TTX, by changes of pipette Na^+ (8–13 mM) (Fig. 6*B*), or by XIP in the recording pipette (Fig. 6*A*). Thus in rat ventricular myocytes contraction is triggered by Ca^{2+} entry through L-type Ca^{2+} channels, which results in Ca^{2+}-induced release of Ca^{2+} (Fabiato, 1985; Cleeman & Morad, 1991).

Relationship between unloaded cell shortening and rate of repolarization

Results obtained with ramp waveforms of repolarization showed that prolonging repolarization increased the amplitude and duration of contraction, suggesting that an enhancement of loading and release of Ca^{2+} from the SR took place during the train of clamp pulses (Fig. 7). Since this slowly developing inotropic effect was not observed in cells superfused with caffeine (Fig. 8*A*) or internally dialysed with XIP (Fig. 8*B*) it appears to be due to reduced Ca^{2+} extrusion by Na^+–Ca^{2+} exchange during the slowed voltage ramp waveform of repolarization. Previously it has been shown that in caffeine-treated guinea-pig ventricular cells most of the voltage-dependent relaxation is abolished following reduction of the transmembrane electrochemical gradient for Na^+, as would be expected if Na^+–Ca^{2+} exchange was involved in an important way (Bridge *et al.* 1988; Bridge, Smolley & Spitzer, 1990). These findings suggest that changes in action potential waveform, for example, those which occur in response to changes in stimulation frequency, can alter contractility in part by altering voltage-dependent sarcolemmal Na^+–Ca^{2+} exchange.

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