The effect of internal sodium and caesium on phasic contraction of patch-clamped rabbit ventricular myocytes

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1. The voltage dependence of phasic contraction was assessed in rabbit ventricular myocytes. Phasic contraction at all potentials was abolished by exposure to ryanodine–thapsigargin, showing that it was due primarily to Ca^{2+} release from the sarcoplasmic reticulum (SR). Experiments were performed at 35 °C, cells were whole-cell patch clamped and contraction was measured optically as unloaded shortening. Cells were held at −40 mV to inactivate the Na^+ current (I_{Na}) and T-type Ca^{2+} current. A standard cellular Ca^{2+} load was established by applying a train of conditioning pulses at 0·5 Hz before each test pulse. The effect of replacing K^+ with Cs^+ in the dialysing pipette solution, and the effect of altering dialysing [Na^+] between 0 and 20 mM, was assessed on contraction.

2. Cells dialysed with a K^+-based, Na^+-free solution exhibited a 'bell-shaped' voltage dependence of the L-type Ca^{2+} channel current ($I_{Ca,L}$), with a maximum $I_{Ca,L}$ at +10 mV. Replacing internal K^+ with Cs^+, or altering pipette [Na^+], did not affect the voltage dependence of $I_{Ca,L}$.

3. The voltage dependence of phasic contraction in cells dialysed with a K^+-based solution was modulated by pipette [Na^+]. The voltage dependence of phasic contraction was bell-shaped with 0 Na^+, became much less bell-shaped with 10 mM Na^+ and with 20 mM Na^+ the phasic contraction elicted at +100 mV was 1·6-fold larger than that at +10 mV.

4. Replacing 80 % of K^+ with Cs^+ in the pipette dialysis solution led to a significant reduction in contraction amplitude and a more rapid decline in contraction amplitude after beginning the dialysis of the cell.

5. Cells dialysed with a Cs^+-based solution displayed a voltage dependence of phasic contraction which was more bell-shaped (i.e. more similar to that of $I_{Ca,L}$) than that obtained with the corresponding K^+-based dialysis solution. The level of pipette [Na^+] still modulated the voltage dependence of phasic contraction in cells dialysed with a Cs^+-based solution.

6. Time-to-peak contraction (t_{pk}) also displayed voltage dependence; it had a minimum value between 0 and +20 mV (the voltage range for maximum $I_{Ca,L}$), but increased at more negative and positive potentials. Alteration of t_{pk} contraction is discussed in relation to the stochastic behaviour of L-type Ca^{2+} channels and SR Ca^{2+} release channels.

7. The shape of the voltage dependence of contraction in rabbit myocytes at 35 °C is modulated by dialysing [Na^+] over the tested range, 0–20 mM. Modulation of voltage dependence of contraction by dialysing [Na^+] is consistent with an influence of reverse Na^+–Ca^{2+} exchange in triggering intracellular Ca^{2+} release, in addition to the trigger Ca^{2+} which enters via $I_{Ca,L}$.

8. The marked effect of dialysing Cs^+ on contraction amplitude, and on the voltage dependence of phasic contraction, does not appear to have been reported previously. Internal dialysis with Cs^+ is a commonly used technique for blocking interfering outward K^+ currents, in order to measure $I_{Ca,L}$ more selectively. The present study suggests that Cs^+ might also interfere with processes involved in excitation–contraction coupling and indicates that it might be wise to exercise caution with the use of internal Cs^+ in experiments investigating excitation–contraction coupling.

The contraction of cardiac muscle in response to an action potential or voltage-clamp pulse can be separated into two components. An initial rapid and transient component which begins shortly after depolarization is commonly called the 'phasic' contraction. This component can be abolished by blocking Ca^{2+} release from the sarcoplasmic reticulum (SR), suggesting that it is due to Ca^{2+} release from the SR which leads to a rapid rise of intracellular Ca^{2+} concentration and myofilament activation (Bers, 1985; Bassani, Bassani & Bers, 1993). However, in response to long and/or positive voltage-clamp pulses, or when cells become loaded with intracellular Na^+, an additional depolarization-induced more slowly rising component can be observed after the phasic contraction, commonly termed 'tonic' contraction. Previous evidence has suggested that tonic contraction is due to a continuous Ca^{2+} entry via 'reverse mode' Na^+–Ca^{2+} exchange which causes a progressive rise in intracellular $[Ca^{2+}]$ and contraction during the depolarization (Eisner, Lederer & Vaughan-Jones, 1981; Beuckelmann & Weir, 1989).

In this study we are concerned primarily with the phasic component of contraction, which under normal physiological conditions may be mainly responsible for ventricular muscle contraction. Via the mechanism of 'Ca^{2+}-induced Ca^{2+} release' (CICR; Fabiato, 1985; Valdeolmillos, O'Neill, Smith & Eisner, 1989; Niggli & Lederer, 1990), a small Ca^{2+} entry into the cell is thought to trigger the SR Ca^{2+} release which gives rise to phasic contraction. Some previous studies have suggested that the trigger Ca^{2+} might enter exclusively via the L-type Ca^{2+} channel and in support of this have shown that phasic contraction (or the intracellular Ca^{2+} transient) has a similar voltage dependence to that of current through the L-type Ca^{2+} channel ($I_{Ca,L}$; London & Krueger, 1986; Cannell, Berlin & Lederer, 1987; Beuckelmann & Wier, 1988; Cleemann & Morad, 1991). The elementary events thought to be involved in $I_{Ca,L}$-induced CICR have been revealed recently by use of Ca^{2+} indicators (Isenberg & Han, 1994) and confocal microscopy (Cheng, Lederer & Cannell, 1993; López-López, Shacklock, Balke & Wier, 1994; Cannell, Cheng & Lederer, 1995). These elegant studies have suggested that stochastic activity of individual L-type Ca^{2+} channels during depolarization controls the opening of closely associated and independently functioning SR release channels, and that the activity of each individual 'SR release unit' sums together to produce the whole-cell Ca_i^{2+} transient.

However a second trigger mechanism which may be able to produce, in addition to $I_{Ca,L}$, a trigger Ca^{2+} entry for CICR is the Na^+–Ca^{2+} exchange working in reverse mode (Berlin, Cannell & Lederer, 1987; Levi, Brooksby & Hancox, 1993*a* for review). The elementary events which might be involved in reverse exchange-induced SR release have not so far been described. However, Leblanc & Hume (1990) found that a residual Ca_i^{2+} transient still remained when $I_{Ca,L}$ was blocked completely. They suggested that Na^+ entering via the fast Na^+ channel might lead to transient Na^+ accumulation under the membrane, thereby activating reverse Na^+–Ca^{2+} exchange and causing Ca^{2+} entry to trigger SR Ca^{2+} release. A similar conclusion for the involvement of fast Na^+ current (I_{Na}) in excitation–contraction coupling was reached by Lipp & Niggli (1994).

In addition to I_{Na}-induced SR release, it is also possible that membrane depolarization *per se* might cause a rapid and sufficient Ca^{2+} entry on reverse Na^+–Ca^{2+} exchange to trigger SR release and a phasic contraction. The reversal potential of the exchange is approximately −30 mV (Bers, 1991) so that membrane depolarization to a more positive potential is anticipated to cause at least an initial period of reverse exchange Ca^{2+} entry which might trigger SR release (Shattock & Bers, 1989; Le Guennec & Noble, 1994). Consistent with this possibility, Levi, Brooksby & Hancox (1993*b*) found in rat myocytes held at −40 mV (to inactivate I_{Na}) that a substantial SR release persisted in the absence of $I_{Ca,L}$. Levi, Spitzer, Kohmoto & Bridge (1994) showed in guinea-pig cells that a rapid block of $I_{Ca,L}$ between individual beats abolished only half the phasic contraction. Kohmoto, Levi & Bridge (1994) showed that the phasic contraction remaining when $I_{Ca,L}$ was blocked appeared to depend on the presence of Na^+–Ca^{2+} exchange activity.

Involvement of both $I_{Ca,L}$ and reverse Na^+–Ca^{2+} exchange as triggers of SR release leads to two hypotheses which can be tested. The first is that the voltage dependence of phasic contraction should depend in a graded fashion on the Na^+ concentration inside the cell. When a cell is dialysed with Na^+-free solution to deplete intracellular Na^+ and thus inhibit reverse exchange, $I_{Ca,L}$ might be anticipated to become the primary SR trigger, thus it might be expected that the voltage dependence of phasic contraction will become similar to $I_{Ca,L}$. On the other hand, dialysis with sufficient Na^+ to activate reverse exchange might be expected to cause the voltage dependence of phasic contraction to become different from $I_{Ca,L}$. The second hypothesis is that with a sufficient dialysing $[Na^+]$, it might be expected that there will be a substantial phasic contraction at positive potentials where there is little $I_{Ca,L}$, since the exchange is expected to reverse to a progressively greater degree with larger depolarization.

Previous studies of the effect of dialysing $[Na^+]$ on the voltage dependence of phasic contraction have produced variable results. Nuss & Houser (1992) investigated the effect of dialysing 0 and 20 mM Na^+ in cat myocytes at 35 °C and found that contraction was influenced significantly by the Na^+ level. However, it is notable in this study that Cs^+ replaced K^+ in the pipette solution which dialysed the cell and, since Cs^+ is known to be a potent blocker of all types of K^+ channels (both in the sarcolemma and the SR; Matsuda & Noma, 1984; Cukierman, Yellen & Miller, 1985), it is possible that intracellular Cs^+ might have affected excitation–contraction coupling. In contrast,

Bouchard, Clark & Giles (1993*a*) using rat myocytes at room temperature dialysed with a K^+-based solution, found no effect on the voltage dependence of contraction of varying dialysing [Na^+] between 8 and 13 mM. On the other hand Vornanen, Shepherd & Isenberg (1994), using guinea-pig myocytes dialysed with a K^+-based solution, found a marked effect on phasic contraction of altering dialysing [Na^+] between 0 and 20 mM. In addition, they also found an important factor that might account for some reported differences in voltage dependence of phasic contraction. At room temperature, the voltage dependence of phasic contraction was bell-shaped and similar to $I_{Ca,L}$, whereas at 37 °C the voltage dependence of phasic contraction was not bell-shaped and could be modulated by dialysing Na^+. They suggested that any SR release triggered by reverse Na^+–Ca^{2+} exchange might be small at room temperature, whereas it might become more substantial at normal physiological temperature.

The objective of this study was to assess the effect of varying dialysing [Na^+] over the normal physiological range on the contraction of rabbit ventricular cells. Experiments were performed at 35 °C to reflect more closely the normal physiological situation. In addition to using a K^+-based dialysing solution, we also tested the effect on excitation–contraction coupling of replacing internal K^+ with Cs^+. The use of internal Cs^+ dialysis to block interfering K^+ currents allows more selective measurement of $I_{Ca,L}$ and is commonly used in studies investigating cardiac excitation–contraction coupling (Beuckelmann & Wier, 1988; Cleemann & Morad, 1991; Lipp & Niggli, 1994; Cannell *et al.* 1995). We found that Cs^+ dialysis had a marked effect on the amplitude of contraction and changed its voltage dependence, and this may have significance for future studies of excitation–contraction coupling.

The preliminary results of this study have been published in abstract form (Levi, Mitcheson & Hancox, 1993*c*).

METHODS

Cell isolation

Myocytes were isolated from both ventricles of the heart of male New Zealand White rabbits (1·8–2·5 kg) using the following method modified from Hancox, Levi, Lee & Heap (1993). Animals were killed by cervical dislocation (a method sanctioned by the Home Office), the heart was quickly removed and the aorta mounted on a cannula for retrograde perfusion at a rate of 6 ml min^{-1} per gram of heart tissue. The basic solution (solution A) contained (mM): NaCl, 130; Hepes, 23; glucose, 21; taurine, 20; creatine, 5; KCl, 4·5; $MgCl_2$, 5; NaH_2PO_4, 1; sodium pyruvate, 5; pH 7·25. The heart was perfused with solution A + 750 μM Ca^{2+} for 2 min, then with solution A + 3·3 μM EGTA for 5 min. After this the heart was perfused for between 12 and 16 min (depending on heart size; the time was increased for larger hearts) with an enzyme-containing solution consisting of solution A + 150 μM Ca^{2+} with 1 mg ml^{-1} collagenase (Type 1, Worthington, Freehold, NJ, USA) and 0·1 mg ml^{-1} protease (Type XIV, Sigma). The enzyme was then washed out by perfusing solution A + 150 μM Ca^{2+} for a further 5 min. At the end of perfusion, the heart was removed from the cannula; both ventricles were dissected and chopped into small pieces which were then shaken for 6 min in a glass conical flask containing 50 ml of solution A + 150 μM Ca^{2+}. The cell suspension was filtered through nylon gauze (200 μm mesh), sedimented in a 50 ml glass beaker for 4 min and the supernatant was then replaced with a higher Ca^{2+}-containing solution; [Ca^{2+}] was increased in two steps up to 500 μM. Cells were kept at room temperature in solution A + 500 μM Ca^{2+} until use and usually survived well for up to 8 h. We stopped using 'KB' solution (Isenberg & Klockner, 1982) for storing cells after isolation since we found in rabbit myocytes, that even a very short incubation (i.e. 2 min) in KB solution, peak $I_{Ca,L}$ amplitude became reduced to 40% ($n = 10$ isolations) and contraction amplitude reduced to less than 20% ($n = 10$ isolations).

Electrical recording

Cells were placed in a Perspex chamber and superfused at 35 °C with Tyrode solution containing (mM): NaCl, 140; Hepes, 5; glucose, 10; KCl, 4; $CaCl_2$, 2·5; $MgCl_2$, 1; titrated to a pH of 7·4 with 4 mM NaOH. Miniature solenoid valves (LFAA1201618H, Lee Products Ltd, Bucks, UK) selected the solution entering the chamber and the superfusate could be changed within 10–20 s. Patch-pipettes (Corning 7052 glass, AM Systems Inc., Everett, WA, USA) were pulled to resistances of 2·5–3 MΩ (Narashige PP 83 pipette puller) and fire polished to between 4 and 5 MΩ (Narashige MF 83 microforge). The basic K^+-based pipette filling solution contained (mM): KCl, 110; K_2ATP, 5; Hepes, 10; $MgCl_2$, 0·4; glucose, 5; titrated to a pH of 7·1 by adding 10 mM KOH. The basic Cs^+-based solution contained (mM): CsCl, 110; TrisATP, 5; Hepes, 10; $MgCl_2$, 0·4; glucose, 5; titrated to a pH of 7·1 by adding CsOH. However, the Cs^+-containing pipette dialysis solution used for the experiments consisted of an 80 : 20 mixture of the Cs^+-based : K^+-based solutions, respectively. Eighty per cent (rather than 100%) of the K^+ was replaced with Cs^+ since we found that membrane conductance tended to increase after a short time with 100% Cs^+ replacement, and this could be prevented by inclusion of 20% K^+ ($n = 15$ cells). Aliquots of 1 M NaCl were added to these 0 Na^+ pipette solutions to give internal solutions containing 10 or 20 mM Na^+.

It is worth noting that since both K^+- and Cs^+-based pipette solutions contained a total of 0·4 mM Mg^{2+}, 5 mM ATP and no added Ca^{2+} (contaminant Ca^{2+} was measured as 3 μM), calculations suggest that [MgATP] in the pipette solution will be 0·39 mM, free [Mg^{2+}] will be 7·7 μM and free [Ca^{2+}], 0·1 μM ('Metlig' program, Biochemistry Department, University of Bristol, Bristol, UK). Pipette solutions with 0·4 mM total Mg^{2+} were used because we found that dialysis with higher total Mg^{2+} regularly caused a rapid decline in contraction amplitude after 2–5 min of internal dialysis. Assuming that the internal environment of the cell is normal shortly after going whole cell (when little dialysis will have occurred), this rapid decline of contraction as dialysis progressed appeared to be an unphysiological consequence of dialysis with a higher internal [Mg^{2+}]. Lowering pipette [Mg^{2+}] to 0·4 mM preserved the contraction amplitude of myocytes for a much longer period during whole-cell recording (sometimes up to 1 h). The possible consequences for intracellular Mg^{2+} regulation of internal dialysis are unknown, but it may not be as simple as the cytoplasmic free [Mg^{2+}] becoming equilibrated with that in the pipette. However, it is useful to consider whether dialysis with these particular pipette solutions might have facilitated CICR, since raising cytoplasmic [Mg^{2+}] is known to block the SR Ca^{2+}

release channel. There are few data for the cardiac SR channel under conditions which approximate physiological, but Smith, Coronado & Meissner (1986) have shown in the skeletal muscle SR channel that it becomes blocked as $[Mg^{2+}]$ is raised, with a half-maximal inhibition ($K_{½}$) of 1·5 mM Mg^{2+}. Since free cytoplasmic $[Mg^{2+}]$ has been measured as 0·5 mM (McGuigan *et al.* 1991), whereas higher levels appear to be required to block the SR channel, it is possible that lowering cytoplasmic $[Mg^{2+}]$ below 0·5 mM may not have a marked effect on the channel. However, it is probable that the local free $[Mg^{2+}]$ in the vicinity of the SR Ca^{2+} channel might be the important factor and there is no information at present about the degree to which free $[Mg^{2+}]$ might be spatially inhomogeneous within the cell cytoplasm. Valdivia *et al.* (1995) have shown recently that adaptation of the SR channel to a constant local activating $[Ca^{2+}]$ is much faster in the presence of cytoplasmic Mg^{2+}, with a $K_{½}$ of 100 μM Mg^{2+}. However, myocytes dialysed with the pipette solutions used in the present study were able to beat repetitively at rates of 2 Hz or greater, and if local $[Mg^{2+}]$ near the channel had fallen below 100 μM this might have been expected to slow the beating rate at which the SR could still effectively release Ca^{2+}. It would therefore appear that dialysis with these pipette solutions might not have reduced local free $[Mg^{2+}]$ near the SR release channel to as low as 100 μM.

The pipette-to-bath liquid junction potential for both the K^+- and Cs^+-based pipette solutions was found to be −2·7 mV. Since this value was small, it was not necessary to make corrections for membrane potential. The vast majority of recordings were made using an Axopatch 200A amplifier with a CV202A head stage (Axon Instruments). However, some preliminary recordings were made using an Axoclamp-2A amplifier (Axon Instruments; ×0·1 head stage) in the single-electrode voltage-clamp mode, either with the discontinuous or continuous clamp mode option. The key advantages of the Axopatch 200A were that recordings were less noisy and series resistance could be compensated, usually up to 85%. A 5 min period for internal dialysis was allowed after attaining the whole-cell configuration, during which voltage-clamp protocols were applied continuously but no voltage dependence measurements were taken. Recordings of conditioning pulses were taken after 4 min (e.g. Fig. 5*A*) to assess the effects of internal Na^+ and Cs^+ on contraction amplitude.

Contraction measurement

The contractile activity (cell shortening) of a myocyte was measured optically using a video-edge detector device (Crescent Electronics, Sandy, UT, USA) coupled to a TV camera mounted on the side port of the microscope. Cursors were positioned on both ends of the cell so that the magnitude of whole-cell shortening was measured, and the accuracy of detection was checked continuously by ensuring that the cursor dots tracked the motion of each end reliably. The system ran at the normal UK frame rate (50 Hz; 1 sample every 20 ms). Rabbit myocytes at 35 °C have a time-to-peak contraction between 160 and 400 ms, thus this system provided between eight and twenty measurement points on the rising phase of contraction. The time resolution of the system was therefore adequate; this was checked by running the system at 240 Hz using a fast-scanning TV camera (Pulnix, Sunnyvale, CA, USA) and the time course of contraction was shown to be similar with both the 50 and 240 Hz sampling rates. The TV camera image was recorded on a video recorder and could therefore be played back later to obtain an off-line record of contraction.

Measurement of $I_{Ca,L}$ amplitude

The amplitude of $I_{Ca,L}$ was measured as the difference between the peak inward current at the start of the test pulse and the steady-state current at the end of an 800 ms test pulse (London & Krueger, 1986; Vornanen *et al.* 1994). This current was sensitive to block by 20 μM nifedipine or 100 μM cadmium (Cd^{2+}) and voltage dependence of the nifedipine- or Cd^{2+}-sensitive current was very similar to that of peak minus steady-state current (London & Krueger, 1986). Additionally, in cells dialysed with a Cs^+-based pipette solution, measurements will not have been contaminated with interfering outward K^+ current. It is possible that in cells dialysed with a K^+-based pipette solution there was some contamination with an overlapping K^+ current but in practice this effect appeared to be very small since the voltage dependence of $I_{Ca,L}$ was almost identical for cells dialysed with either K^+- or Cs^+-based solutions (e.g. Figs 2*A*, 3*B*, 6*B*). This may be partly explained by the fact that $I_{Ca,L}$ is a much larger current than the delayed rectifier K^+ current (I_K) and so any contamination by I_K during an 800 ms duration pulse must be relatively small.

Data analysis and statistics

Voltage-clamp protocols were generated using the program 'VGEN' (written and supplied by John Dempster of Strathclyde University, Glasgow, UK). Data were recorded at 4·5 kHz using a digital data recorder (Instrutech VR 100-8; Great Neck, NY, USA). Data could then be processed 'off-line' by play-back from the data recorder and digitizing at 1 kHz using a CED 1401 A/D interface and the programs 'VCAN' and 'WCP' (also by John Dempster). Data are expressed as means ± S.E.M. To compare observations within the same group, Student's two-sample paired *t* test was used. To compare observations from different groups with unequal sample sizes, Student's two-sample *t* test was used in which sample variance was not assumed equal (Microsoft Excel 5, statistical functions). Significance was taken as that at the 5% level.

RESULTS

The pulse protocol used for all the experiments is shown in Fig. 1*A*. After attaining whole-cell access with the holding potential (V_h) set at −80 mV, compensations were made for series resistance and whole-cell capacitance. The V_h was then depolarized to −40 mV to inactivate the fast Na^+ current (I_{Na}), T-type Ca^{2+} current and transient outward K^+ current, before starting the pulse protocol. Before each test pulse, a train of eight 400 ms conditioning pulses (from −40 to +10 mV) was applied at 0·5 Hz to establish a standard level of SR Ca^{2+} load. The single test pulse which followed each conditioning train was 800 ms in duration and was incremented (in 10 mV steps) to potentials between −30 and +100 mV. Measurements were started 5 min after attaining whole-cell access to allow cells to become dialysed with the Na^+ level of the pipette solution. A separate series of experiments in myocytes dialysed with 10 and 20 mM Na^+-containing solutions showed that the phasic contractions elicited at all test pulse potentials were largely abolished (to 12·5 ± 1·3%) after a 5 min exposure to 20 μM ryanodine–5 μM thapsigargin, showing that

phasic contractions at all pulse potentials were due primarily to SR Ca^{2+} release (Bassani *et al.* 1993). Especially in cells dialysed with 20 mM Na^+, there may have been a substantial and rapid direct Ca^{2+} entry via Na^+–Ca^{2+} exchange at a positive potential, which may have caused a rapid initial phasic-like contraction. However, the sensitivity of phasic contraction to ryanodine–thapsigargin at positive potentials in cells dialysed with 20 mM Na^+ showed that this was not the case and the phasic contraction was instead due to SR Ca^{2+} release.

The voltage dependence of phasic contraction with a K^+-based, 0 Na^+ pipette solution

Figure 1*B* shows the typical effect on contraction of dialysing a cell with a K^+-based, 0 Na^+ pipette solution. Each panel shows the last conditioning pulse in the train followed by the 800 ms test pulse which immediately followed it. Each test pulse activated a rapid phasic contraction that was followed by a small tonic component of contraction which became slightly larger at potentials between +60 and +100 mV. It is notable that after the

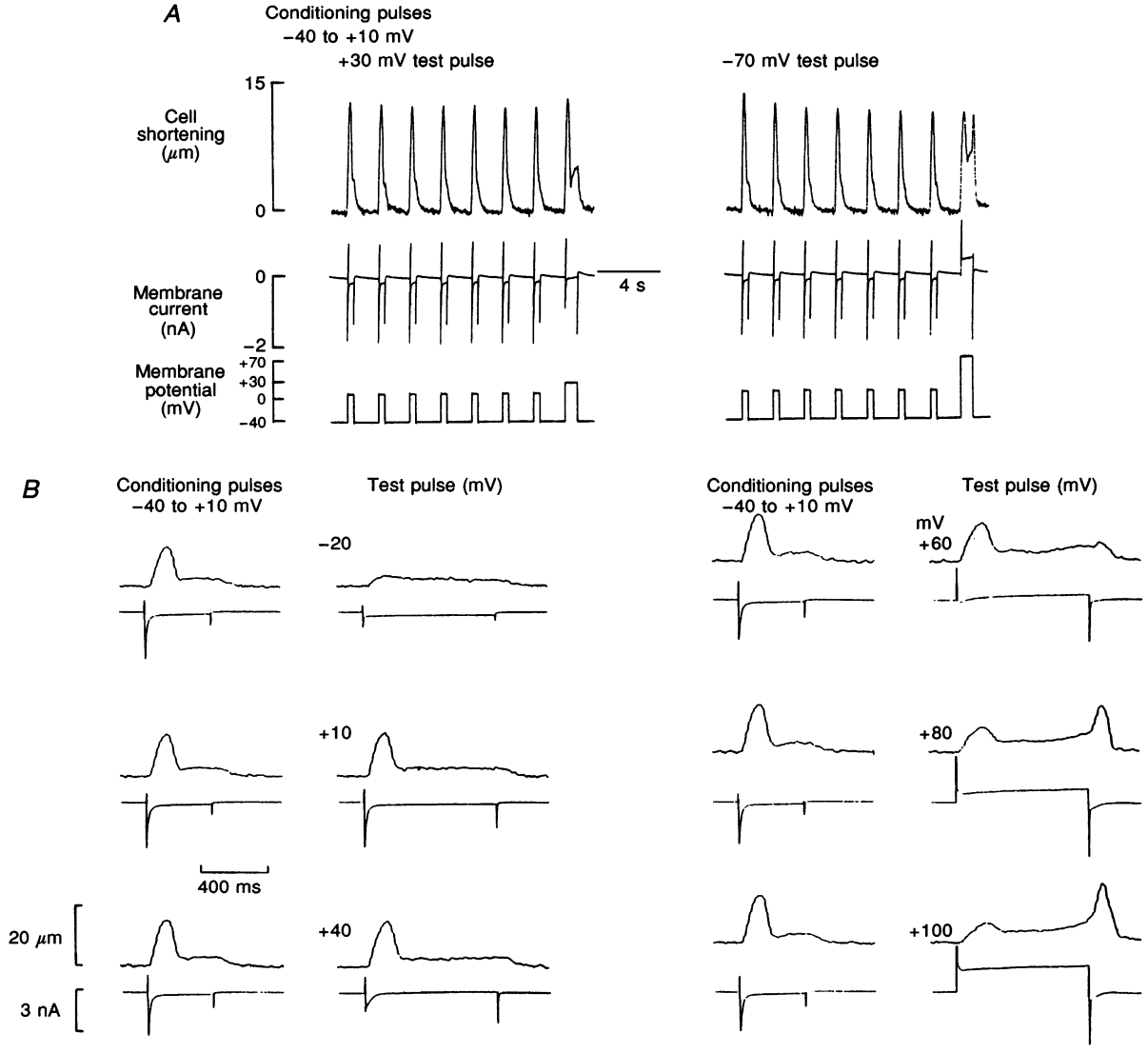

Figure 1

A, pulse protocol used for all the experiments. The cell was held at −40 mV and before each test pulse, a train of eight 400 ms conditioning pulses from −40 to +10 mV was applied at 0·5 Hz, to establish a standard level of SR load. Each test pulse was 800 ms in duration and was applied to potentials between −30 and +100 mV, in 10 mV increments. The left panel shows a test pulse applied to +30 mV, the right panel shows a test pulse applied to +70 mV. The cell was dialysed with K^+-based, 0 Na^+ pipette solution. *B*, typical contractions and membrane currents in a cell dialysed with K^+-based, 0 Na^+ pipette solution. Here and in subsequent figures, the left-hand column of each pair of traces shows the conditioning pulse which immediately preceded each test pulse shown in the right-hand column.

depolarizations to +60, +80 and +100 mV, when membrane potential was step repolarized back to −40 mV at the end of the pulse, there was a spike of inward current which was followed by a second rapid phasic contraction. The initial rapid spike of inward current is due primarily to a deactivating $I_{Ca,L}$ 'tail' current, and the later more slowly declining inward current is due to forward Na^+–Ca^{2+} exchange. Similar 'tail' contractions (or tail Ca_i^{2+} transients) after repolarization from a positive potential have been noted in previous studies (Cannell *et al.* 1987; Beuckelmann & Wier, 1988; Cleemann & Morad, 1991; López-López *et al.* 1994).

Since phasic contraction is largely due to SR Ca^{2+} release, and its magnitude should therefore reflect the degree of SR Ca^{2+} release (London & Krueger, 1986; Cleemann & Morad, 1991), the analysis for this study has concentrated primarily on this component of contraction. The test pulse to −20 mV activated a small $I_{Ca,L}$ and a small phasic contraction (followed by a small sustained contraction during the pulse). A pulse to +10 mV activated a near-maximal $I_{Ca,L}$ and a larger phasic contraction. At more positive potentials the amplitude of $I_{Ca,L}$ declined but it can be seen that phasic contraction did not decline in parallel. A pulse to +40 mV activated a smaller $I_{Ca,L}$ than with the +10 mV pulse, yet phasic contraction was the same as for +10 mV. There was little net inward $I_{Ca,L}$ with the +60 mV pulse, but still a substantial phasic contraction. With test pulses to +80 and +100 mV (which approach the Ca^{2+} equilibrium potential, E_{Ca}; approximately +130 mV under these conditions) there were also small phasic contractions. It is, however, notable that these contractions developed more slowly and had a longer time to peak (t_{pk}) than those elicted by pulses to +10 mV.

Mean results from ten cells for the voltage dependence of $I_{Ca,L}$ and phasic contraction are shown in Fig. 2*A*. To remove any influence of a slow change in $I_{Ca,L}$ or contractile state during a complete pulse sequence, both phasic contraction and $I_{Ca,L}$ activated by each test pulse are plotted as normalized to the contraction activated by the immediately preceding conditioning pulse. $I_{Ca,L}$ had a conventional bell-shaped voltage dependence with a maximum at +10 mV, declining at more positive and negative potentials. There was similarity between the voltage dependence of $I_{Ca,L}$ and contraction up to +10 mV, but it is clear they diverged at more positive potentials. Between +20 and +40 mV, phasic contraction became significantly larger than for a pulse to +10 mV ($P < 0{\cdot}05$), whilst the amplitude of $I_{Ca,L}$ became significantly smaller ($P < 0{\cdot}005$). The contraction at +50 mV was not significantly different from +10 mV, but the amplitude of $I_{Ca,L}$ at +50 mV was only 18% of that at +10 mV. At potentials more positive than +50 mV, contraction amplitude became significantly smaller than for +10 mV ($P < 0{\cdot}005$).

Time-to-peak contraction (t_{pk}) was measured as the time from the start of depolarization to when contraction amplitude reached a maximal value. Figure 2*B* illustrates the mean voltage dependence of t_{pk}. The relation had a U-shape with a minimum t_{pk} between 0 and +20 mV, the potential range over which $I_{Ca,L}$ was maximal. At potentials more negative than 0 and more positive than +20 mV, t_{pk} became significantly prolonged in comparison with +10 mV ($P < 0{\cdot}005$).

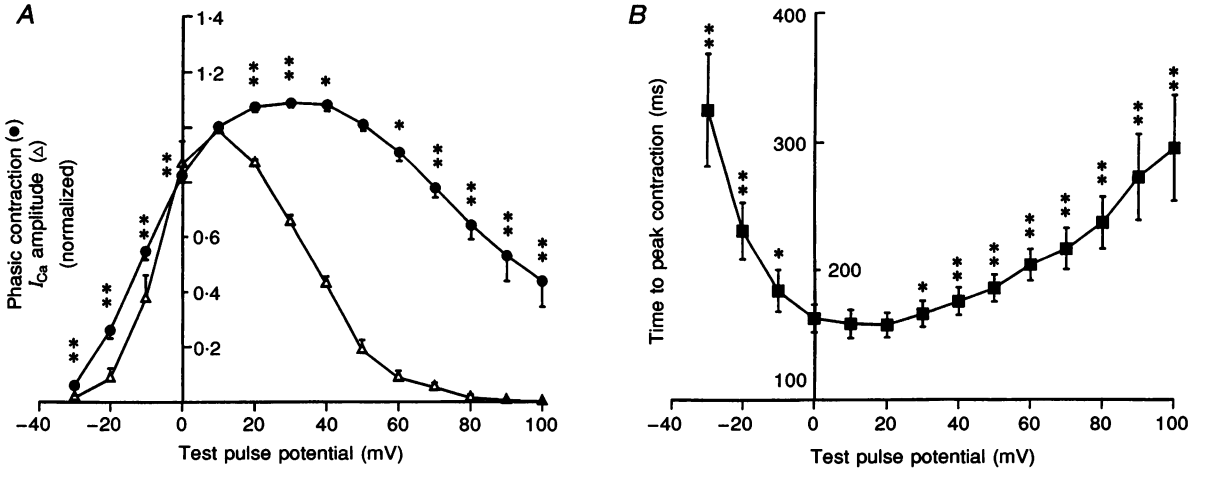

Figure 2

A, mean voltage dependence of contraction and $I_{Ca,L}$ for 10 myocytes dialysed with the K^+-based, 0 Na^+ solution. The amplitude of contraction (normalized to the preceding conditioning pulse) at each potential was tested for a significant difference in comparison with the contraction elicited by a pulse to +10 mV. * $P < 0{\cdot}05$, ** $P < 0{\cdot}005$, no symbol on the data point denotes $P > 0{\cdot}05$. This convention is used in all subsequent figures. *B*, mean voltage dependence of t_{pk} contraction for myocytes dialysed with the K^+-based, 0 Na^+ solution ($n = 8$ cells). Significance is shown in comparison with the +10 mV pulse.

Phasic contraction with a K^+-based pipette solution containing 10 mM Na^+

The typical effect of internally dialysing a rabbit myocyte with a K^+-based solution containing 10 mM Na^+ is presented in Fig. 3*A*. The conditioning pulses which immediately preceded each test pulse are shown on the left of each record. A pulse to +10 mV elicited a maximal amplitude $I_{Ca,L}$ and this was also associated with a large phasic contraction. However, as pulse potential became more positive and $I_{Ca,L}$ declined, there was very little decrease in the amplitude of phasic contraction. A pulse to +60 mV activated little net inward $I_{Ca,L}$ but there was nevertheless a large phasic contraction. Test pulses to +80 and +100 mV (closer to E_{Ca}) also elicited large phasic contractions. It was a typical feature of cells dialysed with the K^+-based, 10 mM Na^+ solution that the 800 ms duration pulses to potentials between +60 and +100 mV elicited, after the initial phasic contraction, 'oscillatory type' phasic

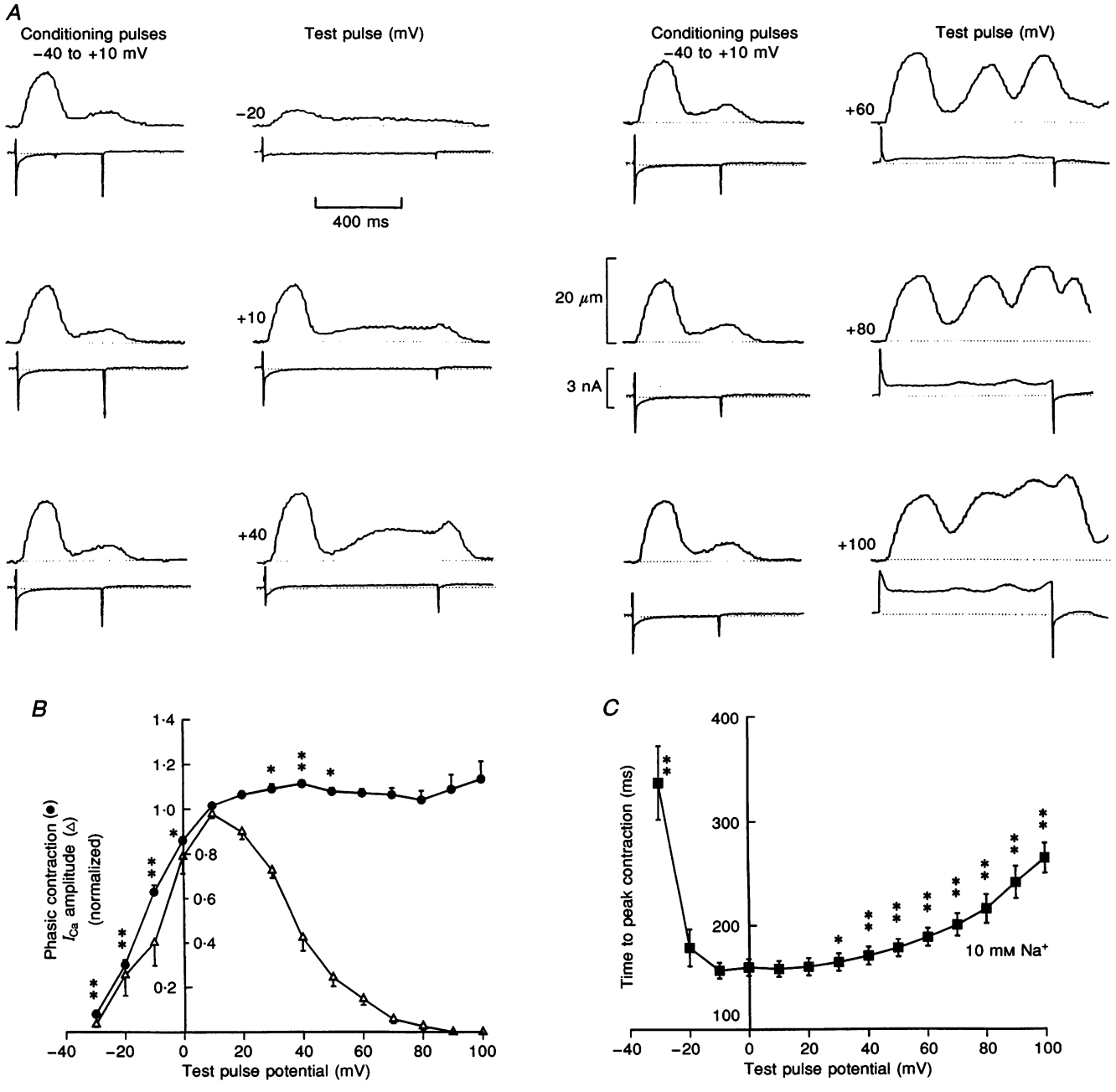

Figure 3

A, contractions and membrane currents elicited at different potentials in a cell dialysed with K^+-based, 10 mM Na^+ pipette solution. *B*, mean voltage dependence of contraction and $I_{Ca,L}$ for eight myocytes dialysed with K^+-based, 10 mM Na^+ solution. Significance is shown in comparison with the +10 mV pulse. *C*, mean voltage dependence of t_{pk} contraction for myocytes dialysed with K^+-based, 10 mM Na^+ solution ($n = 7$ cells). Significance is shown in comparison with the +10 mV pulse.

contractions during the depolarization often superimposed on a slowly increasing tonic contraction.

The mean voltage dependence of $I_{Ca,L}$ and phasic contraction under these conditions is shown in Fig. 3*B* ($n = 8$ cells). $I_{Ca,L}$ once again had a bell-shaped voltage dependence; there was no significant difference between 0 Na^+ and 10 mM Na^+ dialysis for the voltage dependence of $I_{Ca,L}$ ($P > 0.05$). There was a close correspondence between $I_{Ca,L}$ and phasic contraction up to +10 mV, but at more positive potentials the two relations diverged substantially. Contraction was significantly greater at potentials between +30 and +50 mV compared with +10 mV ($P < 0.05$) and yet $I_{Ca,L}$ declined very greatly over the same potential range. Between potentials of +60 and +100 mV, there was no significant difference in phasic contraction amplitude compared with +10 mV ($P > 0.05$), and yet $I_{Ca,L}$ was very small over this voltage range (which progressively approached E_{Ca}). The fact that a pulse to +100 mV activated a large phasic contraction, and at this potential there is expected to be a relatively small Ca^{2+} entry through the L-type Ca^{2+} channel, might indicate the existence of another trigger mechanism for SR release besides $I_{Ca,L}$.

The time course of contraction also showed voltage dependence (Fig. 3*C*). It can be seen in Fig. 3*A* that development of contraction with a pulse to +100 mV was slower than with a pulse to +10 mV. As with 0 Na^+ dialysis, t_{pk} contraction had a U-shaped voltage dependence; it had a minimum value over the range −10 to +20 mV (where $I_{Ca,L}$ is maximal) and at more negative and positive potentials t_{pk} became significantly larger ($P < 0.05$ and 0·005, respectively; t_{pk} at each potential compared with the value at +10 mV).

In summary, with a K^+-based pipette solution, there were clear effects of altering dialysing [Na^+] between 0 and 10 mM on the contraction of rabbit myocytes. The voltage dependence of contraction amplitude became less bell-shaped with 10 mM Na^+. Despite this marked difference however, there was no significant difference in voltage dependence of t_{pk} ($P > 0.05$). The difference in voltage dependence of contraction could not be accounted for by an alteration in $I_{Ca,L}$ with dialysing [Na^+], since $I_{Ca,L}$ had the same voltage dependence with both 0 and 10 mM Na^+.

Phasic contraction with a K^+-based pipette solution containing 20 mM Na^+

Experiments were carried out in seven cells using a KCl-based pipette solution containing 20 mM Na^+, to determine if the voltage dependence of contraction could be further modulated by dialysing Na^+. Figure 4*A* presents a representative recording. The −20 mV pulse activated a small $I_{Ca,L}$ which was accompanied by a small phasic contraction. At potentials more positive than +10 mV, $I_{Ca,L}$ declined but in contrast, phasic contraction became progressively larger at positive potentials. Phasic contraction with the +100 mV pulse was 42% larger than that with a +10 mV pulse and it also had a slightly longer t_{pk}. As for 10 mM Na^+ dialysis, cells dialysed with the K^+based, 20 mM Na^+ solution typically exhibited repetitive phasic contractions during long depolarizations (to potentials more positive than +40 mV), which were superimposed on a slow tonic contraction. The mean voltage dependence of phasic contraction and $I_{Ca,L}$ ($n = 4$ cells) for K^+-based, 20 mM Na^+ is displayed in Fig. 4*B* and the large difference between these two relations is clear. $I_{Ca,L}$ had a bell-shaped voltage dependence with a maximum between 0 and +20 mV, whereas the amplitude of phasic contraction increased progressively as the potentials became more positive. Phasic contraction amplitude elicited by pulse potentials between +60 and +100 mV was significantly larger than the phasic contraction elicited by a +20 mV pulse ($P < 0.005$).

Dialysing [Na^+] and contraction amplitude

Since Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange is expected to be (steeply) dependent on the intracellular Na^+ level (Miura & Kimura, 1989), dialysing [Na^+] may influence the triggering of SR release by reverse exchange. However, in addition, the level of dialysing [Na^+] might also be anticipated to alter the general Ca^{2+} loading status of the cell. As internal Na^+ is increased, the reversal potential of Na^+–Ca^{2+} exchange becomes more negative and this will have the effect of reducing steady-state forward exchange (Ca^{2+} extrusion) and correspondingly increasing steady-state reverse exchange (Ca^{2+} entry). The final result is predicted to be a higher Ca^{2+} loading of the cell and this might also be translated into a greater Ca^{2+} loading of intracellular stores.

Simplistically, it might be expected that the level of cellular Ca^{2+} loading will be reflected in the steady-state contraction amplitude of myocytes, since a greater loading of the SR Ca^{2+} store should lead to a larger Ca_i^{2+} transient and increased phasic contraction. To determine this, the steady-state contraction amplitude of each myocyte produced by trains of conditioning pulses from −40 to +10 mV was measured 240 s after obtaining whole-cell recording conditions and the results are shown to the left of Fig. 5*A*. For thirteen cells dialysed with the K^+-based, 0 Na^+ solution, mean contraction amplitude was 15·67 ± 1·82 μm. For eleven cells dialysed with 10 mM Na^+, mean contraction amplitude was 17·23 ± 2·32 μm and there was no significant difference ($P > 0.05$) for phasic contraction amplitude between 0 Na^+ and 10 mM Na^+. However, the seven cells dialysed with 20 mM Na^+ had a mean contraction amplitude of 23·73 ± 2·25 μm; this was significantly larger than the contraction of cells dialysed with 10 mM Na^+ ($P < 0.05$). These results would seem to indicate that at least for the K^+, 0 Na^+ and for the K^+, 10 mM Na^+ dialysis solutions

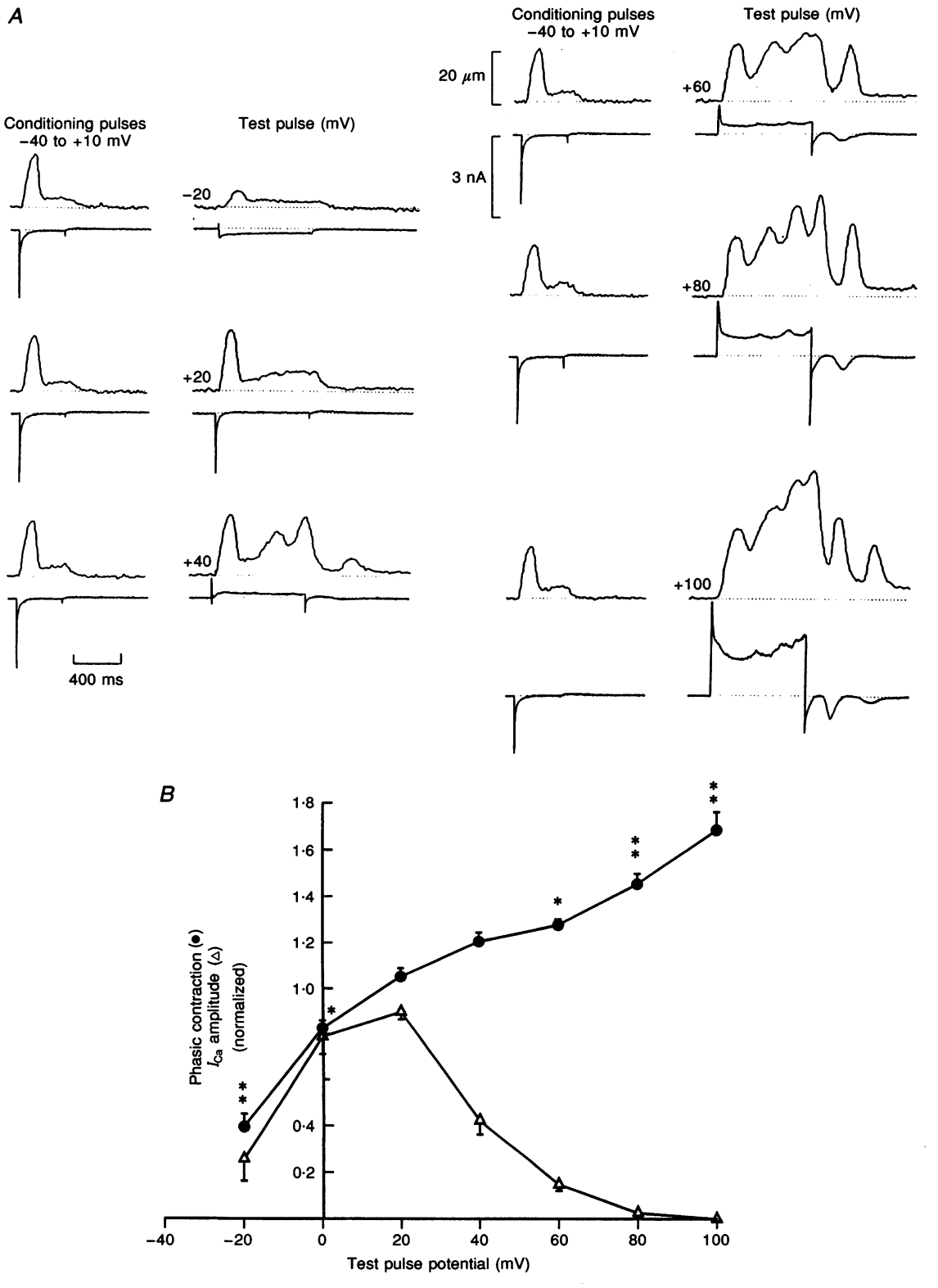

Figure 4

A, typical contractions and membrane currents elicited at different potentials in a cell dialysed with K^+-based, 20 mM Na^+ pipette solution. *B*, mean voltage dependence of contraction and $I_{Ca,L}$ for 4 myocytes dialysed with K^+-based, 20 mM Na^+ solution. Significance is shown in comparison with the +10 mV pulse.

there appears to be a similar level of cellular Ca^{2+} loading, whereas myocytes dialysed with the K^+, 20 mM Na^+ solution have an increased level of Ca^{2+} loading.

Effect of dialysis with a Cs^+-based pipette solution on contraction amplitude

To record the amplitude of $I_{Ca,L}$ selectively with minimal interference from outward K^+ currents, further experiments were carried out with a Cs^+-based pipette solution (see Methods). Cells were dialysed with Cs^+-based solutions which contained either 0 or 10 mM Na^+. Somewhat unexpectedly, we found that dialysis with Cs^+ had a consistent and marked effect on the amplitude of steady-state phasic contraction (to the right of Fig. 5*A*). The steady-state amplitude of contraction with trains of conditioning pulses was once again measured 240 s after attaining the whole-cell configuration. When the Cs^+-based dialysing solution contained 0 Na^+, we found in ten cells that the mean amplitude of contraction was $10{\cdot}2 \pm 1{\cdot}93$ μm. The difference between this and the larger phasic contraction with a K^+-based, 0 Na^+ dialysis solution was highly significant ($P < 0{\cdot}005$). Nine cells were dialysed with a Cs^+-based, 10 mM Na^+ solution and their mean amplitude of contraction was $10{\cdot}36 \pm 2{\cdot}21$ μm. This was also significantly smaller ($P < 0{\cdot}005$) than the mean contraction obtained in eleven cells with the corresponding K^+, 10 mM Na^+ dialysis solution. There was no significant difference in phasic contraction amplitude for the Cs^+-based solutions between 0 and 10 mM Na^+ ($P > 0{\cdot}05$).

The contraction amplitude of cells declines more rapidly with internal Cs^+ dialysis

We also observed that contraction amplitude declined more rapidly when cells were dialysed with a Cs^+-based solution. This was quantified by plotting the steady-state phasic contraction produced by conditioning pulses 400 s after whole-cell access as a proportion of the contraction produced by the same pulses at the start (Fig. 5*B*). For cells dialysed with a K^+-based solution containing either 0 or 10 mM Na^+, contraction amplitude declined to only $88{\cdot}6 \pm 11{\cdot}3\%$ ($n = 13$) and $90{\cdot}1 \pm 5{\cdot}2\%$ ($n = 11$), respectively, during the first 400 s of recording. This did not represent a significant decline during this period ($P > 0{\cdot}05$). However, the decline in phasic contraction observed in cells dialysed with a Cs^+-based solution was very much greater. For ten cells dialysed with a Cs^+-based, 0 Na^+ solution contraction declined to $47{\cdot}5 \pm 7{\cdot}7\%$ over the first 400 s. For eight cells dialysed with a Cs^+-based, 10 mM Na^+ solution contraction declined to $43 \pm 6{\cdot}5\%$ over the first 400 s. The difference in contraction decline between the K^+- and Cs^+-based pipette solutions was highly significant ($P < 0{\cdot}005$). Thus, dialysis with a Cs^+-based solution caused a very substantial and quite rapid decline in the amplitude of phasic contraction.

The voltage dependence of phasic contraction in cells dialysed with Cs^+-based, 0 Na^+ solution

A typical recording obtained from a myocyte dialysed with the Cs^+-based, 0 Na^+ solution is displayed in Fig. 6*A*. The

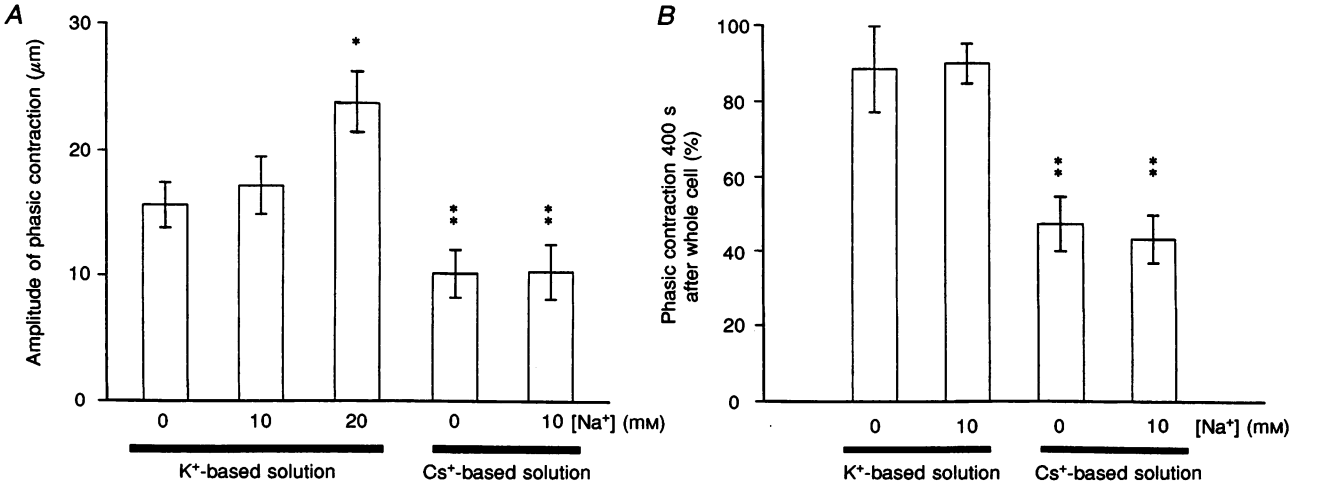

Figure 5

A, mean amplitudes of phasic contraction for myocytes dialysed with different pipette solutions. Contraction amplitude was measured 240 s after rupturing the seal and obtaining whole-cell access and was measured as the steady-state contraction elicited by conditioning pulse trains. Significance is shown in comparison with the contraction amplitude with K^+, 10 mM Na^+ dialysis solution. *B*, decline in phasic contraction amplitude with different internal dialysis solutions. The steady-state amplitude of phasic contraction 400 s after attaining whole-cell conditions was plotted as a percentage of the steady-state contraction just after attaining whole-cell access. There was no significant decline in contraction for the two K^+-based pipette solutions. The Cs^+-based pipette solution showed a much larger decline over the same time period and there was a highly significant difference ($P < 0{\cdot}005$) in decline of contraction between K^+- and Cs^+-based solutions.

amplitude of $I_{Ca,L}$ was maximal with a pulse to +10 mV and phasic contraction was also near-maximal at this potential. As pulse potential became more positive, both the amplitude of $I_{Ca,L}$ and phasic contraction declined. With a pulse to +60 mV, there was a small net inward $I_{Ca,L}$ accompanied by a relatively small phasic contraction. At +100 mV, there was no detectable net inward $I_{Ca,L}$ and very little (if any) phasic contraction. Tail contractions can be seen after repolarization from +80 and +100 mV back to a V_h of −40 mV (similar to those observed with the K^+-based, 0 Na^+ solution). However, one consistent difference between the Cs^+- and K^+-based solutions is that whereas a small phasic contraction still remained with the K^+, 0 Na^+ solution at +100 mV, no phasic contraction remained with the Cs^+, 0 Na^+ solution at this potential.

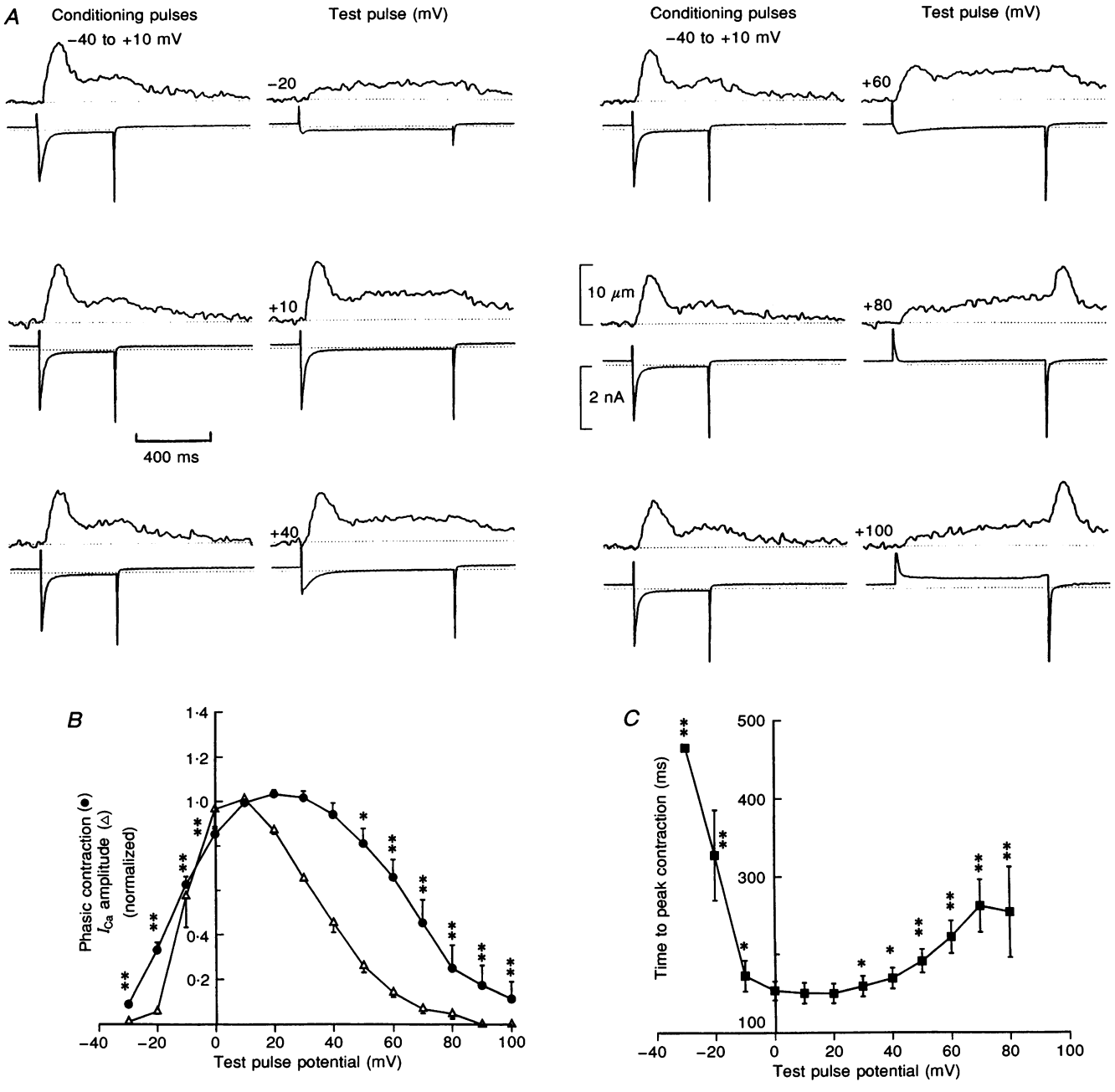

Figure 6

A, contractions and membrane currents in a myocyte dialysed with Cs^+-based, 0 Na^+ pipette solution. *B*, mean voltage dependence of contraction and $I_{Ca,L}$ for eight myocytes dialysed with Cs^+-based, 0 Na^+ solution. Significance is shown in comparison with the +10 mV pulse. *C*, mean voltage dependence of t_{pk} contraction for cells dialysed with Cs^+-based, 0 Na^+ pipette solution ($n = 7$ cells). Significance is shown in comparison with the +10 mV pulse.

The mean voltage dependence of $I_{Ca,L}$ and phasic contraction ($n = 8$ cells) for the Cs^+-based, 0 Na^+ solution is shown in Fig. 6*B*. There was no significant difference in the voltage dependence of $I_{Ca,L}$ between Cs^+- and K^+-based, 0 Na^+ solutions ($P > 0{\cdot}05$). With the Cs^+-based, 0 Na^+ solution, there was a good correspondence between $I_{Ca,L}$ and phasic contraction up to +10 mV. Phasic contraction became maximal between +10 and +40 mV; there was no significant difference in contraction amplitude between each of these potentials ($P > 0{\cdot}05$). In contrast, however, there was a very significant and substantial decline in the amplitude of $I_{Ca,L}$ between +10 and +40 mV. At potentials

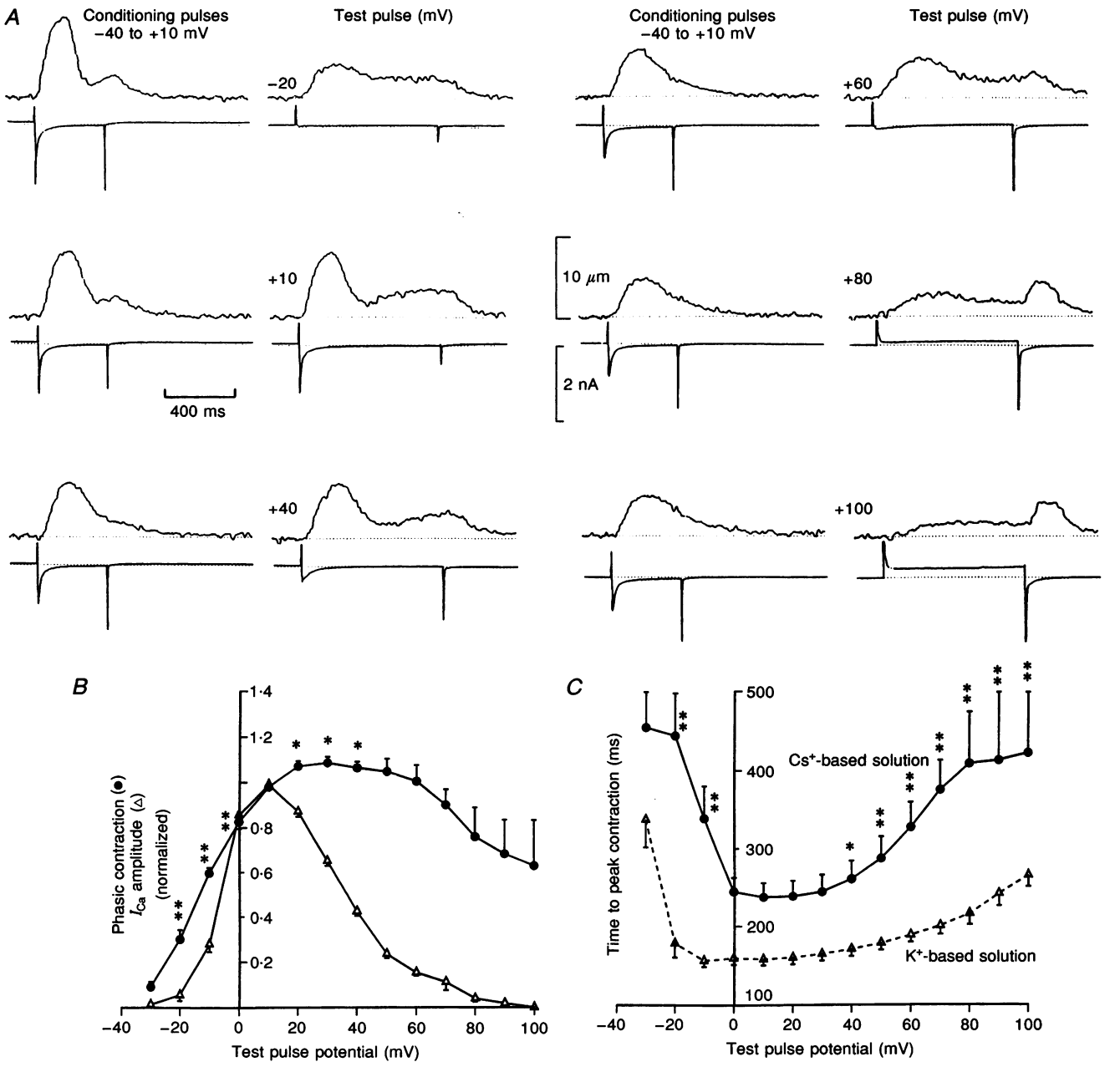

Figure 7

A, typical contractions and membrane currents of a myocyte dialysed with Cs^+-based, 10 mM Na^+ patch pipette solution. *B*, mean voltage dependence of contraction and $I_{Ca,L}$ for 7 myocytes dialysed with Cs^+-based, 10 mM Na^+ solution. Significance is shown in comparison with the +10 mV pulse. *C*, mean voltage dependence of t_{pk} contraction for 7 cells dialysed with Cs^+-based, 10 mM Na^+ solution. Voltage dependence of t_{pk} contraction for K^+-based, 10 mM Na^+ solution is plotted on the same axes to give a comparison. Significance is shown in comparison with the +10 mV pulse.

more positive than +50 mV, phasic contraction became significantly smaller than that at +10 mV ($P < 0.005$) and $I_{Ca,L}$ also declined further.

Figure 6*C* displays the mean voltage dependence of t_{pk} for cells dialysed with the Cs^+-based, 0 Na^+ solution. As for previous dialysis conditions, t_{pk} had a U-shaped relation with test potential. The time course of phasic contraction could only be measured up to +80 mV, since at more positive potentials than this, there was little measurable phasic component. The value of t_{pk} was minimal between 0 and +20 mV and became significantly longer at more positive and negative potentials ($P < 0.05$ and 0.005, respectively). There was no significant difference in the voltage dependence of t_{pk} between the Cs^+, 0 Na^+ and the K^+, 0 Na^+ pipette solutions ($P > 0.05$).

Voltage dependence of phasic contraction with a Cs^+-based dialysis solution containing 10 mM Na^+

A representative recording from a myocyte dialysed with Cs^+-based, 10 mM Na^+ pipette solution is shown in Fig. 7*A*. Under these conditions, it was found consistently that phasic contraction elicited by successive trains of conditioning pulses declined even during a single run of the pulse sequence (compare upper with lower traces). At the same time t_{pk} of the phasic contraction became longer. The amplitude of phasic contraction elicited by a test pulse was near maximal with a +10 mV pulse and declined at more positive potentials as $I_{Ca,L}$ became smaller. It can be observed that the t_{pk} of contraction also appeared to lengthen as pulse potential became more positive. However, since t_{pk} of the conditioning pulses was also lengthening at the same time, this might be partly due to a time-dependent as well as a voltage-dependent effect.

A number of the characteristics of contraction with the Cs^+-based, 10 mM Na^+ dialysis solution were quite different from those observed with the corresponding K^+-based solution. With the K^+-based solution there were always repetitive phasic contractions during the 800 ms test depolarizations to positive potentials (e.g. Fig. 3*A*) and these were never observed in any of the cells dialysed with the Cs^+-based, 10 mM Na^+ solution. It can also be seen that with the Cs^+-based solution there were repolarization tail contractions at the end of the +80 and +100 mV pulses, and these were not seen with the corresponding K^+-based solution.

The mean voltage dependence for $I_{Ca,L}$ and phasic contraction ($n = 8$ cells) with Cs^+, 10 mM Na^+ dialysis are shown in Fig. 7*B*. As for previous plots, both $I_{Ca,L}$ and phasic contraction elicited by the test pulse are shown normalized to the $I_{Ca,L}$ and contraction of the conditioning pulse immediately before. This was particularly important for the Cs^+-based, 10 mM Na^+ solution because of the very substantial decline in steady-state phasic contraction even during a single run of the pulse sequence. The voltage dependence of $I_{Ca,L}$ was bell-shaped as expected and there was no significant difference in the voltage dependence of $I_{Ca,L}$ between the Cs^+- and K^+-based, 10 mM Na^+ dialysis solutions ($P > 0.1$). There was a good correspondence between $I_{Ca,L}$ and phasic contraction up to +10 mV, but they diverged at more positive potentials. Phasic contraction amplitude between +20 and +50 mV was significantly larger than contraction at +10 mV ($P < 0.05$), and yet over this same range there was a substantial decline in $I_{Ca,L}$ amplitude. For potentials between +50 and +100 mV, although there was a trend towards smaller contraction amplitude, there was no significant difference between the phasic contraction over this potential range and that at +10 mV ($P > 0.05$). Over this same range the amplitude of $I_{Ca,L}$ became a small fraction of its value at +10 mV.

Dialysis with the Cs^+-based, 10 mM Na^+ solution had an unexpected and marked effect on t_{pk} of phasic contraction. This is illustrated in Fig. 7*C*, where the mean voltage dependence of t_{pk} for the Cs^+, 10 mM Na^+ solution ($n = 7$ cells) is compared with that for the corresponding K^+-based solution. For each pulse potential level, t_{pk} with the Cs^+-based solution was between 80 and 180 ms longer than that for the K^+-based solution. However, the t_{pk} relation still retained the usual U-shape, with a minimum value of t_{pk} between 0 and +30 mV. At more negative and positive potentials, t_{pk} became significantly longer ($P < 0.05$ and 0.005, respectively). Thus, it appeared that dialysis with this particular solution had a peculiar effect of slowing the time course of phasic contraction at all test potentials.

The effect of dialysing [Na^+] on the voltage dependence of contraction

The plots in Fig. 8*A* and *B* use the data presented in previous figures for each dialysis solution to illustrate the effect of the level of pipette [Na^+] on the voltage dependence of phasic contraction. Figure 8*A* shows, for the K^+-based pipette solution, the difference in voltage dependence of contraction between 0 and 10 mM Na^+ dialysis. (The plot for 20 mM Na^+ dialysis would be further elevated than that for 10 mM Na^+, cf. Fig. 4*B*.) It is clear that dialysing [Na^+] had a marked effect on the voltage dependence of contraction. Although there was similarity between the two relations for potentials up to +30 mV, at more positive potential values they diverged. At +50 mV, normalized phasic contraction with 10 mM Na^+ in the pipette became significantly larger than for 0 Na^+ ($P < 0.05$) and over the range +60 to +100 mV the difference was highly significant ($P < 0.005$). The inset of Fig. 8*A* shows

the difference between 0 and 10 mM Na^+ mean relations, plotted against potential. As potential became more positive than +30 mV, the difference between 0 and 10 mM Na^+ increased progressively in an approximately exponential fashion.

Figure 8*B* shows a similar plot for the two Cs^+-based pipette solutions. The voltage dependence for 10 mM Na^+ is different from 0 Na^+. There was similarity between the two relations up to +30 mV, but divergence at more positive potentials. At +50 mV, the contraction with 10 mM Na^+ was significantly larger than for 0 Na^+ ($P < 0{\cdot}05$) and at more positive potentials than +50 mV the difference became highly significant ($P < 0{\cdot}005$). The inset of Fig. 8*B* shows the difference between 0 and 10 mM Na^+ relations, demonstrating their divergence at potentials more positive than +30 mV.

The effect of dialysis with internal Cs^+ on the voltage dependence of phasic contraction

In Fig. 9*A* and *B*, the effect of replacing K^+ with Cs^+ in the dialysis solution on the voltage dependence of phasic contraction is examined for two different levels of dialysing Na^+. The effect of Cs^+ dialysis in the presence of 10 mM Na^+ is shown in Fig. 9*A*. There was good correspondence between the K^+- and Cs^+-based solutions up to +50 mV, but the voltage dependence diverged at more positive potentials with the relation for the Cs^+-based solution declining, whilst that for the K^+-based solution did not decline. Although the clear trend was for the relations to diverge at positive potentials, the difference was only statistically significant for potentials of +90 and +100 mV ($P < 0{\cdot}05$).

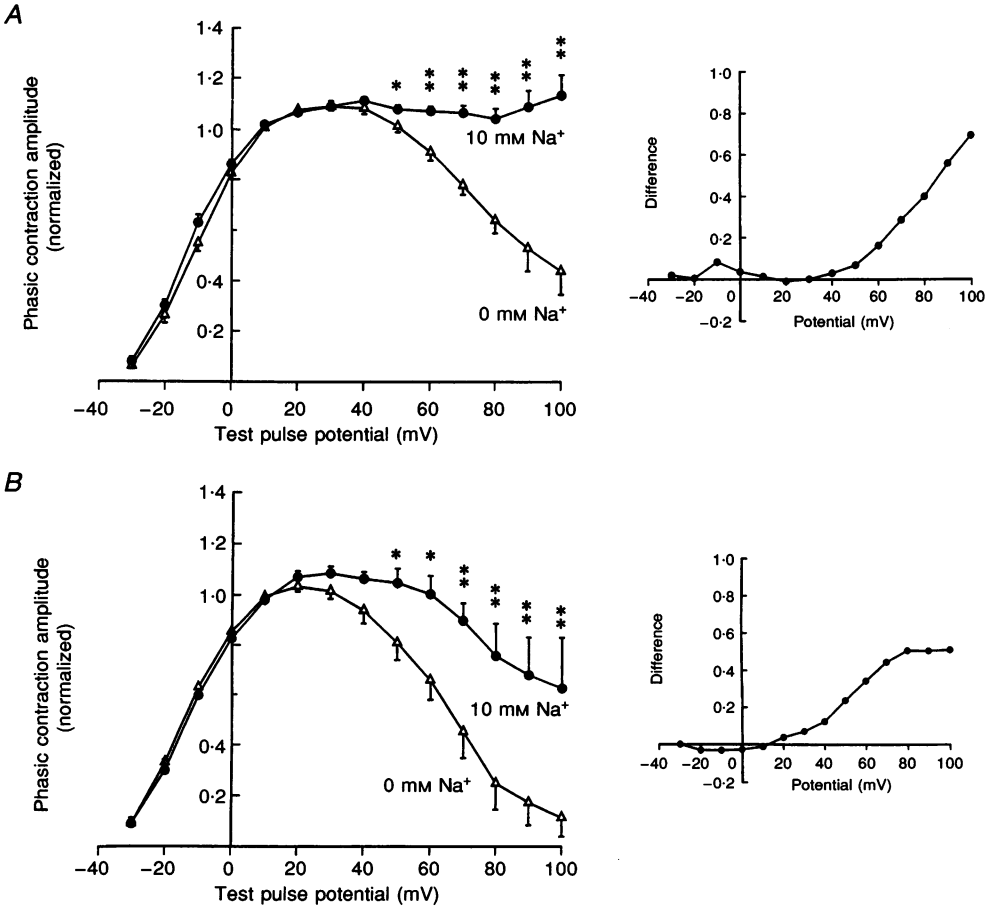

Figure 8

A, the effect of increasing dialysing [Na^+] from 0 to 10 mM on the voltage dependence of contraction; K^+-based pipette solution. The mean voltage dependence of contraction for K^+-based, 0 Na^+ and K^+-based, 10 mM Na^+ are plotted on the same axes for comparison. Significance tests were carried out at each potential level. The inset shows the difference between these two relations (normalized phasic contraction 10 − 0 mM Na_i^+) plotted against membrane potential. *B*, the effect of increasing dialysing [Na^+] with a Cs^+-based pipette solution. The mean voltage dependence of contraction for Cs^+-based, 0 Na^+ and Cs^+-based, 10 mM Na^+ are plotted on the same axes for comparison. Significance tests were carried out at each potential level. The inset shows the difference between these two relations plotted against membrane potential.

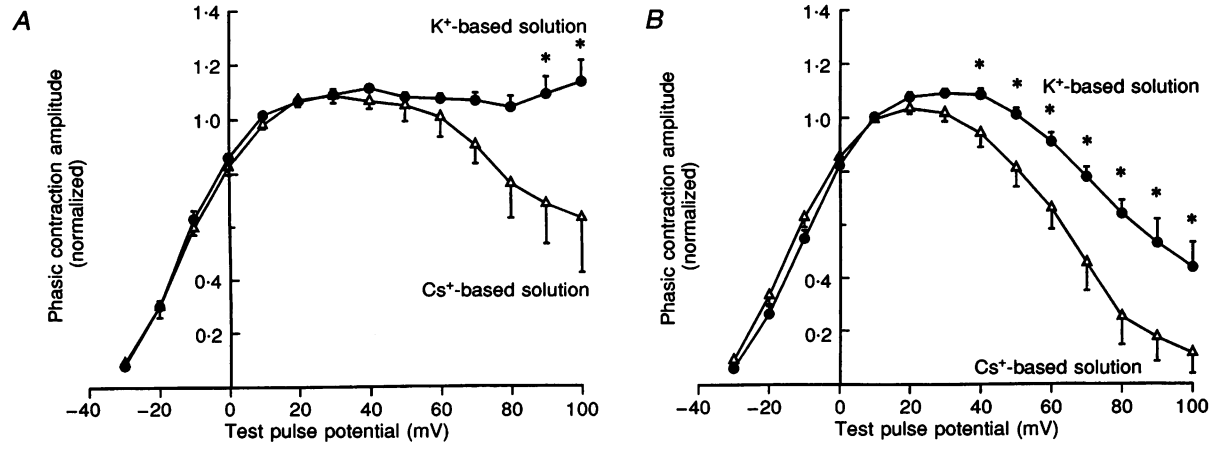

Figure 9

A, the effect of replacing K^+ with Cs^+ in the dialysis solution on the voltage dependence of contraction, in the presence of 10 mM Na^+ dialysis. The mean voltage dependence of contraction for K^+-based, 10 mM Na^+ and Cs^+-based, 10 mM Na^+ are plotted on the same axes for comparison. Significance tests were carried out at each potential level. *B*, the effect of replacing K^+ with Cs^+ in the dialysis solution on the voltage dependence of contraction, in the presence of 0 Na^+ dialysis. The mean voltage dependence of contraction for K^+-based, 0 Na^+ and Cs^+-based, 0 Na^+ are plotted on the same axes for comparison. Significance tests were carried out at each potential level.

Figure 9*B* shows the effect of Cs^+ dialysis on the voltage dependence of contraction in the presence of the 0 Na^+ solution. There was close agreement between the two relations up to +20 mV, but they diverged at more positive potentials with the K^+-based relation declining less steeply than the Cs^+-based relation. At potentials between +40 and +100 mV, normalized phasic contraction with the K^+-based solution was significantly larger than for the Cs^+-based solution ($P < 0{\cdot}05$). The results displayed in Fig. 9*A* and *B* show that replacing K^+ with Cs^+ in the dialysis solution had a clear effect on voltage dependence of phasic contraction, and for either 0 or 10 mM Na^+ tended to make the relation more bell-shaped.

DISCUSSION

There are four main conclusions from this study. First, in rabbit myocytes at 35 °C, the voltage dependence of phasic contraction (largely due to SR release) is altered by replacing 80% of the K^+ in the dialysis solution by Cs^+. In the presence of internal Cs^+, the voltage dependence of phasic contraction became more bell-shaped and more similar to $I_{Ca,L}$. Second, phasic contraction had a different voltage dependence from $I_{Ca,L}$; in fact, the voltage dependence of phasic contraction was not identical to the voltage dependence of $I_{Ca,L}$ with any of the internal dialysis solutions used. Third, the voltage dependence of phasic contraction appears to be modulated by dialysing $[Na^+]$ over the tested range, 0–20 mM, regardless of whether K^+ or Cs^+ was the main dialysing cation. Fourth, t_{pk} of the phasic contraction had a characteristically U-shaped voltage dependence which was similar for all the dialysis conditions employed. The latter three conclusions are consistent with some previous studies which have investigated the voltage dependence of phasic contraction and its modulation by dialysing $[Na^+]$ (Nuss & Houser, 1992; Vornanen *et al.* 1994) but are inconsistent with others which report that voltage dependence of phasic contraction is similar to $I_{Ca,L}$ (London & Krueger, 1986; Cleemann & Morad, 1991) or that it is not modulated by dialysing $[Na^+]$ (Bouchard, Clark & Giles, 1993*b*).

Phasic contraction has been shown previously to be due predominantly to Ca^{2+} release from the SR (Cleemann & Morad, 1991; Bers, 1991; Bassani *et al.* 1993). This was confirmed under the conditions of this study by showing that the phasic contraction at all test potentials was largely (88%) abolished by ryanodine–thapsigargin. However, it is worth pointing out that phasic contraction might not reflect the voltage dependence of the Ca_i^{2+} transient in a precise way, since it is well known that the relation between contraction and Ca_i^{2+} is non-linear (Allen & Kentish, 1985). The voltage dependence of phasic contraction should, however, have a broadly similar shape to voltage dependence of the Ca_i^{2+} transient. The non-linearity may then produce a different slope over which phasic contraction and the transient decline over the negative and positive potential ranges.

The effect of dialysing [Na^+] on the voltage dependence of contraction

Rabbit myocytes were dialysed with a 0 Na^+ pipette solution in order to deplete internal Na^+ and so attenuate any trigger influence of reverse Na^+–Ca^{2+} exchange on SR Ca^{2+} release. If $I_{Ca,L}$ is the only remaining SR trigger in the absence of reverse exchange, then it might be anticipated that the voltage dependence of phasic contraction would become similar to $I_{Ca,L}$. However, in cells dialysed with a K^+-based, 0 Na^+ solution we did not find close agreement between the voltage dependence of contraction and $I_{Ca,L}$. Even at +80 and +100 mV, a small phasic contraction still persisted and at these potentials there is expected to be relatively less Ca^{2+} entry via $I_{Ca,L}$, since these potentials approach the predicted E_{Ca} ($\sim$ +130 mV). In addition, phasic contraction declined less steeply at positive potentials than did $I_{Ca,L}$. There are two possible explanations for these data. First, dialysis with the 0 Na^+ solution may not have completely depleted the Na^+ level directly beneath the sarcolemma (Carmeliet, 1992). Since it is the [Na^+] in this region which directly activates the Na^+–Ca^{2+} exchange, if residual [Na^+] remained under the cell membrane, this might allow some triggering by reverse exchange to be maintained, even with Na^+-free dialysis, and might account for why phasic contraction did not have the same voltage dependence as $I_{Ca,L}$. However, the fact that the voltage dependence of contraction with 0 Na^+ was different from that with 10 mM Na^+ dialysis would seem to suggest that pipette dialysis was able to influence sub-sarcolemmal [Na^+], even if it was not able to precisely control [Na^+] in this region. The second possibility is that there might have been a small residual Ca^{2+} entry via the L-type Ca^{2+} channel at +80 and +100 mV, even though there was no net inward $I_{Ca,L}$ at these potentials. The L-type Ca^{2+} channel is known to have conductance for both K^+ and Cs^+ ions (Matsuda & Noma, 1984) and net $I_{Ca,L}$ reverses at a less positive potential than E_{Ca} because inward Ca^{2+} flux is opposed by outward K^+ or Cs^+ flux through the channel. Therefore, at +100 mV, there may still be a small trigger Ca^{2+} entry via the L-type Ca^{2+} channel (since +100 mV is less positive than E_{Ca}), even though net $I_{Ca,L}$ is outward due to K^+ or Cs^+ efflux being larger than Ca^{2+} entry. Even considering this, however, there was still a large quantitative discrepancy between the phasic contraction remaining at +80 and 100 mV and Ca^{2+} entry via $I_{Ca,L}$. Thermodynamic calculations (Stern, 1992) indicate that at +80 mV, Ca^{2+} entry via $I_{Ca,L}$ is only 3% of that at +10 mV and yet a mean of 64% of maximal phasic contraction was elicited by a +80 mV pulse in cells dialysed with K^+-based, 0 Na^+ solution (Fig. 2*A*). SR Ca^{2+} release and the resulting phasic contraction is expected to be graded with the magnitude of trigger Ca^{2+} entry (Cannell, Berlin & Lederer, 1987). Therefore this large quantitative discrepancy would seem to indicate the possible existence of another SR trigger mechanism, at least with pulses to positive potentials.

The voltage dependence of phasic contraction in rabbit myocytes became strikingly different when 10 mM Na^+ was added to the K^+-based dialysis solution. With test pulses to potentials more positive than +40 mV, normalized phasic contraction was elevated with 10 mM Na^+ dialysis. There are two main possibilities which might account for this. First, higher internal Na^+ is expected to increase the degree to which Na^+–Ca^{2+} exchange reverses with a given depolarization and this might allow it to become a more influential trigger of SR Ca^{2+} release. Second, dialysis with higher [Na^+] might cause a greater general cellular Ca^{2+} loading and in particular a higher Ca^{2+} loading of the SR. If the SR is Ca^{2+} loaded to a greater extent, then it is possible that it may become more sensitive to trigger Ca^{2+} entry and this may affect the voltage dependence of phasic contraction (Cheng *et al.* 1993; Han, Schiefer & Isenberg, 1994). However, increased SR Ca^{2+} loading would also be expected to cause a larger steady-state contraction amplitude. It is relevant that we found no significant difference between the steady-state phasic contraction amplitude of myocytes dialysed with 0 or 10 mM Na^+ (similar results were found both for K^+- and Cs^+-based dialysis solutions, Fig. 5*A*). This suggests either an absence or only a small increase in the SR Ca^{2+} load of cells dialysed with 10 mM Na^+ and tends to indicate that this might have played a relatively minor role in the change of voltage dependence of phasic contraction with 10 mM Na^+.

Dialysis with the K^+-based, 20 mM Na^+ solution produced a further change in voltage dependence of phasic contraction. Instead of contraction amplitude reaching a plateau between +20 and +100 mV, as for the K^+, 10 mM Na^+ dialysis, the amplitude of phasic contraction increased progressively with more positive depolarizations. In addition, however, the amplitude of steady-state phasic contraction was increased in cells dialysed with K^+, 20 mM Na^+ and this would seem to reflect a higher cellular Ca^{2+} and SR loading with 20 mM Na^+ dialysis. Thus the change in voltage dependence of phasic contraction may also have been influenced by an altered sensitivity to trigger Ca^{2+} entry of a more highly loaded SR, as well as an enhanced SR triggering by reverse exchange. However, it is not entirely clear how an altered sensitivity of CICR alone might produce phasic contractions which increase with greater depolarizations. If the only SR trigger mechanism is $I_{Ca,L}$, there will be relatively little trigger Ca^{2+} entry with a pulse to +100 mV (see previous discussion). Yet when cells were dialysed with 20 mM Na^+, the phasic contraction with +100 mV was significantly larger than with a pulse to +10 mV. The simplest explanation for this result would seem to be a mechanism which is able to generate a larger trigger Ca^{2+} entry with greater depolarization, and the

voltage-dependent properties of reverse Na^+–Ca^{2+} exchange are consistent with such behaviour.

The effect of internal Cs^+ on phasic contraction

Dialysis of rabbit myocytes with an 80% Cs^+ : 20% K^+ pipette solution had an unexpected and marked effect on contraction characteristics. The phasic contraction amplitude of cells dialysed with Cs^+-based solution fell to between 40 and 45% of their starting levels within 400 s after attaining whole-cell access. (There was no significant decline of phasic contraction in cells dialysed with a 100% K^+ solution.) This rapid decline resulted in the phasic contraction of Cs^+-dialysed cells being much smaller when measured 240 s after attaining the whole-cell configuration. In fact, we frequently found that the phasic contraction of Cs^+-dialysed cells had become almost abolished after 10 min of dialysis. In contrast, this was very rarely observed in K^+-dialysed cells. Dialysis with Cs^+ also changed other contractile features; the voltage dependence of phasic contraction became more bell-shaped in cells dialysed with a Cs^+ solution and this occurred independently of the dialysing [Na^+]. Repetitive phasic contractions during a prolonged positive depolarization were never observed in Cs^+, 10 mM Na^+-dialysed cells. In myocytes dialysed with K^+, 10 mM Na^+, the level of tonic contraction during the test pulse always increased progressively with greater depolarization, consistent with previous data for myocytes and multicellular preparations (see Fig. 3*A*; and Eisner *et al.* 1981). In myocytes dialysed with Cs^+, 10 mM Na^+, tonic contraction appeared to be less dependent on potential and sometimes showed little dependence at all (cf. Fig. 7*A*). A unique feature of Cs^+, 10 mM Na^+ dialysis was that t_{pk} phasic contraction became significantly lengthened for all values of membrane potential. This was not a consequence of Cs^+ dialysis itself since it was not observed with Cs^+, 0 Na^+ dialysis. Instead, it appeared to depend on the presence of both Cs^+ and Na^+ together in the dialysis solution.

A number of possibilities exist which might account for the marked effect of internal Cs^+ on excitation–contraction coupling. One mechanism is that Cs^+ might reduce myofilament Ca^{2+} sensitivity. However Cs^+ is not known to have an effect on the myofilaments and a previous study which measured contraction and Ca_i^{2+} transient simultaneously did not report that Cs^+ dialysis changed the relation between Ca_i^{2+} and contraction (Cleemann & Morad, 1991). In any case, a change of myofilament Ca^{2+} sensitivity would seem unlikely to account for the altered voltage dependence of contraction with Cs^+ dialysis. Han *et al.* (1994) commented briefly in the Methods section of their paper that they found Cs^+ dialysis to depress the amplitude of Ca_i^{2+} transients by 50% in guinea-pig cells. This suggests that the effect of Cs^+ might be on SR release rather than the myofilaments. In addition, there are known effects of Cs^+ on the SR which might account for the findings we have observed. It seems likely that each large Ca^{2+} release from the SR into the cytoplasm will be accompanied by compensatory charge movement back into the SR in order that a large intra-SR potential does not develop with each Ca^{2+} release (Williams, 1992). The SR membrane is known to contain K^+ channels and these provide one route for this charge compensation, so that Ca^{2+} release under normal conditions might be expected to be accompanied by simultaneous K^+ uptake into the SR. However, the SR K^+ channels have been shown to have a very low conductance for Cs^+ (Cukierman *et al.* 1985). Thus when myocytes are dialysed with Cs^+, it is possible that the much lower Cs^+ flux through these channels might become inadequate and provide insufficient charge compensation. A progressively increasing negative potential might be expected to develop across the SR membrane with each Ca^{2+} release, and this will tend to inhibit further Ca^{2+} release. This can account for the decline in phasic contraction (and presumably Ca_i^{2+} transient; Han *et al.* 1994) observed with Cs^+ dialysis, but it is less easy to conceive how this mechanism might be responsible for the altered voltage dependence of phasic contraction with Cs^+, or for the absence of repetitive phasic contractions during long pulses to positive potentials.

The effects of Cs^+ on excitation–contraction coupling may have relevance for a number of previous studies which have used a Cs^+-based dialysis solution (Beuckelmann & Wier, 1988; Leblanc & Hume, 1990; Cleemann & Morad, 1991; Lipp & Niggli, 1994; Lopez-Lopez *et al.* 1994; Cannell *et al.* 1995). Although we cannot be certain that Cs^+ dialysis might have a similar effect in other species, the comments of Han *et al.* (1994) do seem to indicate that the same effect is present in guinea-pig myocytes. Cs^+ dialysis makes the voltage dependence of phasic contraction more bell-shaped, and thus appear as if it depends more on SR triggering via $I_{Ca,L}$ and less on possible SR triggering due to Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange.

Dependence of t_{pk} phasic contraction on pulse potential

Rabbit myocytes showed a U-shaped dependence of t_{pk} phasic contraction regardless of whether they were dialysed with K^+ or Cs^+, or whether Na^+ was present in the pipette solution. t_{pk} was smallest over the voltage range where the amplitude of $I_{Ca,L}$ was maximal and at more negative and positive potentials t_{pk} became larger. A similar voltage dependence of t_{pk} was also reported by Vornanen *et al.* (1994). In addition, it seems relevant that at least for K^+-based dialysis solutions, the voltage dependence of t_{pk} was not modulated by adding 10 mM Na^+ to the dialysis solution. This would seem to indicate that the degree of activation of reverse Na^+–Ca^{2+} exchange might not contribute to the voltage dependence of t_{pk} and that it might be governed primarily by the kinetics and voltage

dependence of $I_{Ca,L}$. Recent studies using the confocal microscope to detect local Ca_i^{2+} transients (Ca^{2+} sparks; Cheng *et al.* 1993; López-López *et al.* 1994; Cannell *et al.* 1995) might provide a possible mechanism for the voltage dependence of t_{pk}. López-López *et al.* (1995) have shown that the probability of evoking a local Ca_i^{2+} transient is highest at +10 mV (where macroscopic $I_{Ca,L}$ is greatest) and the probability reduces at more negative and positive potentials. The lower probability of local Ca_i^{2+} transients negative to +10 mV may be related to the lower probability for stochastic opening of L-type Ca^{2+} channels over this range, whereas the lower probability for local Ca_i^{2+} transients at positive potentials may be related to smaller unitary L-type Ca^{2+} channel currents as the potential approaches E_{Ca} (López-López *et al.* 1994). Therefore, it is possible that a smaller number of individual Ca^{2+} release units might become activated with a longer latency at negative and positive potentials and this might lead to a slowed rate of rise of the Ca_i^{2+} transient and a slowed rate of phasic contraction over these potential ranges.

However, it is worth noting that one piece of data does not appear to be entirely consistent with this hypothesis. A complete and rapid block of $I_{Ca,L}$ by high-dose nifedipine did not lengthen the t_{pk} phasic contraction in guinea-pig myocytes (Levi *et al.* 1994) and if $I_{Ca,L}$ is the major determinant of t_{pk} contraction, then it would have been expected to become lengthened. Similar results on the Ca_i^{2+} transient, showing that rapid application of nifedipine does not lengthen t_{pk} of the transient, have also been obtained recently (A. J. Levi, unpublished observations). It is not clear at present how to account for these results. However, it is possible that, with a sufficient level of internal Na^+ and a normal SR Ca^{2+} content, reverse Na^+–Ca^{2+} exchange occurring at the start of depolarization might be adequate to cause a rapid activation of CICR and an unchanged rate of SR Ca^{2+} release.

Implications of these results for normal physiology?

It would seem likely that the behaviour of cells dialysed with a K^+-based solution at 35 °C might reflect normal physiology more closely, since K^+ is the normal major intracellular cation. An intracellular Na^+ level of 10 mM is probably within the normal range, especially considering that the normal heart rate of rabbits is between 3 and 4 Hz, which will produce a raised internal Na^+ compared with a rate of 0·5 Hz.

With a K^+-based, 10 mM Na^+ dialysis solution, the voltage dependence of phasic contraction was not bell-shaped and it had a quite different shape to the voltage dependence of $I_{Ca,L}$. It seems likely that there might be another SR trigger mechanism functioning under these conditions and one clear possibility is Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange. The normal cardiac action potential upstroke depolarizes the membrane rapidly to +50 or +60 mV and under normal conditions it is presumably this event which triggers the SR to release Ca^{2+}. The magnitude of trigger Ca^{2+} entry via $I_{Ca,L}$ will be smaller at +60 mV compared with +10 mV, since +60 mV approaches E_{Ca} (theoretical calculations suggest that at +60 mV, Ca^{2+} entry via $I_{Ca,L}$ is 10% of that at +10 mV). The results of this study suggest that at +60 mV, although $I_{Ca,L}$ may still be the major trigger mechanism, reverse exchange might also play a role in triggering SR release. The precise proportion of SR triggering which is carried out by either of these mechanisms, and the possible nature of any interaction between them, remains to be determined.

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