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HIGH SELECTIVITY OF CALCIUM CHANNELS IN SINGLE DIALYSED HEART CELLS OF THE GUINEA-PIG

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SUMMARY

1. Membrane currents and action potentials were recorded in single ventricular cells obtained from guinea-pig hearts by enzymatic dissociation.

2. Ca^{2+} channel currents carried by Ba^{2+} or Ca^{2+} were recorded with a suction pipette (5–10 μm diameter) for voltage clamp and internal dialysis. Currents through Na^+, K^+ and non-selective monovalent cation channels were suppressed by suitable holding potentials and external and internal solutions.

3. The dialysis method allowed exchange within minutes of alkali metal cations (e.g. Cs^+) and small molecules (e.g. quaternary derivatives of lidocaine and verapamil). Nevertheless, Ca^{2+} channels remained functional for considerable periods, typically 20 min and sometimes more than 1 h.

4. With Ba^{2+} outside and Cs^+ inside, current flow through Ca^{2+} channels changed from inward to outward at strongly positive levels beyond a clear-cut reversal potential E_{rev}. Several methods for defining E_{rev} were in close agreement: (1) zero-crossing of leak-subtracted peak current, (2) inversion of time-dependent current changes during channel activation or inactivation, (3) inversion of drug-sensitive current as defined by channel blockers such as Cd^{2+} or D-600.

5. E_{rev} varied with external Ba^{2+} or internal Cs^+. E_{rev} increased by 29 mV per 10-fold increase in Ba^{2+}. Interpreted with constant-field theory, E_{rev} values correspond to P_{Ba}/P_{Cs} of $\sim$1360.

6. With 5 mM-Ca^{2+} outside and 151 mM-Cs^+ inside, Ca^{2+} channel current reversed near +75 mV, corresponding to $P_{Ca}/P_{Cs} \sim 6000$. Earlier measurements of E_{rev} (Lee & Tsien, 1982) suggest that $P_{Ca}/P_K > 1000$.

7. At strongly positive membrane potentials where channel activation is maximal, the Ca^{2+} channel current–voltage relationship is strongly non-linear, with conductance increasing on either side of an inflexion point near E_{rev}.

8. Activation of inward or outward currents through Ca^{2+} channels follows a sigmoid time course, as expected if activation were a multi-step process.

INTRODUCTION

Ca^{2+} channels are important in the electrical activity of many excitable cells. They remain less well understood than Na^+ or K^+ channels, despite much effort over the last ten years (Reuter, 1979, 1983; Coraboeuf, 1980; Hagiwara & Byerly, 1981, 1983;

Kostyuk, 1981; McDonald, 1982; Tsien, 1983). One major obstacle has been the lack of an experimental system as suitable for studying Ca^{2+} channels as a squid axon is for studying Na^+ channels. Specific criteria for such a system were set forth by Hagiwara & Byerly (1981): (1) the preparation should have a strong Ca^{2+} current, (2) the voltage clamp should be spatially uniform and rapidly settling, (3) the composition of solutions on both sides of the membrane should be under experimental control, (4) the Ca^{2+} current should be separable from other ionic currents, (5) the reversal potential should be clearly defined if possible.

Meeting these criteria seems particularly worthwhile in the heart, where Ca^{2+} channels participate in impulse initiation, conduction, and excitation–contraction coupling and respond to modulation by neurotransmitters, hormones, and cardiotonic drugs. Progress has come with micro-electrode voltage-clamp recordings of Ca^{2+} currents in single heart cells (e.g. Isenberg & Klöckner, 1980, 1982; Powell, Terrar & Twist, 1981). In these studies, no special attempt was made to satisfy the criteria (3)–(5) as listed above. It seems particularly important to separate Ca^{2+} channel current from overlapping outward currents through other channels, as already attempted in multicellular cardiac preparations (Marban & Tsien, 1982; Kass, Scheuer & Malloy, 1982). Fortunately, this problem is amenable to newly developed suction pipette methods (Lee, Weeks, Kao, Akaike & Brown, 1979; Lee, Akaike & Brown, 1980; Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Hume & Giles, 1981; Lee & Tsien, 1982, 1983; Irisawa & Kokubun, 1983).

This paper presents experiments using the internal dialysis method of Lee *et al.* (1980) to study Ca^{2+} channels in single ventricular cells from guinea-pig heart. In an earlier paper (Lee & Tsien, 1982), we briefly described Ca^{2+} channel currents in this system with external Ca^{2+} and Na^+ and internal K^+ at near-physiological concentrations. Here we take a more biophysical approach. To minimize concern about possible ion movements through Na^+ channels, K^+ channels, Ca^{2+}-activated non-selective cation channels, or Na^+–Ca^{2+} exchange, Na^+ and K^+ ions were replaced by Cs^+ or sucrose; Ca^{2+} was also replaced by Ba^{2+} in many experiments. We carried out a number of experimental tests to establish that Ca^{2+} channel current can be studied in isolation from other time-dependent currents. The dialysis system allows relatively rapid control of the internal concentration of ions and drugs with only a slow run-down of Ca^{2+} channel current. Contrary to the view that Ca^{2+} channels are essentially one-way pathways (Kostyuk, 1981), large outward currents through Ca^{2+} channels can be recorded beyond a clearly defined reversal potential (see also Reuter & Scholz, 1977; Hino & Ochi, 1980). We find that Cs^+ moves outward through the channel with either Ba^{2+} or Ca^{2+} outside. The reversal potential responds appropriately to changes in both external and internal ion concentrations. On either side of its reversal potential, the Ca^{2+} channel current activates with a sigmoid time course and its peak amplitude increases non-linearly with driving force.

Some aspects of this work have been reported in preliminary communications (Lee, Lee & Tsien, 1981; Lee & Tsien, 1982).

METHODS

Isolation of single heart cells

Heart cells were obtained by enzymatic dispersion with zero Ca^{2+} medium, as described in detail by Powell & Twist (1976) and subsequently modified by Kao, Christman, Luh, Krauhs, Tyers & Williams (1980). We have used the method given by Kao *et al.* (1980) with some slight modifications.

Female guinea-pigs (Charles River) 200–700 g in weight were killed by cervical dislocation. The heart was quickly removed and immersed in Tyrode solution containing 1 mM-Ca^{2+} at 37 °C. The heart was mounted immediately on the cannula of a perfusion apparatus for retrograde perfusion through the aorta (Langendorff) at a pressure of 40 cmH_2O. Three different solutions were perfused sequentially for 5 min each: (1) Tyrode solution containing 1 mM-Ca^{2+}, (2) nominally Ca^{2+}-free Tyrode solution, (3) Tyrode solution containing 50 μM-Ca^{2+} plus 0·1 % collagenase (Type 1, Sigma), 0·1 % hyaluronidase (Type 1-S, Sigma), 0·1 % bovine serum albumin (fraction V, Sigma) and 20 mM-taurine (Sigma). At the end of the perfusion, the ventricles were cut away from the rest of heart, scored every 0·5 mm with a razor blade, and incubated at 37 °C in solution (3) with bovine serum albumin increased to 2 %. Every 5–10 min the ventricular tissue was placed in fresh incubation solution, while dissociated cells were collected from the previous fluid by centrifugation at 200 rev/min for 2 min. Pelleted cells were washed in nominally 1 mM-Ca^{2+} Tyrode solution containing 30 % Medium 199 (Gibco) and 10 % horse serum, then resuspended in the same solution for storage at room temperature until they were used in electrophysiological experiments.

Suction pipette method for voltage clamp and internal dialysis

The method was essentially that described by Lee, Brown and colleagues in earlier papers (Lee *et al.* 1979, 1980), with minor modifications (Lee & Tsien, 1982, 1983). In these experiments, the suction pipette used for voltage clamp and internal dialysis had a tip diameter of 5–10 μm.

Experimental solutions

External solutions were made up as follows. Tyrode solution contained (mM): NaCl, 120; KCl, 4·8; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $NaHCO_3$, 25; glucose, 5·5; variable $CaCl_2$. '0 Na^+, 0 K^+ solution' contained Cs aspartate, 85; $MgCl_2$, 2·85; variable $CaCl_2$ or $BaCl_2$; sucrose, 146; glucose, 5·5; pH 7·6 with Tris.

Internal solutions were made up as follows. 'CsF + Cs aspartate solution' contained CsF, 37; Cs aspartate, 114; K_2HPO_4, 5; glucose, 5·5; pH 7·2 with Tris. 'Cs phosphate + Cs aspartate solution' contained Cs aspartate, 75·5; Cs_2HPO_4, 29; CsH_2PO_4, 17·5; glucose, 5·5; pH 7·0.

The drugs D-600 and D-575 were kind gifts from Knoll AG (Ludgwigshafen, F.R.G.). QX-314 was a kind gift of Astra Pharmaceuticals (Worcester, MA).

For calculations of membrane permeability, activity coefficients for Ba^{2+} or Ca^{2+} in the various bathing solutions were estimated as follows. The mean activity coefficient (e.g. γ_{BaCl_2}) was calculated using the empirical formula provided by Pitzer & Mayorga (1973). Single ion activity coefficients were obtained with the Guggenheim convention,

$$\gamma_{Ba^{2+}} = (\gamma_{BaCl_2})^2.$$

Values for Ba^{2+} ranged from 0·378 (for $[Ba^{2+}]_o$ of 1 mM) down to 0·288 (for 40 mM).

Experimental conditions where Ca^{2+} channel currents were obscured by other ionic currents

In all the experiments on Ca^{2+} channel current reported in this paper, Cs^+ was used in place of K^+ in the internal dialysate to reduce the possibility of outward currents through K^+ channels.

A transient outward current (I_{to}) could be observed under certain conditions when the external medium contained Ca^{2+} and the internal dialysate contained K^+. Preliminary analysis showed that I_{to} resembled the outward current described by Josephson & Sanchez-Chapula (1982) in rat ventricular cells. I_{to} was activated in a strongly voltage-dependent fashion by step depolarizations beyond −30 mV; the outward current reached a peak within a few milliseconds, then decayed with a time constant of about 20 ms. When measured at a fixed test potential, I_{to} was progressively inactivated at less negative holding potentials, with half-inactivation at about −55 mV and full inactivation at −40 mV. The outward current transient was completely abolished by replacing internal K^+ with Cs^+, as expected from previous work (e.g. Marban & Tsien, 1982).

In our earlier paper (Lee & Tsien, 1982), interference from I_{to} was largely avoided by the use of a holding potential of −40 mV. This approach was also used in most of the experiments in the present study, as an additional precaution to substitution of Cs^+ for internal K^+.

All experiments were carried out at room temperature (21–23 °C).

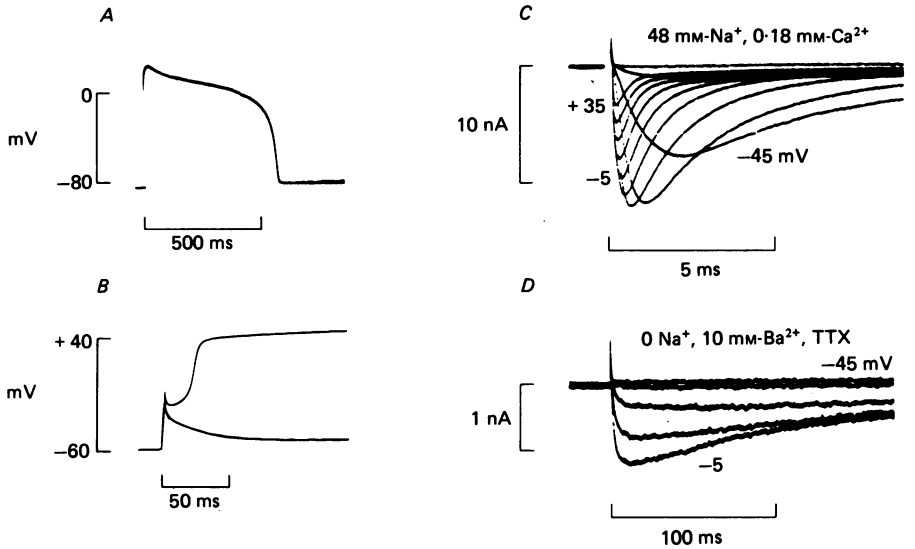


Fig. 1. Activity dependent on Na^+ channels and Ca^{2+} channels in single guinea-pig ventricular cells. Action potentials (*A* and *B*) and membrane currents under voltage clamp (*C* and *D*). *A*, intracellular micro-electrode recording. Action potential was evoked by a 1 ms extracellular stimulus pulse. Bathing solution was 1·8 mM-Ca^{2+} Tyrode solution. Cell 42C. *B*, suction pipette recording. Slow response action potential was elicited by an intracellular current pulse (7 nA, 4 ms). The superimposed subthreshold response followed a 6 nA pulse. Pipette contained 151 mM-Cs^+ internal solution. External fluid was 0 Na^+, 0 K^+, 85 mM-Cs^+ solution with 10 mM-Ba^{2+} and 10 μM-TTX. Cell 8C. *C* and *D*, Na^+ channel and Ca^{2+} channel currents recorded from the same single heart cell, separated with appropriate external solutions. Total membrane currents were recorded during step depolarizations from a holding potential of −75 mV to levels spaced 10 mV apart. Dialysate was CsF + Cs aspartate internal solution. *C*, steps to levels between −65 mV and +35 mV. Cell was bathed in 48 mM-Na^+, 57 mM-Cs^+, 97 mM-sucrose, 0·18 mM-Ca^{2+} external solution. *D*, steps to levels between −45 mV and −5 mV. 0 Na^+, 85 mM-Cs^+, 146 mM-sucrose, 10 mM-Ba^{2+} external solution with 10 μM-tetrodotoxin. Cell 9C.

RESULTS

Action potentials

As a preliminary to studies of Ca^{2+} channel activity, we looked at action potentials in standard physiological solution without internal dialysis to check against possible damage during the cell isolation procedure. Micro-electrode recordings were carried out in myocytes with cylindrical shape and regular striation patterns. Such cells displayed resting potentials near −85 mV and gave full blown, all-or-none action potentials when driven by extracellular stimuli (Fig. 1*A*). Accompanied by clearly visible twitch contractions, the action potentials displayed a fully developed plateau like that recorded from multicellular guinea-pig ventricular myocardium. Thus, in

these cells, as in other single heart cell preparations, Ca^{2+} channels seem to function normally despite the enzymatic dispersion.

Fig. 1*B* illustrates another type of electrical activity that more directly reflects Ca^{2+} channel function. In this case, the recording was carried out with a suction pipette filled with dialysate containing CsF + Cs aspartate; Na^+ currents were eliminated by

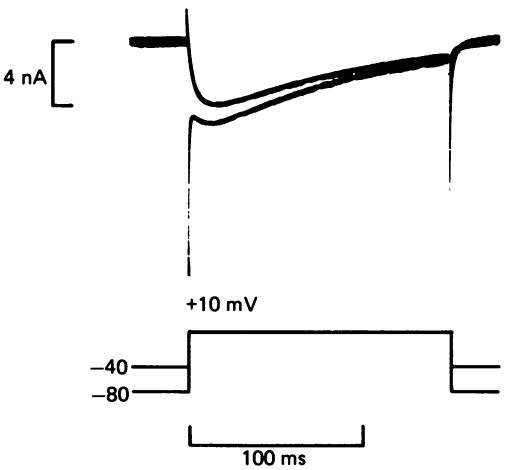


Fig. 2. Separation of Na^+ current from Ba^{2+} current through Ca^{2+} channels by an inactivating holding potential. I_{Na} appears as a downward spike of current during the step depolarization from −80 mV, but is absent with depolarization from −40 mV. External solution was Na^+ Tyrode containing 5·4 mM-Ba^{2+}. Cell 2.

the external medium, a 0 Na^+, 10 mM-Ba^{2+} solution with 10 μM-tetrodotoxin. Ca^{2+}-channel-dependent 'slow response' action potentials were evoked by intracellular current pulses, applied through the suction pipette. The slow response activity showed a clear threshold, as indicated by the response to a slightly weaker stimulus pulse. The action potential overshoot (+40 mV) and maximal rate of rise (~6 V/s) were similar to values observed for slow responses in multicellular preparations.

Separation of currents carried by Na^+ channels and Ca^{2+} channels

In our first voltage-clamp experiments, we used an internal dialysate containing CsF + Cs aspartate. We began with a brief comparison between Na^+ channel currents and Ca^{2+} channel currents recorded from the same cell. Fig. 1*C* and *D* illustrates the separation of the two inward currents by means of different external solutions. All records were obtained by step depolarizations from a −75 mV holding potential. In the presence of 48 mM-Na^+, 0·18 mM-Ca^{2+} solution (Fig. 1*C*), a series of step depolarizations produced a characteristic pattern of inward currents through Na^+ channels. Activation is noticeable at about −60 mV, and peak current is maximal near −25 mV. These values are typical for Na^+ currents in single heart cells (e.g. Lee *et al.* 1979; Brown, Lee & Powell, 1981). The graded voltage dependence of the current time course and peak amplitude of inward Na^+ current provide some indication of the quality of voltage-clamp control.

Fig. 1*D* shows currents recorded from the same cell after changing the external

media. Current through Na^+ channels was eliminated by removing the external Na^+ and by tetrodotoxin; current through Ca^{2+} channels was enhanced by the presence of 10 mM-Ba^{2+}. Under these conditions, the large, relatively rapid inward current was completely eliminated; seen instead are inward current signals of smaller amplitude

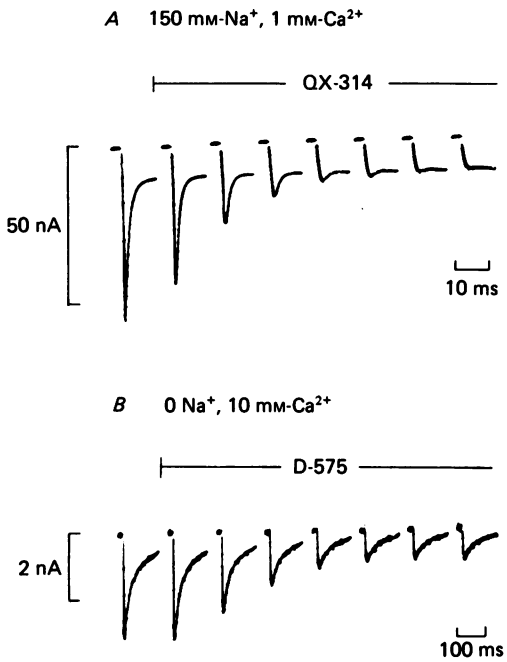


Fig. 3. Response of I_{Na} and I_{Ca} to quaternary drugs in the internal solution. *A*, effect of internal QX-314 on I_{Na}. Traces taken before, and 1, 2...7 min after introducing 500 μM-QX-314 into the dialysis fluid. Recordings of membrane current associated with step depolarizations from −60 mV to −10 mV. Linear leak and capacity current were determined with −10 mV hyperpolarizing pulses and have been subtracted. External solution was 1 mM-Ca^{2+}, 150 mM-Na^+ Tyrode solution; internal fluid was 0 Na^+, 0 K^+, 151 mM-Cs^+ solution. Cell 126E. *B*, effect of internal D-575 on I_{Ca}. Traces taken before, and 3, 3½, 4...6 min after introducing 100 μM-D-575 into the dialysis fluid. Recordings of membrane current associated with step depolarizations from −40 mV to +20 mV. Linear leak and capacity current were determined with −10 mV hyperpolarizing pulses and have been subtracted. External solution was 10 mM-Ca^{2+}, 0 Na^+, 0 Cs^+ solution; 0 Na^+, 0 K^+, 151 mM-Cs^+ solution inside. Cell 148C.

and slower time course. The inward Ba^{2+} current becomes detectable with depolarization to about −35 mV, and reaches its maximal peak amplitude near −5 mV. These values are in line with earlier work on Ca^{2+} channel currents (see Reuter (1979) and Coraboeuf (1980) for reviews).

Fig. 2 illustrates the isolation of Ca^{2+} channel current by means of changes in holding potential. In this case, the external solution contains permeant ions for both Na^+ and Ca^{2+} channels (151 mM-Na^+, 5·4 mM-Ba^{2+}). Starting from a holding potential of −80 mV, step depolarization to +10 mV evokes a brief surge of inward current and a rapid initial decay, followed by a secondary rise to a smaller inward peak and

a slower current decay. From a holding potential of −40 mV, depolarization to the same test level fails to evoke the initial inward current surge, but leaves the slower inward current component only slightly reduced in magnitude. Apparently, holding at −40 mV completely inactivates Na^+ channels, but only slightly inactivates Ca^{2+} channels. This pattern is as expected from previous studies of the inactivation properties of Na^+ channels and Ca^{2+} channels (see Reuter, 1979, for review).

Evidence for effectiveness of intracellular dialysis

Although measurements of Na^+ current were not the main focus of this paper, changes in I_{Na} provided a convenient means for assessing the effectiveness of the intracellular dialysis in allowing small molecules to reach the inside surface of the cell membrane. One such molecule is QX-314, the quaternary derivative of lidocaine. Nearly all the Na^+ current in ventricular cells was blocked by QX-314 at an internal concentration of 0·5 mM, a submaximally effective dose in other excitable cells (e.g. Schwarz, Palade & Hille, 1977). As Fig. 3*A* illustrates, block was more than half-complete within minutes after starting the exchange of dialysate. Likewise, Ca^{2+} channel current responded within a few minutes to D-575, the quaternary derivative of the Ca^{2+} channel blocking drug verapamil (Fig. 3*B*). Hescheler, Pelzer, Trube & Trautwein (1982) have already shown block of Ca^{2+} channels by intracellular ionophoretic injection of D-890, the quaternary derivative of D-600.

The accessibility of Na^+ and Ca^{2+} channels to internal drugs seems consistent with their responsiveness to changes in intracellular monovalent cations. Brown *et al.* (1981) measured changes in Na^+ channel currents expected for a prompt exchange of myoplasmic Na^+ in dialysed rat heart cells; similarly prompt changes in Ca^{2+} channel current were seen when internal Cs^+ was varied (Fig. 9).

Survival of Ca^{2+} channel current

Run-down of Ca^{2+} channels has been a serious problem in a number of systems where the internal milieu is experimentally manipulated (e.g. Fedulova, Kostyuk & Veselovsky, 1981; Fenwick, Marty & Neher, 1982). The effectiveness of the present internal dialysis system makes it particularly interesting to ask how well Ca^{2+} channel function can be maintained. We have encountered a broad spectrum of behaviour, but our general impression is that the rate of run-down depends much more on the state of the cells after isolation than on the diameter of the suction pipette or the strength of the suction. Survival of Ca^{2+} channel function is favoured by keeping the frequency of depolarizing pulses low, and by using Ba^{2+} as the permeant divalent cation. Under these conditions, it was possible to record Ca^{2+} channel currents that remained stable for an hour or more (Fig. 4).

Although encouraging, this degree of viability was found only occasionally in our earlier recordings, obtained with fluoride and aspartate as the main anions in the internal dialysate. Survival of Ca^{2+} channel current was more consistent in later experiments using dialysate containing phosphate + aspartate (see Methods). On the other hand, substitution of chloride for fluoride seemed to accelerate Ca^{2+} channel run-down. The viability of the Ca^{2+} channel seemed to be improved somewhat by including 5–10 mM-EGTA in the internal dialysate, but not by oxygenating the internal and external solutions, or by adding millimolar amounts of ATP. Although

Ca^{2+} currents were often increased by addition of cyclic AMP to the perfusate (B. P. Bean, K. S. Lee & R. W. Tsien, unpublished), inclusion of exogenous cyclic AMP or ATP was not needed for the maintenance of Ca^{2+} channel function (cf. Irisawa & Kokubun, 1983).

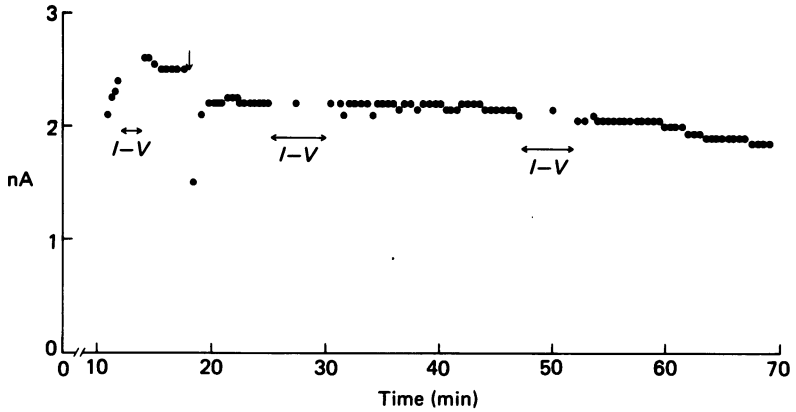
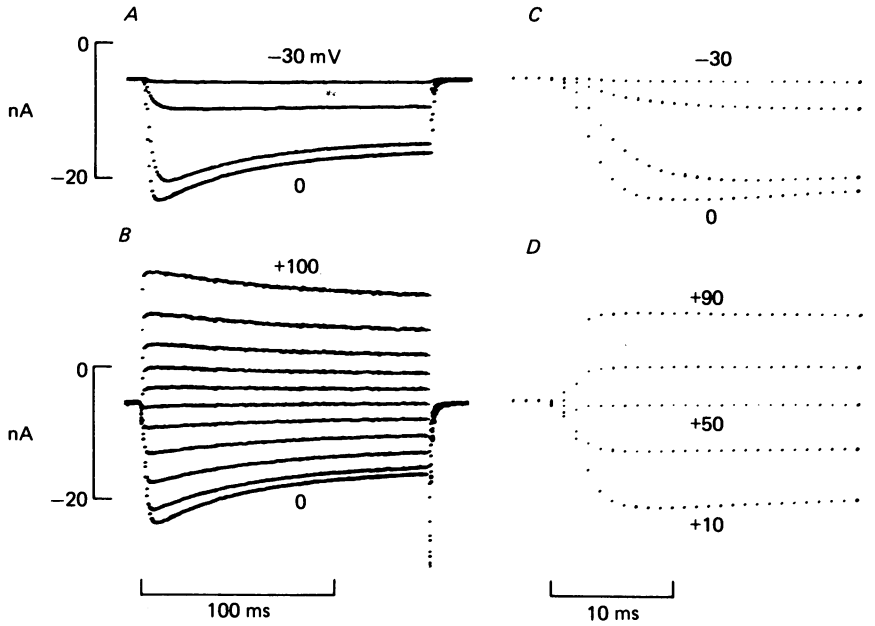


Fig. 4. Survival of Ca^{2+} channel function during internal dialysis. Individual measurements of the magnitude of the peak inward Ba^{2+} current (ordinate) are plotted against the time elapsed after cell was sucked up onto the dialysis pipette (abscissa). Pipette contained CsF + Cs phosphate internal solution. 5·4 mM-Ba^{2+}, 0 Na^+ external solution was applied at $t = 6$ min. Recording at high amplification of Ba^{2+} current through Ca^{2+} channels was begun at $t = 11$ min, as indicated. Horizontal arrows indicate periods when *I–V* relationships were determined. Vertical arrow marks the passage through the dialysis system of a large air bubble which produced a large but temporary inward holding current and the temporary decrease in Ca^{2+} channel current. At $t = 70$ min, a double-pulse protocol was applied for studying inactivation and systematic examination of Ca^{2+} channel run-down was discontinued. Cell 2.

Properties of Ba^{2+} movements through Ca^{2+} channels

We began our analysis of Ca^{2+} channel properties with the aim of reducing possible interference from other time-dependent currents to a bare minimum. Currents through Na^+ channels were eliminated by removing Na^+ ions from both external and internal solutions and by holding the membrane potential at −40 mV between depolarizations as described in Figs. 3 and 4. The possibility of interference from voltage- or Ca^{2+}-activated K^+ channels was minimized by using Cs^+ instead of K^+ in the internal dialysate, and Ba^{2+} instead of Ca^{2+} in the external medium. Cs^+ is much less permeable than K^+ in most K^+ channels and can block K^+ channels when applied inside cells. Ba^{2+} fails to activate Ca^{2+}-activated K^+ channels and is also a potent blocker of K^+ channels when applied externally.

Fig. 5 shows current signals taken during a series of depolarizing pulses in the presence of 2 mM-external Ba^{2+}. Linear leak and capacity currents were determined with −10 mV hyperpolarizing pulses and have been digitally subtracted. As Fig. 5*A* illustrates, activation of inward Ba^{2+} current becomes significant with depolarization beyond −30 mV; the inward current is maximal near 0 mV. With depolarizing pulses beyond 0 mV (Fig. 5*B*), the current signal changes progressively from a decaying



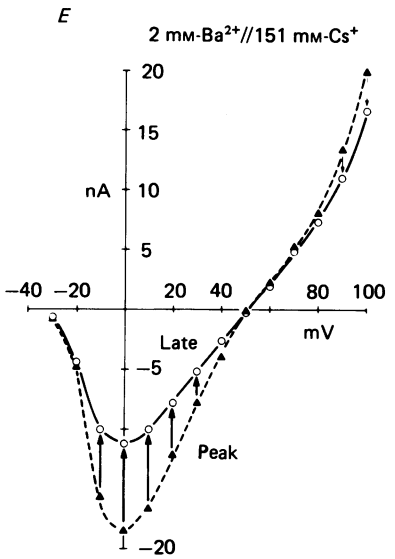


Fig. 5. Voltage-dependence of Ca^{2+} channel current. 2 mM-Ba^{2+}, 0 Na^{+}, 85 mM-Cs^{+} external solution, 151 mM-Cs^{+} internal solution. *A–D*, membrane current traces after subtraction of linear leak and capacity currents. *E*, peak current (▲) or current at end of depolarizing pulse (○) plotted against the level of the depolarization. Arrows indicate current relaxation during the depolarizing pulse. Cell 111A.

inward current to a decaying outward current. Fig. 5*E* shows analysis of this experiment in a current–voltage diagram. The early peak current and the late current are plotted relative to the holding current at −40 mV. It can be seen that the early peak current (filled triangles) and the time-dependent current decay (arrows) both disappear at the same null potential, near +50.

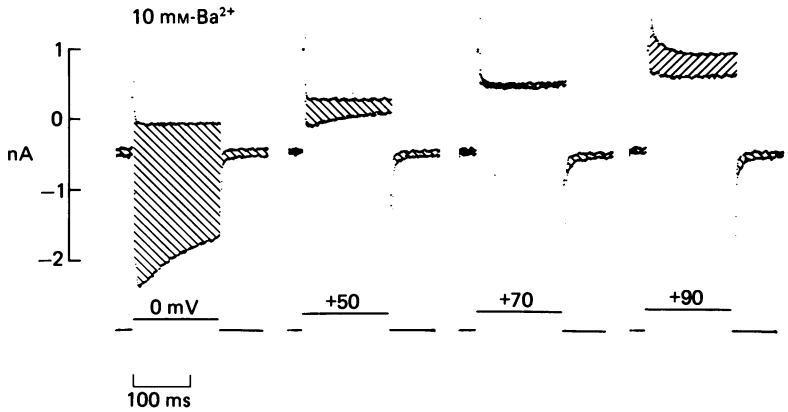


Fig. 6. Cadmium block of inward and outward currents through Ca^{2+} channels. Records of total membrane current (no leak subtraction). Shaded areas show Cd^{2+}-sensitive inward current (0, +50 mV) and Cd^{2+}-sensitive outward current (+90 mV).

When displayed on an expanded time base (Fig. 5*C* and *D*), records from the same experiment show an inversion of the time-dependent current during the rise to peak. The time course of activation is strongly accelerated by increasing depolarization over the range between −30 mV and 0 mV (Fig. 5*C*), but is much less steeply voltage dependent between 0 mV and +100 mV (Fig. 5*D*). The rising phase of inward current at +30 mV is mirrored by the activation of outward current at +70 mV; the current signal is nearly flat at +50 mV. Thus, the same reversal potential (E_{rev}) is indicated by three operationally different measurements: (1) the level of leak-subtracted early current, (2) time-dependent current during the rising phase of transient current, (3) time-dependent current during the decay from peak current. These results support the view that all the time dependence arises from changes in the conductance of Ca^{2+} channels, and that the only other current pathway is a linear leak conductance.

This interpretation was tested pharmacologically by experiments using the inorganic Ca^{2+} channel inhibitor Cd^{2+} (Figs. 6 and 7*A*). The records in Fig. 6 are shown without leak subtraction to allow inspection of the total current signal and its response to Cd^{2+}. In this experiment, the extracellular Ba^{2+} concentration was 10 mM, and reversal of the time-dependent current takes place near +70 mV. This is considerably more positive than E_{rev} in Fig. 5, as might be expected from the 5-fold higher $[Ba^{2+}]_o$ (see Fig. 8). Exposure to Cd^{2+} inhibits time-dependent current changes at all potentials. The Cd^{2+}-sensitive current (shaded) is decaying inward current below +70 mV, and a decaying outward current above this level. At +70 mV, where the current trace was flat in the control run, there is nearly perfect superposition of traces taken before and after exposure to Cd^{2+}. The disappearance of Cd^{2+}-sensitive current

provides a pharmacological method for defining the reversal potential that is independent from the other approaches but in good agreement with them.

Partial block of Ca^{2+} channels by submaximally effective concentrations of Cd^{2+} provide an even more stringent test of the idea that Ca^{2+} channels can carry current

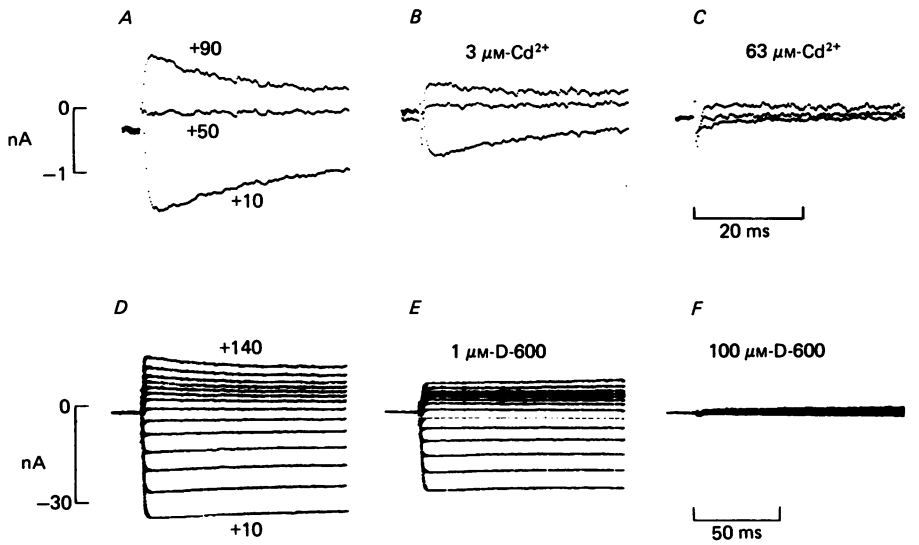


Fig. 7. Graded Ca^{2+} channel blockade as a test of the genuineness of E_{rev}. Membrane current records after subtraction of linear leak and capacity currents. *A–C*, response to Cd^{2+}. 10 mM-Ba^{2+}, 0 Na^+, 85 mM-Cs^+ solution outside, 151 mM-Cs^+ solution inside. Cell 37G. *D–F*, D-600 effect. 20 mM-Ba^{2+}, 0 Na^+, 85 mM-Cs^+ solution outside, 151 mM-Cs^+ solution inside. Cell 98E.

in either direction, and are solely responsible for all the time-dependent current, whether it be inward or outward. Fig. 7 *A–C* shows an experiment where 3 μM-Cd^{2+} reduced both the peak inward and peak outward currents, without significantly changing the current signal at the kinetically defined reversal potential. The same is true for experiments like the one illustrated in Fig. 7 *D–F*, which shows partial or full block of Ba^{2+} currents through Ca^{2+} channels by the organic agent D-600. Blockade of outward and inward currents through Ca^{2+} channels has also been demonstrated with verapamil, diltiazem and nitrendipine (Lee & Tsien, 1983).

Response of E_{rev} to changes in external Ba^{2+}

The reversal potential was dependent on the external Ba^{2+} concentration as already mentioned. Fig. 8 shows E_{rev} determinations from a total of thirty-five cells, plotted against the log of the external Ba^{2+} activity (a^o_{Ba} upper scale) or the external Ba^{2+} concentration ($[Ba^{2+}]_o$, lower scale). The activity coefficient γ_{Ba} ($=a^o_{Ba}/[Ba^{2+}]_o$) was calculated for each solution as described in Methods. The external Ba^{2+} was varied by adding appropriate amounts of $BaCl_2$ to the basic 0 Na^+, 0 K^+ external solution without compensation for changes in ionic strength or tonicity. The collected

results were fitted reasonably well by a straight line with a slope of 29 mV per 10-fold increase in a^{o}_{Ba} (dashed line a_2 in Fig. 8).

Constant-field theory provides a starting point for interpreting these results, even though it requires assumptions about the mechanism of ion permeation which might not be justified (see Discussion). Such theory is helpful for comparing our results with

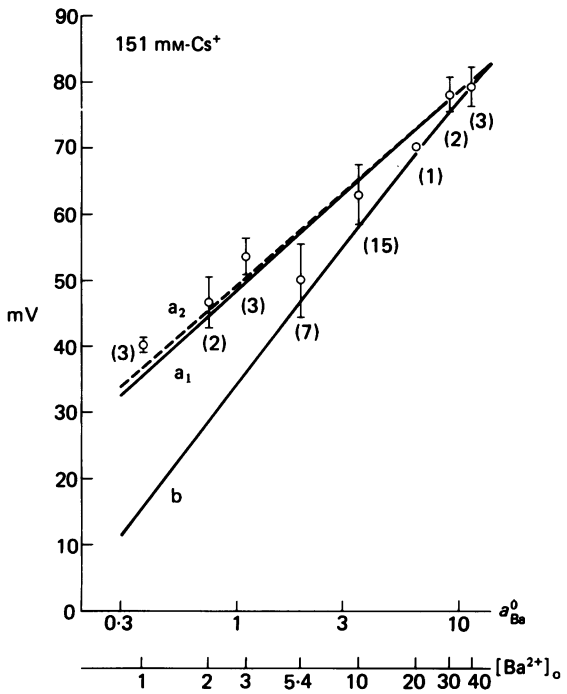


Fig. 8. Collected measurements of Ca^{2+} channel reversal potential from thirty-five cells. Means ± S.E. of means are plotted (number of determinations given in parentheses). Continuous curve a_1 obeys eqn. (1), with $P_{Ba}/P_{Cs} = 1356$ and $V' = 0$. Values for activity coefficients are assigned as described in text. Dashed straight line a_2 plots eqn. (2), with the same value of P_{Ba}/P_{Cs}. Curve b is given by eqn. (1) with $P_{Ba}/P_{Cs} = 106$ and $V' = +80$ mV.

previous analysis of membrane permeabilities to divalent and monovalent cations (Fatt & Ginsborg, 1958; Meves & Vogel, 1973; Jan & Jan, 1976; Reuter & Scholz, 1977; Lewis, 1979).

According to the constant-field theory, the zero-current potential E_{rev} can be related to the permeability coefficients P_{Ba} and P_{Cs} as follows:

$$E_{rev} = \frac{RT}{F} \ln \frac{4P'_{Ba}a^{o}_{Ba} + P_{Cs}a^{o}_{Cs}}{P_{Cs}a^{i}_{Cs}}, \quad (1)$$

where $P'_{Ba} = P_{Ba}\,[1+\exp((E_{rev}-V')F/RT)]^{-1}$. The contribution of intracellular Ba^{2+} ions is considered negligible and is not explicitly included (see below). V' represents the difference between internal and external surface potentials (Meves & Vogel, 1973). An activity coefficient $\gamma_{Cs} = 0{\cdot}75$ was used in the estimates $a^{i}_{Cs} = 113{\cdot}25$ mM and $a^{o}_{Cs} = 63{\cdot}75$ mM.

Curve a_1 shows a fit to the data with $P_{Ba}/P_{Cs} = 1356$ and $V' = 0$. The curve deviates only slightly from the dashed straight line with 29 mV/decade slope (a_2), which satisfies the equation

$$E_{rev} = \frac{RT}{2F} \ln \frac{4P_{Ba}a^{o}_{Ba}}{P_{Cs}a^{i}_{Cs}}, \quad (2)$$

also with $P_{Ba}/P_{Cs} = 1356$. The straight line turns out to be a very good approximation to eqn. (1) because the contribution of external Cs^+ is swamped by the effect of external Ba^{2+}, and because $\exp(E_{rev}F/RT) \gg 1$ in the expression for P'_{Ba}. Under such conditions, eqn. (1) reduces to eqn. (2).

Estimates of P_{Ba}/P_{Cs} and of V' are interrelated. In our case, the simple approach of setting $V' = 0$ seems reasonable because it gives a good fit to the data. In earlier studies of Meves & Vogel (1973) and Reuter & Scholz (1977), it was assumed that $V' = +80$ mV, as though there were a large negative surface potential at the inner membrane surface. There are good reasons (Chandler, Hodgkin & Meves, 1965) to expect such a surface potential in squid axons perfused with the 25 mM-CsF + sucrose (Meves & Vogel, 1973); much less so for heart cells with myoplasmic solutions of higher ionic strength. Thus, it may not be so surprising that assuming that $V' = +80$ gives a much poorer fit to our data. Curve b in Fig. 8 is drawn for $V' = +80$ and $P_{Ba}/P_{Cs} = 106$ in eqn. (1). This relation fits the data at high a^{o}_{Ba}, but at low a^{o}_{Ba} it veers off away from the data, toward a 58 mV/decade slope that is clearly inappropriate.

Response of E_{rev} and outward current to changes in internal Cs^+

Dialysed single cells offer the opportunity of manipulating intracellular ion concentrations in a way not possible with multicellular preparations. Unfortunately, addition of Ba^{2+} to the internal dialysate did not give consistent results. In one cell, the outward current at strong depolarizations relaxed from its outward peak to a greater degree; in two other cells, the addition of internal Ba^{2+} seemed to enhance a slowly increasing outward current.

We also looked for an organic ion which might serve as an inert substitute for K^+ or Cs^+ as the main monovalent cation. Tetramethylammonium, tetraethylammonium, Tris, and *N*-methylglucamine were tested. None of the four ions seemed to be inert: in each case, replacement of internal K^+ or Cs^+ by the organic ion not only diminished outward Ca^{2+} channel current, as expected, but also decreased inward currents carried by Ca^{2+} or Ba^{2+}. One possibility is that these ammonium derivatives share a common pharmacological action in blocking Ca^{2+} channels. Another possibility is that Ca^{2+} channel function requires the presence of monovalent alkali metal cation inside the cell (see Almers & Palade, 1981; Byerly & Hagiwara, 1982). This explanation seems less likely here since replacement of even a fraction of the internal Cs^+ by ammonium derivatives (e.g. 33% *N*-methylglucamine) diminished both inward and outward currents through the Ca^{2+} channel.

Since none of the cation substitutes seemed to be inert, we turned to sucrose as a replacement for internal Cs^+. Fig. 9*A* shows an experiment where the dialysate Cs^+ was reduced to 50% for 2 min, and then restored for 3 min. Comparison between the bracketing control runs shows good reversibility. Several effects of reducing internal Cs^+ are apparent: (1) outward current at +100 mV is decreased, (2) inward current

at 0 mV is somewhat increased, and (3) the reversal potential is displaced to a more positive potential. Effect (1) was found in each of five experiments; the outward current was roughly halved by the 50% reduction in internal Cs^+. Changes in the magnitude of the time-dependent inward current at 0 mV were less consistent, possibly because of run-down.

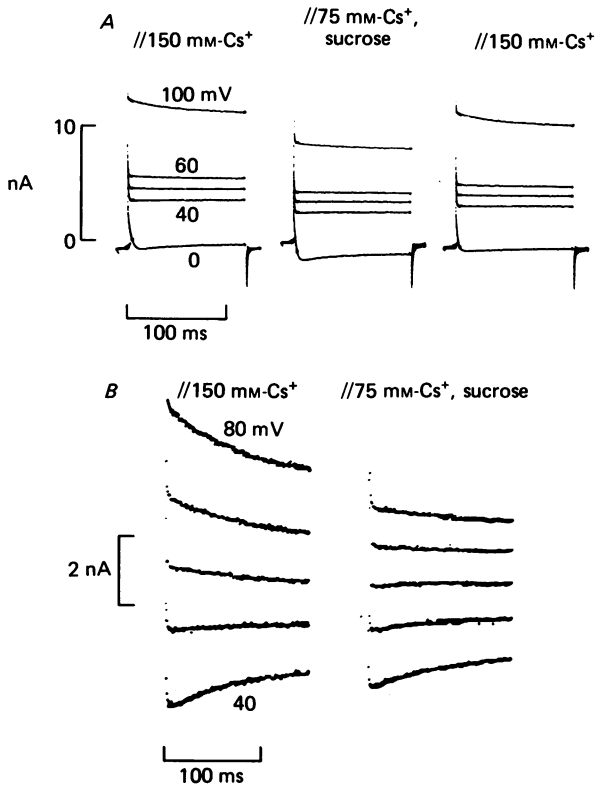


Fig. 9. Response of Ca^{2+} channel current to changes in internal Cs^+. 2 mM-Ba^{2+} external solution, internal solution as labelled. *A*, total membrane currents recorded at low amplification. Depolarizations to indicated membrane potentials from a holding potential of −40 mV. Cell 113A. *B*, total membrane currents recorded at high amplification in a different experiment. Vertical spacing between traces has been reduced arbitrarily to allow inspection of time-dependent current changes. Cell 111A.

Fig. 9*B* considers changes in E_{rev} in more detail, using records taken at higher amplification from another cell. E_{rev} was roughly +53 mV with 100% internal Cs^+, and about +64 mV with 50% internal Cs^+. The +11 mV displacement is roughly consistent with $\Delta E_{rev} = +9$ mV predicted theoretically from eqn. (1) or eqn. (2).

Experiments with external Ca^{2+} and internal Cs^+

Inversion of current through Ca^{2+} channel was not as obvious with Ca^{2+} instead of Ba^{2+} as the permeant divalent cation in the external medium. Fig. 10 illustrates two kinds of behaviour that we observed with depolarizing pulses beyond 0 mV. In

panel *A*, the magnitude of the inward Ca^{2+} current becomes smaller and smaller with increasingly strong pulses, but there is no potential where the time-dependent current disappears; the current signal creeps upward, even at the strongest depolarizations. This pattern was typical of most of the experiments. However, several cells demonstrated the type of behaviour illustrated in Fig. 10*B*. In this case, the time-dependent current was completely suppressed near +70 mV or so, and the outward current decayed slightly at even stronger depolarizations.

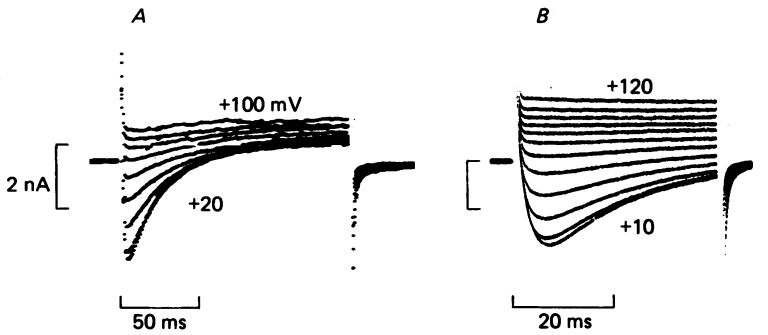


Fig. 10. Variable appearance of outward current with 10 mM-Ca^{2+} external solution. Total membrane current records without leak subtraction, evoked by depolarizations from a −70 mV holding potential to levels spaced by 10 mV as indicated. Cells 16C (*A*) and 18B (*B*).

Fig. 11*A* shows another example of the second pattern. Here, as in Fig. 10*B*, the outward current decays only slightly. However, additional arguments indicate that a true reversal of Ca^{2+} channel current takes place. After the current signals in Fig. 11*A* had been corrected for linear leak and capacity current, they yielded the peak *I–V* relationship indicated by the open symbols in Fig. 11*D*. The *I–V* relation crosses the voltage axis near +75 mV, very close to its inflexion point. Outward currents at depolarizations beyond the reversal activated with a sigmoid time course similar to the activation of inward current negative to E_{rev}, as expected if inward and outward currents were controlled by the same gating process.

This kinetic argument for the genuineness of the reversal potential was buttressed by pharmacological evidence. Another *I–V* relationship was taken after the cell had been exposed to 0·1 mM-Cd^{2+} to block some of the Ca^{2+} channels (filled symbols). The *I–V* relation in Cd^{2+} crosses that for the control near the voltage axis, between +70 and +80 mV; Cd^{2+} reduced outward current at depolarizations beyond +75 mV.

If this reversal potential is interpreted provisionally using a constant-field expression for E_{rev}, with external Ca^{2+} and internal Cs^{+} like eqn. (1), one may estimate P_{Ca}/P_{Cs}. Using values of a^{i}_{Cs} and a^{o}_{Cs} given above, and $\gamma_{Ca} = 0{\cdot}341$ (Pitzer & Mayorga, 1973), $E_{rev} = +75$ mV translates to $P_{Ca}/P_{Cs} = 6326$. The main point here is that P_{Ca}/P_{Cs} is very large, being even greater than P_{Ba}/P_{Cs}.

The *I–V* curves in Fig. 11*B* are far from ohmic. In both runs, the *I–V* curve shows a clear inflexion close to the point of reversal, with conductance increasing rather steeply with driving force on either side of the inflexion. Similar non-linearity has

been found in adrenal chromaffin cells with external Ca^{2+} and internal Cs^+ (Fenwick *et al.* 1982), and in ventricular cells with Ba^{2+} outside and Cs^+ inside (Fig. 5*E*, this paper). In an earlier report (Lee & Tsien, 1982, Fig. 3), the peak *I–V* relation with 1 mM-Ca^{2+} outside and 150 mM-K^+ inside also showed a marked upward curvature positive to E_{rev}; an increase in conductance negative to E_{rev} was not evident, but may have been obscured by the influence of voltage-dependent channel opening.

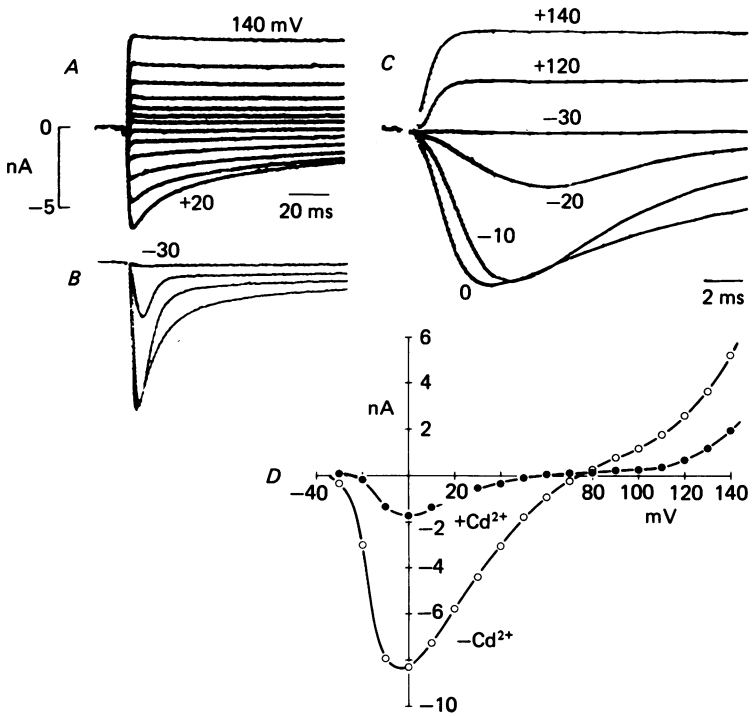


Fig. 11. Voltage dependence of Ca^{2+} channel current with Ca^{2+} as the external divalent cation. 5 mM-Ca^{2+}, 0 Na^+, 0 K^+ external solution, 151 mM-Cs^+, 10 mM-EGTA internal solution. *A* and *B*, records taken with step depolarizations from −40 mV to the levels indicated, displayed on a slow time base. *C*, records on a fast time base, selected from the same sequence of pulses as in *A* and *B*. *D*, relationship between membrane potential and leak-subtracted membrane current, before and after exposure to 100 μM-Cd^{2+}.

DISCUSSION

Internally dialysed heart cells as a system for studying Ca^{2+} channel currents

The combination of suction pipettes and single heart cells provides an experimental system for studying Ca^{2+} channels that satisfies all the requirements listed by Hagiwara & Byerly (1983; see Introduction). The internal dialysis allows intracellular levels of ions or drugs to be changed within a few minutes, while leaving the cells robust enough to show Ca^{2+} channel activity, often for 20 min or more. Inward or outward currents through Ca^{2+} channels were recorded with little or no interference from other time-dependent membrane currents, including Na^+ channels and various

voltage-dependent or Ca^{2+}-dependent cation channels. With appropriate series-resistance compensation, the single suction pipette voltage clamp settled rapidly enough to reveal the sigmoid kinetics of Ca^{2+} channel activation, although not rapidly enough to resolve the full time course of deactivation during inward tail currents.

Suction pipettes allow cardiac Ca^{2+} channel activity to be recorded more quickly and easily than with micro-electrode or sucrose-gap techniques. A large number of cells can be studied over a wide range of clamp potentials. Analysis of Ca^{2+} channel current in single cardiac cells complements recordings of the activity of individual cardiac Ca^{2+} channels (Reuter, Stevens, Tsien & Yellen, 1982; Cavalie, Ochi, Pelzer & Trautwein, 1983), obtained with the patch-clamp technique of Hamill *et al.* (1981).

Comparisons between Ca^{2+} channels in heart cells and neurones

Previous work in cardiac and neuronal systems has led to fundamentally different views of the kinetics and rectifier properties of Ca^{2+} channels (see Reuter, 1979; Hagiwara & Byerly, 1981; Tsien, 1983). The present experiments may help to resolve some of the controversial points. The over-all conclusion is that Ca^{2+} channels in heart are more similar to their counterparts in other tissues than previously thought.

Activation of cardiac Ca^{2+} channels was described as a simple first-order process in earlier studies in multicellular cardiac preparations (e.g. Reuter & Scholz, 1977; McDonald, 1982) and single ventricular cells (e.g. Isenberg & Klöckner, 1980). These studies ran contrary to most of the reports from neuronal preparations, where a multi-step process was indicated by the sigmoid onset of Ca^{2+} channel current following voltage steps (see Kostyuk (1981) and Hagiwara & Byerly (1981) for reviews). In accord with the neuronal results, our results show a clear delay in the activation of cardiac Ca^{2+} channels (e.g. Figs. 5 and 11; Lee & Tsien, 1983).

In the earlier cardiac work, insufficient speed of voltage control or overlap from capacity current or transient outward current may have obscured the time course of Ca^{2+} channel activation. On the other hand, the delay we observe seems consistent with recent observations that openings of cardiac Ca^{2+} channels often occur in bursts (Reuter *et al.* 1982; Cavalie *et al.* 1983), much the same as in other excitable cells (e.g. Lux & Nagy, 1981; Fenwick *et al.* 1982). Sigmoid activation kinetics and bursts of single channel activity can both be explained by the existence of intermediate state(s) between resting and open states of the channel.

Ca^{2+} channels can carry outward current but are not ohmic

Characteristics of open Ca^{2+} channels have also been described very differently in heart and nerve cells. In neurones, the prevailing view has been that Ca^{2+} channels are strongly rectifying pathways, essentially incapable of carrying outward current (Kostyuk, 1981; Hagiwara & Byerly, 1981). Cardiac Ca^{2+} channels, on the other hand, have been described most often as showing a genuine reversal potential and a voltage-independent conductance in accord with Ohm's law (see Reuter & Scholz, 1977; Hino & Ochi, 1980; Coraboeuf, 1980). According to our results, neither of these views is entirely correct. Although we find evidence for a genuine reversal potential, the I–V relationship for the open channel is strongly inflected rather than ohmic. In both of these respects, this paper and an earlier study (Lee & Tsien, 1982) are in good agreement with findings of Fenwick *et al.* (1982) in adrenal chromaffin cells.

Ca^{2+} channels are extremely selective against K^+ or Cs^+

A 100-fold selectivity for Ca^{2+} over Na^+ or K^+ has been estimated for Ca^{2+} channels in cow ventricular muscle (Reuter & Scholz, 1977). Our values for P_{Ba}/P_{Cs} or P_{Ca}/P_{Cs} in guinea-pig ventricular cells are more than 1000:1 (see pp. 265 and 267). The same is true for P_{Ca}/P_K according to E_{rev} values of Lee & Tsien (1982). The wide difference in selectivity can be appreciated without use of constant-field theory by considering values of E_{rev} itself. For example, Reuter & Scholz (1977) found that E_{rev} averaged +18 mV in 0·45 mM-Ca^{2+} Tyrode solution and +33 mV in 1·8 mM-Ca^{2+} Tyrode solution; on the other hand, Lee & Tsien (1982) found that E_{rev} was +65 in 1 mM-Ca^{2+} Tyrode solution. Possible explanations for the discrepancy are species-dependent differences in Ca^{2+} channel selectivity (see Reuter & Scholz, 1977, p. 33), or varying amounts of interference from other ionic channels in the two preparations.

However it is expressed, the degree of selectivity carries very different implications for the amount of monovalent cation transfer through Ca^{2+} channels. Under physiological conditions, where Ca^{2+} ions are so heavily outnumbered by monovalent cations, a 100-fold selectivity for Ca^{2+} implies roughly equal fluxes of Ca^{2+} and K^+ at 0 mV (Reuter & Scholz, 1977). If the selectivity were really an order of magnitude greater, as our results suggest, the flux through the channel would be dominated by Ca^{2+} ions, to about the same extent as the flux through Na^+ channels is dominated by Na^+. The high selectivity of Ca^{2+} channels is noteworthy in view of their large single channel conductance and ion throughput (Reuter *et al.* 1982; Tsien, 1983). Observations of ion–ion interactions within the Ca^{2+} channel (Hess, Lee & Tsien, 1983; Hess & Tsien, 1983) lead to a model for ion permeation that can account for the high selectivity and rapid ion transfer (Tsien, Bean, Hess & Nowycky, 1983; Hess & Tsien, 1983, 1984).

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