Inactivation of single Ca^{2+} channels in rat sensory neurons by extracellular Ca^{2+}

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1. Single Ca^{2+} channels conducting 20 mM Ba^{2+} from adult rat dorsal root ganglion cells were characterized using the two-electrode patch-clamp technique configuration.
2. Channels demonstrating specific characteristics of conductance, voltage dependence and dihydropyridine sensitivity were classified as high-threshold or L-type Ca^{2+} channels.
3. Mean single-channel current in 20 mM Ba^{2+} did not show inactivation, but inactivation occurred when using Ca^{2+} as a permeating ion.
4. Stimulus protocols were delivered alternately in the cell-attached and whole-cell electrode, while recording single-channel activity and total Ca^{2+} current simultaneously.
5. A mean single-channel Ba^{2+} current from a stimulated patch did not show inactivation. However, stimulation of a physiological whole-cell Ca^{2+} current induced a marked inactivation of mean single-channel Ba^{2+} current.
6. Complete Ca^{2+} current block by the addition of 200 μM Cd^{2+} in the external solution removed single-channel inactivation in patches stimulated through a whole-cell electrode.

The variety of Ca^{2+} conductances in excitable cells is tangible evidence of the importance of Ca^{2+} permeation in different cell functions. Ca^{2+} channels have two main purposes: providing charges to trigger and shape the action potential, and furnishing the cytoplasm with divalent ions to be used as second messengers. Among the various Ca^{2+} channel types, several hypotheses have been put forward concerning their functions. The T-type, or low-threshold, Ca^{2+} channel (Carbone & Lux, 1984) contributes to the action potential formation (Llinás & Yarom, 1981; Llinás, 1988; Wanke, Becchetti, Biella, Del Bo & Ferroni, 1992). N-type Ca^{2+} channels appear to be associated with neurotransmitter release (Hirning *et al.* 1988; Lemos & Nowycky, 1989). Speculation about high-threshold or dihydropyridine-sensitive channels suggests that they have a role in maintaining high-frequency voltage oscillation during neuronal bursts (Llinás & Yarom, 1981; Pietrobon & Hess, 1990; Mazzanti, Galli & Ferroni, 1992) and are possibly involved in substance release (Perney, Hirning, Leeman & Miller, 1986; Rane, Holz & Dunlap, 1987; Holz, Dunlap & Kream, 1988; Lemos & Nowycky, 1989). One peculiarity of the high-threshold Ca^{2+} channel is the persistence of its current during long-lasting stimulation. The inactivation process, which terminates the Ca^{2+} accumulation in the cell, is composed of two components. The first is a voltage-dependent inactivation factor, and the second is the intracellular level of the divalent ion (Yue, Backx & Imredy, 1991; Mazzanti, DeFelice & Liu, 1991).

Does inactivation of the L-type single channel depend on the concomitant activity of the calcium channels in the cell (DeFelice, 1993)? In other words, does the build-up of intracellular Ca^{2+}, due to the simultaneous opening of all the Ca^{2+} channels on the membrane, increase L-type channel inactivation?

Ca^{2+}-dependent inactivation of Ca^{2+} current is a well studied phenomenon in neuronal cells (Eckert & Tillotson, 1981; Brown, Moromoto, Tsuda & Wilson, 1981; Chad & Eckert, 1984; Dupont, Bossu & Feltz, 1986; Kasai & Aosaki, 1988; Akaike, Tsuda & Oyama, 1988; Morad, Davies, Kaplan & Lux, 1988; Gutnick, Lux, Swandulla & Zucker, 1989; Kay, 1991; Peres, Bertollini, Camagni & Wanke, 1991; Yue *et al.* 1991). The concept that one component of the Ca^{2+} current is inactivated by the same calcium ions that permeate from outside is generally accepted.

Some neuronal cells produce Ca^{2+} spikes that effect oscillations of the transmembrane potential (Jahnsen & Llinás, 1984; Llinás & Yarom, 1986). Repetitive stimulation increases the probability of Ca^{2+} channels opening (Artalejo, Mogul, Perlman & Fox, 1991). Strong depolarization before testing the potential increases the probability of long openings, called 'high-P_o' or 'mode 2' (Pietrobon & Hess, 1990). These two mechanisms for increasing Ca^{2+} current co-exist in spontaneously firing neurons. By coupling the intrinsic 'high-P_o' kinetics with the extrinsic firing rate dependence, both of which influence single-channel functions, neuronal cells are able to increase the level of

intracellular Ca^{2+} during action potential bursts (Mazzanti *et al.* 1992).

All of the Ca^{2+} permeabilities are probably influenced by repetitive voltage oscillations; thus, we should be able to study the kinetics of a particular component of the Ca^{2+} permeability system, while leaving all the others unaltered. Using classical whole-cell recording methods, it is impossible to observe only one current type without manipulating the others. We attempted to solve the problem by utilizing a two-electrode patch-clamp technique (Mazzanti & DeFelice, 1987), in which we recorded single-channel and whole-cell currents at the same time in rat dorsal root ganglion cells. Using this technique, we were able to activate every Ca^{2+} current present on the cell membrane and at the same time observe the function of a single L-type Ca^{2+} channel, whose mean value represents a miniature version of the whole-cell L-current. Moreover, using low doses of Ba^{2+} as the permeating ion in the cell-attached pipette, we were able to separate the calcium-dependent inactivation component from the voltage-dependent inactivation component of the high-threshold Ca^{2+} current. Recent experiments in heart cells have demonstrated that increased density of Ca^{2+} channels is crucial for the inactivation of the current (Mazzanti *et al.* 1991). The progressive accumulation of Ca^{2+} ions close to the internal face of the membrane could interfere with Ca^{2+} permeability, enhancing channel inactivation.

The data presented in this study demonstrate pronounced inactivation of the single-channel current upon whole-cell stimulation. Results showed that inactivation was absent in the channel if the voltage stimulus was applied to the cell-attached pipette. The cumulative influence of intracellular Ca^{2+} ions on Ca^{2+} permeability suggests that the density of channels may play an important role in action potential firing pattern duration and, consequently, in neuron excitability.

METHODS

Dorsal root ganglion cells, from anaesthetized (with sodium pentobarbitone, 30–35 mg kg^{-1} I.P.) and decapitated adult rats, were prepared by enzymatic digestion, following the procedure of Ferroni, Mancinelli, Camagni & Wanke (1989). The cells removed were bathed for 12–24 h in tissue culture medium. Immediately preceding the experiments, the cells were washed in a bath solution consisting of (mM): 142 NaCl, 5 KCl, 2 $CaCl_2$, 0·5 $MgCl_2$, 5 tetraethylammonium (TEA), 2,4-aminopyridine (4-AP), 10 Hepes and 0·003 TTX, adjusted to pH 7·35.

The cell-attached electrode solution, used to record patch Ba^{2+} current through high-threshold Ca^{2+} channels, contained (mM): 20 $BaCl_2$, 70 NaCl, 1 EGTA, 0·003 TTX, 20 TEA, 10 4-AP and 10 Hepes, at pH 7·35. In some experiments Ba^{2+} was replaced with 20 mM Ca^{2+}. The whole-cell electrode contained an intracellular-like solution comprised of (mM): 120 potassium aspartate, 20 TEA, 10 NaCl, 10 Hepes, 2 BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) and 2 MgATP, at pH 7·35. We used 2 mM BAPTA to facilitate study of the local effect of Ca^{2+} ions entering from outside and instantly buffering any Ca^{2+} increase in the cytoplasm. In some experiments we also used 100 μM BAPTA, or alternatively, 1 mM EGTA plus 0·1 mM Ca^{2+} to obtain a final Ca^{2+} concentration in the whole-cell solution of 10^{-7} M. We observed no substantial difference in the behaviour of Ca^{2+} channels using either solution.

In all the experiments we applied the two-electrode recording method. One electrode was in the whole-cell configuration, and the other was in the cell-attached configuration (see Mazzanti & DeFelice, 1987, 1990). The two electrodes were first placed in a cell-attached configuration. In the pipette containing intracellular-like solution, we applied a negative suction pulse and changed the configuration to whole-cell recording to control the cell membrane potential. Consequently, we could measure and control the cell-attached patch membrane voltage. In several experiments we observed the channel before and after whole-cell patch breaking. The channel maintained similar conductance, voltage dependency and open probability to those observed during the time span of a single experiment.

Single-channel analysis followed the procedure used in Mazzanti *et al.* (1991). We fitted each histogram to two exponential least-squared regressive curve-fitting routines. The faster of the two time constants obtained with this method depends on the filter setting, which in these experiments was 1000 Hz. The minimum resolvable open time was 160 μs, and the double-exponential fit gave the filter frequency for the fast time constant. Although the fast time constant could offer information about channel gating, e.g. the rapid flickering process, we have not analysed it in detail. Instead, we used the slower time constant (τ) as an index to compare the kinetics of the channel.

The electrodes were made from borosilicate glass using a programmable pipette-puller (Flaming–Brown P-87, Sutter Instrument Co., Novato, CA, USA). After coating with Sylgard (Dow Corning, Midland, MI, USA) and fire polishing the tips to an approximate internal diameter of 1–2 μm, the electrodes had resistances ranging from 4 to 10 MΩ. Cell-attached and whole-cell currents were measured with List EPC-7 (Darmstadt, Germany) and Axopatch-1D (Axon Instruments Inc., Foster City, CA, USA) amplifiers, respectively.

Data were filtered at 1000 Hz unless otherwise noted. We stored the data on a video cassette recorder and analysed it on a Nicolet 310 (Nicolet Inc., Madison, WI, USA) oscilloscope and Mitsuba 386SX computer, using programs developed by William Goolsby (Department of Anatomy and Cell Biology, Emory University, Atlanta, GA, USA).

RESULTS

Adult rat dorsal root ganglion neurons contain at least three types of Ca^{2+} channel that can be delineated by voltage sensitivity, pharmacology and conductances (Nowycky, Fox & Tsien, 1985; Fox, Nowycky & Tsien, 1987; Ferroni *et al.* 1989). However, characterization of single channels using low concentration divalent cations as a charge carrier has not been successful. Filling the patch pipette with 20 mM Ba^{2+} probably reduces the channel's conductance, narrowing the difference for this parameter among different channel types. During our experiments, using a voltage range between −20 and 10 mV, we observed only one Ca^{2+} pathway in 60 % of the patches.

The remaining 40 % of the patches were either empty or contained ionic channels demonstrating characteristics not corresponding to the L-type Ca^{2+} channels and were disregarded. We characterized this channel based on conductance, voltage dependence and pharmacology. Figure 1*A* shows single-channel current recordings obtained in the cell-attached configuration on isolated dorsal root ganglion cells at four test potentials.

The membrane potential was clamped at −80 mV by a second electrode, in the whole-cell configuration (see Methods). Figure 1*B* shows single-channel current–voltage (i–V) relationships. Experimental data points were calculated from amplitude histogram analysis of single-channel traces from holding potentials of −80 mV. The straight line is a least-squared fit through the experimental points. The relationship is:

$$i = \gamma\ (V - E),$$

where the single-channel conductance $\gamma = 18$ pS and the current reversal potential $E = 38$ mV. The calculated single-channel conductance is very close to that obtained for L-type Ca^{2+} channels, under the same conditions, in embryonic chick ventricular cells (Mazzanti *et al.* 1991).

The second criterion used to characterize the channel was its sensitivity to the holding potential. The easiest way to isolate L-type current is to deliver the test protocol from a more depolarized holding potential (Hess, Lansman & Tsien, 1984; Fox *et al.* 1987; Ferroni *et al.* 1989). The procedure was to voltage clamp the cell at −80 mV with a whole-cell electrode during a step-voltage stimulation of the cell-attached pipette to three different potentials (−10, 0, +10 mV). The same cell was then clamped at −50 mV, and an equal voltage protocol was used to stimulate the membrane patch.

Figure 2 demonstrates two current traces elicited from different holding potentials for each of the three test voltages. Amplitude histograms of the current recordings ($n = 16$) were identical despite different holding potentials.

The channel was also tested for its sensitivity to dihydropyridine compounds. To evaluate the action of these drugs, we clamped the cell at −50 mV holding potential and increased the voltage to 0 mV to activate only L-type Ca^{2+} channels. For the pharmacological characterization we used the L-type Ca^{2+} channel agonist 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylic acid methyl ester (Bay K 8644) at a concentration

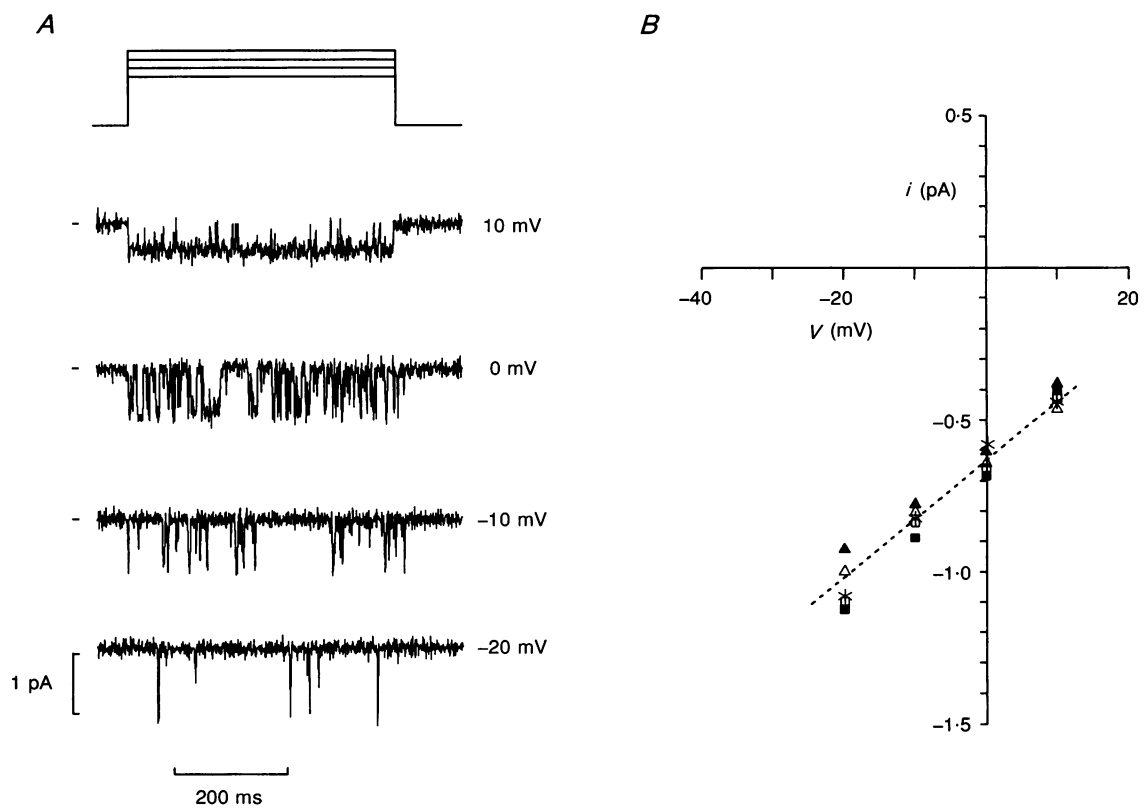

Figure 1. Characterization of single L-type Ca^{2+} channels in dorsal root ganglion neurons
A, single-channel current records obtained in the cell-attached configuration at four test potentials of −20, −10, 0 and 10 mV. The current values from five experiments were used to plot single-channel current–voltage relationships (*B*; holding potential −80 mV). The conductance of the channel, in 20 mM Ba^{2+}, was 18 pS, and the extrapolated reversal potential was 38 mV.

of 1 μM and the antagonist nifedipine at a concentration of 5 μM. The addition of the agonist to the cell-attached pipette prolonged the open time of the channel. Figure 3 illustrates single-channel openings at a test potential of 0 mV without (left) and with (right) Bay K 8644. The calculated mean open time increased from $1{\cdot}38 \pm 0{\cdot}4$ ms ($n = 5$) to $13 \pm 0{\cdot}7$ ms ($n = 7$).

We also performed experiments adding 1 μM nifedipine to the pipette solution. Only one experiment in over thirty cell-attached patches showed single-channel activity with a conductance similar to L-type Ca^{2+} channels, but such a low open probability would reduce the mean single-channel current to zero. Taking into account the probability of observing the channel (60 %), the likelihood of viewing the channel using nifedipine is less than 5 %.

Single calcium channels that are activated with voltage steps from a holding potential of −50 mV to test voltages between −20 and 10 mV, have a conductance of around 18 pS in 20 mM external Ba^{2+}, and are sensitive to dihydropyridine compounds are classified as high-threshold, or L-type, Ca^{2+} channels (Yue & Marban, 1990; Mazzanti *et al.* 1991). It is possible that in some experiments, not tested with dihydropyridine compounds, a contamination of N-type Ca^{2+} channels exists (Plummer & Hess, 1991). However, the voltage sensitivity is one of the main criteria used to distinguish between the two different channel types (Plummer, Logothetis & Hess, 1989).

The experimental method required simultaneous Ca^{2+} current activation. Thus, the holding potential had to be −80 mV. To avoid contamination in the cell-attached pipette by Ca^{2+} conductances other than those of the L-type, each single-channel experiment was performed using different holding potentials (−50 and −80 mV), with subsequent measurement of the single-channel conductances and analysis of the kinetic parameters. In fact, neuronal L-type Ca^{2+} channels revealed kinetic parameters that are similar to those seen in cardiac tissue high-threshold channels. The Ca^{2+} pathway shows an increased duration in the open state at more depolarized potentials. The increments in both the opening probability (P_o) and channel

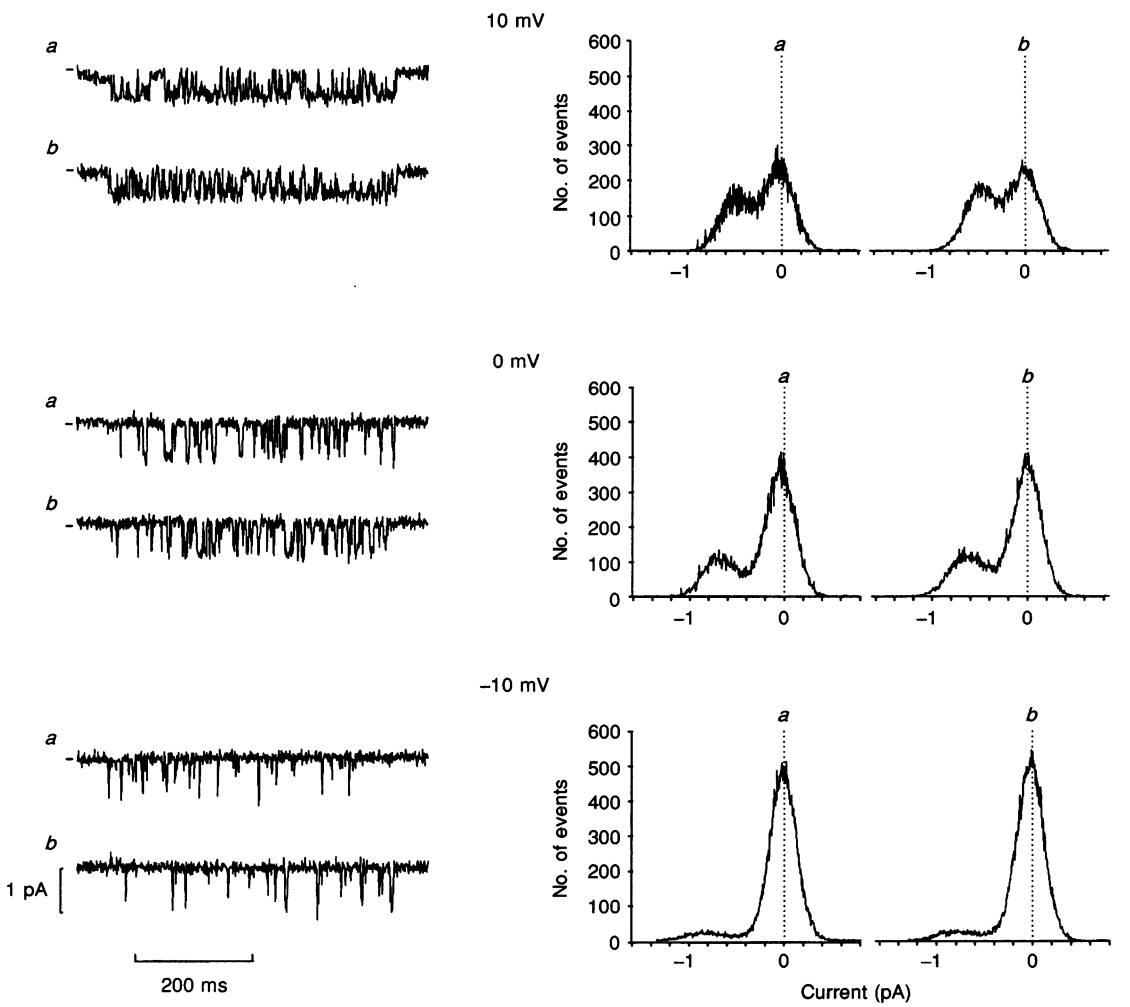

Figure 2. Effects of different holding potentials on the L-type single-channel current
On the left, single current traces elicited from holding potentials of −80 mV (*a*) and −50 mV (*b*) for each of three test voltages. On the right, the comparison of amplitude histograms obtained from 16 current recordings at each test voltage.

open time, with associated membrane voltages, are summarized in Fig. 4.

The two plots show single-channel open-state probability (P_o) and open-time distribution as a function of membrane potential. Single-channel data acquired at different holding potentials (−50 mV, triangles; and −80 mV, squares) appear equivalent with respect to the kinetic parameters. In Fig. 4*A* the dashed line represents a non-linear least-squared fit of the equation:

$$P_o = a \times \exp[b(V - c)]/\{1 + \exp[b(V - c)]\},$$

where $a = 0{\cdot}5$ and $0{\cdot}48$, $b = 0{\cdot}09$ and $0{\cdot}08$, and $c = -2{\cdot}7$ and $-2{\cdot}2\ mV^{-1}$, respectively. In Fig. 4*B* the points plotted were obtained from a two-exponential fitting of open-time distribution histograms. Although the fast time constant offers information about the channel kinetics, we have not analysed it. The absence of values at the −20 mV membrane potential is due to the extensive flickering of the channel at this potential and, consequently, the presence of only one time constant. Both kinetic parameters increase to a more positive membrane voltage, indicating a marked voltage dependency, and both are independent of the holding potential.

The replacement of Ca^{2+} as a permeating ion with Ba^{2+} modifies the channel function. If we compare single-channel current recordings using 20 mM Ba^{2+} and 20 mM

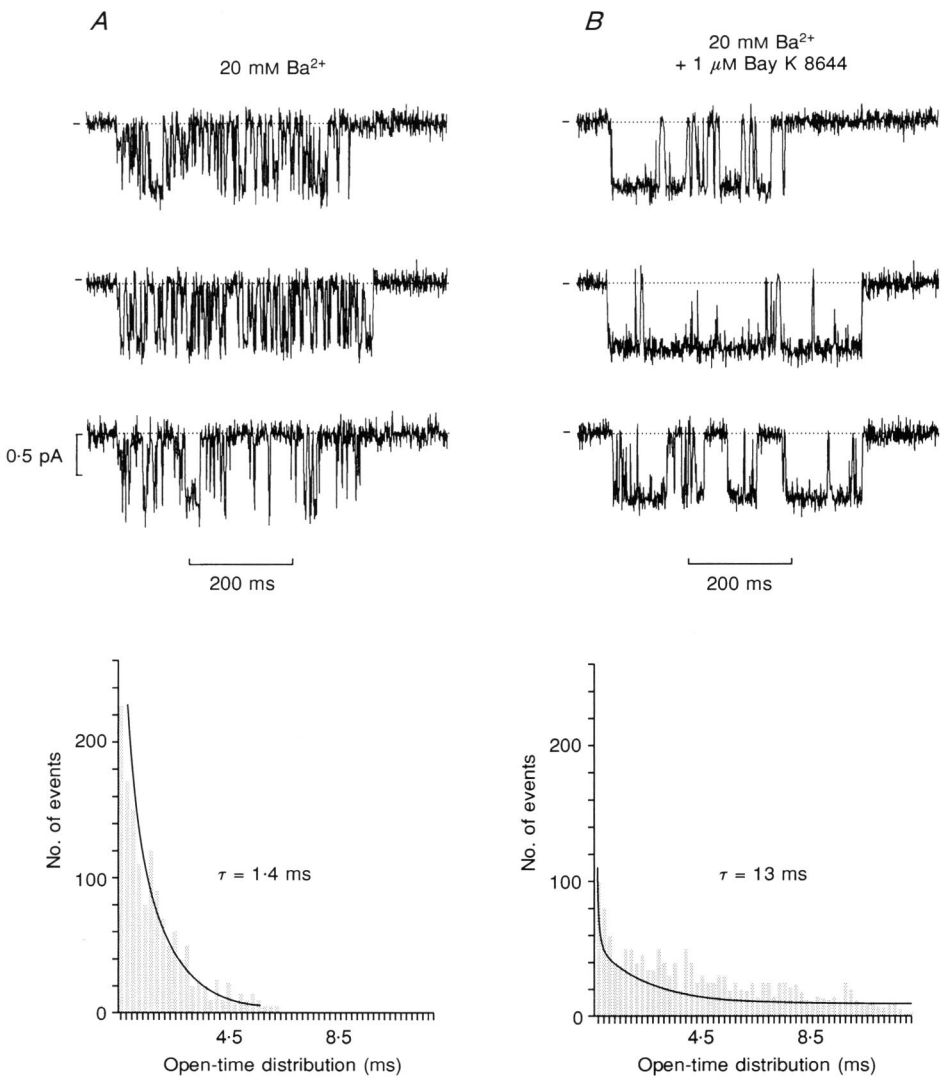

Figure 3. Effects of 1 μM Bay K 8644 on the open time of a single Ca^{2+} channel conducting 20 mM Ba^{2+}

The open-channel kinetic (*A*) shows fast transitions at a test potential of 0 mV from 50 mV holding potential. The addition of 1 μM dihydropyridine agonist (*B*) prolongs the open time of the channel. The effect is quantified in the apparent open-time distribution (shown below) from 2 s data, in which τ increases from 1·4 to 13 ms after addition of Bay K 8644. These time constants were derived from double-exponential fits to each histogram, and they represent the slower time constant (see Methods). The relative histogram area of the 1·4 ms time constant was 27 %, whereas for 13 ms it was 78 %.

Ca^{2+}, it is evident that parameters such as conductance and inactivation are modified. In the mean single-channel results ($n = 14$) obtained from single-channel recordings during stimulus from −50 mV holding potential to −20 mV test voltage (Fig. 5), the mean Ba^{2+} current value is sustained during a 500 ms voltage step (Fig. 5*A*). In contrast, when the channel conducts Ca^{2+} ions, the mean current shows marked inactivation (Fig. 5*B*). The sensitivity of the channel conducting Ca^{2+} ions to dihydropyridine compounds was tested at the end of the experiment. Single-channel recordings and mean current after addition of 1 μM Bay K 8644 are illustrated (Fig. 5*C*).

In this study we based our experimental method on the relative lack of inactivation in the mean single-channel current that was obtained with low [Ba^{2+}]. Using this experimental configuration it was possible to examine the influence of Ca^{2+} ions coming from other sources on the internal face of the channel and observe the reduction in effect that divalent ions had on the same channel under observation.

The diverse effect of various permeable ions on current inactivation has already been described (Hess & Tsien, 1984; Yue & Marban, 1990), implicating a minor affinity of the Ca^{2+} channel for Ba^{2+} ions. Inactivation not present in single-channel experiments using low external [Ba^{2+}] may be due to the absence of a critical divalent ion concentration that modulates the channel. However, the Ca^{2+} pathway maintains its intrinsic characteristics even when Ba^{2+} is the permeating ion (Peres *et al.* 1991). Probably, the Ba^{2+} current passing through a single channel is not sufficient to promote inactivation. If there are multiple channels in the cell-attached patch area, time-dependent inactivation occurs in the presence of external Ba^{2+}, suggesting co-operation among channels. Channel clustering contributes to the increase in concentration of the divalent ions on the internal side of the membrane and, consequently, enhances the inactivation process. Figure 6 is an illustration of a cell-attached experiment with one, two, and three Ca^{2+} channels conducting Ba^{2+}. The mean current values show a gradual increase in inactivation.

Experimental observation of a single Ca^{2+} channel that does not inactivate offers the possibility to study the effect of physiological Ca^{2+} permeation entering from the vicinity of the single-channel internal face. Using a different stimulation procedure, we investigated the influence of whole-cell Ca^{2+} influx on the high-threshold Ca^{2+} channel. The experimental protocol and the various stimulation procedures are illustrated in Fig. 7. In Fig. 7*A* the membrane

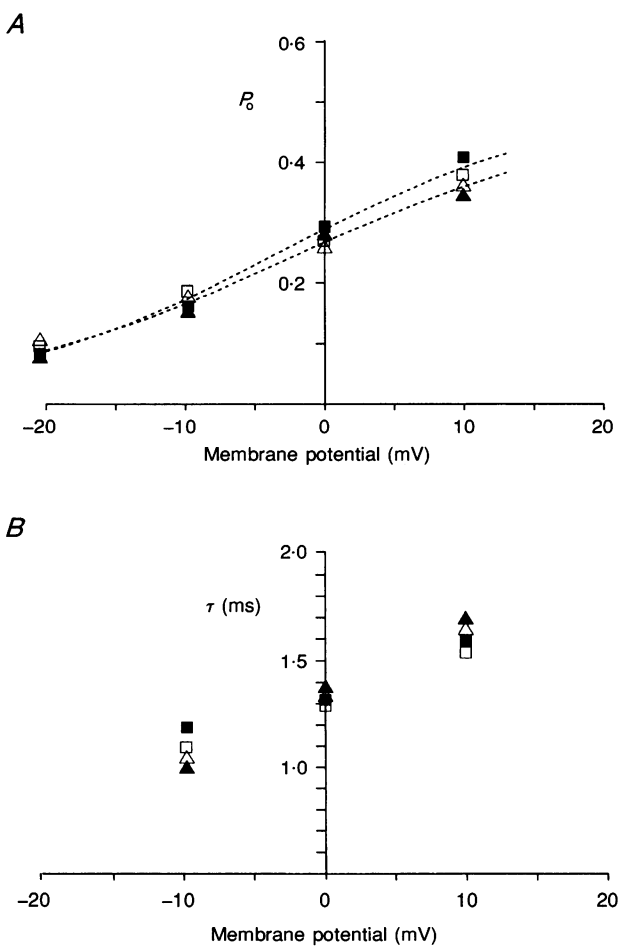

Figure 4. Kinetic parameters of a single-channel open state
Open probability (*A*) and open-time constant (*B*) obtained from open-time histograms are plotted as a function of membrane potential. The triangles reflect data from two experiments in which the voltage protocol started from a holding potential of −50 mV, and the squares reflect a holding potential of −80 mV.

voltage was held at a negative potential by the whole-cell electrode, and a voltage-step protocol was delivered in the on-cell patch pipette to stimulate only the single channel. The on-cell experiment generated a mean single-channel current ($n = 20$) that remained constant during the entire test. In Fig. 7*B* the same cell as in Fig. 7*A* was used; the voltage was clamped in the single neuron at various test potentials and macroscopic Ca^{2+} current(s) recorded.

Single-channel openings were recorded simultaneously with the cell-attached pipette. When the stimulus was applied using the whole-cell electrode (Fig. 7*B*), the cell-attached pipette recorded single-channel currents similar to the three examples. The mean ($n = 20$) single-channel Ba^{2+} current, depicted at the bottom of Fig. 7*B*, shows pronounced inactivation. If the stimuli in the whole-cell and in the cell-attached pipette were identical, the voltage experienced by the patch was, in both cases, the same. The voltage challenge used for whole-cell stimulation (Fig. 7*B*) activates all of the various Ca^{2+} conductances present on the dorsal root ganglion cell membrane, in addition to the single channel in the patch (Fox *et al.* 1987; Ferroni *et al.* 1989). Divalent ions enter the cell and alter the Ca^{2+}

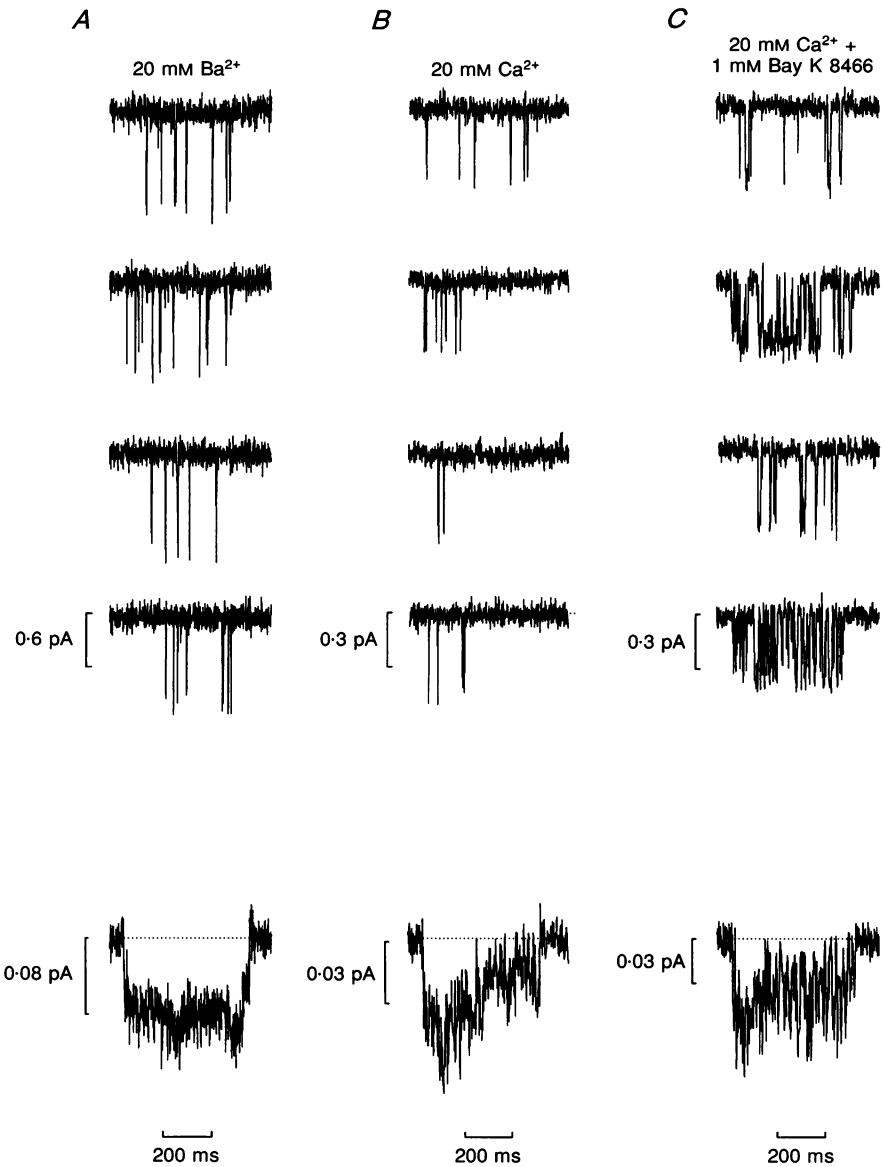

Figure 5. Comparison of Ca^{2+}- and Ba^{2+}-induced inactivation
Comparison of a single channel conducting 20 mM Ba^{2+} (*A*) and 20 mM Ca^{2+} (*B*). The calculated conductances are 18 and 8–9 pS, respectively. The bottom traces are two mean currents obtained from 14 current recordings, each showing a pronounced difference in their kinetic properties. The inactivation process is evident when the channel conducts Ca^{2+}, but is absent when Ba^{2+} is the charge carrier. On the right (*C*) are shown single-channel traces and mean current ($n = 10$) in 20 mM Ca^{2+} after addition of 1 μM Bay K 8644. A 600 Hz filter was used to record the single-channel Ca^{2+} currents.

concentration in the cytosol. The time course of the single-channel inactivation in a whole-cell stimulated cell was not constant. Calculated time constants from twelve experiments ranged between 50 and 200 ms, suggesting varying degrees of Ca^{2+} permeability through the membrane. As illustrated in Fig. 6, it is evident that the number of channels per unit area is not constant for various membrane regions. It is not unusual to find patches containing three, four, or even five channels, indicating the presence of clusters of similar ionic pathways.

These results allow one to speculate on the role of external calcium ion influx in the inactivation process of a single channel. When a single Ca^{2+} channel conducts Ba^{2+}, the inactivation due to the divalent ions flowing through the channel is absent. Since the voltage-dependent inactivation should be the same, it is possible that the current decay results from a build-up of internal calcium during stimulation of the entire cell.

It is confirmed that Ca^{2+} ion influx is, in part, responsible for single-channel inactivation as illustrated in Fig. 8. Using equivalent patch-clamp configurations and stimulation patterns, we first stimulated the patch and then the whole-cell.

Our results showed that on-cell electrode stimulation elicited a non-inactivating mean single-channel current ($n = 14$). Stimulation of the entire cell activated a significant whole-cell Ca^{2+} current and induced channel inactivation. The addition of 0·2 mM Cd^{2+} to the external solution was sufficient to eliminate the whole-cell Ca^{2+} current produced by various types of Ca^{2+} channels. Preventing Ca^{2+} ion permeation prohibited inactivation of the mean single-channel current ($n = 16$).

DISCUSSION

Single-channel conductance and kinetics

The experiments described above emphasize the importance of using low concentrations of divalent ions to study the physiology of Ca^{2+} channels. L-type Ca^{2+} permeability has already been characterized in heart cells and in dorsal root ganglion neurons. Using 90 or 110 mM Ba^{2+}, dihydropyridine-sensitive channels show 23–27 pS conductances (Hess *et al.* 1984; Nowycky *et al.* 1985; Fox *et*

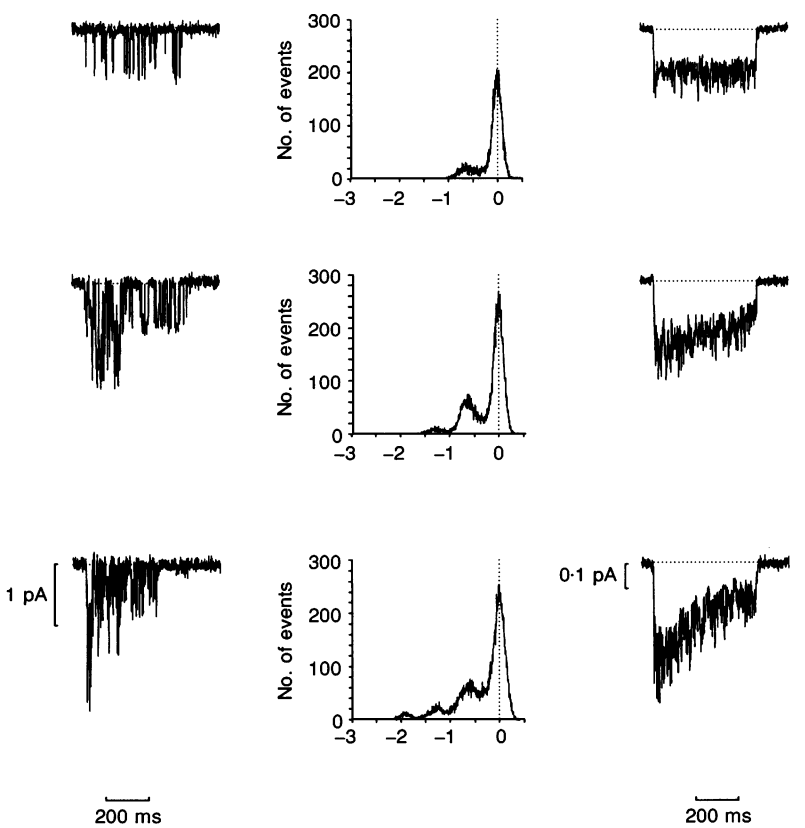

Figure 6. The inactivation property of a Ca^{2+} channel conducting 20 mM Ba^{2+}
The figure shows three different single-channel experiments in which one (upper traces), two (middle traces), and three (lower traces) Ca^{2+} channels conducting Ba^{2+}, elicited by voltage steps from −50 to −10 mV holding potential, are present. The left panel shows examples of single-channel recordings. The central panel depicts amplitude histograms obtained from 5 s recordings. On the right, three mean current values are compared from 10 single-channel traces in each experiment. It is evident that the inactivation process appears when the patch contains more than one Ca^{2+} channel.

al. 1987; Yue & Marban, 1990; Artalejo et al. 1991). At a low Ba^{2+} concentration (10–20 mM), heart cells (Mazzanti & DeFelice, 1990; Yue & Marban, 1990; Mazzanti et al. 1991) and dorsal root ganglion neurons (Mazzanti et al. 1992) demonstrated a conductance around 20 pS. Disregarding minor variations in the conductance values, single Ca^{2+} channel kinetic characteristics of both heart cells and dorsal root ganglion neurons are modified based on the concentration of the divalent ion. It is known that the reversal potential of the channel in cardiac myocytes increases by about 10 mV for every 30 mM increase in Ba^{2+} concentration (McDonald, Cavalie, Trautwein & Pelzer, 1986; Pietrobon & Hess, 1990) and shifts the open probability curve to the right. As a consequence, the channel's voltage sensitivity to the transition rates between the closed and the open state changes in 90–110 mM Ba^{2+}. Current traces that cause long openings of the channel in low Ba^{2+} concentration at 10 mV membrane potential would have the same kinetics at 40–50 mV in high Ba^{2+} concentration. This behaviour becomes marked when the concentration of divalent ions, in particular Ca^{2+} ions under physiological conditions, is less than 5 mM. Ca^{2+} channel opening would also be more likely to occur at membrane voltages less than 0 mV, suggesting an additional role of L-type Ca^{2+} channels during the repolarization phase of the nervous action potential (Mazzanti et al. 1992).

Ca^{2+}-dependent inactivation

The ability to measure single calcium channel openings, using the two-electrode technique, when the calcium concentration inside the cell was increased allowed us to separate the calcium-dependent from the voltage-dependent

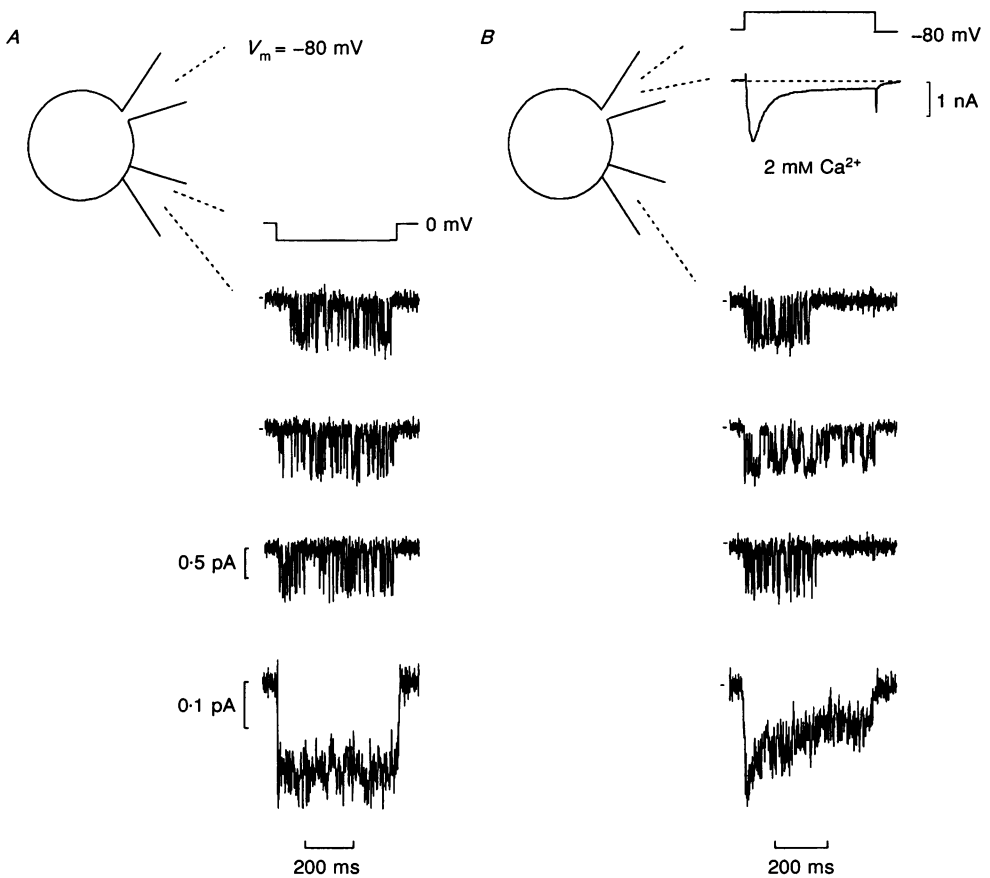

Figure 7. Two different procedures used to trigger the opening of L-type Ca^{2+} channels
A, voltage protocol from −80 to 0 mV membrane potential was applied to the membrane patch isolated by the on-cell electrode. In this case, it was possible to record a single-channel Ba^{2+} current, while the whole-cell pipette clamped the membrane potential (V_m) at −80 mV. A mean of 20 single-channel current traces, like the three examples shown, produces the trace depicted at the bottom of the panel. *B*, the same stimulus protocol, delivered via the whole-cell electrode, activated macroscopic Ca^{2+} current(s) recorded by the same electrode. The cell-attached pipette detected single L-type Ca^{2+} channels conducting 20 mM Ba^{2+}. Three single-channel traces are shown. The lowest trace is the result of single-channel averaging ($n = 20$) and shows inactivation of the current. Using this procedure on the same cell, it was thus possible to compare the influence of whole-cell Ca^{2+} current(s) on the kinetic parameters of the single channel.

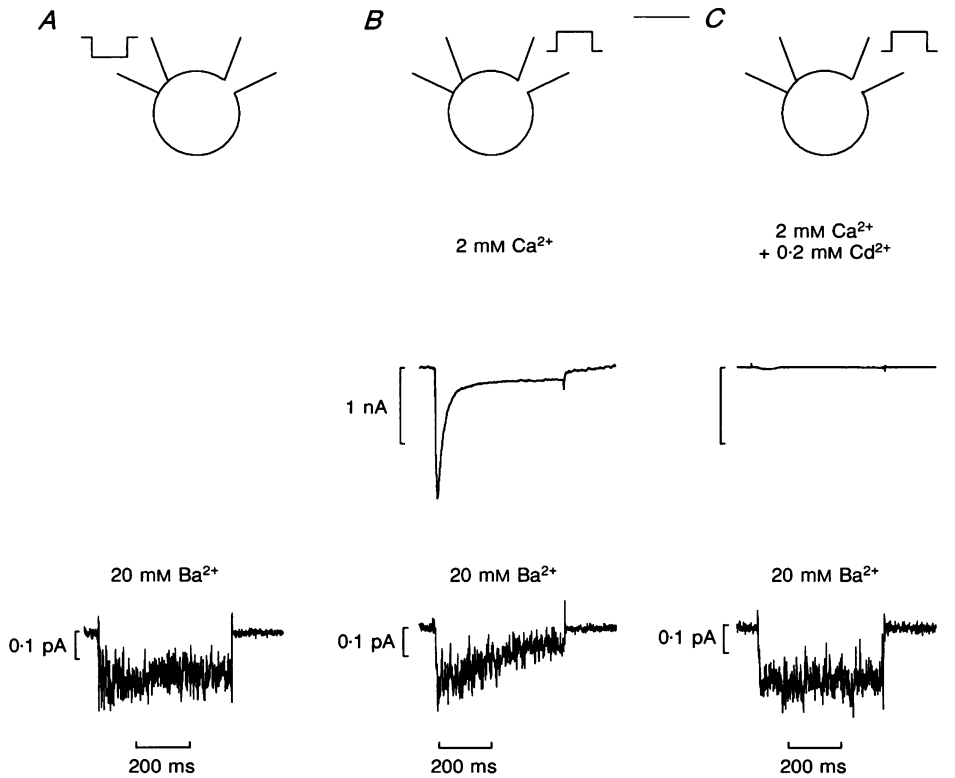

Figure 8. Effect of whole-cell Ca^{2+} current block on the inactivation of a single-channel Ba^{2+} current

The different experimental configurations are illustrated above each current recording. *A*, mean single-channel current ($n = 14$) was obtained from cell-attached stimulation at 0 mV membrane potential with the cell potential clamped at −80 mV by the whole-cell electrode. *B* demonstrates macroscopic Ca^{2+} current (middle panel) and mean single-channel activity (lower panel, $n = 16$), triggered by a voltage step from −80 to 0 mV delivered in the whole-cell pipette. In this case, the mean current shows inactivation. *C*, the addition of 200 μM Cd^{2+} to the external solution eliminates whole-cell Ca^{2+} current (middle panel). The mean current (lower panel, $n = 16$) does not show inactivation and reverts its kinetics to that of *A*.

components of the dihydropyridine-sensitive current inactivation. More specifically, it permitted the separation of the intrinsic and extrinsic inactivation processes of the L-type Ca^{2+} channel. Voltage-dependent inactivation and the resulting decrease in current, due to the same ions being transported by the pore, represent intrinsic characteristics; whereas the influence of intracellular Ca^{2+} accumulation, due to the concurrent functioning of many channels, is considered an extrinsic factor that enhances channel inactivation. The use of Ba^{2+} instead of Ca^{2+} ions in cell-attached experiments as charge carriers allows us to observe directly the influence of Ca^{2+} ions in physiological concentration coming from adjacent pathways on a single channel. Ba^{2+} current flowing through a single L-type Ca^{2+} channel shows little or no voltage-dependent inactivation, and the low affinity of Ba^{2+} for Ca^{2+} channels (Hess & Tsien, 1984) reduces current-dependent inactivation (Yue *et al.* 1991; Mazzanti *et al.* 1991). In general, using Ba^{2+} as a permeating ion through a Ca^{2+} channel minimizes the intrinsic inactivation factors of the pore. During whole-cell stimulation, the adjacent channels conduct calcium at physiological concentration, with the observed effect that single-channel inactivation is directly dependent on the amount of intracellular Ca^{2+} (Fig. 8*B*). Lux & Brown (1984) found that there was no correlation between the flow of Ca^{2+} through a single calcium channel and the time spent in a subsequent closed state. From their experiments, in which they used 40 mM Ca^{2+} as the conducting ion, they concluded that calcium ions do not inactivate individual Ca^{2+} channels. In contrast, Yue *et al.* (1991) showed that, in 160 mM Ca^{2+}, calcium ions inactivate the very channels through which they have passed. The experiments of both investigative groups focused on patches that contained one or two channels. Furthermore, as mentioned in the first section of the Discussion, a high concentration of divalent cations disturbs the kinetics of the channel. The absence of Ca^{2+} inactivation in the Lux & Brown experiments may be explained by the theory that current through one channel might never establish the concentration necessary to block the pore. Moreover, 40 mM external Ca^{2+} is sufficient to shift

the open probability curve to the right, producing flickering kinetics at test potentials of 0–10 mV. The absence of long openings, together with a low single-channel conductance, appears inadequate to provide a sufficient rise in the internal Ca^{2+} concentration to enable interaction with the channel. In the case of Yue *et al.* (1991), even if 160 mM Ca^{2+} produces a wider shift to the right of the open probability curve, a higher single-channel conductance is probably sufficient to achieve the critical intracellular Ca^{2+} concentration that enhances inactivation.

Our data suggest that the inactivation of the L-type single channel, during a depolarization step, depends on the concomitant activity of all types of calcium channels present at the cell surface. The theory that co-operation among ionic channels plays a role in modulating transmembrane Ca^{2+} currents (Mazzanti *et al.* 1991; Imredy & Yue, 1992; DeFelice, 1993) may represent a dynamic cellular control of ion movement through the plasma membrane. Therefore, Ca^{2+}-dependent Ca^{2+} current inactivation could be part of a feedback system in association with Ca^{2+}-activated K^+ channels to terminate action potential bursts. Ca^{2+} channel density would play an important role in both processes. Low quantities of functioning channels would maintain membrane voltage oscillations. The repetitive firing increases the open probability of Ca^{2+} channels, providing a rise in intracellular Ca^{2+} that promotes inactivation of the current and increases K^+ conductances.

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Acknowledgements

We would like to thank Mr Gaspare Mostacciuolo for his excellent technical assistance, Drs Enzo Wanke & Enzo Mancinelli and particularly Dr Louis J. DeFelice for their critical reading of the manuscript, and Dr Linda Cairns and Cathy Alden for editing the paper.

Received 2 June 1993; accepted 13 October 1993.