Activation of Three Types of Membrane Currents by Various Divalent Cations in Identified Molluscan Pacemaker Neurons

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ABSTRACT We investigated membrane currents activated by intracellular divalent cations in two types of molluscan pacemaker neurons. A fast and quantitative pressure injection technique was used to apply Ca^{2+} and other divalent cations. Ca^{2+} was most effective in activating a nonspecific cation current and two types of K^+ currents found in these cells. One type of outward current was quickly activated following injections with increasing effectiveness for divalent cations of ionic radii that were closer to the radius of Ca^{2+} ($Ca^{2+} > Cd^{2+} > Hg^{2+} > Mn^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+} > Pb^{2+} > Sr^{2+} > Mg^{2+} > Ba^{2+}$). The other type of outward current was activated with a delay by $Ca^{2+} > Sr^{2+} > Hg^{2+} > Pb^{2+}$. Mg^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} were ineffective in concentrations up to 5 mM. Comparison with properties of Ca^{2+}-sensitive proteins related to the binding of divalent cations suggests that a Ca^{2+}-binding protein of the calmodulin/troponin C type is involved in Ca^{2+}-dependent activation of the fast-activated type of K^+ current. The sequence obtained for the slowly activated type is compatible with the effectiveness of different divalent cations in activating protein kinase C. The nonspecific cation current was activated by $Ca^{2+} > Hg^{2+} > Ba^{2+} > Pb^{2+} > Sr^{2+}$, a sequence unlike sequences for known Ca^{2+}-binding proteins.

INTRODUCTION

Membrane conductances activated by intracellular Ca^{2+} ions, such as potassium- and chloride-selective types as well as nonspecific cation conductances have been described in a variety of preparations (for reviews see e.g., Meech, 1976; Schwarz and Passow, 1983; Latorre et al., 1984; Hockberger and Swandulla, 1987; Partridge and Swandulla, 1988). Little is known, however, about channel activation by Ca^{2+} ions. One way to characterize the Ca^{2+}-binding sites involved in the activation process is to test the ability of other divalent cations to substitute for Ca^{2+} in activating these channels.

We investigated the membrane currents induced by fast intracellular injection of various divalent cations in two types of pacemaker neurons, in which Ca^{2+}-activated

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cation channels and potassium channels play a key role in generating characteristic spontaneous activity patterns (Lux and Hofmeier, 1982*a*, *b*; Swandulla and Lux, 1985; Partridge and Swandulla, 1987, 1988). Our results show that the ability of divalent cations to activate Ca^{2+}-dependent currents in these neurons varies significantly for different current types. One type of Ca^{2+}-dependent K^+ current is activated by all divalent cation species tested. The effectiveness of the injected ions in activating this current correlates with their ionic radius. While Ca^{2+} activates the largest currents, the effectivity of the other divalent cations decreases with a smaller or larger ionic radius. A second type of Ca^{2+}-dependent K^+ current is activated only by Ca^{2+}, Sr^{2+}, Hg^{2+}, and Pb^{2+}. Finally, the Ca^{2+}-dependent nonspecific cation current found in bursting pacemaker neurons is activated by Ca^{2+}, Hg^{2+}, Zn^{2+}, Ba^{2+}, Pb^{2+}, and Sr^{2+} in order of decreasing effectivity.

We conclude that different types of binding sites are involved in Ca^{2+}-dependent activation of these membrane conductances. The ability of divalent cations in activating the first type of Ca^{2+}-dependent K^+ current agrees qualitatively with the change in tyrosine flourescence of calmodulin induced by, or the affinity of troponin C for, divalent cations. This suggests that a Ca^{2+}-binding protein similar to calmodulin and troponin C is involved in Ca^{2+}-dependent activation of this channel. The long time course of the second type of Ca^{2+}-dependent K^+ current, in comparison with the time course of intracellular $[Ca^{2+}]$ after Ca^{2+} injection, already indicated that Ca^{2+}-dependent activation of these channels involves one or more enzyme-controlled steps (Hofmeier and Lux, 1981*b*). The set of divalent cations that activate this current is compatible with the set of cations that activate protein kinase C.

METHODS

Preparation and Recording

Subesophageal ganglia were dissected from the snail *Helix pomatia* and the capsules were removed. The remaining inner sheath of connective tissue was also removed after being exposed for 3 min to pronase (1 mg/ml Ringer solution) (Swandulla and Lux, 1985). The use of pronase does not significantly affect membrane behavior (Eckert and Lux, 1975). Experiments were carried out on the F1 burster cell (Kerkut et al., 1975), also known as RPa1 (Sakharov and Salanki, 1969), and on U-cells (Lux and Hofmeier, 1982*a*, *b*; Swandulla and Lux, 1985) in the right parietal ganglion. The preparation was kept in Ringer solution (see below) at 18°C.

The voltage-clamp circuit was as described previously (Heyer and Lux, 1976; Hofmeier and Lux, 1981*b*). Intracellular potential and current electrodes were filled with 3 M KCl. Data were recorded on magnetic tape (Store 4 DS; Racal Electronic Systems, München, FRG) and analyzed using a digital oscilloscope (VKS 22-16; Wuntronic, München, FRG) and a chartwriter (7402A; Hewlett-Packard Co., Palo Alto, CA).

Intracellular Injections

Various solutions (see below) were applied intracellularly by fast and quantitative pressure-injection (Hofmeier and Lux, 1981*b*). This method provides a resolution of 10^{-11} liter and, considering the average cell volume of $0.5–1 \times 10^{-8}$ liter, is suitable for measured injection quantities of <1% of cell volume.

The injection pipettes were as described previously (Hofmeier and Lux, 1981*b*). Diameters of the solution reservoir and the tip were 50 and 1–2 μm, respectively. After filling the pipette with ion exchanger (477317; Corning Glass Works, Corning, NY) from the top, the injection solution was sucked up from the tip (Fig. 1 *D*). A constant negative holding current of 10 nA was applied to the ion exchanger of the injection pipette to prevent cation drift out

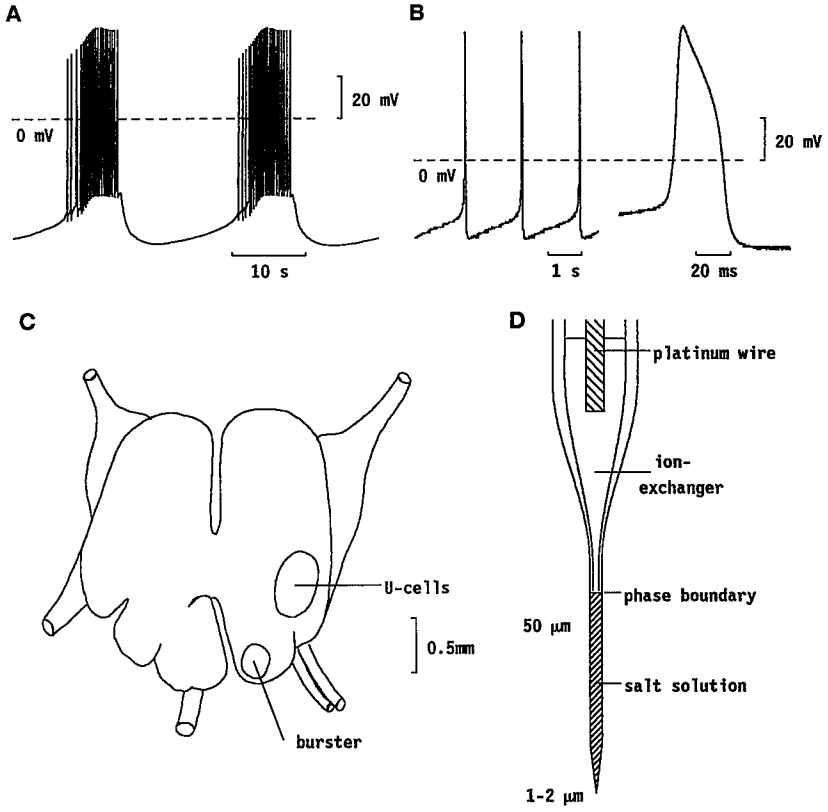

FIGURE 1. (*A*) The spontaneous activity of F1 burster neurons shows action potentials grouped in bursts of 10–25, separated by silent phases during which membrane potential reaches values as negative as −60 mV. During the first half of each burst the overshoot increases up to ~40 mV and decreases during the second half. (*B*) U-cells fire regularly with a frequency of ~0.5 Hz. The individual action potential is characterized by a long duration (20–40 ms) and large overshoot (60–70 mV). (*C*) Dorsal view of the subesophageal ganglion complex of the snail *Helix pomatia* after preparation. The characteristic locations of the two types of pacemaker neurons studied, F1 bursters and U-cells, are indicated. (*D*) Schematic of the injection pipette used for intracellular pressure injection (see text).

of the tip. This measure also provided a means to verify proper penetration of the tip through the cell membrane and it was found to prevent or at least delay clogging of the pipette.

Injections were quantified by observing the shift of the phase boundary between the aqueous injection solution and the ion exchanger during injection, and by calculating the cylindri-

cal volume of injected solution. Taking into account the fact that the pipette was tilted ~45° when the length of the injection cylinder was measured, this leads to

$$V_{inj} = 1/\sin 45° \cdot \pi/4 \cdot d^2 \cdot l, \quad (1)$$

where d is the inside diameter of the pipette and l is the length of the cylinder as measured under the microscope.

The cell volume was approximated using the formula of an ellipsoid volume. Two perpendicular diameters were measured with an ocular micrometer and their geometric mean was substituted for the third diameter, which was perpendicular to the viewing plane of the microscope and could not be measured. This amounts to the formula

$$V_{cell} = \pi/12 \cdot (d_1 \cdot d_2)^{1.5}, \quad (2)$$

where d_1 and d_2 are the two measured diameters.

Quantities of up to 5% of cell volume can be injected by single pressure pulses of ~0.1 s in duration, a short interval compared with the time scale of the effects studied here. Numerous tests have been performed ruling out an artificial origin of these effects (Hofmeier and Lux, 1981*b*; Swandulla and Lux, 1985).

Solutions

The Ringer solution contained (in millimolar): 80 Na^+, 4 K^+, 10 Ca^{2+}, 5 Mg^{2+}, 114 Cl^-, 10 glucose, and 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) for buffering (pH 7.8). Injection solutions contained one of the following substances in 100 mM concentration: $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, $ZnCl_2$, $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$, $MnCl_2$, $CoCl_2$, and $NiCl_2$. $Pb(NO_3)_2$ was used instead of $PbCl_2$ because $PbCl_2$ is not sufficiently soluble in water. To prevent dilution of internal K^+ by the injected volume, possibly resulting in a local K^+ gradient inversion and K^+ influx (Heyer and Lux, 1976), 66 mM KCl was added to each injection solution. A control solution containing 66 mM KCl did not induce inward or outward currents even when quantities exceeding 10% of cell volume were injected into neurons clamped at −50 mV. The pH was adjusted using KOH or HCl as close to the probable intracellular value of 7.4 (Thomas and Meech, 1982; Swandulla and Lux, 1985) as possible without causing precipitation of the dissolved salts.

Since even small quantities of Ca^{2+} induce large current responses, some experiments were carried out using lower Ca^{2+} concentrations.

Injection solutions containing divalent cations other than Ca^{2+} could have been contaminated by Ca^{2+} due to impurities of the distilled H_2O or the salts. With the injection volumes applied, transient increases in intracellular Ca^{2+} concentration of maximally 0.5 μM would occur. To exclude activating effects of these small amounts we injected a control solution containing 1 mM $CaCl_2$ and 66 mM KCl. Injections of up to 5% of cell volume were found to be ineffective.

Intracellular Spreading of Injected Divalent Cations

Divalent cations were injected intracellularly using a technique allowing large amounts (up to 5 mM based on cell volume) to be applied within a fraction of a second, i.e., a very short interval compared with the time scale of the current responses. There was no indication of intracellular precipitation of the injected salts. Three processes determine the intracellular spreading of the injected cations: (*a*) diffusion, (*b*) buffering by high-affinity Ca^{2+}-binding proteins in the cytosol, and (*c*) sequestration by Ca^{2+} transport systems (e.g., Na^+/Ca^{2+} exchangers, Ca^{2+}-ATPase) found in plasma membrane and membranes of mitochondria and other organelles. Diffusion together with intracellular buffering determine the rise of intracellular $[Ca^{2+}]$ near the plasma membrane, while the decay most likely reflects the kinetics of

the sequestration process. Based on an estimated apparent diffusion coefficient of $2 \cdot 10^{-6}$ cm^2 s^{-1} for Ca^{2+} ions in the cytosol (which includes the effect of cytosolic buffers, see Connor and Nikolakopoulou, 1982), the average injected Ca^{2+} ion should cover the distance between pipette tip and cell membrane (estimated to average about 100 μm in U-cells) within ~8 s. Experimental values obtained from the somewhat larger bursting neurons using ion-sensitive microelectrodes averaged ~12 s (Hofmeier and Lux, 1981*b*).

RESULTS

The location of both types of neurons in the ganglia of the snail *Helix pomatia* and their spontaneous activity patterns are shown in Fig. 1. In burster neurons action potentials are grouped in bursts of 10–25, separated by intervals during which the cell is silent and membrane potential reaches values as negative as −60 mV. This characteristic activity pattern can be explained by the interaction of a Ca^{2+}-activated nonspecific cation current, which provides the depolarizing drive underlying the burst, and Ca^{2+}-dependent outward currents found in these neurons (Swandulla and Lux, 1985; Partridge and Swandulla, 1987).

A regular firing pattern with a nearly constant frequency of ~0.5 Hz is observed in the other type of pacemaker neuron investigated (U-cells, Lux and Hofmeier, 1982*a*, *b*). The individual action potential is 20–40 ms in length and has an overshoot of 60–70 mV. Under voltage clamp inward Ca^{2+} and outward K^+ currents can be activated by depolarization. Blocking the Ca^{2+} current by external application of Ni^{2+}, Cd^{2+}, or Co^{2+} results in almost complete suppression of the total membrane current. This indicates that in these neurons Ca^{2+} conductances and Ca^{2+}-activated K^+ conductances form the basis of excitability.

Currents Induced by Ca^{2+} Injection

For Ca^{2+} injections, neurons were voltage clamped near resting potential at −50 mV and short hyperpolarizing pulses of 10 mV amplitude were applied to measure changes in overall membrane conductance during the injection-induced currents. Conforming to previous reports (Swandulla and Lux, 1985), only an outward current was activated in U-cells after Ca^{2+} injection, whereas a sequence of currents was observed in bursters (Fig. 2 *A*). This sequence consisted of an inward current that immediately followed the injection and an outward current that showed a fast and a slow component. Inward and outward currents were paralleled by reversible increases in membrane conductance.

Detailed studies of the injection-induced inward current, which revealed a linear *I-V* relation with a reversal potential near −20 mV, have shown that these channels are permeable for many cations including the larger species, such as choline, TEA, and Tris, whereas Cl^- does not contribute significantly to this current (Swandulla and Lux, 1985; Partridge and Swandulla, 1987). The outward currents reversed at ~−65 mV, near the estimated reversal potential for K^+ currents (−70 mV). In the presence of external TEA (10 mM) outward currents were suppressed in both types of pacemaker neurons (Fig. 3). These findings indicate that the conductances involved are selective for K^+ ions. This is consistent with previous results obtained in *Helix* neurons (Meech and Standen, 1975; Hofmeier and Lux, 1981*a*, *b*; Hermann and Hartung, 1982; Lux and Hofmeier, 1982*a*).

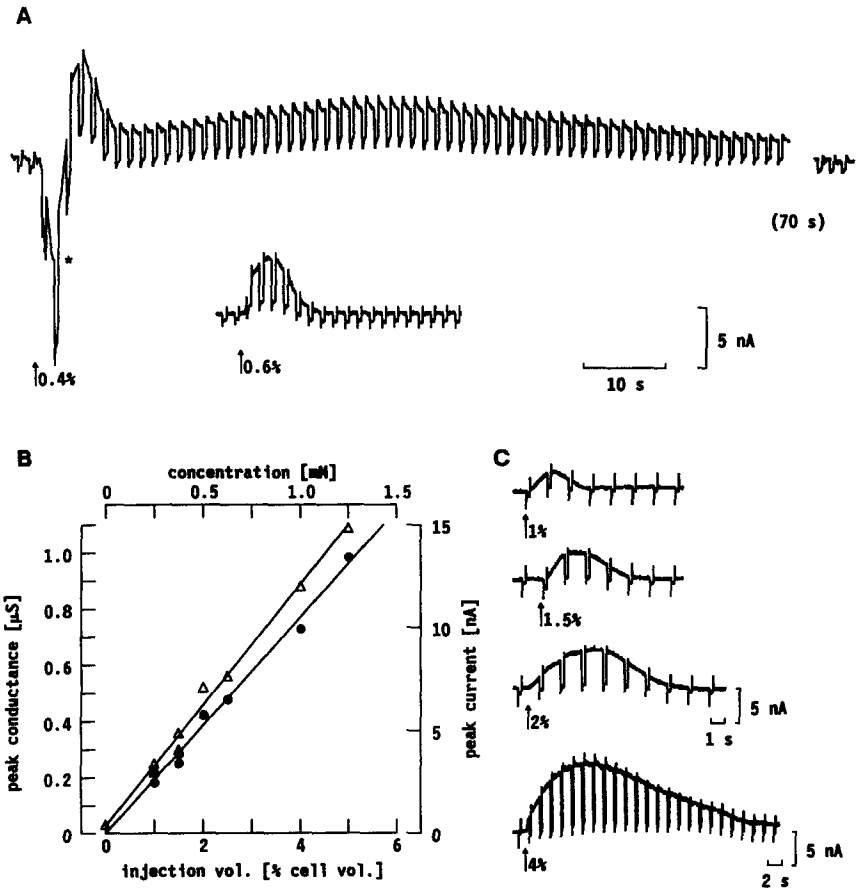

FIGURE 2. Current responses induced by Ca^{2+} injections. (*A*) Ca^{2+} (100 mM solution) was injected into a burster (*top*) and a beater neuron under voltage-clamp conditions, and current responses were recorded. Holding potential was −50 mV and overall membrane conductance was measured every 1.5 s by applying hyperpolarizing pulses of 10 mV amplitude and 0.5 s duration. Injections are marked by arrows and the injected volumes in percent of cell volume are indicated. The peak of the inward current is marked (*). The current deflection below the asterisk is due to one of the hyperpolarizing pulses. (*B* and *C*) Quantitative dependence of Ca^{2+}-activated K^+ current on intracellular [Ca^{2+}] elevation. Different amounts of Ca^{2+} (25 mM solution) were injected into the same voltage-clamped beater neuron. Only current responses to four of a total of eight injections are shown (*C*). Both the peak outward current (*circles*) and the largest conductance value (*triangles*) measured after each injection show linear dependence on the injected amount of Ca^{2+} solution relative to cell volume (*B*). The second horizontal axis shows the injected Ca^{2+} concentration. Peak Ca^{2+} activities near the cell membrane caused by the injections are probably much lower (see text).

A Ca^{2+}-induced TEA-resistant outward current has been described in bursting neurons of *Aplysia* and is thought to reflect Ca^{2+}-dependent inactivation of Ca^{2+} channels (Kramer and Zucker, 1985*b*). Although the time course of this current is similar to the slow outward current described above, it is unlikely that both currents are identical. Whereas a Ca^{2+}-induced inactivation of Ca^{2+} channels should result in

a decrease of membrane conductance, the increase observed here (Fig. 2 *A*) agrees well with the assumption that the outward currents are carried by K^+ ions.

Outward Currents Activated by Divalent Cations

The fast component of the outward current in burster neurons and the outward current in U-cells showed similar time courses, peaking ~10 s after the injection (Fig. 2 *A*). Further similarities shared by these conductances (see below) suggest that similar types of Ca^{2+}-activated K^+ channels are involved in both neurons. However, in burster neurons the slow component often predominated in the total current response, masking the fast component, which, furthermore, may have overlapped

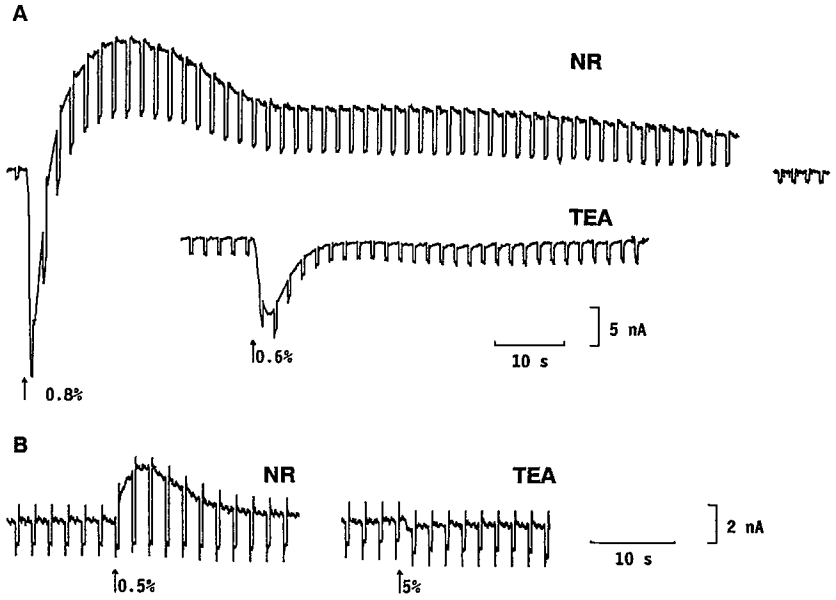

FIGURE 3. Effect of TEA on currents activated by Ca^{2+} injections. Ca^{2+} (100 mM) was injected into voltage-clamped neurons (*A*, burster; *B*, beater neuron) bathed in normal ringer solution (*NR*) and then in ringer solution containing 10 mM TEA-Cl (replacing 10 mM NaCl). The current responses show that Ca^{2+}-activated outward currents are suppressed in both cell types. Membrane conductance was measured by applying short hyperpolarizing pulses (*A*, 10 mV; *B*, 20 mV).

the decaying inward current. Investigation of the fast outward current therefore focused on U-cells, thereby avoiding contamination by other Ca^{2+}-activated currents. As shown in Fig. 2 *C*, the amplitude of this current depends on the injected amount of Ca^{2+}. Different quantities of a solution containing 25 mM $CaCl_2$ were injected into a U-cell clamped at −50 mV. In Fig. 2 *B* the peak outward current and the peak conductance are plotted vs. the injected quantity (related to cell volume), showing a linear dependence for both (see also Gorman and Hermann, 1979; Hermann and Hartung, 1982). Solutions containing various divalent cations in 100 mM concentration were injected into both types of pacemaker neurons as described for Ca^{2+} injections. No additional types of outward currents other than those induced

by Ca^{2+} injections were observed after injections of other divalent cations. The injection-induced outward currents were always paralleled by reversible increases in membrane conductance.

Injections of all divalent ions tested consistently activated the fast outward current in U-cells. For most cation species, the current activated by injections on the order of 3–5% of cell volume peaked within ~5 s. Currents induced by Sr^{2+} exhibited a slower onset of ~15 s. Repeated injections of all divalent cations other than Ba^{2+} had the same effect as the first injection. However, injection-induced currents decayed more slowly as the total injected amount increased (Fig. 4), which was possibly due to exhaustion of the intracellular sequestration mechanisms, and eventually current amplitudes decreased. By contrast, only the first injection of Ba^{2+} induced a small outward current (Table I), whereas subsequent injections failed to

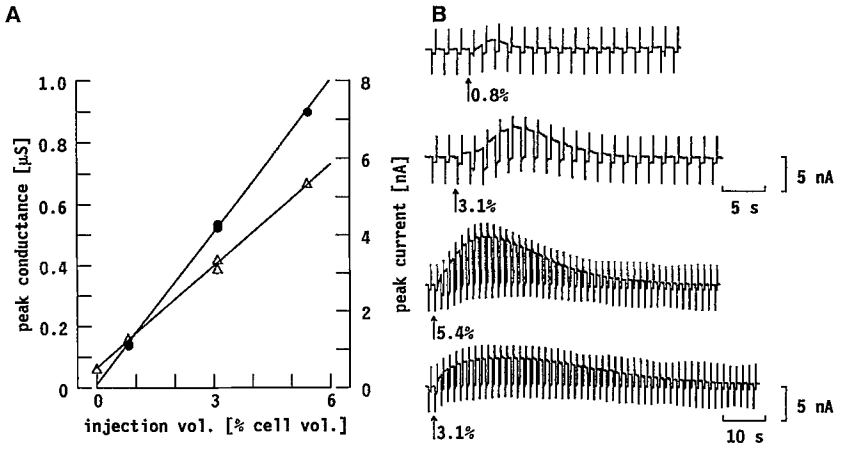

FIGURE 4. Cd^{2+}-activated outward currents in beater neurons. Different quantities of Cd^{2+} (100 mM solution) were injected into a single voltage-clamped U-cell. The current responses to four injections are shown in order (*B*). The peak outward current (*circles*) and the largest conductance value (*triangles*) measured after each injection are plotted vs. injection volume (*A*).

produce any response (see also Meech and Thomas, 1980). No recovery was observed.

While the largest currents were observed after Ca^{2+} injections, the amplitudes varied significantly for other divalent cations. Since current amplitudes also varied with the injected quantities, peak amplitudes were divided by the ratio of injected volume to cell volume, yielding values equivalent to injections of 1 mM (i.e., 1 mmol per liter of cell volume) of each divalent cation. The average of values obtained from several injections was taken as the measure of effectivity for the cation species in activating the particular current (Table I). Ca^{2+} was most effective in activating the fast outward current seen in U-cells, followed by Hg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+}, Sr^{2+}, Mg^{2+}, and Ba^{2+}.

The ability of divalent cation species in activating the fast outward current correlates with the ionic radius (Fig. 5). Divalent cations with ionic radii close to the

TABLE 1

Outward Currents Activated by Divalent Cations in Beating Pacemaker Neurons

Ion	Outward current		N	Ion radius
	nA	I_X/I_{Ca}		*pm*
Mg^{2+}	0.68 ± 0.004	0.09	4	65
Ni^{2+}	1.43 ± 0.03	0.18	6	69
Zn^{2+}	1.88 ± 0.05	0.24	3	74
Co^{2+}	1.60 ± 0.12	0.20	4	74
Mn^{2+}	2.03 ± 0.06	0.25	5	93
Cd^{2+}	5.30 ± 0.30	0.67	5	97
Ca^{2+}	7.87 ± 0.27	1.00	6	99
Hg^{2+}	2.48 ± 0.29	0.32	3	110
Sr^{2+}	1.27 ± 0.14	0.16	4	113
Pb^{2+}	1.40 ± 0.23	0.18	4	121
Ba^{2+}	0.52 ± 0.12	0.07	3	135

The peak outward current amplitudes induced by injections of the above divalent cations into voltage-clamped U-cells were measured and normalized to injections of 1 mM (see text). The mean value from several injections, the standard error, and the relative effectivity based on Ca^{2+} is indicated for each ion species. The ionic radii (Pauling) were taken from Wiberg (1976).

radius of Ca^{2+} are more effective in activating this current than cations with much smaller or larger ionic radius.

The fast outward current component induced by injections of Ca^{2+} in burster neurons was activated by most divalent cations. Outward currents activated by Cd^{2+} (Fig. 6), Co^{2+}, Ni^{2+}, and Ba^{2+} showed only the fast but not the slow component. In the case of Cd^{2+} and Co^{2+}, onset and decay of the current responses, which also lacked the inward current, were similar to those seen in U-cells. Outward currents

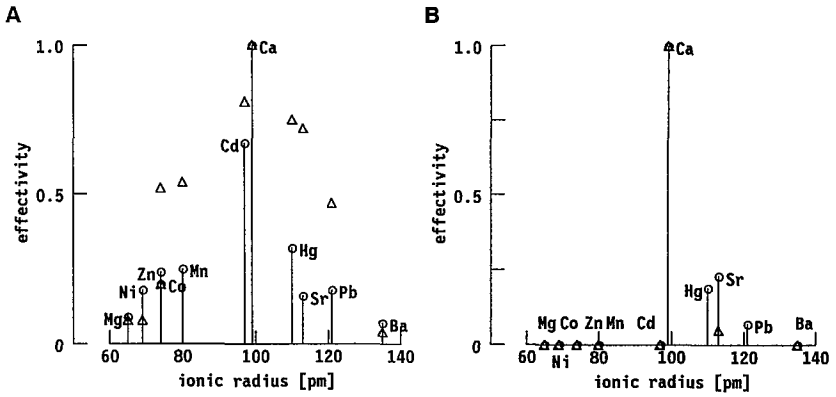

FIGURE 5. Effectivity of divalent cations in activating outward currents (data from Tables I and II). The effectivity of divalent cations, based on Ca^{2+}, in activating the fast (*A*) and the slow (*B*) component of the outward current is plotted vs. the ionic radius (*circles*). For comparison, the effectivity of divalent cations in increasing tyrosine fluorescence of calmodulin (*A*, from Chao et al., 1984, values determined in saturation) and in activating protein kinase C (*B*, see Takai et al., 1979, cation concentration: 0.3 mM) is also indicated (*triangles*).

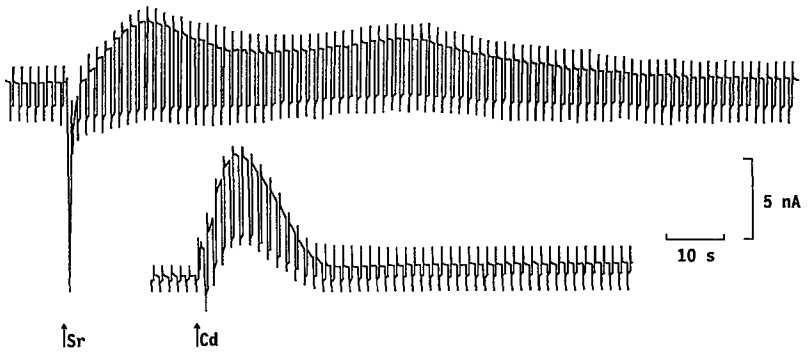

FIGURE 6. Currents induced by Sr^{2+} and Cd^{2+} in burster neurons. Injections of Sr^{2+}, like Ca^{2+}, into voltage-clamped burster neurons induced an inward current followed by an outward current consisting of a fast and a slow component. Only the fast component of the outward current was observed after injections of Cd^{2+}.

with fast onset were also observed after injections of Sr^{2+} (Fig. 6), Hg^{2+}, and Pb^{2+}. However, these cations also activated a sizeable slow component, and a distinct peak of the fast component was only observed in a few cases. While all three were certainly less effective than Ca^{2+}, as could be determined from peak amplitudes of the total outward currents, no sequence could be established. Of those divalent cations that activated the fast but not the slow component, Cd^{2+} was most effective, inducing currents almost as large as Ca^{2+}, followed by Co^{2+}, Ba^{2+}, and Ni^{2+} (Table II). No outward currents were observed after injections of Mg^{2+}, Zn^{2+}, and Mn^{2+}. Since the fast current component in burster neurons and the outward current in U-cells are

TABLE II

Currents Activated by Divalent Cations in Bursting Pacemaker Neurons

Ion	Inward current		Outward current		N	Ion radius
	nA	I_X/I_{Ca}	*nA*	I_X/I_{Ca}		*pm*
Mg^{2+}	—		—		4	65
Ni^{2+}	—		0.54 ± 0.07*		3	69
Zn^{2+}	6.13 ± 0.81	0.25	—		3	74
Co^{2+}	—		2.77 ± 0.36*		5	74
Mn^{2+}	—		—		4	93
Cd^{2+}	—		6.31 ± 0.49*		4	97
Ca^{2+}	24.8 ± 2.3	1.00	22.8 ± 1.4	1.00	8	99
Hg^{2+}	8.38 ± 1.84	0.34	4.40 ± 0.06	0.19	3	110
Sr^{2+}	1.44 ± 0.39	0.06	5.20 ± 0.38	0.23	4	113
Pb^{2+}	1.90 ± 0.28	0.08	1.48 ± 0.16	0.07	3	121
Ba^{2+}	4.81 ± 1.13	0.19	1.67 ± 0.28*		2	135

The peak amplitudes of inward and outward currents induced by injections of different divalent cations were measured and normalized to injections of 1 mM (see text). The average, standard error, and relative effectivity based on Ca^{2+} are listed for each ion species. Outward current values marked with an asterisk refer to the fast component and indicate that the slow component was not observed following injections of these cations. The other values in the same column refer to the slow component.

both activated by most divalent cations and show very similar time courses (e.g., Cd^{2+}, Fig. 4 *B* and 6), similar channel types are likely to be involved.

While the Ca^{2+}-activated K^+ permeability underlying the slowly activated outward current has been shown not to vary with membrane potential (Hofmeier and Lux, 1981*a*), the voltage dependence of the fast activated outward current induced by injection has not yet been determined. By injecting large amounts of 100 mM Cd^{2+} (~10% of cell volume) into U-cells, a response with a sustained plateau could be induced allowing measurements at various clamp potentials under nearly identical conditions (Fig. 7 *A*). The *I-V* plot in Fig. 7 *B* shows that the activated K^+ current (after subtracting the resting conductance) reverses at ~−65 mV. The solid line drawn in the same plot shows the least-squares fit of a Goldman-Hodgkin-Katz *I-V* relation for a potential-independent permeability. The deviation of the experimental data from this curve suggests that the K^+ permeability activated by divalent

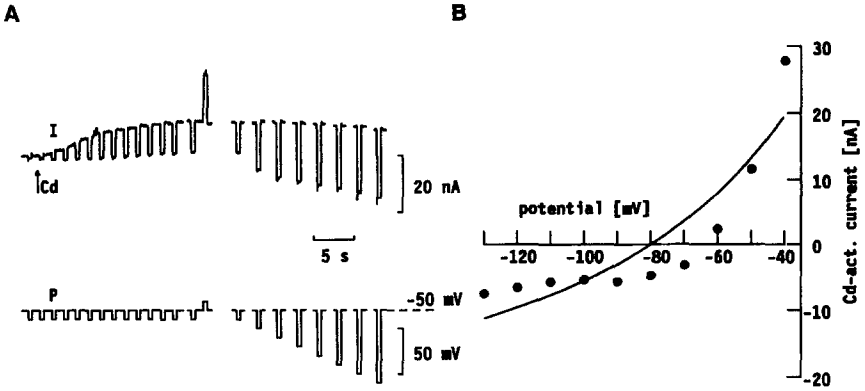

FIGURE 7. *I-V* relation of the Cd^{2+}-activated current in beater neurons. (*A*) A comparatively large amount of Cd^{2+} (~10 mM based on cell volume) was injected into a voltage-clamped U-cell to obtain a long plateau in the resulting outward current. During this plateau current amplitudes were measured at various potentials. (*B*) The current amplitudes are plotted vs. membrane potential after subtraction of currents due to the neuron's resting conductance. The solid line represents the Goldman-Hodgkin-Katz *I-V* relation for a voltage-independent permeability obtained by least-squares approximation (see text).

cations shows some voltage dependence and increases with less negative membrane potentials. This agrees with results obtained from activation of the Ca^{2+}-dependent K^+ conductance by voltage steps (Lux and Hofmeier, 1982*a*).

After Ca^{2+} injection, i.e., when intracellular [Ca^{2+}] was elevated above the resting level, the onset of the outward current activated by depolarization was much faster than in the control situation, where intracellular [Ca^{2+}] was initially low (Fig. 8 *A*). This indicates that Ca^{2+} entry through activated Ca^{2+} channels and possibly the Ca^{2+}-dependent activation mechanism are rate-limiting, whereas the potential-dependent activation, once Ca^{2+} is present in sufficient concentration near the Ca^{2+}-sensitive sites, is much faster. Furthermore, current amplitudes were much larger after Ca^{2+} injection (Fig. 8 *C*), suggesting that Ca^{2+} influx during depolarization activates only ~50% of the available K^+ channels.

The slow outward current component, which was only seen in burster neurons, usually peaked 30–60 s after Ca^{2+} injection and decayed within several minutes. Measurements using ion-sensitive microelectrodes have shown that intracellular [Ca^{2+}] decays significantly before this current reaches peak amplitude (Hofmeier and Lux, 1981*b*). Slow outward currents were observed after injections of Ca^{2+}, Sr^{2+} (Fig. 6), Hg^{2+}, and Pb^{2+}. Peak current values were measured and values equivalent to injections of 1 mM were calculated as described above. (Table II). The ionic radius does not appear to be the crucial parameter for the effectivity of divalent cations in activating this current since Cd^{2+} is ineffective even though its ionic radius is very close to that of Ca^{2+} (Fig. 5 *B*).

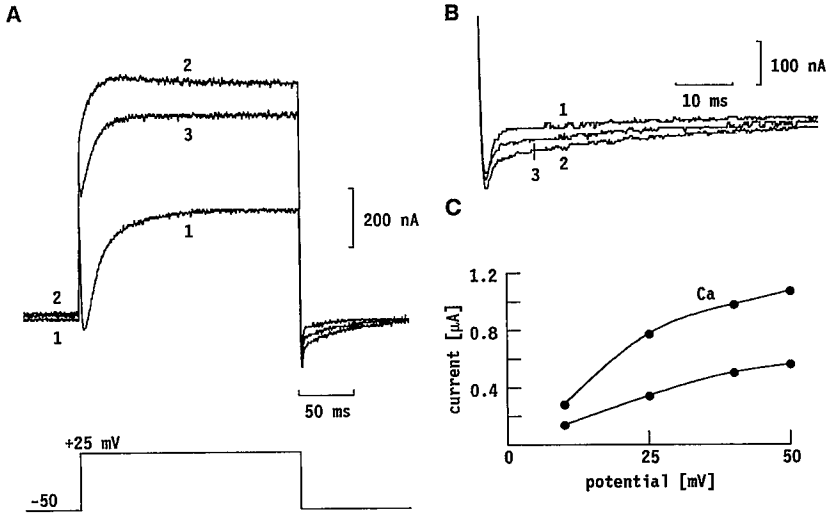

FIGURE 8. Outward currents during depolarization after Ca^{2+} injections. (*A*) Depolarizing pulses of 200 ms duration to +25 mV were applied before (*1*) and after (*2*) injection of Ca^{2+}. Elevation of intracellular [Ca^{2+}] before depolarization caused the outward current to activate more rapidly and reach much higher values. Sweep 3 shows an intermediate state ~10 s after sweep 2. Full recovery occurred within ~30 s. (*B*) Enlarged tail currents from *A* after repolarization. (*C*) The outward current activated by depolarizing steps to various membrane potentials before and after Ca^{2+} injections was measured 200 ms after depolarization. The values before and after Ca^{2+} injection are plotted vs. membrane potential. Approximately 3% of cell volume was injected in all cases (100 mM solution).

Inward Currents Activated by Divalent Cations

Inward currents showing a fast onset were observed after injections of Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Hg^{2+}, and Pb^{2+}. All currents except for those induced by Zn^{2+} injections were paralleled by increases in membrane conductance. Only Zn^{2+}-activated currents decayed more slowly (50% after 10 ± 3 s) and no significant change in membrane conductance was observed. Peak current amplitudes were measured, and values equivalent to injections of 1 mM were calculated and averaged as described above (Table II). However, Ca^{2+}, Sr^{2+}, Ba^{2+}, Hg^{2+}, and Pb^{2+} also induced outward

currents (see above), which may have partially overlapped the inward current. It is therefore possible that peak currents were underestimated. However, since the onset of the inward current was much faster than the onset of the outward current after, for example, Cd^{2+} injections (see below), this should not drastically affect the effectivity sequence. No inward currents were observed after injections of Mg^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}. In these cases, it is unlikely that inward currents as large as those observed with other divalent cations were entirely masked by the activated outward current since current responses to short hyperpolarizing pulses indicate that the reversal potential of the total injection-induced current was below −60 mV (Fig. 6).

While initial injections of Ba^{2+} induced fast inward currents as described above, subsequent injections were followed by long-lasting inward currents during which overall membrane conductance was increased. Long-lasting inward currents have also been reported following prolonged ionophoretic injection of Ba^{2+} into R15 neurons of *Aplysia* (Gorman and Hermann, 1979). The effectivity of divalent cations in activating inward currents showed no simple dependence on the ionic radius (Fig.

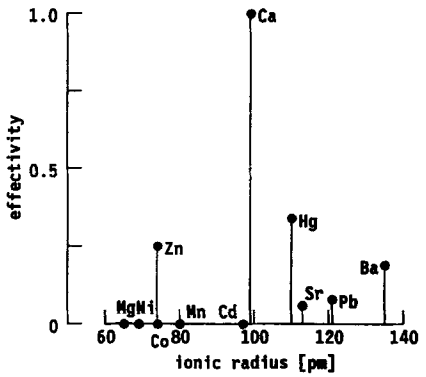

FIGURE 9. Effectivity of divalent cations in activating inward currents in burster neurons (data from Table II). The effectivity, based on Ca^{2+}, is plotted vs. the ionic radius.

9). Comparing the data for the alkaline earth cations with the Eisenman selectivity series, one finds that sequence III ($Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$, from Diamond and Wright, 1969) describes the effectivity of these cations in activating the fast inward current. However, the Eisenman theory of selectivity cannot be applied easily to the transition metals (Mn^{2+}, Co^{2+}, etc.) since nonCoulomb forces also become important for these ions (see Diamond and Wright, 1969 for a review).

DISCUSSION

The Fast Activated K^+ Conductance

Our data show that different types of Ca^{2+}-activated membrane conductances vary in their ability to be activated by other divalent cations. The effectiveness of different divalent cations in activating one type of Ca^{2+}-dependent K^+ conductance correlates with the ionic radius of the applied cation. For comparison, the effective-

ness of divalent cations both in activating this conductance and in increasing tyrosine fluorescence of calmodulin (values taken from Chao et al., 1984) are indicated in Fig. 5. It should be noted that neither parameter corresponds directly to the mere binding properties of the protein involved, but instead represents the ability of divalent cations to substitute for Ca^{2+} in inducing certain (putative) conformational changes. Similar results have, nevertheless, been reported for the binding of divalent cations to troponin C (Fuchs, 1971). This suggests that Ca^{2+}-dependent activation of a class of K^+ channels found in both types of pacemaker neurons studied involves a Ca^{2+}-binding protein similar to calmodulin or troponin C either as an activator protein or as part of the channel protein complex.

A correlation between the effectivity of divalent cations in activating K^+ channels with the ionic radius has also been observed for reconstituted Ca^{2+}-dependent K^+ channels from skeletal muscle (Oberhauser et al., 1988). These authors observed that some divalent cations (e.g., Co^{2+}, Ni^{2+}) enhance the Ca^{2+} sensitivity of the channel rather than activating the channel by themselves. The Ca^{2+} concentration applied to observe these effects (1 μM) is about three times the resting level reported for the neurons studied here (<0.3 μM, see Hofmeier and Lux, 1981*b*). Concentrations on the order of 1 μM could have been reached in our experiments if Ca^{2+} contamination of the injection solutions is considered (see Methods). Hence, Ca^{2+}-binding proteins similar to calmodulin or troponin C that are altered in their Ca^{2+} sensitivity by other divalent cations could be involved in Ca^{2+}-dependent activation of the K^+ channels found in skeletal muscle (see Oberhauser et al., 1988) or the fast activating K^+ channels studied here. However, the activation mechanism of Ca^{2+}-binding proteins is still controversial and is likely to vary among the different proteins (see Ebashi and Ogawa, 1988 for a review).

Preliminary results using the calmodulin antagonist W-7, which appears to bind to the hydrophobic region of calmodulin and also inhibits protein kinase C (Levine and Williams, 1982), show that this drug does not block the fast activating outward current (Müller, T. H., and D. Swandulla, unpublished). Previously observed effects of W-7 and neuroleptic drugs on the Ca^{2+}-activated K^+ conductance in erythrocytes (Lackington and Orrego, 1981) may be due to a direct block of the Ca^{2+} channel, which is independent of the drugs' action on calmodulin as has been shown in paramecium (Ehrlich et al., 1988) and which is also observed in the molluscan neurons studied here (Müller, T. H., and D. Swandulla, unpublished). Thus, the Ca^{2+}-binding site involved in activating this K^+ current is more likely to be located on the channel protein complex (in analogy to troponin C) rather than on a separate activator protein such as calmodulin.

The Slowly Activated K^+ Conductance

Another type of Ca^{2+}-dependent K^+ conductance, characterized by delayed activation following Ca^{2+} injection and found in the bursting, but not the beating type of pacemaker neuron, is only activated by a few divalent cation species: $Ca^{2+} > Sr^{2+} > Hg^{2+} > Pb^{2+}$, while Mg^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} show no significant effect or activate only the fast component (see above). Slow outward currents following iontophoretic injections of divalent cations have also been reported in the R15 burster of *Aplysia* (Gorman and Hermann, 1979). The largest currents were

induced by injections of Ca^{2+}, followed by Cd^{2+}, Hg^{2+}, Sr^{2+}, Mn^{2+}, and Fe^{2+}, while Mg^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} had no effect. However, when iontophoresis is used to inject divalent cations, long intervals on the order of 10 s are required to apply sufficient amounts in both *Aplysia* (Gorman and Hermann, 1979) and *Helix* neurons (Hermann and Hartung, 1982). In this case, although the fast and slow outward current components both contribute to the total response, the injection itself is probably the rate-limiting factor that determines the onset of the activated current. Apparently, outward currents were only observed after iontophoretic injection of those cations that activate the (presumably predominant) slow component, Ca^{2+}, Sr^{2+}, and Hg^{2+}, as well as Cd^{2+}, which is a potent activator of the fast component.

The slowly activated K^+ conductance is very sensitive to Ca^{2+} but not to Cd^{2+}. Hence, the ionic radius does not appear to be the primary parameter determining the effectivity of divalent cations in activating this type of channel. When activated by fast Ca^{2+} injection, this conductance lags and significantly outlasts the elevation of intracellular [Ca^{2+}], as measured using ion-sensitive microelectrodes (Hofmeier and Lux, 1981*b*). This suggests that Ca^{2+}-dependent activation of the slowly activated K^+ channel relies on a Ca^{2+}-dependent enzyme rather than on a direct effect of Ca^{2+} ions on the channel protein. In accordance with the set of divalent cations that activate this conductance (see above), the Ca^{2+}-dependent enzyme protein kinase C can also be activated by Sr^{2+}, but not by Mg^{2+}, Ba^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+} (Takai et al., 1979), and could therefore be involved in Ca^{2+}-dependent activation of this channel.

The Ca^{2+}-activated Cation Conductance

The Ca^{2+}-activated nonspecific cation conductance, which has also been described in burster neurons of *Aplysia* (Kramer and Zucker, 1985*a*), appears to be activated by Hg^{2+}, Ba^{2+}, Pb^{2+}, and Sr^{2+} in order of decreasing effectiveness. This sequence was not found for known Ca^{2+}-binding proteins. It could be reconciled with the set of cations that activate protein kinase C, although Ba^{2+} induces a significant inward current and has no effect on the kinase. However, the fact that these channels are functional in cell-detached patches suggests a direct action of divalent cations on the channel itself or a closely attached protein (Partridge and Swandulla, 1987).

The current induced by Zn^{2+} injections differs from the Ca^{2+}-activated inward current in that it is not paralleled by an increase in overall membrane conductance. Similar currents have been observed after injections of cyclic AMP, resulting from an increase in a cation conductance occurring simultaneously with a decrease in K^+ conductance (Swandulla and Lux, 1984).

Influence of Intracellular Buffers and Sequestration

When comparing the effects of different divalent cations, one must consider the possibility that intracellular buffers and sequestration mechanisms may behave differently for each ion species. In fact, the most abundant intracellular high-affinity Ca^{2+}-binding protein, calmodulin (Carafoli, 1987), has a higher affinity for the divalent cations that match Ca^{2+} in ionic radius (Chao et al., 1984), possibly causing these cations to appear less effective in activating membrane currents. The true

effectivity profile for the activation of the fast outward current by divalent cations would then be even more accentuated in favor of Ca^{2+} than the one shown in Fig 5. However, the time course of fast outward currents induced by injections of most divalent cations showed very little variability, suggesting that factors varying with ion species exert at most a minor influence on the intracellular spreading of the injected cations. Only responses to injections of Cd^{2+} and Sr^{2+} decayed more slowly, possibly because sequestration may not be as efficient for these cations.

The displacement of Ca^{2+} from cytosolic buffers caused by injections of excess divalent cations other than Ca^{2+} constitutes another possible source of error since the released Ca^{2+} ions, rather than the injected ions, could activate membrane currents (see Meech and Thomas, 1980). The macroscopic dissociation constant of calmodulin is estimated to be 10^{-4} to 10^{-6} M under conditions similar to those found in cells (Stoclet et al., 1987). Hence at intracellular Ca^{2+} concentrations of 10^{-7} M only a small fraction of the binding sites are loaded, amounting to an estimated 10 mM of bound Ca^{2+} in squid axons (Carafoli, 1987). Furthermore, of the divalent cations studied here, only Cd^{2+}, Pb^{2+}, and to a lesser degree Mn^{2+}, Sr^{2+}, and Zn^{2+}, but not, for example, Co^{2+} or Hg^{2+} are able to significantly displace Ca^{2+} from these binding sites (Chao et al., 1984). Finally, if displacement of Ca^{2+} from cytosolic buffers or from negatively charged phospholipids in the plasma membrane contributed significantly to the effect of other divalent cations, then both inward and outward currents should be equally affected. This is not the case since injections of Cd^{2+} or Co^{2+} into burster neurons induced a significant outward current but no inward current (Table II).

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