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Both barium and calcium activate neuronal potassium currents

(calcium-dependent potassium current/transient potassium current/tetraethylammonium ion/whole-cell voltage clamp/subtraction protocol)

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ABSTRACT **Amphibian spinal neurons in culture possess both rapidly inactivating and sustained calcium-dependent potassium current components, similar to those described for other cells. Divalent cation-dependent whole-cell outward currents were isolated by subtracting the voltage-dependent potassium currents recorded from *Xenopus laevis* neurons in the presence of impermeant cadmium (100–500 μM) from the currents produced without cadmium but in the presence of permeant divalent cations (50–100 μM). These concentrations of permeant ions were low enough to avoid contamination by macroscopic inward currents through calcium channels. Calcium-dependent potassium currents were reduced by 1 μM tetraethylammonium. These currents can also be activated by barium or strontium. Barium as well as calcium activated outward currents in young neurons (6–8 hr) and in relatively mature neurons (19–26 hr *in vitro*). However, barium influx appeared to suppress the sustained voltage-dependent potassium current in most cells. Barium also activated at least one class of potassium channels observed in excised membrane patches, while blocking others. The blocking action may have masked and hindered detection of the stimulatory action of barium in other systems.**

The discovery of a voltage-dependent potassium current requiring intracellular calcium for its activation (1, 2) was followed rapidly by descriptions in other cells of this virtually ubiquitous current (3–13). It is found in erythrocytes, hepatocytes, and a variety of gland cells as well as in muscle and nerve. Although analyses of the properties of single channels have revealed the existence of pores exhibiting a range of unitary conductance (20–300 pS) (8, 14–16), the currents have in common a stringent requirement for intracellular calcium, and they are generally maintained or only slowly inactivated in response to sustained stimuli (15–18).

Cultured amphibian spinal neurons possess a calcium-dependent potassium current that is characterized by two components, one that inactivates rapidly (I_{Kci}) and another that is sustained (I_{Kcs}) (19). They can be distinguished by their voltage dependence of activation and inactivation. Here we report that both of these currents can be activated by barium or strontium and reduced by tetraethylammonium (Et_4N) ions at micromolar concentrations. We show also that single potassium channels can be activated by barium. The observation that barium or strontium in addition to calcium can activate potassium currents raises the possibility that these currents may be activated by endogenous divalent cations other than calcium. Some of these findings have been reported in abstract form (20).

METHODS

Dissociated cell cultures were prepared from neural plate stage *Xenopus laevis* embryos; cells were grown in a fully defined medium according to procedures previously reported (21, 22). In brief, the posterior region of the neural plate was allowed to dissociate in Ca,Mg-free saline, without enzymatic digestion, and cells were plated on tissue culture plastic in modified Steinberg's solution [in mM: NaCl, 58.2; KCl, 0.7; $CaCl_2$, 9.6; $Ca(NO_3)_2$, 0.4; $MgSO_4$, 1.3; Tris base, 4.6; titrated to pH 7.8 with NaOH]. The cells survive and differentiate as they metabolize endogenous supplies of yolk and lipid. The cultures contain a heterogeneous population of cell types, including sensory neurons, motor neurons, and interneurons (21, 23, 24).

Standard whole-cell voltage-clamp techniques (25) were used to record from isolated neurons 25 μm in diameter having neurites less than 50 μm long, to ensure adequate voltage control (19); most neurons with branched processes were rejected, to avoid autaptic interactions. During an experiment the neuron was continually superfused with saline (in mM: NaCl, 80; KCl, 3; $MgCl_2$, 5; Hepes, 5; titrated to pH 7.4 with NaOH; tetrodotoxin, 0.1 μg/ml) to which the indicated divalent cation (Ca, Ba, Sr, Cd, or Co) had been added. The pipet solution consisted of a 10% hypotonic dilution of a solution containing 100 mM KCl, 3.5–5.5 mM KOH, and 5 mM Hepes at pH 7.4; a calcium chelator was not added. Pipet resistances ranged from 2 to 6 MΩ when measured in the bath.

Whole-cell currents were recorded by a Dagan 8900 amplifier with a 1-GΩ head-stage. Leak subtraction was routinely employed. A change in the leak current during the recording in different salines could give anomalous results; data were rejected when there was a change in the current level at the standard holding potential of −80 mV. Divalent cation-dependent currents were obtained by digital subtraction of the currents obtained in their absence from those obtained in their presence. When series resistance compensation was applied, the residual errors were estimated to be only a few millivolts. Under these conditions, the divalent cation-dependent current consisted of an inactivating and a sustained component, suggesting that the current profile is not attributable to large series resistance errors. Data were filtered at 2.5 kHz, sampled on-line at 0.2-ms intervals, and stored by a PDP 11/23 computer with a Cheshire A-D interface. Capacitative transients at the onset and termination of voltage jumps were removed either digitally or by hand for analysis of the ionic currents.

Single channel currents were recorded from inside-out excised membrane patches (25). Pipets had resistances ranging from 11 to 22 MΩ when filled with the standard solution (in mM: KCl, 20; NaCl, 60; $MgCl_2$, 5; Hepes, 5; titrated to pH 7.4 with NaOH). A 10-GΩ head-stage was employed; data were filtered at 2 kHz, digitized at 0.1-ms intervals, and recorded by a PDP 11/23. Currents were sampled continuously for several seconds at voltages depolarized from the

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Abbreviations: I_{Kci}, inactivating calcium-dependent potassium current; I_{Kcs}, sustained calcium-dependent potassium current; Et_4N, tetraethylammonium.

holding potential of −80 mV . Typically, data were first obtained in a "0 Ca" bath solution (in mM: KCl, 100; NaCl, 3; EGTA, 10; Hepes, 5; titrated to pH 7.4 with KOH). The patch was then exposed to similar solutions that contained only 1 mM EGTA and (*i*) 100 μM free Ba, or (*ii*) 100 μM free Ca, or (*iii*) 100 μM free Ba, 50 mM KCl, and 50 mM NaCl. Data were analyzed for current amplitudes and channel open times with computer programs. The threshold for event detection was 50% of the mean amplitude.

RESULTS

Divalent Cation-Dependent Current. The whole-cell voltage-clamp method (25) was used to study outward currents of *Xenopus* spinal neurons developing *in vitro* (19). Inward Na currents were blocked by tetrodotoxin. Ca- and voltage-dependent currents were activated by depolarization that allowed Ca influx, using a Ca concentration low enough to avoid marked reduction of outward currents by inward Ca current (Fig. 1*b*; $n = 5$). Omission of Ca and addition of Cd to block Ca channels then permitted recording of voltage-dependent currents. The Ca-dependent component of outward currents was isolated, as described elsewhere (19), by subtracting the voltage-dependent currents from those recorded in the presence of Ca (Fig. 1*a*). The resulting currents exhibit an initial fast rising transient peak (I_{Kci}) followed by a sustained plateau (I_{Kcs}). The two Ca-dependent current components are present in young neurons (6–8 hr *in vitro*; Fig. 2*b*; $n = 11$) and in old neurons (1 day; Fig. 1*a*; $n = 24$; ref. 19).

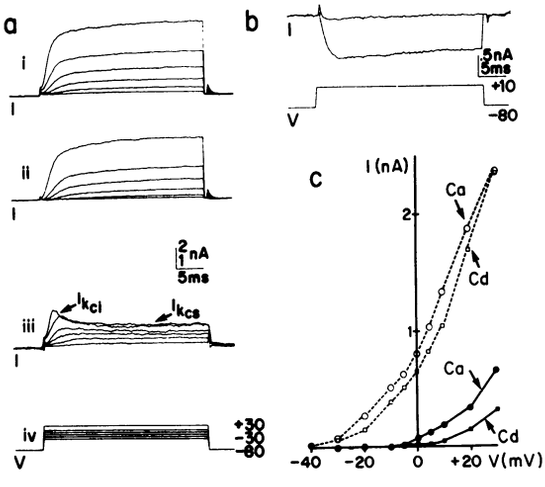

FIG. 1. (*a*) Isolation of Ca-dependent whole-cell outward currents from a neuron 23 hr *in vitro*. Currents were evoked at 1-s intervals by 30-ms voltage commands ranging from −30 to +30 mV from a holding potential of −80 mV. Series resistance was 8.6 MΩ and was compensated 70%. (*i*) Composite Ca- and voltage-dependent outward currents recorded in the presence of 100 μM Ca. (*ii*) Voltage-dependent currents obtained in the presence of 500 μM Cd without added Ca. Cd does not appear to permeate Ca channels, since in control experiments no inward current could be detected with 10 mM Cd in the bath. (*iii*) Ca-dependent outward currents obtained by subtraction. The current is characterized by an initial peak (I_{Kci}) followed by a plateau (I_{Kcs}). This subtraction protocol was used to derive the divalent cation-dependent outward currents illustrated in subsequent figures. (*iv*) Voltage stimulus template for *i–iii*. On the current scale bar, "2" refers to upper traces and "1" refers to lower traces. (*b*) Ca-dependent inward current (I_{Ca}) from a neuron at 20 hr with outward currents blocked by internal Cs and external Et_4N; the current recorded in 10 mM Ca (lower trace) is reduced to less than 100 pA in 100 μM Ca (upper trace). I_{Ca} is maximum at +10 mV (19). (*c*) Voltage dependence of currents elicited in the presence of either 100 μM Ca (circles) or 500 μM Cd (squares). Values are the initial (2 ms, filled symbols) and plateau (25 ms, open symbols) amplitudes during the 30-ms step, corrected for residual series resistance error. Data from a neuron at 22 hr. The two currents cannot be superimposed by displacement along the abscissa. Further, Ca-dependent currents show rundown with a time course similar to that of Ca currents (19), and the magnitude of the outward currents recorded in Ca depends on the external Ca concentration.

These two components are unlikely to be the result of any of several possible artifacts. The magnitude of the Ca-dependent outward current decreases as the external Ca concentration is reduced (19), suggesting that the difference current does not result primarily from monovalent cation flux through Ca channels. The decline in outward Ca-dependent current is not due simply to a time-dependent decrease in Ca influx, since Ca current inactivates more slowly, declining by 15% at most during a 30-ms voltage step (19). Examination of the voltage dependence of the currents recorded in Ca and in Cd indicates that surface charge effects predicted from differences in the identity and concentration of divalent cations cannot completely account for the increase in currents recorded in the presence of Ca (Fig. 1*c*; ref. 26). Similar conclusions are reached from comparison of the conductance–voltage relationships of these currents. Subtraction of a Cd current elicited by a step to 0 or +10 mV from a Cd current elicited by a step to +30 mV did not result in a difference current that had an initial inactivating peak followed by a sustained current. Furthermore, the profile of the Ca-dependent current obtained when the Ca solution contained 1 mM Ca instead of 0.1 mM Ca is similar to that shown here ($n = 7$); under these conditions, one would expect the subtraction protocol to exhibit surface charge effects opposite to those possible when 0.1 mM Ca is used. In addition, the Ca-dependent current isolated with a 10 mM Co rather than a 0.5 mM Cd solution also activated rapidly ($n = 7$) and generally showed rapid inactivation ($n = 6/7$). Finally, even though the identity and concentration of one divalent cation differed in the various external solutions, 5 mM Mg was always present.

These two currents can be distinguished by several criteria. For example, I_{Kcs} is activated at potentials positive to −30 mV and does not show inactivation. I_{Kci} is activated at more depolarized potentials (approximately −10 mV) and is susceptible to inactivation as discussed below.

Ba Activates an Outward Current. The specificity of the requirement for Ca was examined by performing the same experiments with Ba (50–100 μM) rather than Ca in the bath. These concentrations of Ba did not produce a detectable inward current in control experiments. However, with 10 mM Ba the inward Ba current is sustained during the 30-ms voltage step. Ba-dependent outward currents consist of a peak and a plateau at the more depolarized potentials (Fig. 2*a*); again the sustained plateau is apparent at potentials negative to those at which the peak appears. The Ca- and Ba-dependent currents could be elicited from the same cell (Fig. 2*b*). The Ba-dependent current also has a peak and plateau profile in young and mature cells ($n = 7/16$ total of both ages), indicating that Ba influx leads to the activation of currents that are macroscopically similar to those activated by Ca (I_{Kci} and I_{Kcs}). The current–voltage relations in the presence of Ba and Cd again reveal that the transient current is not the result of surface charge effects (Fig. 2*e*). Similar results were obtained when Sr replaced Ba or Ca in the bath ($n = 3$).

In some cells, isolation of the Ba-dependent current by subtraction of the underlying voltage- (but not divalent cation-) dependent component yielded only the transient current (Fig. 2 *c* and *d*). The late sustained outward current, typical of Ca-dependent currents, was either absent (Fig. 2*c*; $n = 5/16$ total) or appeared to be inward (Fig. 2*d*; $n = 4/16$ total). These results indicate that, in the presence of Ba, the total plateau outward current was smaller than the total

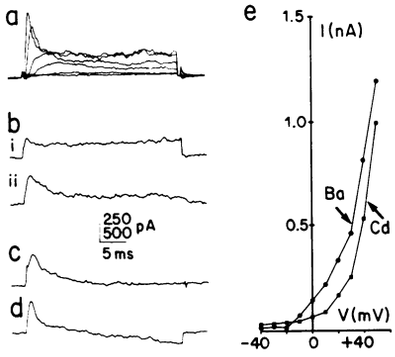

FIG. 2. (*a*) Ba-dependent currents resulting from 30-ms depolarizing commands to −30, −20, −10, 0, +20, and +30 mV isolated by subtraction as illustrated in Fig. 1. Neuron 27 hr in culture; 100 μM Ba. (*b–d*) Divalent cation-dependent outward currents elicited by a 30-ms depolarizing command to +30 mV in the presence of either 100 μM Ca (*b i*) or 100 μM Ba (*b ii–d*). The recordings are from three different neurons at 7, 25, and 22 hr *in vitro*. On the current scale bar, "250" refers to the upper traces and "500" refers to the lower traces. (*e*) Voltage dependence of currents elicited in the presence of either 100 μM Ba (circles) or 100 μM Cd (squares). Values are the initial (2-ms) amplitudes during the 30-ms step. Data from a neuron at 26 hr. Repeated runs in Cd resulted in superimposed Cd curves. Ba activated a transient outward current at depolarized potentials.

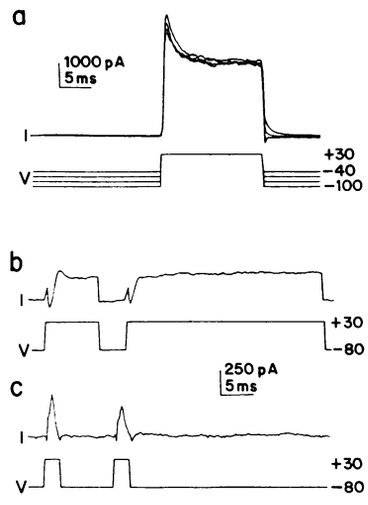

FIG. 3. Inactivation properties of I_{Kci} and I_{Kcs}. Recordings from three neurons at 1 day *in vitro*; currents isolated by subtraction. (*a*) Steady-state inactivation of I_{Kci}. The cell was held at potentials of −80, −60, −100, and −40 mV for 20 ms and then commanded to +30 mV for 30 ms. (*b*) Inactivation of I_{Kci} by a double-pulse protocol. (*c*) Inactivation of Ba-dependent transient current by a depolarizing prepulse.

plateau outward current observed in Cd. Although 100 μM Ba had either no (n = 4/6) or minimal (n = 2/6) effect extracellularly on voltage-dependent outward currents recorded in the presence of Cd, it is possible that the intracellular levels of Ba achieved during an experiment may have affected outward currents. Ba is known to block K currents intracellularly and extracellularly (27–30) and can be more effective when applied to the intracellular face of the membrane (31, 32).

Inactivation Properties of Divalent Cation-Dependent Currents. In addition to their similar amplitude, time course, and voltage of activation, the Ba- and Ca-dependent transient currents share inactivation properties. The divalent cation-dependent peak current inactivates, while the plateau is sustained. Depolarized holding potentials (−60, −40 mV) lead to steady-state inactivation of I_{Kci}, since there is a reduction in the peak but not plateau current; hyperpolarization (−100 mV) results in a larger peak current (Fig. 3*a*; n = 4). Comparable inactivation was observed for the transient current elicited by Ba (n = 3). Inactivation of I_{Kci} was also produced by a double-pulse protocol. A prepulse reduces the peak but not the plateau of the Ca-dependent current (Fig. 3*b*; n = 4); in control experiments, the prepulse protocol did not lead to inactivation of the Ca current elicited by the second pulse (n = 4). The Ba-dependent transient current is similarly inactivated by short prepulses (Fig. 3*c*; n = 4). The effect of activity (prepulse) or holding potential on the plateau current elicited by Ba could not be reliably examined due to the apparent action of Ba on the voltage-dependent current (Fig. 2 *c* and *d*).

Ionic Dependence of Divalent Cation-Dependent Currents. The ionic dependence of I_{Kci} and I_{Kcs} on K was defined by examination of tail currents. I_{Kci} or I_{Kcs} was maximally activated and the membrane was then repolarized to different holding potentials. These experiments were repeated in salines of different K concentration to alter E_K. I_{Kci} was activated by brief (2.4-ms) voltage steps. In normal saline (3 mM KCl), E_K is −86 mV and the tail currents are observed to reverse near −80 mV; in high-K saline (6 mM), E_K equals −68 mV and the tail currents reverse near −60 mV (Fig. 4*a*). Thus the reversal potential of I_{Kci} follows both the K concentration and the membrane potential as expected from the Nernst equation (n = 5). I_{Kcs} was similarly shown to be a K current by using 30-ms pulses (n = 4; data not shown; however, see Fig. 3*a*).

Millimolar levels of Et_4N block Ca-dependent K currents in some systems (33–35). Much lower concentrations of Et_4N reversibly inhibited I_{Kci} and I_{Kcs}. Bath application of 1 μM Et_4N led to a substantial reduction; however, the relative effects on I_{Kci} and I_{Kcs} varied in each cell studied (Fig. 4*b*; n = 3). Thus Et_4N does not distinguish between the two Ca-dependent K current components in *Xenopus* spinal neurons.

Single Channel Recordings. Single channel currents were recorded from inside-out patches excised from neurons at 1 day in culture to examine directly the activation of K channels by Ba. Depolarization activated only relatively small conductance channels (≤40 pS) (36) in the absence of divalent cations. When 100 μM Ba was then introduced into

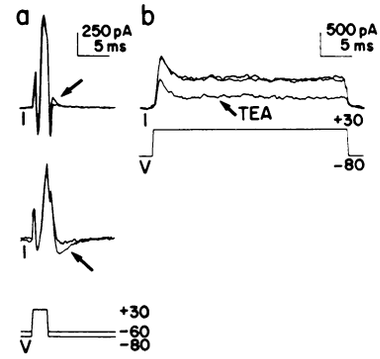

FIG. 4. (*a*) Ionic dependence of I_{Kci}. Currents obtained by subtraction, as previously; recordings from two neurons at 1 day *in vitro*. Ca-dependent outward currents were activated by a 2.4-ms voltage command to +30 mV and then returned to the holding potential of either −80 or −60 mV. Cells were held at −60 mV for only 3 ms before the voltage jump to limit steady-state inactivation. Top traces, Ca-dependent outward currents and tail currents (arrow) recorded in normal saline (3 mM KCl; E_K = −86 mV). Middle traces, Ca-dependent outward currents and tail currents (arrow) obtained in high-K saline (6 mM KCl; E_K = −68 mV). Tail currents reverse near E_K. (*b*) Effect of Et_4N (TEA) on Ca-dependent outward currents. Records from a cell at 1 day. Et_4N at 1 μM substantially and reversibly reduces but does not completely block both I_{Kci} and I_{Kcs}.

the bath, larger conductance channels were observed (26 out of 131 patches). Sufficient data were available for analysis of slope conductance in 17 patches.

One class of channels had a slope conductance of 51 ± 4 pS and an extrapolated reversal potential (V_{rev}) of −31 ± 3 mV (mean ± SEM; n = 12; E_K = −42 mV). Openings were brief (≈1 ms) and occurred in bursts (Fig. 5*a*). The permeability ratio P_{Na}/P_K calculated from the Goldman–Hodgkin–Katz equation has a mean value of 0.15. These channels carry predominantly K current, since a reduction in $[K]_i$ by a factor of 2 causes a positive shift of the reversal potential by 13 ± 4 mV (n = 3). An 11-mV shift is predicted for a channel with this calculated P_{Na}/P_K. Application of 100 μM Ca led to activation of channels with a similar conductance (49 ± 3 pS; n = 4) and burst pattern. These currents had an extrapolated reversal potential of −30 ± 5 mV.

Larger conductance channels were also activated by Ba (79 ± 5 pS; n = 5). These channels were further distinguished by an extrapolated reversal potential near zero (−3 ± 4 mV; n = 5), suggesting a Cl channel (E_{Cl} = 3 mV) or a nonselective cation channel (P_{Na}/P_K = 1, E_{rev} = −8 mV). Reduction in $[K]_i$ did not cause a positive shift of their reversal potential. Under conditions of whole-cell voltage clamp, a divalent cation-dependent Cl or nonselective cation current would contribute less than 10% of the total divalent cation-dependent outward current, given the conductances and relative frequencies of the K and larger conductance channels.

The activity of channels present in divalent cation-free solution was frequently reduced or absent in the presence of Ba. Since channel activity can disappear spontaneously during a recording, the reversibility of the effect was examined. Ba reversibly blocked voltage-dependent K channels (n = 5; Fig. 5*b*; ref. 36), consistent with known actions of Ba on whole-cell K currents (27–32).

DISCUSSION

Our principal finding is the demonstration that Ba and Sr as well as Ca activate K currents in amphibian spinal neurons. This result contrasts sharply with the strict requirement for Ca reported for other preparations. These ions all permeate Ca channels in amphibian neurons, as elsewhere. When low external concentrations were employed, the inward current elicited by depolarization was sufficient to activate the outward currents, while small enough to avoid appreciable reduction of them. The divalent cation-dependent current was then isolated from the purely voltage-dependent current by subtraction of the current obtained from the same neuron in the absence of Ba, Sr, or Ca.

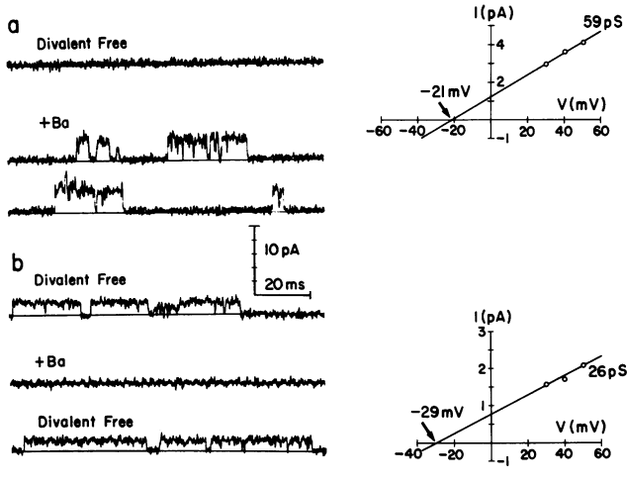

FIG. 5. Divalent cation sensitivity of K channels in two inside-out membrane patches at +30 mV (*Left*), and *I–V* plots for these channels (*Right*). (*a*) Application of Ba to the intracellular face of a patch activates channels (lower traces; 59 pS; V_{rev} = −21 mV) that are not observed in the presence of a divalent cation-free solution (uppermost trace). (*b*) Smaller conductance channels (top trace; 26 pS; V_{rev} = −29 mV; ref. 36) are blocked by Ba (middle trace). Channel activity returns after removal of Ba (bottom trace).

An initial transient peak current is followed by a sustained plateau current. These appear to constitute two separate currents, since they are activated at different potentials and differ in the extent of their inactivation. Subtraction protocols have been employed effectively in previous studies (37, 38), even though their use introduces several potential sources of artifact, among which are a change in the leak current and a difference in screening of surface charge in salines of various compositions. These difficulties were surmounted as described in *Results*. Furthermore, the absence or presence of a divalent cation may alter the Ca-independent current and invalidate the assumption that the voltage-dependent current is constant. In addition, if the divalent cation-dependent current were extremely voltage dependent, substantial errors due to a time-dependent voltage drop across the series resistance would result in an apparent inactivation of the current. However, this factor cannot account for the observations that an outward current component is activated more rapidly in the presence of Ca, Ba, or Sr, and that this component is inactivated by a prepulse. Although there was variability in the amplitude of the peak, and the plateau was often suppressed by Ba, both currents were activated by Ba and Ca in both young and physiologically relatively mature neurons.

These outward currents are the most sensitive to Et_4N so far reported, to our knowledge, since they are substantially reduced by a concentration of 1 μM. It was not possible to use Et_4N for isolation of the divalent cation-dependent currents, however, since higher concentrations reduced the voltage-dependent K current as well. Analysis of tail currents confirmed that the transient and the sustained outward current are carried by K, consistent with their reduction by Et_4N.

Both transient and sustained Ca-dependent K currents have been described in neurons (1, 2, 5, 6, 39–42), but we have found no other report of their coexistence in single nerve cells. In contrast, both currents are found in cardiac and striated muscle (3, 4, 7, 43), and their coexistence has been clearly demonstrated (44). The functional significance of the transient current activated by Ca remains to be clarified, but it provides a mechanism of rapid activation of outward current that may be useful in terminating depolarizations involving Ca influx. Selective pharmacological blockade would be a useful approach to this issue.

The direct observation of single channel K currents activated by Ba confirms the conclusions from analysis of whole-cell currents. K channel density would be predicted to be low. Given the amplitudes of the macroscopic and single channel currents and the ionic solutions used in their measurement, calculation indicates that there are only ≈50 divalent cation-activated K channels per cell. These channels appear to be the same as those activated by Ca, by the criteria of conductance, reversal potential, and burst pattern. They may be the channels previously described by Blair and Dionne (45), with a lower conductance as a result of the presence of Na ions and a reduced concentration of K ions in the internal solution (45, 46). The P_{Na}/P_K for these channels is similar to that determined by Blair and Dionne. The nonzero value indicates that the channels are largely but not completely K selective (see also refs. 47 and 48) if the current–voltage relation is linear in the region of E_K. These channels are likely to contribute to the sustained component

of the whole-cell K current activated by Ca (I_{Kcs}), since they were observed during prolonged depolarization.

The relaxation of the restriction to Ca for activation of K currents prompts investigation of a role for Mg, an endogenous divalent cation. Mg alters the kinetics, and in some cases the conductance, of channels activated by *N*-methyl-D-aspartate, a glutamate analogue (49, 50). Examination of Ca-activated K channels in artificial lipid bilayers reveals that Mg enhances their affinity for Ca and the cooperativity of the action of Ca; Mg itself does not activate these channels, however (51).

This appears to be the first description of K currents activated by Ba and Sr in addition to Ca in vertebrate neurons. Ba has recently been shown to be effective in activating K channels in porcine pancreatic acinar cells (52) and in a molluscan egg (53). There is apparently only one previous report of Ba activation of a neuronal K current (54). Many investigations have used higher concentrations of Ba that may have suppressed the delayed K current, as evidenced by the reversible suppression of voltage-dependent K channels. If such an effect predominated it could obscure the activation reported here. The study of a rapidly activated current, not masked by suppression of a delayed outward current, has contributed to the appreciation of the stimulatory action of Ba observed at low concentrations.

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