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A Transient Outward Current Dependent on External Calcium in Rat Cerebellar Granule Cells

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Summary. The outward potassium current of rat cerebellar granule cells in culture was studied with the whole-cell patch-clamp method. Two voltage-dependent components were identified: a slow current, resembling the classical delayed rectifier current, and a fast component, similar to an I_A-type current. The slow current was insensitive to 4-aminopyridine and independent of external Ca^{2+}, but significantly inhibited by 3 mM tetraethylammonium. The fast current was depressed by external 4-aminopyridine, with an $ED_{50} = 0.7$ mM, and it was abolished by removal of divalent cations from the external medium. The sensitivity of the transient outward current to different divalent cations was investigated by equimolar substitution of Ca^{2+}, Mn^{2+} and Mg^{2+}. In 2.8 mM Mn^{2+}, the transient potassium conductance was comparable to that in 2.8 mM Ca^{2+}, while in 2.8 mM Mg^{2+} the transient component was drastically reduced, as in the absence of any divalent cations. However, when Ca^{2+} was present, Mg^{2+} up to 5 mM had no effect. The transient current increased with increasing concentrations of external Ca^{2+}, $[Ca^{2+}]_o$, and the maximum conductance *vs.* $[Ca^{2+}]_o$ curve could be approximated by a one-site model. In addition, the current recorded with 5.5 mM BAPTA in the intracellular solution was not different from that recorded in the absence of any Ca^{2+} buffer. These results suggest that divalent cations modulate the potassium channel interacting with a site on the external side of the cell membrane.

Key Words cerebellar granule neurons · potassium conductance · calcium · manganese · magnesium · 4-aminopyridine

Introduction

Previous studies demonstrated that neural membranes contain several populations of potassium channels, differing in kinetics, pharmacology and Ca^{2+} sensitivity. In a wide variety of cells the outward potassium current activated by depolarization can be separated into two components: a fast transient, low-voltage-activated current, which depends on the holding potential and a slowly rising and slowly inactivating current, with properties similar to the "classical" squid axon potassium current. The fast transient current was first described by Connor and Stevens (1971*a*) and by Neher (1971) in molluscan neurons and called I_A current. This current has been shown to be important in spike repolarization (Belluzzi, Sacchi & Wanke, 1985), in controlling the firing frequency (Connor & Stevens, 1971*b*) and in modulating the threshold response (Segal, Rogawski & Barker, 1984). Despite the constancy of functions attributed to the current, many subtypes of this channel, differing in kinetics, voltage-dependence and pharmacological sensitivity, have been described (Rogawski, 1985; Rudy, 1988).

One major question concerns the role played by Ca^{2+} in the control of voltage-dependent transient potassium currents. Although in most cases the I_A has been proved to be Ca^{2+}-independent, Ca^{2+}-dependent transient outward currents have been also reported in calf cardiac Purkinje fibers (Siegelbaum & Tsien, 1980), in sympathetic neurones from bullfrog (MacDermott & Weight, 1984) and rat (Galvan & Sedlmeir, 1984), neurosecretory cells from the supraoptical nucleus (Bourque, 1988), in *Aplysia* (Junge, 1985), and snail neurones (Kostyuk & Martynyuk, 1988).

In a previous study (Robello, Carignani & Marchetti, 1989), we reported that the outward current of granule cells can also be separated into two components: a slowly activating and inactivating current and a fast transient current, which was inactivated when the holding potential was more positive than −60 mV. Both components are mainly carried by potassium ions, as revealed by reversal potential measurements and by the observation that they are greatly reduced by internal cesium. In the present study, these currents are further characterized both

kinetically and pharmacologically, and in terms of regulation by divalent cations. In particular, we propose that the transient potassium current is dependent on the external Ca^{2+} concentration.

Materials and Methods

Cell Culture

Dissociated cell cultures of rat cerebellar granule cells were prepared by trypsin digestion and mechanical trituration from 8-day-old neonatal rats, following the procedure of Levi et al. (1984), as previously described (Robello et al., 1989). Cells were plated at a density of 2×10^6 per dish, on 35-mm plastic dishes or on glass coverslips, coated with 10 μg/ml poly-L-lysine and kept at 37°C in humidified 95% air/5% CO_2 atmosphere. Experiments were performed between day 5 and 12 after plating.

Electrophysiology

Membrane currents were measured with the standard whole-cell patch-clamp configuration (Hamill et al., 1981). Patch electrodes were manufactured from glass capillaries (Type 7052, Garner Glass) with a programmable Sachs and Flaming puller and had a resistance of 5–10 MΩ, when filled with the standard internal solution. Cell responses were amplified and filtered at 3 kHz by an EPC-7 (List Electronic, Darmstadt, FRG). Capacitance transient neutralization and series resistance compensation were optimized. Both stimulation and data acquisition were performed with a Labmaster D/A A/D converter driven by Pclamp software (Axon Instruments, Burlingame, CA). Currents were corrected for leak and residual capacitance transients by a P/4 paradigm. Data were fitted to models using the commercial laboratory software Asystant+ (Asyst Software Technologies, Rochester, NY).

Solutions

The standard external solution contained (in mM): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10 glucose and 300 nM tetrodotoxin to block the sodium current. The pH was adjusted to 7.4 with NaOH and the osmolarity was close to 290 mosM. In some experiments the concentration of the different divalent cations was varied (*see* the Table). When all divalent cations were omitted from the external solution, 1 mM ethylene-glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) was added. The pipette filling solution contained (in mM): 142 KCl, 2 $MgCl_2$, 10 HEPES, 2 EGTA. The pH was adjusted to 7.3 with Trizma base. The standard internal solution was in some cases modified by substituting EGTA with 5.5 mM 1,2-bis (2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) or by omitting both. We verified that the pipette solution rapidly and efficiently diffused into the cell because when the pipette contained cesium, the outward potassium current was eliminated in <3 min.

Tetraethylammonium (TEA) and 4-aminopyridine (4-AP) were made up in the external solution and applied by steady perfusion (3 ml/min gravity flow). All chemicals were purchased from Sigma Chemical, St. Louis, MO.

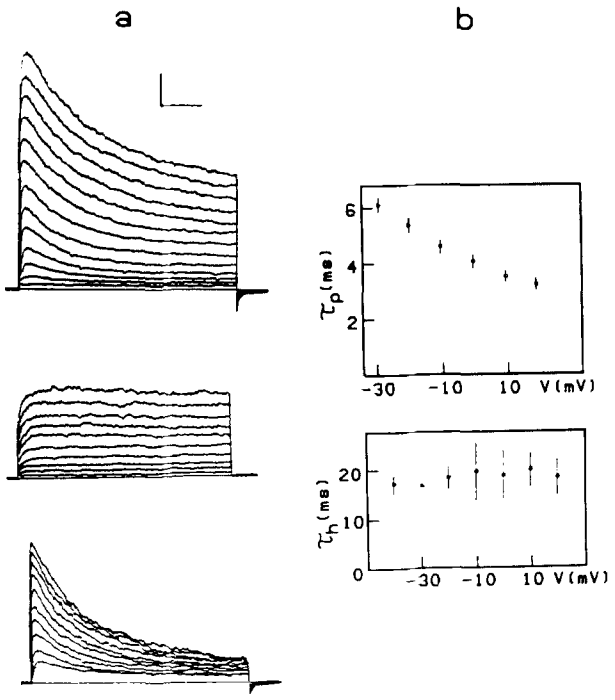

Fig. 1. Outward potassium currents in cerebellar granule cells. (*a*) Currents evoked in standard solutions from the holding potentials of −80 mV (top), −50 mV (middle) and traces obtained by digital subtraction of the middle traces from the top traces (bottom). Step depolarizations were delivered in 10-mV increments. Calibration bars: 500 pA, 20 msec. (*b*) Time to peak (top) and time constant of inactivation, τ_h (bottom) of the transient potassium current as a function of the test potential. Points are mean values ± SD of four cells. Inactivation was described by Eq. (1)

Results

As previously reported (Robello et al., 1989), in a granule cell held at −80 mV, step depolarizations elicit two outward components (Fig. 1*a*, top). The

current activates rapidly and then decays to roughly 50% of its peak value in 100 msec. In contrast, from the holding potential of −50 mV, the current has a monotonic time course with no inactivation (Fig. 1*a*, middle). To isolate the transient component, the current traces from −50 mV holding potential were digitally subtracted from the current traces from −80 mV. The resulting current (Fig. 1*a*, bottom) activated quickly, and the time to peak decreased with increasing depolarizations (from 6.1 msec at −30 mV to 3.3 msec at +20 mV, Fig. 1*b*). The current was almost entirely transient. The inactivation time course could be described by a single exponential curve

$$I(t) = I_0 \exp(-t/\tau_h) + I_{ss} \quad (1)$$

where I_0 is the current value at the beginning of inactivation (peak current) and I_{ss} is the current value at the end of the 100-msec test pulse. The time constant τ_h was found to be independent of the test voltage and close to 20 msec (Fig. 1*c*). The residual current at 100 msec (I_{ss}) indicates that separation of the two components was not complete, as previously reported (Cull-Candy, Marshall & Ogden, 1989).

To attempt a better separation, we investigated the pharmacological properties of the two components. 4-AP caused a rapid and potent inhibition of the transient portion of the current elicited from −80 mV (Fig. 2*b*, left) while it was almost ineffective on the steady-state component of the current (Fig. 2*b*, right). The effect was entirely reversible after a few minutes of wash in all cells tested (n = 25). Finally, the inhibition of 4-AP was dose dependent, with ED_{50} = 0.7 mM (Fig. 3).

In contrast to the 4-AP effect, the transient current was insensitive to 3 mM TEA, while the steady-state value was reduced by 20% in a reversible manner (Fig. 4*a*). The same amount of TEA caused a 30% inhibition of the current from −50 mV (Fig. 4*b*), indicating a specific block of the slow outward component.

Sensitivity of the outward current to divalent cations was investigated by bathing the cells in the divalent-free solution. In this condition, the transient portion of the current elicited from −80 mV was totally abolished and the current assumed a non-inactivating time-course (Fig. 5). The value of the current at 100 msec was instead slightly increased, probably as a consequence of the change in surface potential (Frankenhaeuser & Hodgkin, 1957). The whole effect was reversible as divalent cations were replaced.

We tested the role of different divalent cations by equimolar substitutions of Ca^{2+}, Mg^{2+} and Mn^{2+}. The relative conductance of the transient outward component was calculated from the relation

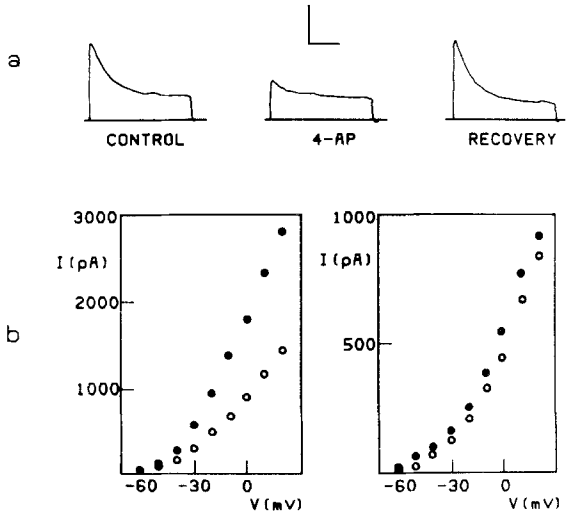

Fig. 2. Effect of 3 mM 4-AP on the outward potassium current. The holding and test potentials were −80 and 0 mV. (*a*) Current traces before (*control*), during perfusion with 4-AP and after wash (*recovery*). Calibration bars: 1 nA, 25 msec. (*b*) Current-voltage relationship for the peak (left) and the steady-state (right) current from the holding potential of −80 mV. (●) control; (○) 4-AP

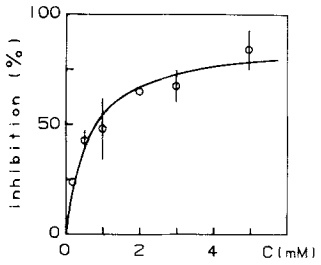

Fig. 3. Concentration dependence of 4-AP effect. Points are the mean values ± SD of 4–7 experiments except the values for 0.2 and 2 mM, which were obtained from one cell. The percentage of inhibition of the current ($I\%$) was calculated from the relation:

$$I\% = 100\,(1 - I_{4AP}/I_{control})$$

where $I_{control}$ and I_{4AP} are the differences between the peak and the steady-state current before and during 4-AP application, respectively. The theoretical dose-dependence curve was obtained from the relation:

$$I\% = I\%_{max}/(1 + ED_{50}/C)$$

where C is the concentration of 4-AP (in mM), $I\%_{max}$ is the maximum percentage inhibition and ED_{50} is the concentration of 4-AP that gives half maximum inhibition. The best fit gave $I\%_{max}$ = 88 and ED_{50} = 0.7 mM

$$G_t(V) = (I_{peak} - I_{ss})/(V - 80) \quad (2)$$

where I_{peak} is the maximum current during a test pulse to the potential V and I_{ss} is the current just before the end of the same pulse. The potassium

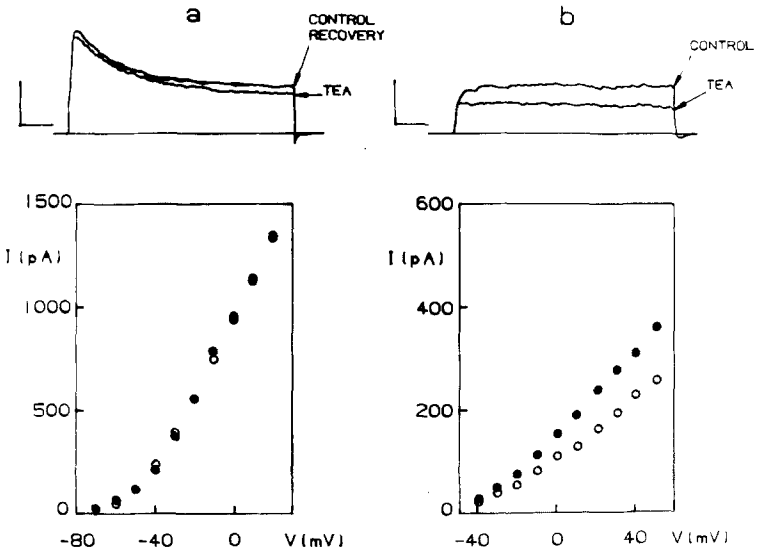

Fig. 4. Effect of 3 mM TEA on potassium currents. (*a*) *Top*: current traces from the holding potential of −80 to 0 mV, before (*control*), during perfusion with TEA and after wash (*recovery*); calibration bars: 200 pA, 15 msec. *Bottom*: current-voltage relationship for the peak current from −80 mV. (●) control; (○) TEA. (*b*) *Top*: current traces from the holding potential of −50 to +10 mV, before and during perfusion with TEA. Calibration bars: 200 pA, 15 msec. *Bottom*: current-voltage relationship for the current from −50 mV. (●) control; (○) TEA

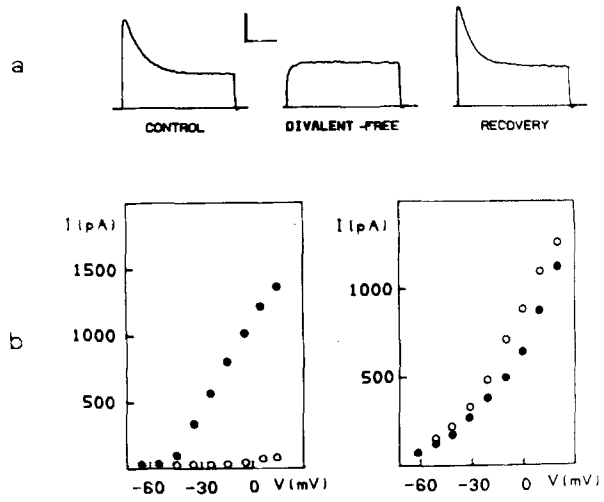

Fig. 5. Effect of external divalent cations on the potassium current. The holding potential was −80 mV and the test potential was 0 mV. (*a*) Current traces before (*control*), during perfusion with the divalent-free solution and after wash (*recovery*). Calibration bars: 500 pA, 25 msec. (*b*) Current-voltage relationship for the peak (left) and steady-state (right) current in standard (●) and in divalent cation-free external bath (○)

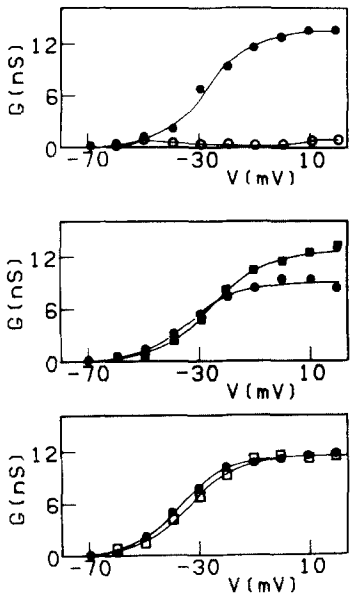

Fig. 6. Potassium conductance as a function of the test voltage in the presence of different divalent cations in the external solution. Experimental points were fitted by Eq. (3). Three different representative cells are shown. *Top*: (●) 2.8 mM Ca^{2+}; (fit parameters were: G_{max} = 13.6 nS, $V_{1/2}$ = −27.6 mV). (○) 2.8 mM Mg^{2+}; (no fit). *Middle*: (●) 2.8 mM Ca^{2+}; (G_{max} = 9.2 nS, $V_{1/2}$ = −33.8 mV). (■) 2.8 mM Mn^{2+}; (G_{max} = 13.2 nS, $V_{1/2}$ = −24.9 mV). *Bottom*: (●) 2.8 mM Ca^{2+}; (G_{max} = 11.7 nS, $V_{1/2}$ = −33.8 mV). (□) 2.8 mM Ca^{2+} + 5 mM Mg^{2+}; (G_{max} = 11.7 nS, $V_{1/2}$ = −36.8 mV)

reversal potential was taken as −80 mV (Robello et al., 1989). $G_t(V)$ was fitted to the equation

$$G_t(V) = G_{max}/1 + \exp[-(V - V_{1/2})/K] \quad (3)$$

where G_{max} is the maximum conductance, $V_{1/2}$ is the potential value at which $G_t = G_{max}/2$ and K is a form factor. When Mg^{2+} was the only divalent cation present in the external bath (in a concentration of 2.8 mM), the transient component of the current was abolished, as in the absence of divalent cations. The transient component conductance G_t was close to 0 at all test potentials (Fig. 6, top). On the contrary, when the external bath contained 2.8 mM Mn^{2+}, the transient conductance was not abolished but often slightly bigger than in 2.8 mM Ca^{2+} (Fig. 6, middle). The ratio $G_{max}(Mn^{2+})/G_{max}(Ca^{2+})$ was 1.3 ± 0.3 in 10 cells. In addition, Mn^{2+} caused a voltage shift of

Table. Transient potassium conductance in the presence of different divalent cations

[Dival. cat.]$_{ext}$ (mM)			G_{max} ± SD (nS)	$V_{1/2}$ ± SD (mV)	n
Ca^{2+}	Mg^{2+}	Mn^{2+}			
1.8	1	—	11.0 ± 3.4	−31.3 ± 5.4	14
—	—	—	0.7 ± 0.3	—	5
2.8	—	—	12.2 ± 3.6	−31.3 ± 3.7	10
—	2.8	—	0.8 ± 0.4	—	4
—	—	2.8	16.2 ± 6.1	−24.2 ± 4.7	10
2.8	5	—	12.6 ± 4.5	−30.4 ± 5.6	3

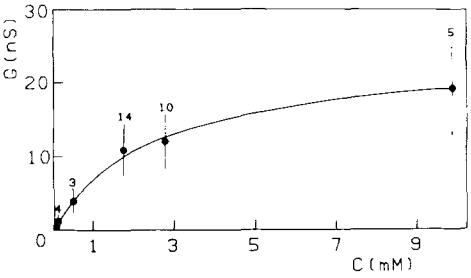

Fig. 7. Potassium conductance as a function of external Ca^{2+} concentration. Points represent the mean value ± SD in as many cells as indicated by the number above error bars. The value for 10^{-5} M was obtained from one cell. The solid line is the best fit of experimental points by Eq. (4) with $\bar{G}_{max}$ = 24 nS and ED_{50} = 2.3 mM

the activation in the positive direction. The voltage shift was $V_{1/2}$ (in Mn^{2+}) − $V_{1/2}$ (in Ca^{2+}) = 7.0 ± 2.7 mV in 10 cells.

When Mg^{2+} (from 0.2 to 5 mM) was added to the 2.8 mM Ca^{2+} external solution, the transient current was not altered in any respect (Fig. 6, bottom). This experiment shows that in the presence of Mg^{2+} alone, the transient current is not blocked, but rather fails to activate.

The results just described are summarized in the Table.

Parallel experiments with specific blockers of the voltage-dependent calcium current gave negative results. Neither 100 μM $CdCl_2$ nor 10 μM verapamil had any effect on the granule cell outward current (*not shown*), whereas the same dose of verapamil inhibited the voltage-dependent calcium current by 30% (Marchetti, Carignani & Robello, 1991).

The dependence of the transient current on the external Ca^{2+} was studied, varying its concentration from 10^{-5} to 10^{-2} M in the absence of other divalent cations (Fig. 7). The maximum transient conductance to potassium (Eq. (3)) as a function of Ca^{2+} concentration was calculated and experimental points were fitted to one-site model equation

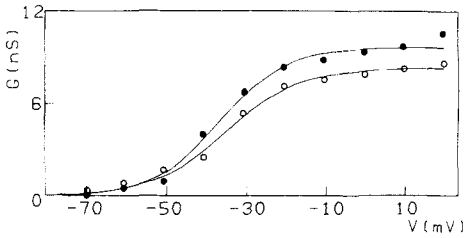

Fig. 8. Potassium conductance as a function of test potential in two representative cells in different conditions of internal Ca^{2+} buffering. (●) 5.5 mM BAPTA; the best fit with Eq. (3) gave G_{max} = 9.7 nS, and $V_{1/2}$ = −36 mV; (○) 0 mM EGTA; G_{max} = 8.4 nS and $V_{1/2}$ = −34 mV

$$G_{max}(C) = \bar{G}_{max}/(1 + ED_{50}/C) \quad (4)$$

where C is the external Ca^{2+} concentration and ED_{50} is the Ca^{2+} concentration that gives $G_{max} = \bar{G}_{max}/2$. The values of G_{max} and ED_{50} obtained from the best fit of the experimental points were 24 nS and 2.3 mM, respectively.

The potassium conductance was not affected either by replacing the normal internal Ca^{2+} buffer (2 mM EGTA) with a more effective one (5.5 mM BAPTA; *see* Marty & Neher, 1985) or by omitting it (Fig. 8). Therefore, in contrast with the external Ca^{2+} requirements, we were unable to demonstrate a dependence of the potassium current on the internal Ca^{2+} concentration.

Discussion

The outward potassium current of rat cerebellar granule cells can be separated into two components: a slow, noninactivating, TEA-sensitive component, and a transient, 4-AP-sensitive component. These currents have been described previously in granule cell in explant cultures (Hockberger, Tseng & Connor, 1987; Cull-Candy et al., 1989) and in dissociated cells (Robello et al., 1989). The transient component shares several properties of I_A-type potassium current (Connor & Stevens, 1971*a*; Rogawski, 1985), including a low threshold of activation, a fast activation and inactivation kinetics and the already mentioned 4-AP sensitivity. In this study we have shown that it differs from the "classical" I_A current because it is rapidly and totally abolished when divalent cations are removed from the external medium. We will discuss a possible explanation of this effect and then compare our findings with previous data on neuronal potassium channels.

Divalent cations modulate potassium channels by different mechanisms. In the first place, they influence the gating of all voltage-dependent chan-

nels by controlling the surface potential (Frankenhaeuser & Hodgkin, 1957). Removing Ca^{2+} from the external medium results in a shift of the activation curve towards more negative potentials, an effect which was actually observed for the delayed component of the current (Fig. 5). However, the transient current was not simply shifted, but rather abolished in the absence of divalent cations, a typical behavior of Ca^{2+}-dependent potassium channels (review in Rudy, 1988), which are sensitive to intracellular Ca^{2+} concentration and are dependent on the Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Several observations indicate that this transient current is, instead, dependent on external divalent cations.

The first observation is that substitution of Ca^{2+} with Mn^{2+} did not cause a depression of the transient current, which was even slightly bigger in Mn^{2+} than in Ca^{2+}. Therefore, a Ca^{2+} current is unlikely to be involved because Mn^{2+} permeates less than Ca^{2+} through Ca^{2+} channels (Nelson, 1986; Hess, Lansman & Tsien, 1986), and it is also less effective than Ca^{2+} in activating potassium channels from the inside (Oberhauser, Alvarez & Latorre, 1988; Müller, Swandulla & Lux, 1989). In Mn^{2+}, the potassium activation curve was shifted by 7 mV in the depolarizing direction, an effect similar to that reported in cultured rat sensory neurons for an I_A-type potassium current (Mayer & Sugiyama, 1988). On the other hand, the drastic reduction of the transient current in the presence of Mg^{2+} alone is unlikely to reflect a block of the potassium channel by Mg^{2+} because: (i) when a sufficient concentration of Ca^{2+} was present in the medium, even a relatively high concentration of Mg^{2+} (5 mM) did not reduce the transient current; (ii) lowering the total divalent concentration had the same effect of Mg^{2+}.

A role of internal Ca^{2-} is also excluded by the experiments with different concentrations of Ca^{2+} chelating agents in the pipette. In the presence of 5.5 mM BAPTA, which is known to be at least five times more efficient than EGTA (Marty & Neher, 1985), the internal Ca^{2+} concentration is estimated to be lower than 10^{-9} M, while in the absence of any buffer, the internal Ca^{2+} concentration can be as high as 10^{-6} M. The transient potassium currents recorded in these two conditions were virtually indistinguishable.

Another, albeit indirect, proof that the transient current is not dependent on Ca^{2+} influx arises from the observation that the current-voltage relationship was not N-shaped and did not even show a plateau at high voltages (*not shown*), where the driving force on Ca^{2+} approaches zero. This observation is in agreement with the ineffectiveness of specific calcium blockers such as Cd^{2+} and verapamil on the outward current.

Finally, although we have recently measured a high voltage-activated Ca^{2+} current in cells from the same cultures (Marchetti, Carignani & Robello, 1990), we were unable to resolve Ca^{2+} currents in the voltage range of -60 to -40 mV, where the transient current is already activated.

The Ca^{2+} dependence was further investigated quantitatively by measuring the transient current in the presence of different concentrations of Ca^{2+}. The current *vs.* concentration curve could be approximated by a one-site mode. However, points at low concentrations are poorly resolved because the current was very small and the possibility of a higher order reaction kinetics cannot be ruled out. Single-channel experiments would clarify this issue better. From the present data, we make the hypothesis that one Ca^{2+} ion interacts with one potassium channel site. As Ca^{2+} and Mn^{2+} have similar ionic radii (99 and 93 pm, respectively), while Mg^{2+} is relatively smaller (65 pm), this external site could be selective upon ion dimension bases, as proposed by Müller et al. (1989) for a fast outward current activated by internal divalent cations in molluscan pacemaker neurons.

Several authors have reported a modulatory action of external divalent cations on potassium channel activity in nerve cells (Gilly & Armstrong, 1982; Begenisich, 1988; Mayer & Sugiyama, 1988). However, to our knowledge, only a recent report by Kostyuk and Martynyuk (1988) has provided evidence of a fast potassium current activated by external Ca^{2+} in snail neurons. Our results stress the importance of investigating in detail the source of Ca^{2+} when studying Ca^{2+}-dependent potassium currents.

Previous studies on cerebellar granule cells in explant cultures have ruled out the presence of Ca^{2+}-dependent potassium currents (Cull-Candy et al., 1989). It is possible that certain characteristics, including Ca^{2+} sensitivity, appear at a particular stage of development. Developmental changes in a potassium-sensitive channel have been reported in cerebellar Purkinje cells from rat embryos (Yool, Dionne & Gruol, 1988). In 8-day-old postnatal (P8) rats, cerebellar granule cells are still in the proliferative phase and are not well differentiated, a condition that is very important to their adaptation in culture (Levi et al., 1984). We used cultures at least 5 days after dissociation, and we have not investigated if the Ca^{2+}-dependent current was present at earlier stages. Cull-Candy et al. (1989) have used relatively younger cells (from P8 rats, but only during the first 5 days in explant cultures),

which might express different potassium channels. The potassium current pattern described in this study correlates well with that described by Hockberger et al. (1987) in "intermediate" cells, where "intermediate" refers to a stage of development and means granule cells from P5 rats which have grown from one to two weeks in explant culture. However, these authors did not study the Ca^{2+} requirements of the potassium current. It would be interesting to investigate if development of neuronal cells induces changes in the Ca^{2+} requirements of potassium channels.

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