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Development of Voltage-Activated Potassium Currents in Cultured Cerebellar Granule Neurons Under Different Growth Conditions

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SUMMARY AND CONCLUSIONS

1. The functional expression of two potassium currents in cultured cerebellar granule cells was investigated with the whole cell patch-clamp technique in relation to development and growth condition. Cells were grown in medium containing different concentrations of potassium: 25 mM (K25) and 40 mM (K40), together referred to as "high K^+ "; 10 mM (K10) or "low K^+ "; and K10 with 100 μ M *N*-methyl-D-aspartate (KNMDA). All conditions are known to influence maturation and survival of granule cells in culture.

2. At 2 days in vitro (DIV) the membrane capacitance, taken as index of membrane surface area, was the same for cells grown in each growth condition. At 7–9 DIV it had increased in each condition, but to a substantially larger extent in cells grown in KNMDA, K25, and K40 than in cells grown in K10. During development the input resistance only decreased in cells grown in KNMDA and high K^+ .

3. A delayed potassium current (I_K) and a fast transient potassium current (I_A) could both be recorded at 2 DIV in each growth condition, although a few neurons only expressed the I_K . The I_K was partially suppressed by tetraethylammonium (5 mM), whereas I_A was predominantly sensitive to 4-aminopyridine (5 mM).

4. Normalized for cell capacitance, the specific I_A conductance hardly changed during development in cells grown in high K^+ and KNMDA. Cells in K10, however, displayed an I_A with totally different properties in 23 of 24 cells; the specific I_A conductance in these cells was considerably smaller at 7–9 DIV, suggesting a deletion of these channels during development.

5. At 2 DIV, I_A inactivated monoexponentially with a time constant that did not significantly differ between growth conditions. The time constant was slightly voltage dependent. At 7–9 DIV the voltage dependence became more prominent, in particular at voltages more positive than +10 mV. In many cells grown in K10 in which a transient current still could be detected, the voltage dependence was even stronger, with the fastest inactivation at ~ 0 mV.

6. The voltage of half-maximal steady-state inactivation of I_A was -90.8 ± 0.3 (SE) mV at 2 DIV for cells in each growth condition. It shifted to more depolarized levels at 7–9 DIV, dependent on growth condition: -80 mV in K10 and KNMDA, -77 mV in K25, and -72 mV in K40. At 2 DIV the slope factor of the Boltzmann function describing the voltage-dependent removal of inactivation was 8.2 ± 0.9 mV, which did not change at 7–9 DIV, when it was 8.9, 8.4, and 8.0 mV in K10, K25, and K40, respectively.

7. The activation of the I_K was voltage dependent and half-maximal at ~ 0 mV in all growth conditions and independent of time in culture. During development the specific I_K conductance increased in the cells grown in the K10 condition but not in cells grown in the other conditions. Because I_A was greatly reduced in this condition, there seems to be a reciprocal modulation of these two currents in neurons grown in K10.

8. These findings suggest that the functional expression of I_A

and I_K in cerebellar granule cells in culture can be regulated by activity-dependent processes during a critical period of development. Because the high K^+ and KNMDA conditions could mimic the early afferentiation of granule cells, similar mechanisms may play a role in the voltage gated potassium channel expression in cerebellar granule cells in situ.

INTRODUCTION

As key determinants of membrane excitability, potassium channels are well placed to regulate neuronal and synaptic function, and therefore alteration in potassium channel activity has been implicated in the mechanisms of neuronal plasticity (Kaang et al. 1992; Nelson et al. 1990). There is evidence from both in vivo and in vitro studies that potassium current expression can be regulated by extrinsic factors varying from different growth factors (Dourado and Dryer 1994; McFarlane and Cooper 1992, 1993; Wu and Barish 1994) to hormones (Joëls and De Kloet 1992).

In this study we have focused on the development of voltage-dependent potassium currents in cerebellar granule cells during maturation in vitro and its modulation by external conditions. Maturation and survival of cultured cerebellar granule cells grown in serum-containing media are activity dependent and require chronic depolarization, usually provided by 20–40 mM K^+ (Gallo et al. 1987). Granule cell maturation and survival in vitro can also be promoted by *N*-methyl-D-aspartate (NMDA) added to low- K^+ -containing medium. The dependence on high K^+ or low K^+ and NMDA develops within a limited period, between 2 and 4 days in vitro (DIV), and this effect of depolarization is mediated through Ca^{2+} influx through voltage-sensitive calcium channels and NMDA receptors (Balázs et al. 1988b; Gallo et al. 1987). These observations led to the proposal that high K^+ and low K^+ with NMDA mimic an in vivo event in the history of granule cells. This type of culture is an attractive model to study the influences of environmental factors on the expression of receptors and ion channels (Aronica et al. 1993; Balázs et al. 1988a,b; Condorelli et al. 1993). Granule cells receive the first afferent input from the glutamatergic mossy fibers only after reaching the internal granular layer. In vivo it takes ~ 2 days before the immediately postmitotic granule cells arrive at their final destination (Altman 1982).

In the present study we show that the environmental factors that mimic early afferentiation are important for the functional expression of potassium channels in cerebellar granule cells.

METHODS

Cell cultures

Cultures were derived from cerebella of 7- to 8-day-old Wistar rats and grown in a 10% fetal calf serum containing medium under conditions resulting in a nerve cell enriched preparation (see Balázs et al. 1988a). Briefly, cerebella were sliced ($450 \times 450 \mu\text{m}$) and the tissue was dissociated after trypsinization (0.025% trypsin solution, 37°C , 15 min) by trituration in the presence of DNase (0.01%) and trypsin inhibitor (0.05%). Isolated cells were collected by centrifugation, and, after resuspension in basal Eagle's medium (Gibco) containing 10% fetal calf serum and 2 mM glutamine, plated onto 35-mm-diam Nunc Petri dishes (precoated with $10 \mu\text{g/ml}$ poly-L-lysine) at a density of $2\text{--}2.5 \times 10^5$ cells per square centimeter. The growth of nonneuronal cells was inhibited by the addition of cytosine β -D-arabinofuranoside ($10\text{--}20 \mu\text{M}$) ~ 19 h after seeding. These cultures contain 90% granule cells, 4–6% GABAergic neurons, and a small number (2–3%) of glial and endothelial cells (Kingsbury et al. 1985; Thangnipon et al. 1983). During time in culture many neurons cluster together and increase slightly in size. We only made whole cell recordings from isolated neurons. It has been shown that these cells stain positively to neuron-specific enolase and not to glial fibrillary acidic protein (Thangnipon et al. 1983; see also Cull-Candy et al. 1988). Cultures were either grown in a "suboptimal" medium in terms of promotion of cell survival ["low" K^+ : 10 mM K^+ (K10)] or in the presence of "high" K^+ [25 mM K^+ (K25) or 40 mM K^+ (K40)] and K10 with $100 \mu\text{M}$ NMDA (KNMDA; NMDA added at 1 DIV). The latter three conditions, which promote cell survival and maturation, have also been referred to as "trophic" conditions (Balázs et al. 1992). The effect of the growth conditions was examined in cultures at 2 DIV and at 7–9 DIV. In terms of most biochemical parameters measured hitherto in this type of culture, the maturation is slow during the first days in culture but then accelerates, reaching a plateau at 7–8 DIV (Gallo et al. 1987; Moran and Patel 1989).

Electrophysiology

Cell cultures were thoroughly washed with the extracellular solution containing (in mM) 130 NaCl, 5.4 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 25 glucose, pH adjusted to 7.4 (all chemicals obtained from Sigma, unless mentioned otherwise). In some experiments we superfused the cells with an extracellular solution containing tetrodotoxin ($1 \mu\text{M}$), which completely abolished the fast inward sodium current but did not affect the outward current pattern. In a few experiments potassium currents were manipulated with 5 mM 4-aminopyridine (4-AP) or 5 mM tetraethylammonium (TEA). Recordings were carried out with patch pipettes that had 3–4 M Ω resistance when filled with the following solution (in mM): 140 KF, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, and 10 1.1-ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, pH adjusted to 7.4. Intracellular calcium was buffered to $\sim 10^{-8}$ M. Whole cell patch recordings were performed as described by Hamill et al. (1981). The recording pipette was advanced with a hydraulic manipulator to the granule cell soma. Negative pressure was applied to form a gigaseal and further gentle suction broke the membrane and provided the whole cell configuration. Capacitive electrode transients and the series resistance were compensated in the whole cell mode. Cell capacitance and series resistance were read from the dials of the patch-clamp amplifier (Axoclamp 200A). Granule cells had a diameter of 8–11 μm ; this is in agreement with the observed membrane capacitance, if we estimate the surface area of the cell assuming a sphere and a specific membrane capacitance of $1 \mu\text{F}/\text{cm}^2$. Apart from a smaller size, neurons recorded at 2

DIV had the same shape as neurons recorded at 7–9 DIV. Stable voltage-clamp measurements could be obtained ≥ 1 min after breakthrough, which in these small cells should allow equilibration of the pipette content with the cytosol. After completion of the recording (15–20 min) the electrode was withdrawn from the cell and the voltage offset was checked. All recordings were performed at room temperature ($20\text{--}23^\circ\text{C}$). Data acquisition and voltage control were performed with a computer-controlled interface (Atari Mega ST). Data were stored on hard disk for off-line analysis with custom-made software. Correction for nonspecific linear leak was performed on all traces. Leak conductance was estimated from a series of scaled hyperpolarizing voltage steps. Values are given as means $\pm$ SE. Statistical comparisons were made with Student's *t*-test unless mentioned otherwise.

RESULTS

Input resistance and membrane capacitance

The input resistance (R_{mem}) of the neurons was measured under voltage clamp from a hyperpolarizing voltage step from -70 to -140 mV. At 2 DIV, cells grown in K10 medium had an R_{mem} that was not different from those grown in the KNMDA and K25 conditions (Fig. 1A). The membrane capacitance (Fig. 1B), assumed to be proportional to membrane surface, was the same for cells in each growth condition at 2 DIV. At 7–9 DIV, R_{mem} of cells grown in KNMDA and K25 was decreased to 350 M Ω , and capacitance was enhanced by 90%. The resistance of cells grown in K10 was slightly higher (20%) at 7–9 DIV than at 2 DIV (Fig. 1A); their membrane capacitance was 30% lower than that of the cells grown in KNMDA or K25. Even when corrected for smaller cell size, neurons grown in K10 therefore had a 36% higher R_{mem} at 7–9 DIV than at 2 DIV.

At 7–9 DIV, neurons were also investigated under current-clamp conditions. Spontaneous action potentials (APs) were not observed, but when the membrane potential was adjusted to -70 mV by constant current injection, APs could be evoked with 2-ms superimposed depolarizing current pulses in cells from all conditions (Fig. 1C). Cells grown in K10 had a lower threshold (-46 ± 5 mV, mean $\pm$ SE) for evoking APs than those grown in high K^+ (-30 ± 6 mV, $P < 0.05$), whereas the threshold in the NMDA-treated cells was between these two values (-40 ± 5 mV).

Voltage-clamp analysis of potassium currents

Depolarization of cerebellar granule cells from a hyperpolarized level of -140 mV evoked an outward potassium current with a transient and sustained component (see also Cull-Candy et al. 1989). Although Ca^{2+} -dependent potassium currents have been described in mouse cerebellar granule cells (Fagni et al. 1991), we did not observe these currents in our preparation using a voltage protocol with a short hyperpolarization and the high-calcium buffering solution in the pipette. In a few experiments Ba^{2+} was substituted for Ca^{2+} , which hardly affected the outward current pattern; from this we also concluded that little Ca^{2+} -dependent potassium current is activated (in agreement with Cull-Candy et al. 1989). Figure 2Aa shows a typical example of current traces recorded in a cell grown in K25 at 7–9 DIV. The membrane was depolarized for 200 ms to a level between -60 and $+60$ mV after a 100-ms conditioning hyperpolariza-

FIG. 1. Mean input resistance (R_{mem} ; A) and membrane capacitance (C_{mem} ; B) of cerebellar granule cells in primary culture grown in 10 mM K^+ (K10), K10 with 100 μ M *N*-methyl-D-aspartate (KNMDA), and 25 mM K^+ (K25) media at 2 days in vitro (DIV) ($n = 10, 6$, and 6 , respectively) and 7–9 DIV ($n = 23, 25$, and 21 , respectively). Bars: means $\pm$ SE. C: action potential threshold of cerebellar granule cells at 7–9 DIV grown in K10, KNMDA, and K25 medium. Under current clamp the membrane potential was conditioned at -70 mV by constant injection of a small current. Action potentials were evoked with depolarizing current pulses of 2 ms ($n = 12$).

tion to -140 mV that removed voltage-dependent inactivation of the transient component. Separation of the transient and sustained current was based on the difference in their

voltage-dependent properties: insertion of a 50-ms voltage step to -40 mV between the hyperpolarizing and depolarizing step will inactivate the transient current before it can be

FIG. 2. Aa–Ac: voltage-gated potassium current (type A) in a cerebellar granule cell at 7 DIV grown in K25 medium elicited with the voltage protocols shown in the insets. Aa: depolarizing voltage commands preceded by a conditioning prepulse of 100 ms to -140 mV; holding potential -70 mV. Ab: similar protocol as in Aa, except for a 50-ms step at -40 mV inserted between the prepulse and the depolarizing pulse. Ac: traces in Ab subtracted from those in Aa isolate the transient current. In these recordings tetrodotoxin (TTX) was not applied; the fast negative deflection at the start of all depolarizations represents a fast sodium current. Ba–Bc: potassium currents from a cerebellar granule neuron recorded at 7 DIV grown in K10. Cell with a small transient current and a prominent delayed outward current (type B). Ba and Bb were recorded with same voltage protocols used in Aa and Ab; Bc is the subtracted trace. Ca–Cc: cell at 7 DIV grown in K10 medium displaying mainly a delayed outward current (type C). Traces in Ca–Cc are obtained using the same procedures shown in Aa–Ac.

FIG. 3. A: application of 5 mM 4-aminopyridine (4-AP) to a cerebellar granule cell grown in K25 at 7 DIV suppressed the transient current (I_A) by 91%. The current was evoked with a step to +60 mV and a duration of 200 ms, and a prepulse at -140 mV for 2 s. In comparison with control the peak current is reduced, with an almost complete recovery after 12 min of washout. The delayed potassium current was not affected. B: application of 5 mM tetraethylammonium (TEA) to a cerebellar granule cell grown in K25 at 7 DIV, using the same protocol as in A. Only the late component is reduced by 62%, with no reduction of the transient current. The reduction was completely reversible after washout.

activated. Therefore this protocol will only evoke the slowly activating delayed potassium current (I_K ; protocol in Fig. 2Ab). Subtraction of the current traces (Fig. 2Ab) from the responses without the step to -40 mV (Fig. 2Aa) isolates the fast transient potassium current (I_A ; Fig. 2Ac).

All cells grown in high K^+ and KNMDA expressed a delayed current and a fast transient current at 7–9 DIV. In accordance with the classification used by Beck et al. (1992), we have called this most common current pattern type A (Fig. 2, Aa–Ac). From the cells grown in K10, however, only 1 of 24 recorded neurons displayed this type A current pattern, whereas the remaining cells were classified in the two other patterns described by Beck. In 16 cells (67%) we found a small transient current with a prominent delayed rectifier (type B; Fig. 2, Ba–Bc) and in 7 cells (29%) we found a completely dominating delayed current and no transient potassium current (type C; Fig. 2C). The B and C patterns were never observed in the KNMDA, K25, or K40 group. The distinction between types B and C was, however, more gradual than absolute, and it was not used in the quantitative analysis. The amplitude of the transient current in the seven cells of type C was very small; therefore no attempt was made to quantify the kinetics of this transient current.

The transient current could be reversibly blocked by 5 mM 4-AP (reduced by 94%, $n = 3$; Fig. 3A), which is a characteristic feature of all A currents (Hockberger et al. 1987; see also review in Rudy 1988). I_K was present in all four growth conditions; it was specifically and reversibly reduced by the application of 5 mM TEA (reduced by 68%, $n = 4$); TEA hardly affected the transient component (Fig. 3B).

Fast transient current

At 2 DIV most cells displayed I_A , with a threshold for activation around -50 mV. The voltage dependence of the activation of the current is shown as the specific conductance (Fig. 4, A and B). The latter was calculated from the current amplitude and the driving force for potassium ($V_m - E_K$) with V_m the membrane potential and E_K the reversal potential for potassium according to the Nernst equation; the value was normalized to cell capacitance taken as a measure of total membrane surface. The specific conductance of I_A was smaller in cells grown in K10 than in KNMDA and K25 (Fig. 4A; K40 was not recorded at 2 DIV). At 7–9 DIV in culture, however, the specific conductance of I_A of cells grown in K10 showed a different pattern than at 2 DIV. In this condition the specific I_A conductance was virtually zero, and only at membrane potentials above +30 mV did we

observe some outward current. We cannot exclude that at these very positive voltages a small additional transient current is recruited, but because this current was only detected in a subset of cells grown in K10 no further analysis was performed. In the other three conditions the specific conductance did not change significantly, suggesting that cells grown in these conditions already had reached their maximum density of I_A channels at 2 DIV (Fig. 4B). The data did not allow a more detailed analysis of the slight decrease observed in K40 compared with K25.

The inactivation of I_A was described by a single-exponential function with a voltage-dependent time constant (Fig. 5A). At 2 DIV the time constants of inactivation of I_A in K25 were between 17 and 24 ms and only slightly voltage dependent. At 7–9 DIV the time constant of inactivation of the I_A in K25 showed a stronger voltage dependence, especially at membrane potentials more depolarized than 10 mV, where inactivation became considerably slower (Fig. 5B). The voltage dependence of inactivation of I_A at 7–9 DIV of cells grown in K10 was different: between threshold of activation and 0 mV the inactivation became faster (from 30 to 13 ms). Above 0 mV it slowed down to values for the time constant as high as 40 ms, which is close to the ones found in K25 (Fig. 5B). The values of the time constants of inactivation recorded in cells grown in KNMDA and K40 were similar to the ones obtained in K25 (data not shown).

The voltage dependence of steady-state inactivation of I_A was investigated with a fixed depolarization to +30 mV lasting 200 ms and preceded by a 50-ms hyperpolarizing pulse to a potential between -20 and -140 mV. The depolarization evoked a progressively larger transient current with more negative conditioning potentials. The voltage dependence of the measured removal of steady-state inactivation could be fitted with the Boltzmann equation

$$I(V_m) = I_{\max} / \{1 + \exp[(V_m - V_h)/V_c]\}$$

where V_h is the membrane potential at half-maximal inactivation and V_c is proportional to the slope of the curve at V_h . The steady-state inactivation of I_A as a function of membrane potential is shown in Fig. 5C. To emphasize the age-dependent effects, the data are shown for the K25 group at 2 DIV and at 7–9 DIV; the curves in Fig. 5C were constructed from the mean values of V_h and V_c of each group. The voltage of half-maximal inactivation in cells at 2 DIV was 90.8 ± 0.8 mV with a slope factor V_c of 8.2 ± 0.8 mV for all growth conditions. At 7–9 DIV V_h shifted to more depolarized levels to reach a value of -80 mV in cells grown in K10 and KNMDA, -77 mV in cells grown in K25, and -72 mV in

FIG. 4. *A*: voltage dependence of activation of the specific conductance of I_A . Specific conductance was calculated from the peak current amplitude and the driving force for potassium and normalized to cell capacitance. Mean specific conductances were calculated for 2-DIV granule cells grown in different conditions ($\blacktriangle$: K10, $n = 10$; $\circ$: KNMDA, $n = 6$; $\bullet$: K25, $n = 6$). Error bars: means $\pm$ SE. *B*: voltage dependence of activation of the specific conductance of I_A in cerebellar granule cells at 7–9 DIV. The numbers of granule cells at 7–9 DIV are: K10, $n = 23$; KNMDA, $n = 25$; K25, $n = 21$; and 40 mM K^+ (K40), $n = 19$. Symbols as in *A* ($\triangle$: K40).

cells grown in K40. The values of V_c at 7–9 DIV were not significantly different: 8.9, 8.4, and 8.0 mV for K10, K25, and K40, respectively.

The time course of removal of inactivation (τ_{removal}) was investigated using a protocol in which cells were hyperpolarized from -70 mV to -100 mV with steps of increasing duration (10-ms increments) and then depolarized to evoke a current at $+30$ mV. The increase in amplitude of the transient A current as a function of duration of the hyperpolarizing prepulse was fitted with a single-exponential function

$$I(t) = I_{\text{max}} \{1 - \exp(-t/\tau_{\text{removal}})\}$$

where τ_{removal} represents the time constant of the removal of inactivation. At 2 DIV, τ_{removal} did not significantly differ

between the cells grown in the three conditions (41 ± 10 ms), and the values were comparable with values found in other cell culture preparations (Cull-Candy et al. 1989; Ficker and Heinemann 1992); at 7–9 DIV the time constants observed in our cells were significantly smaller (12.1 ± 1.3 ms). This observation also confirmed that a conditioning prepulse of 100 ms is sufficient for removal of steady-state inactivation in the protocols used in Fig. 2.

Delayed current

Depolarization from -70 mV, evoked a voltage-dependent delayed potassium current (I_K). Our test pulses 200 ms in duration did not allow a detailed analysis of the very slow

FIG. 5. *A*: example of monoexponential fit of the inactivation of the isolated transient current evoked at 0 and $+60$ mV in a cerebellar granule cell grown in K25 and measured at 7 DIV. *Inset*: type of protocol comparable with the ones used in Fig. 2. Subtraction of currents isolated the fast transient current used for the fits. Symbols: data points used for fitting. Small dots: fitted curve, $I(t) = I_{\text{max}} \exp[(t_0 - t)/\tau]$, where I_{max} is the amplitude of the current at t_0 , the moment of the voltage step; τ denotes the time constant of inactivation. Note that τ is larger at $+60$ mV than at 0 mV (values are 50 and 28 ms, respectively). *B*: voltage dependence of the time constant of inactivation of the transient current in granule cells at 2 DIV ($\circ$: K10; $\square$: K25) and 7–9 DIV ($\bullet$, $\blacksquare$). Numbers of cells are the same as mentioned in Fig. 4, *A* and *B*, except for K10, 7–9 DIV ($n = 16$). *C*: voltage dependence of steady-state inactivation of the fast transient current at 2 DIV and 7–9 DIV for cells grown in K25 indicates a clear shift of the membrane potential at half-maximal inactivation (V_h). Curves are constructed from the mean values of V_h and V_c (which is proportional to the slope of the curve at V_h). Points: locations of the observations.

FIG. 6. Voltage-dependent activation of the delayed potassium current recorded at 2 DIV (A) and 7–9 DIV (B). The specific conductance of the slowly inactivating delayed potassium current (I_K) was calculated using the same procedure as in Fig. 4. Means $\pm$ SE are based on the same number of cells as used in Fig. 4, A and B.

decay of this current (time constants of 1–3 s). Recent reports (McFarlane and Cooper 1991, 1992) suggest that this delayed potassium current consists of two components: the delayed rectifier current, as described in this paper, and a very slowly inactivating current with an activation threshold around -40 mV and a time constant of 1–3 s. Figure 6 shows the voltage dependence of the specific conductance of I_K when the current is evoked by a depolarization from -70 mV at 2 DIV (Fig. 6A) and 7–9 DIV (Fig. 6B) for the various growth conditions. The threshold for activation was around -30 mV and half-maximal activation was at 0 mV for all conditions. During development the specific conductance of I_K was the same in K25 and K40 cells and increased slightly in cells grown in KNMDA, but became almost twice as large in cells grown in K10 (Fig. 6B).

DISCUSSION

In the present study we investigated the development of I_A and a delayed potassium current in cultured cerebellar granule cells grown in media containing different elevated concentrations of K^+ (10, 25 or 40 mM) or 100 μ M NMDA in K10 medium. We demonstrated a specific functional diversity of potassium channels during development and we showed that this pattern is influenced by the external growth conditions that resembles early afferent cerebellar granule cell input. In contrast to the cells grown in KNMDA, K25 and K40, there is a decrease in the functional expression of the transient current in cells grown in K10. The alteration of the properties and practical disappearance of the transient current could be the result of the lack of activity-dependent influences of this treatment during a critical period of development. Previous studies in ciliary ganglion neurons show that elevation (25 mM) of extracellular potassium per se does not affect the I_A , although it enhances survival of these neurons. I_A in these neurons is, however, affected by other extrinsic (trophic) factors (Dourado and Dryer 1994). It has been reported that the relative deficit of A currents in hippocampal neurons after a short period in culture can be associated with the loss of factors that are normally present in the in vivo environment (Ficker and Heinemann 1992; Wu and Barish 1992). In later

development I_A is the prominent outward current in cerebellar granule cells, as shown in a whole cell voltage-clamp study in cerebellar slices (Bardoni and Belluzzi 1993). The fact that transient currents in cerebellar granule cells were decreased in low K^+ might be due to the fact that the extrinsic factors provided by the elevated K^+ or low K^+ with NMDA, but not by low K^+ alone, mimic the conditions that affect neurons during development in situ.

Modulation of I_A and I_K and relation to cell excitability

An interesting observation is the fact that there seems to be a reciprocal modulation of I_A and I_K (Fig. 7). Although I_A had almost disappeared in K10, the specific I_K conductance was considerably increased. Similar observations have been observed when mouse hippocampal neurons are cultured for 5–7 days on glial cells (which provide an environment rich in trophic factors). These cells express a larger A current density than sister cells grown without glia. In contrast, a slow transient D current is larger in neurons seeded on poly-lysine-coated glass than in neurons grown

FIG. 7. Summary of the development of specific conductance at +60 mV of the I_A and I_K in cells grown in K10 and K25 at 2 DIV and at 7–9 DIV. Note that in time the specific I_K conductance increased by 64%, whereas the specific I_A conductance decreased to 15% in cells grown in K10.

on glial cells (Wu and Barish 1994). There is also evidence from rat sympathetic neurons that a fast transient current is expressed only when the neurons are cocultured with their ganglionic nonneuronal cells. These neurons also exhibit a reciprocal modulation of their transient currents and I_K (McFarlane and Cooper 1992). Thus, dependent on growth conditions, cerebellar granule neurons can coordinate the expression of different potassium channels. Variation in the relative proportions of the I_A and I_K will have consequences for electrical behavior of the cells. In fact, we could evoke APs with lower firing thresholds in K10 cells than in cells grown in the other conditions, indicating that the excitability had increased and that the disappearance of I_A is not simply due to degeneration of the cells in this low- K^+ medium. We cannot exclude that changes in inward currents play a role in this increased AP threshold in the high- K^+ and KNMDA conditions, but it is interesting to note that Nerbonne and Gurney (1989) observed a higher AP threshold in neonatal rat superior cervical ganglion cells that have an A current in contrast to younger cells that only have a delayed outward current.

Changes in the expression of potassium currents

Despite the fact that survival of the cerebellar granule cells becomes dependent on the level of extracellular potassium between 2 and 4 DIV (Gallo et al. 1987), potassium currents in cells grown in K10 are already different from cells in high K^+ and KNMDA at 2 DIV. Although the kinetics and voltage dependencies were comparable in cells grown in either low K^+ or in high K^+ /KNMDA, the conductance of the transient current was smaller in the K10 cells, suggesting that I_A channels are deleted. Twenty hours after addition of NMDA, cells in KNMDA already displayed a significantly larger transient current at 2 DIV than cells grown in K10 without NMDA, suggesting that the changes start to occur quickly after the environmental change. Whether the diversity of potassium channel functions are brought about by posttranslational modifications (Levitan 1988), mechanisms of alternative splicing of potassium channel genes (Timpe et al. 1988), and/or via changes in the assembly of subunits (Ruppersberg et al. 1990) has to be determined (see also Jan and Jan 1990). Relatively fast changes in the mRNAs for potassium channels have been observed after seizures that are accompanied by transient elevations of extracellular potassium. Seizure activity caused a transient reduction of Kv1.2 (delayed rectifier type of potassium channel gene) and Kv4.2 (a fast-inactivating type of K^+ channel gene) mRNAs in the dentate granule cells of the hippocampus within 90 min and 3 h, respectively (Tsaur et al. 1992).

Comparison of potassium currents with other type of neurons

The fast transient current in mature cells grown in high K^+ and KNMDA had kinetics and voltage-dependent properties of inactivation that were similar to the fast A current detected in cerebellar granule cells of 20-day-old rats (Bardoni and Belluzzi 1993) and in other mammalian neurons (Cull-Candy et al. 1989; Ficker and Heinemann 1992; Numan et al. 1986; Wu and Barish 1992). It activated rapidly,

and at voltages more depolarized than -50 mV, inactivated with a monoexponential time course and could be suppressed by 4-AP (5 mM). However, in contrast with the I_A time constants of inactivation measured in hippocampal neurons, which do not vary systematically with voltage (see for example Wu and Barish 1992), at 7–9 DIV the inactivation of the I_A in the cultured cerebellar granule cells became slower at more depolarized potentials. The steady-state inactivation curve fitted by a Boltzmann equation gave values for V_h that are comparable or slightly more hyperpolarized than the estimates obtained in the preparations mentioned above. Especially, the V_h at 2 DIV is at an extremely hyperpolarized level. Because cerebellar granule cells in vivo have resting membrane potentials around -70 mV (Slesinger and Lansman 1991), the function of the I_A at this stage of development remains obscure. Although the properties of the currents of cells grown in high K^+ - and KNMDA-supplemented media are rather similar, minor differences can be detected, e.g., cells grown in K25 have a larger specific I_A conductance than those grown in K40. Whether this is related to the higher level of chronic depolarization and therefore higher baseline levels of intracellular Ca^{2+} in cells grown in K40 is presently unknown (but see also Bessho et al. 1994).

The delayed potassium current had voltage-dependent properties similar to those described for I_K in other mammalian neurons (Cull-Candy et al. 1989; Ficker and Heinemann 1992; Segal and Barker 1984). However, as described above, we cannot exclude that this current also contains a slowly inactivating component with an activation threshold of -40 mV and a time constant of 1–3 s (McFarlane and Cooper 1991). At 7–9 DIV the specific potassium conductance was the same in cells grown in high K^+ and KNMDA but was significantly larger in K10-grown cells. The altered properties and decrease of the transient current might be partly compensated for by an increase in K^+ channel expression.

Possible mechanisms in the expression of I_A and I_K

There is good evidence indicating that the effects of high K^+ or NMDA on cell survival are mediated by calcium influx either through dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (Gallo et al. 1987) or NMDA-receptor-gated ion channels (Balázs et al. 1988b). Blockers of these channels block the effects on cell survival and receptor expression (Balázs et al. 1992; Gallo et al. 1987). It has been shown that NMDAR2A subtypes are specifically upregulated in cerebellar granule cells grown in elevated K^+ media or KNMDA through an increase in resting intracellular calcium levels as measured with calcium imaging techniques, suggesting that the increase in Ca^{2+} is the key event leading to these effects (Bessho et al. 1994). Calcium may therefore also play a crucial role in the expression of potassium channels during development. Several reports support this hypothesis: it has been demonstrated that calcium is required for the normal maturation of activation kinetics of a delayed rectifier in spinal cord neurons of *Xenopus* during a critical period of transcription (Desarmenien and Spitzer 1991; Ribera and Spitzer 1989). Increasing functional calcium channel expression in the primary cerebellar cultures has been reported from 2 DIV onward (Marchetti et al. 1991), and also, NMDA receptors increase rapidly between

2 and 4 DIV in each growth condition (Resink et al. 1992), providing a mechanism for early Ca^{2+} modulation of potassium channels. However, during the first 4 DIV the morphological maturation and growth of the cells were similar in the presence or absence of calcium blockers in these cells (Gallo et al. 1987). Future studies should clarify whether calcium influx at an early stage is crucial for the functional development of these potassium channels.

Conclusions

The fast transient current plays a role in the control of repolarization and synaptic transmission (Rogawski 1985; Rudy 1988). It contributes to spike frequency adaptation and repolarization and therefore to the shortening of APs (Storm 1987), which in turn could modulate transmitter release (Klein et al. 1980). We report that environmental factors like high K^+ and/or addition of NMDA, conditions that mimic physiological stimulation during a critical period in granule cell development in vivo, are necessary for the expression of the transient current (I_A) in cultured cerebellar granule cells. These results provide an example of how modulation of ion channel function could be generated and suggest that epigenetic factors are involved in the expression of potassium channels and that potassium channels may change in relation to the ongoing synaptic activity.

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