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VOLTAGE-ACTIVATED MEMBRANE CURRENTS IN RAT CEREBELLAR GRANULE NEURONES

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SUMMARY

1. Voltage-activated currents have been recorded from cerebellar granule neurones in explant cultures from young rats (1–9 days old). Cells were examined with whole-cell patch-clamp methods. Depolarizing pulses from a pre-pulse potential of −100 mV evoked a rapidly activated transient inward current, and an outward current which decayed in two phases. The ionic dependence, kinetics and pharmacological properties of these currents have been studied.

2. Peak inward Na^+ currents in cells from 7-day-old rats were in the range 350–450 pA. No evidence was found for the presence of calcium currents. Thus, inward current was unchanged in zero Ca^{2+}, 1 mM-EGTA solution. No inward current was obtained in medium containing 10 mM-Ba^{2+} and tetrodotoxin (TTX). Supplementing the pipette (i.e. intracellular) solution with Mg-ATP did not reveal any Ca^{2+} current.

3. Depolarizing steps (from −100 mV) in TTX-containing solution gave an early transient outward current and a late outward current. The transient current resembled I_A described in other cells, and reversed close to E_K in both normal and elevated potassium concentrations, indicating that K^+ is the predominant charge carrier. Depolarizing steps from −50 mV failed to give a transient outward current, and gave only a slowly rising current which resembled the late potassium current, I_K.

4. Inactivation of the transient current was examined by applying test depolarizations from increasingly negative pre-pulse potentials (−50 to −120 mV): half-inactivation occurred at −72 mV. Transient outward currents decayed exponentially with time constants, τ, of 7·3–25·3 ms at 0 mV. The time course of removal of inactivation in cells held at −50 mV, and given increasingly long pre-pulses to −100 mV, was exponential with $\tau = 35$ ms.

5. Both transient and late outward currents were reversibly abolished by addition to the bathing medium of 10 mM-Ba^{2+} or 1 mM-quinine. Outward K^+ current was not dependent on external calcium. Tetraethylammonium (20 mM) selectively reduced the late outward current; the peak transient current was reduced by less than 20%. 4-Aminopyridine (2 mM) showed little selectivity between transient and late outward currents.

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6. It is concluded that cerebellar granule cells from young rats possess voltage-activated inward Na^+ current as well as two types of K^+ current, I_A and I_K. In terms of neuronal functioning, the properties of the transient outward current may confer a role in regulating excitability and in repolarization, but a definitive statement will require knowledge of the cellular location and relative densities of channels in granule cells *in vivo*.

INTRODUCTION

Despite constituting by far the largest population of neurones in the mammalian brain, little was known about the membrane properties of cerebellar granule cells until recently (Cull-Candy & Ogden, 1985; Hirano, Kubo & Wu, 1986; Hockberger, Tseng & Connor, 1987), since they are too small to readily accommodate microelectrodes. Large numbers of granule cells can be obtained in relatively pure cultures by mechanical or enzymatic dispersal of rat cerebellum. This has led to a number of studies on granule cells, including measurement of $^{22}Na^+$ flux (Beale, Dutton & Currie, 1980), uptake and release of glutamate (Levi & Gallo, 1986), turnover of inositol phospholipids (Nicoletti, Wroblewski, Novelli, Alho, Guidotti & Costa, 1986) and intracellular calcium measurement using Fura-2 (Connor, Tseng & Hockberger, 1987). Ion channels linked to amino acid receptors in granule cells have also been examined with patch-clamp methods (Cull-Candy, Howe & Ogden, 1988). The small size of these cells (5–10 μm) is an advantage for whole-cell recording, as good voltage control is possible. We have investigated the ionic dependence, kinetics and pharmacological properties of the voltage-activated whole-cell currents in granule cells maintained in explant culture. In particular, a transient outward potassium current has been identified and studied in some detail. The present description extends the knowledge of membrane properties of mammalian central neurones, and is a necessary prerequisite for studies on synaptic transmission between cerebellar neurones. Preliminary reports of these experiments have been presented (Cull-Candy, Dilger, Marshall & Ogden, 1986; Cull-Candy, Marshall & Ogden, 1987).

METHODS

Preparation and maintenance of cells

Cerebella were removed aseptically from Sprague–Dawley rat pups, killed by decapitation after cervical dislocation, and were transferred to warmed Dulbecco's medium (Gibco Cat. No. 041-1965) containing 2 mM-L-glutamine, 10% fetal calf serum (heat-inactivated), 100 units/ml penicillin and 100 units/ml streptomycin. The meninges were removed as completely as possible, and each cerebellum minced into small pieces (1–2 mm) in 2 ml of medium. The tissue was then drawn into a 2 ml syringe, and gently dispersed through a 200 μm nylon mesh (Cadisch & Sons, London N3) placed over the end of a 30 ml sterile polypropylene bottle. No enzymes were used in the dissociation procedure. Further medium was used to rinse the mesh to give a final volume of 15 ml, and the explants were allowed to settle for several minutes. The upper 10 ml was then aspirated away, and the remainder was plated onto glass cover-slips (Chance Propper No. 1, 13 mm diameter) which had previously been coated with poly-L-lysine hydrobromide (Sigma) at 10 μg/ml in distilled water (see Cohen, Balazs, Hajos, Currie & Dutton, 1978). In some instances poly-L-lysine-coated cover-slips were also treated with a solution of laminin (BRL-Gibco) at 20 μg/ml in borate buffer, pH 8·6. This was applied for 2 h at room temperature, aspirated away, and the cover-slips rinsed

with distilled water. Neurones extended processes and adhered to laminin-coated cover-slips more quickly than to poly-L-lysine cover-slips, allowing recordings to be made at earlier times in culture. As an indication of plating density, a ratio of one cerebellum to eight cover-slips was used for 7-day-old rat pups. Explants were maintained in the above culture medium with four cover-slips per 35 mm Petri dish (Falcon, 3001F), at 37 °C in a humidified incubator gassed with 5% CO_2:95% air. The medium was replaced every second day.

Identification and cytology

Granule cells were identified in cerebellar explants under Nomarski (differential interference contrast) optics. They were characterized by their small size (diameter 5–10 μm) and spherical shape, and greatly outnumber other larger cell types present. The granule cell nucleus occupies most of the cell, and the nucleolus is large and prominent. Cytological identification of granule cells as neurones was confirmed with the neurofilament-selective monoclonal antibody RT97 (Wood & Anderton, 1981), as described previously (Cull-Candy, Dilger, Ogden & Temple, 1985; Cull-Candy *et al.* 1986). Neurofilament was clearly detectable in granule cell processes within 6–8 h of plating, and fine neurites contained large amounts of neurofilament by 20 h. Outgrowth was sparse even after several days in culture, and the extent of arborization was clearly much less than in a mature granule cell *in situ*.

Whole-cell recordings have been made from granule cells obtained from rats between 1 day before birth (E21) and 10 days after birth (P10), but all experimental traces presented in this paper are from cells taken from 7-day-old rats. Although we have recorded within 3 h of plating, experiments were generally performed after 16 h, by which time explants are more firmly attached to the cover-slip. Records presented here were obtained at times between 16 and 120 h *in vitro*. Granule cells had a capacitance of $2{\cdot}9 \pm 0{\cdot}7$ pF (mean $\pm$ S.D., $n = 45$). This value is consistent with the surface area of the cell body estimated as a sphere of diameter 10 μm, and an assumed membrane capacitance of 1 μF cm^{-2}.

Pipettes and solutions

Patch pipettes were made from borosilicate (hard) glass capillary tubing containing a filament (Clark Electromedical) with a List LM 3P-A puller, and were back-filled. The outside diameter of pipettes was 1·5 mm, and both thick-walled (0·32 mm) and thin-walled (0·16 mm) glass was used. Although thick-walled pipettes often sealed onto cells more easily, thin-walled pipettes were generally preferred because of the lower series resistance possible. To reduce their electrical capacitance, all pipettes were coated to within a few hundred micrometres of their tips with Sylgard 184 resin (Dow Corning). The tips of the pipettes were polished using a heated platinum filament. Pipettes were filled with an 'intracellular' solution of the following composition: KCl, 140 mM; NaCl, 4 mM; $CaCl_2$, 0·5 mM; EGTA, 5 mM; HEPES, 10 mM, titrated to pH 7·2. Patch pipettes fabricated from thin-walled glass had impedances in the range 2–8 MΩ when filled with this solution.

For electrophysiological experiments, cells on cover-slips were transferred to a glass-bottomed dish, and viewed with a water-immersion objective lens (Nomarski optics, Zeiss 40×, numerical aperture 0·75) at a total magnification of ×640. The normal extracellular solution was as follows: NaCl, 150 mM; KCl, 2·8 mM; $CaCl_2$, 1·0 mM; HEPES, 10 mM; at pH 7·2. Test solutions were maintained at constant osmolarity by reducing NaCl. All experiments were performed at room temperature, 20–23 °C. In order to apply test solutions, a fine plastic nozzle was advanced under the water-immersion objective, and solution changes were made using a two-way Hamilton tap.

Whole-cell recording

Membrane currents were recorded with a List EPC-7 (List Medical, Darmstadt, FRG) or an Axopatch-1B amplifier (Axon Instruments, CA, USA), both employing a 500 MΩ feedback resistor in the headstage. Seals of 20–50 GΩ were routinely obtained. The series resistance in these experiments was 10–25 MΩ; input resistance was typically 10 GΩ. Voltage pulses were generated by the calibrated output of a Digitimer pulse generator. Current, voltage and triggering pulses were recorded on an FM tape-recorder (Racal, Store 4).

RESULTS

Voltage-activated inward currents

Figure 1*A* shows whole-cell inward currents evoked over a wide range of depolarized potentials (−50 to +80 mV) from a pre-pulse potential of −100 mV. In this experiment, potassium within the patch pipette was replaced by caesium to reduce the voltage-activated outward current. The peak inward Na^+ current initially increased with increasing depolarization, then declined towards zero as its reversal potential was approached. At potentials positive to their reversal potential, the outward currents (carried by Na^+ and Cs^+) through sodium channels inactivated rapidly and were difficult to resolve. Peak inward current is plotted against voltage in Fig. 1*B*. Inward current is maximal at −20 mV, and the current–voltage relationship is linear beyond this potential. The extrapolated reversal potential of +53 mV deviates from the calculated E_{Na} of +87 mV because the outward current is not carried exclusively by Na^+. Maximal inward currents of 350–450 pA were routinely obtained in cells from 7-day-old rats examined 16–120 h after dissociation. These currents were abolished by 100 nM-tetrodotoxin, and in Na^+-free medium. We have not studied inactivation of the sodium current in detail, but peak inward currents were reduced by approximately 20% at a pre-pulse potential of −50 mV, compared with those evoked after long hyperpolarizing pre-pulses.

No evidence was found for an inward current carried by Ca^{2+} ions. Neither a late component of inward current nor any inflexion on the falling phase of the Na^+ current were observed. The current–voltage curve in zero Ca^{2+}, 1 mM-EGTA solution was displaced such that activation of fast sodium current occurred at slightly more negative levels than in control solution, but other features such as the time course of currents were unaffected ($n = 6$). Furthermore no inward current was seen in solutions containing Ba^{2+} and TTX, even though barium currents through divalent cation channels are often larger than the calcium currents under the same conditions (e.g. Hagiwara & Ohmori, 1983). Barium suppresses voltage-activated outward currents in granule cells, thereby improving the likelihood of observing any small component of late inward Ca^{2+} current.

In many types of cells, calcium currents have been reported to decline with time during whole-cell recording (e.g. Byerly & Yazejian, 1986; Kay & Wong, 1987). This has been ascribed to removal of soluble intracellular constituents, essential to calcium channel function. In granule cells, we have consistently observed only voltage-activated fast inward current, even in recordings taken as soon as possible (i.e. less than 60 s) after establishing the whole-cell conformation. Further, we have recorded with 2 mM-Mg-ATP and 1 mM-$MgCl_2$ added to the pipette (i.e. intracellular) solution, a procedure which has been effective in slowing run-down of calcium currents in other preparations. Although no TTX-resistant inward current was evident in these conditions, it remains possible that the Ca^{2+} current was labile in whole-cell recordings.

Voltage-activated outward currents

In all the experiments described below the outward currents were investigated with 100 nM-TTX present in the extracellular solution to abolish the inward

currents. A depolarizing voltage step to +30 mV, from a holding potential of −120 mV, evoked an outward current which clearly decayed in two phases, as depicted in Fig. 5*A*; the current activates rapidly and reaches an early peak, then declines over tens of milliseconds to leave a late current which decays more slowly, with a half-time of the order of seconds. Initial observations revealed that the early transient outward current showed a marked voltage-dependent inactivation, and could only be evoked from holding potentials more negative than −50 mV. In this respect it appeared to resemble the transient outward current (A-current) previously described in a variety of excitable cells (reviewed by Rogawski, 1985). We have therefore attempted to characterize these currents, and to discriminate between the transient and late outward currents on the basis of the voltage dependence of activation and inactivation and susceptibility to potassium conductance blockers.

Reversal of the transient outward current

The reversal potential for the transient outward current was first investigated to provide information on the ionic basis of the current. A transient outward current was evoked from a holding potential of −120 mV by a short (3 ms) test pulse to +20 mV. The cell was then repolarized to various potentials (−10 to −140 mV), and the decay of current examined (Fig. 1*C*). In normal extracellular K^+ (2·8 mM) an outward instantaneous current can be seen following repolarization to −10 mV and the magnitude decreased upon repolarization to more negative potentials. Beyond the reversal potential, an inward instantaneous current is evident on repolarizing to −140 mV (Fig. 1*C*). From the current–voltage relation plotted in Fig. 1*E*, a reversal potential of −93 mV is obtained. This is near the equilibrium potential calculated for K^+ (−98 mV) suggesting that K^+ is the predominant charge carrier of the transient outward current.

The instantaneous current–voltage relation in Fig. 1*E* is not linear, probably due to underestimation of the currents at negative potentials where they decline rapidly. To investigate further the ionic species carrying transient current, the extracellular K^+ concentration was raised from 2·8 to 28 mM and a similar voltage protocol was followed (Fig. 1*D*). Clear inward current is seen immediately on repolarization to potentials more negative than −40 mV. The current–voltage relationship plotted in Fig. 1*E* shows a reversal potential at −37 mV and increased slope conductance compared with data in 2·8 mM-K^+. A similar reversal potential was noted in two other cells. The 56 mV change in reversal potential agrees well with that predicted for a ten-fold elevation in external K^+, and further supports the conclusion that K^+ is the main charge carrier.

Voltage dependence of inactivation of transient outward current

When depolarizing voltage pulses were applied from a holding potential between −50 and −60 mV, the transient outward current was absent. Only a more slowly rising outward current was seen, which showed little inactivation with time. This current resembled the late, slowly decaying outward current, I_K, described in other preparations. The voltage dependence of inactivation of the transient current in the steady state was investigated by applying pre-pulses of 1 s duration over a range of potentials (−50 to −120 mV), followed by a fixed test pulse to 0 mV to evoke

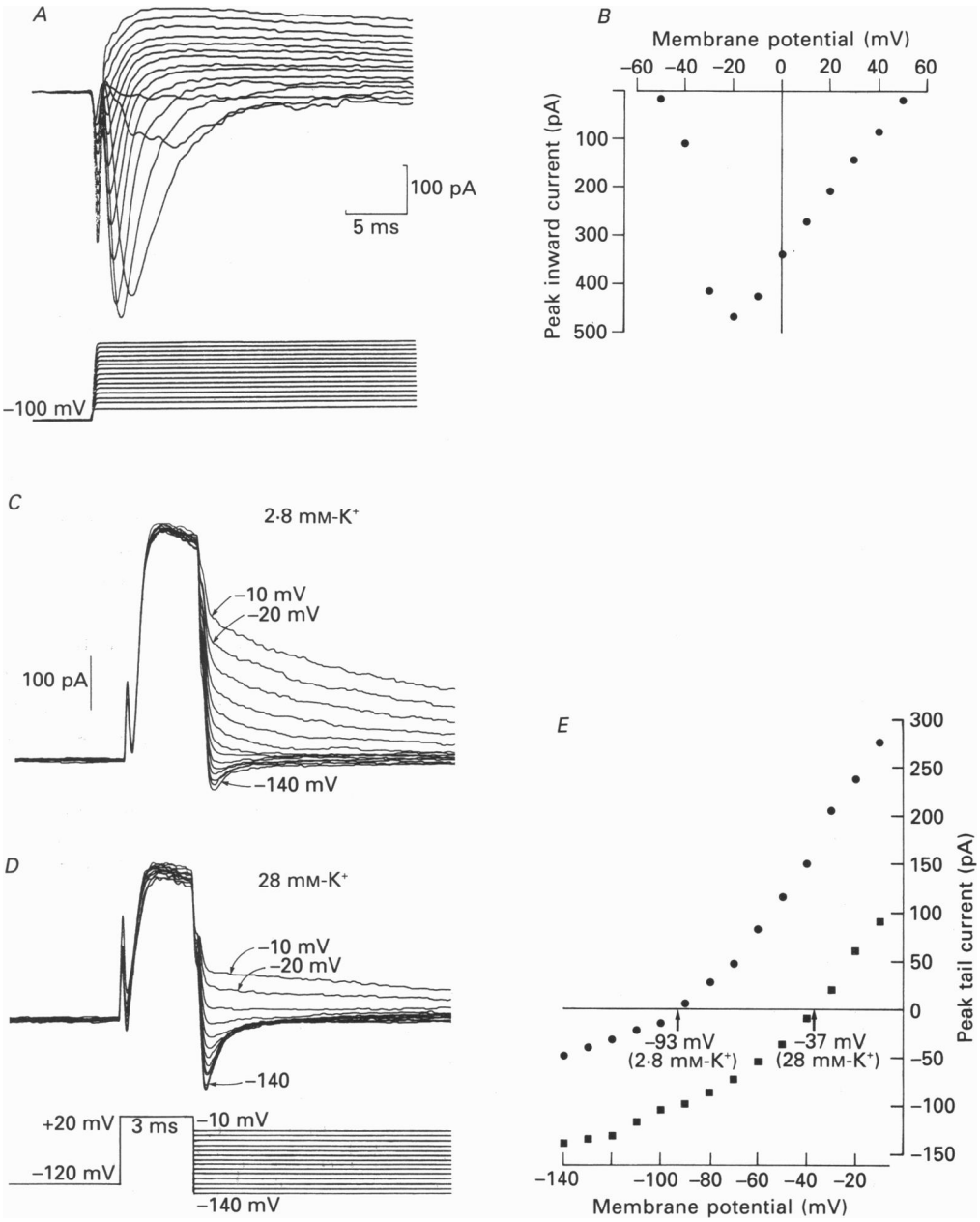

Fig. 1. *A* and *B*, voltage-activated membrane currents recorded under whole-cell voltage clamp from a cerebellar granule cell. The cell was clamped at a holding potential of −50 mV, and a hyperpolarizing conditioning pre-pulse of 1 s duration at −100 mV applied before test depolarizing pulses. *A*, whole-cell currents were recorded using a patch pipette containing Cs^+. Pre-pulse protocol, followed by depolarizing test pulses between −50 and +80 mV. Calibration, 100 pA and 5 ms. *B*, current–voltage relationship for peak inward current. The extrapolated reversal potential is +53 mV. *C–E*, experiment to determine

outward current. This is illustrated in Fig. 2*A*. Test depolarizations from increasingly negative potentials evoked a progressively larger peak of transient current.

The current evoked from each holding potential, normalized as a fraction of the maximum current, is plotted to give a steady-state inactivation curve in Fig. 2*B*. The curve is sigmoid, and has been fitted with an expression derived from the Boltzmann distribution, for the proportion of channels inactivated as a result of the depolarizing pre-pulse:

$$\frac{I}{I_{max}} = \frac{1}{1+\exp\left(\frac{V_h - V}{V_c}\right)},$$

where I is the current evoked from holding potential V, expressed as a fraction of the maximum current, I_{max}. V_h is the potential at which $I/I_{max} = 0{\cdot}5$, and V_c is a constant which is related to the steepness of the curve. The expression was linearized, and data fitted by the least-squares method to yield values for V_h and V_c. The data shown in Fig. 2*B* give half-inactivation (V_h) at -72 mV and a slope factor (V_c) of 7·2 mV for an e-fold potential change. Experiments on three other cells, using holding potentials between -60 and -100 mV, gave values of -70, -72 and -75 mV for V_h, and 7·2 mV in each case for V_c.

Separation of transient and delayed currents

In order to investigate the time course of inactivation of the transient outward current it is necessary to identify an appropriate steady level towards which the current decays and which can be taken as an asymptote. The transient outward currents in other preparations have generally been obtained in isolation by using one of the following three procedures. (1) The transient outward current has been evoked at potentials where the delayed outward current is not activated; or (2) other outward currents have been suppressed pharmacologically; or (3) currents have been subtracted graphically.

We have investigated the possibility of separating the two types of outward current in the granule cells by subtraction of families of outward currents evoked

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the reversal potential of the transient outward current. The voltage protocol is illustrated beneath *D*: the cell was held at -50 mV, and transient outward current evoked every 6 s by a 1 s hyperpolarizing pre-pulse to -120 mV followed by a 3 ms test pulse to $+20$ mV. At the end of the test pulse, the cell was repolarized to a range of potentials between -10 and -140 mV, and tail currents observed. *C*, currents evoked in normal extracellular solution containing 2·8 mM-potassium. Note the large size of the tail currents at -10 and -20 mV; these reverse at about -90 mV, and are clearly inwards at more negative potentials. *D*, K^+ was raised tenfold to 28 mM (NaCl reduced to maintain constant osmolarity), and the same voltage protocol applied. Note the reduction in size of the peak outward current at -10 mV and the increase at -140 mV. The currents reverse between -30 and -40 mV. Calibration, 100 pA. *E*, instantaneous current–voltage relationships for the transient outward currents, plotted from experimental traces shown in *C* and *D*. Currents were measured 500 μs after the repolarizing step to allow the clamp to settle fully. In normal external potassium (●, 2·8 mM), the reversal potential was estimated to be -93 mV by linear interpolation. On raising K^+ to 28 mM (■), the outward current was estimated to reverse at -37 mV, representing a shift of 56 mV.

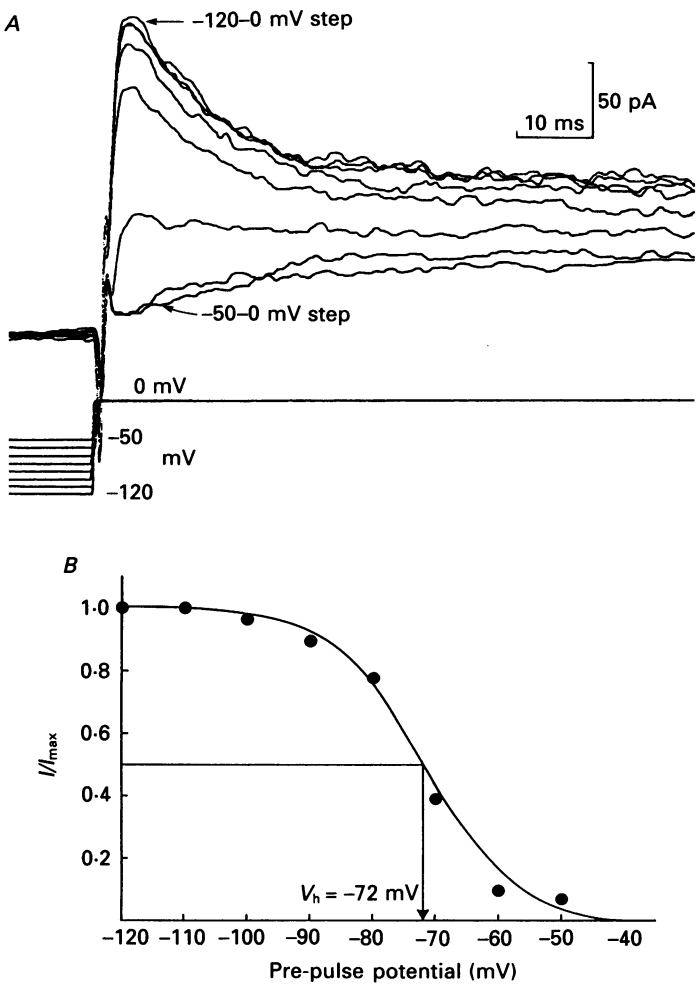

Fig. 2. The transient outward current shows voltage-dependent inactivation. Lower traces in *A* show the voltage protocol: the cell was clamped at −50 mV, and pre-pulses of 1 s duration (over a range of hyperpolarized potentials up to −120 mV) preceded a test pulse to 0 mV. *A*, large transient inward currents are activated by steps from hyperpolarized potentials (e.g. −120 to 0 mV step); transient inward current is absent on steps from depolarized potentials (e.g. −50 to 0 mV step), although a delayed outward current is visible. TTX is present throughout; calibration, 10 ms and 50 pA. *B*, the steady-state inactivation curve was obtained by plotting peak outward current as a fraction of maximal evoked outward current *versus* pre-pulse potential. The curve was fitted with a Boltzmann relationship (see text), and inactivation was half-maximal at −72 mV.

from different holding potentials. Currents evoked from a holding potential of −120 mV arise from both transient and delayed rectifier conductance (Fig. 3*A*). On the other hand, the transient current is virtually inactivated at −60 mV, so current evoked from a holding potential of −60 mV consisted mainly of the delayed outward current (Fig. 3*B*). However, it is clear that subtraction of the currents in Fig. 3*B*

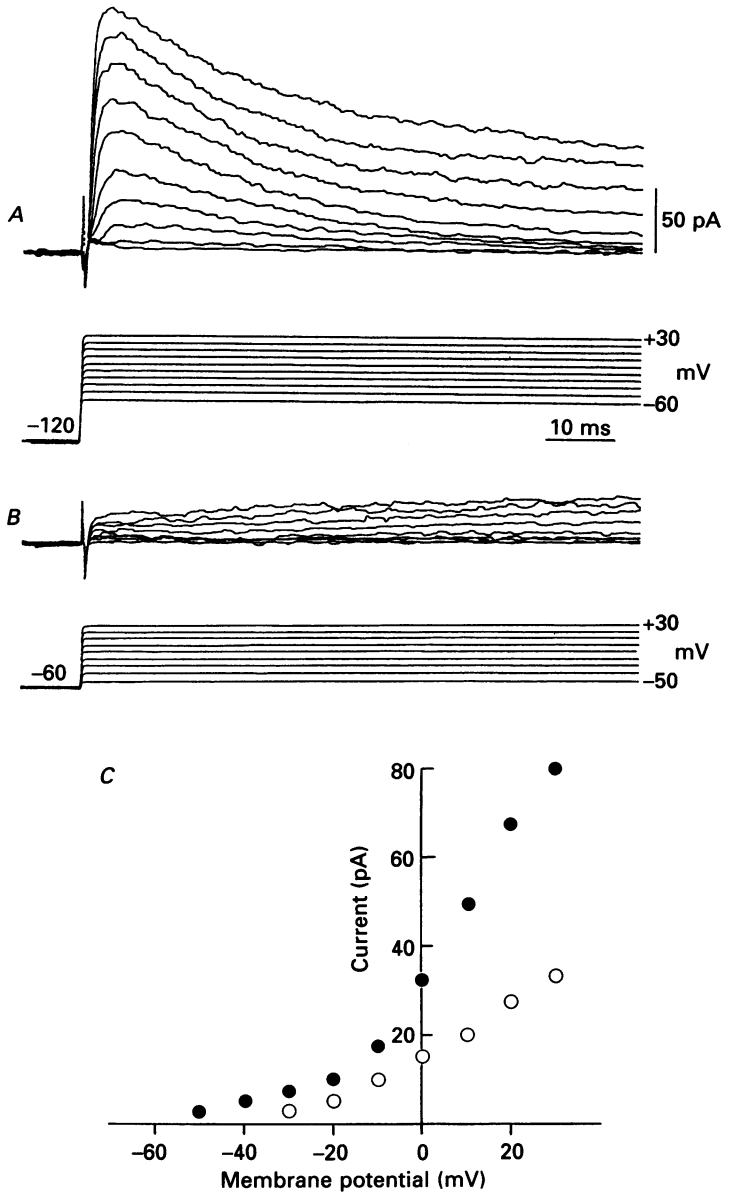

Fig. 3. Components of the outward current evoked by depolarizing pulses from different holding potentials. *A*, outward currents evoked between −60 and +30 mV, after a 1 s hyperpolarizing pre-pulse to −120 mV (lower records in *A* indicate voltage protocol). *B*, currents were evoked between −50 and +30 mV from a holding potential of −60 mV. Note that the transient outward current is now absent, and that outward current at late times is also reduced. TTX present throughout; calibration, 10 ms and 50 pA. *C*, current–voltage relationships for the late current measured 80 ms after depolarizing steps from −60 (○) and −120 mV (●).

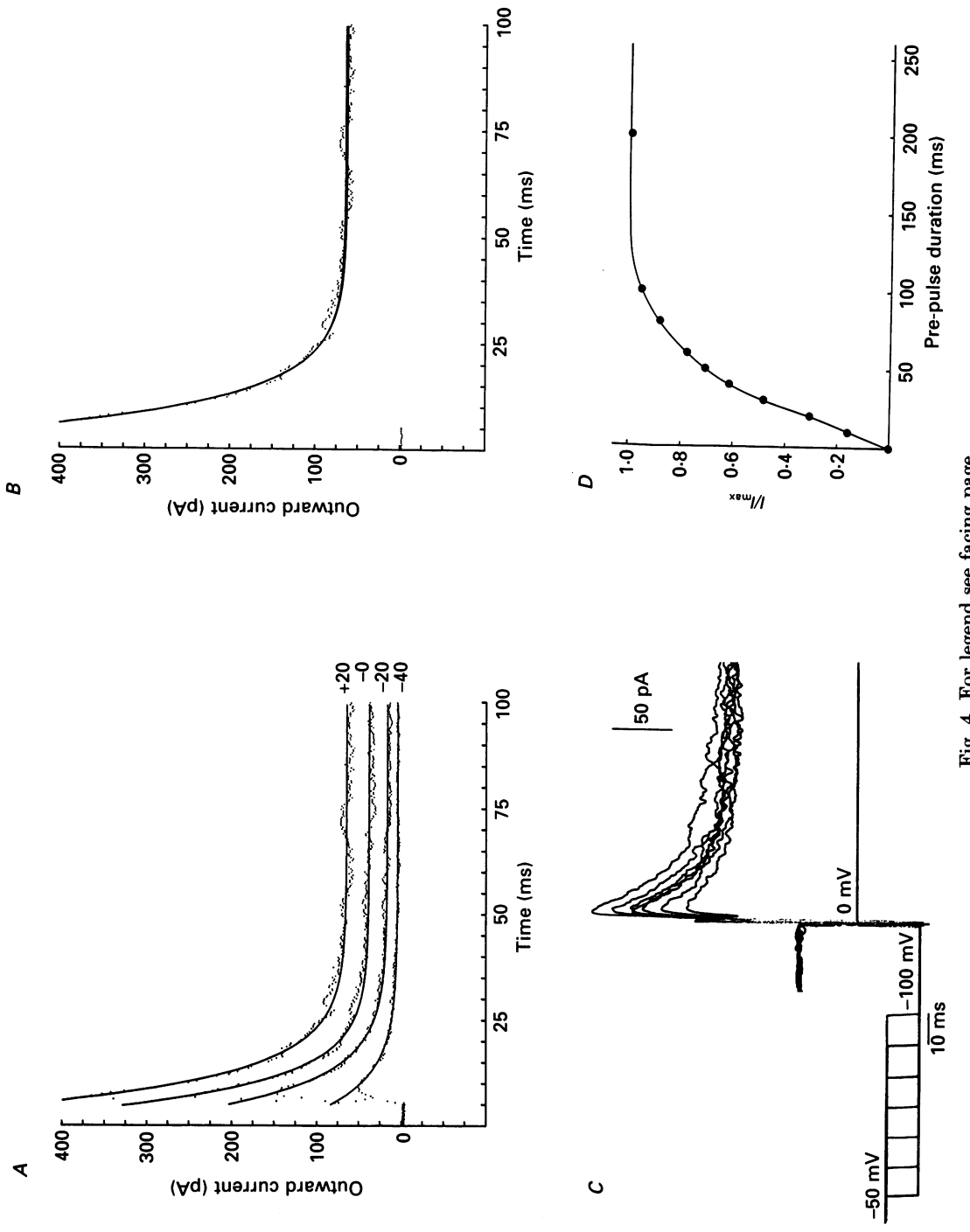

Fig. 4. For legend see facing page.

from those in Fig. 3*A* will not yield a transient which decays to zero, since at late times there is an additional component which shows voltage-dependent inactivation. This is apparent from Fig. 3*C*, which shows current-voltage relationships for the late current (at 80 ms) evoked from pre-pulse potentials of −60 and −120 mV. Similar results were obtained for outward current measured at 1 s (not shown). Two explanations are possible: the late delayed rectifier current may show a marked voltage-dependent inactivation, or the transient also has a second more slowly decaying component. Since neither alternative can be excluded, distinct components could not be distinguished by a subtraction procedure.

Time course of decline of transient outward current

We have obtained measurements of the initial rapid phase of decline of outward current, which may be compared with data obtained from other cell types. Figure 4*A* shows a set of outward currents evoked from −120 mV, by stepping to −40, −20, 0 and +20 mV. The decay phases were fitted with a single-exponential function which was not constrained to an asymptotic value. It can be seen that the fit to the data points is good, and the asymptote coincides well with the late current. This is confirmed in Fig. 4*B*, where this fit is compared to a fit with the asymptote constrained as the apparent steady level. These curves are virtually identical, and are therefore superimposed. Furthermore, decays could not be fitted better by two exponentials.

We have found that the ratio of the peak transient current to late current (at, say, 100 ms) at any test potential varied considerably from cell to cell, even within the same culture. However, in no instance have we observed a second peak of current larger than the peak transient current, or any upward inflexion on the falling phase of the late current (as might be expected if the late current was large). In general, the time constant of decay became faster at more depolarized potentials. The mean time constant of decay at 0 mV was 18·9 ms (range 7·3–25·3 ms, $n = 8$ cells). Since no subtraction procedure has been performed, this decay time may represent inactivation of the transient superimposed on a slowly activating maintained current. However, such an activation would prolong the apparent time constant of

Fig. 4. *A* and *B*, time course of inactivation of the transient outward current in rat cerebellar granule cells. In each experiment the cell was held at −50 mV, and a 1 s hyperpolarizing pre-pulse to −120 mV was applied to remove resting inactivation before stepping to the potentials indicated. *A*, outward currents evoked by step depolarizations to −40, −20, 0 and +20 mV were fitted with single exponential functions to give time constants of 8·2, 8·0, 7·3 and 7·0 ms, respectively. *B*, comparison of unconstrained exponential fit with that obtained when the asymptote was set equal to the current at 100 ms. The two exponentials cannot be distinguished on the reduced figure. Time constants of 7·7 and 7·9 ms were obtained, respectively. *C* and *D*, time course of removal of inactivation of the transient outward current. *C*, the cell was clamped at −50 mV, and a hyperpolarizing pre-pulse to a constant potential of −100 mV applied for durations varying between 10 and 200 ms. This was followed in each case by a test depolarization to 0 mV in order to elicit outward currents (as shown on the voltage records in the lower part of *C*). The size of the peak current increased with longer duration pre-pulses. TTX present; calibration, 50 pA and 10 ms. *D*, normalized peak transient outward current plotted against pre-pulse duration. A logarithmic fit to this curve gives the time constant of removal of inactivation as 35 ms at −100 mV.

inactivation with increasing depolarization, whereas a clear shortening is seen. It therefore seems likely that the decay times observed represent the time course of fast inactivation of the transient current, and that this varies over an approximately threefold range from cell to cell.

Removal of inactivation of transient current

The time course of removal of inactivation was investigated using the double-pulse protocol illustrated in Fig. 4*C*. Cells were held at −50 mV, and a hyperpolarizing pre-pulse of variable duration applied before a constant test pulse to 0 mV. As described above, the inactivation curve is steepest between −50 and −100 mV. Conditioning pre-pulses to −100 mV were prolonged in 10 ms increments. Peak transient current at 0 mV increased as the pre-pulse was lengthened, as shown by plotting peak current against pre-pulse duration (Fig. 4*D*). The curve is exponential in form, and was fitted by linear least squares to semilogarithmic plots of the data with the expression:

$$\frac{I}{I_{\max}} = 1 - \exp\left(\frac{-t}{\tau}\right),$$

where I is the current evoked by a test depolarization to 0 mV after a pre-pulse of duration t at −100 mV; $I_{\max}$ is the maximum current evoked at 0 mV; and τ is the time constant for removal of inactivation. A mean value for τ of 35 ± 2 ms at −100 mV was obtained from five cells. Longer pre-pulses, between 1 and 10 s, did not produce any further increase in peak current. On the basis of these results, maximal transient outward currents were routinely evoked after a 1 s conditioning pre-pulse to −120 mV.

Cs^+, Ba^{2+} and quinine on outward currents

To investigate further the nature of the two phases of outward currents we employed a number of agents known to block potassium currents. Replacement of potassium in the patch pipette with equimolar caesium reduced both transient and late outward current. It is unlikely that the residual outward current resulted from incomplete equilibration of the pipette solution with the cell interior, and it seems likely that the potassium channels are permeable to caesium to some degree. In contrast, external caesium (up to 10 mM) was found to have no effect on outward current.

Removal of external calcium did not reduce or increase outward current, and we found no evidence for a calcium-activated potassium current in cerebellar granule cells. We did not study the effect of external cobalt or manganese. External application of 10 mM-barium to the cells (Fig. 5*C*) rapidly and completely abolished all outward current. The effect was fully and rapidly reversible, and the outward current regained its control amplitude within 60 s of resuming perfusion with normal solution. A similar complete block of outward current was observed when 1 mM-quinine was added to the external solution (Fig. 5*D*), but this effect was only partially reversible (to approximately 60% of control) after 10 min of washing with control solution in each of three cells studied.

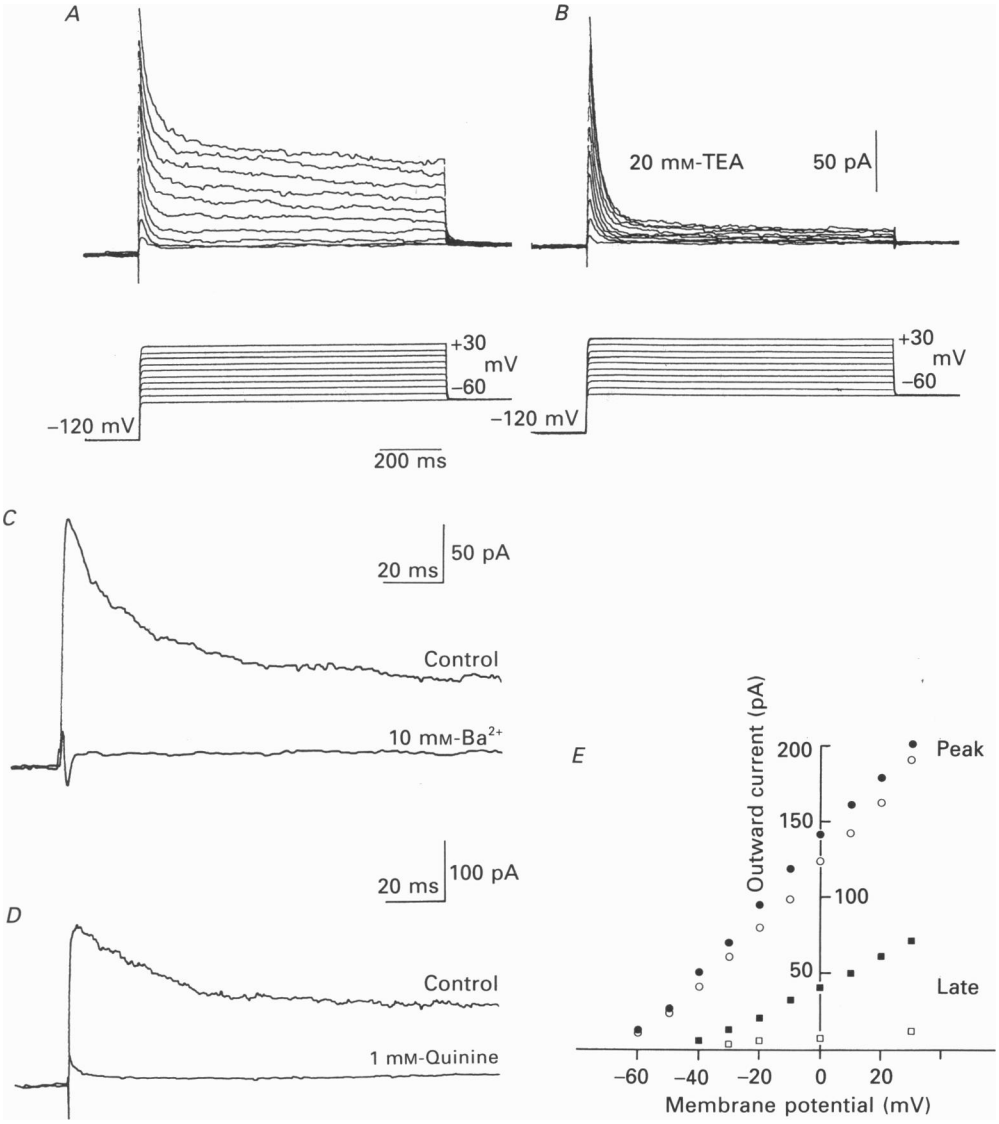

Fig. 5. Effects of TEA, barium and quinine on outward currents. *A*, upper records: outward currents evoked in control solution (containing 100 nM-TTX). A 1 s hyperpolarizing pre-pulse to −120 mV preceded depolarizing test pulses to potentials between −60 and +30 mV. *B*, outward currents evoked by voltage steps (same protocol as *A*) with 20 mM-TEA present. Calibration, 50 pA and 200 ms. *C* and *D*, effects of external barium and quinine on outward current in granule neurones. Currents were evoked by a depolarizing pulse to 0 mV, following a 1 s pre-pulse to −100 mV to remove resting inactivation. *C*, outward currents in normal solution, and 20 s after application of solution containing 10 mM-barium. Calibration, 50 pA and 20 ms. *D*, outward current in control solution, and 30 s after application of solution containing 1 mM-quinine (different cell from *C*). Calibration, 100 pA and 20 ms. TTX present throughout. Outward current was completely abolished by both barium and quinine. *E*, current–voltage relationships for peak outward current in control (●) and TEA (○) solutions, and late current in control (■) and TEA □) solutions. The predominant effect of TEA is to reduce the late current, although a small but measurable effect on the peak current is also apparent.

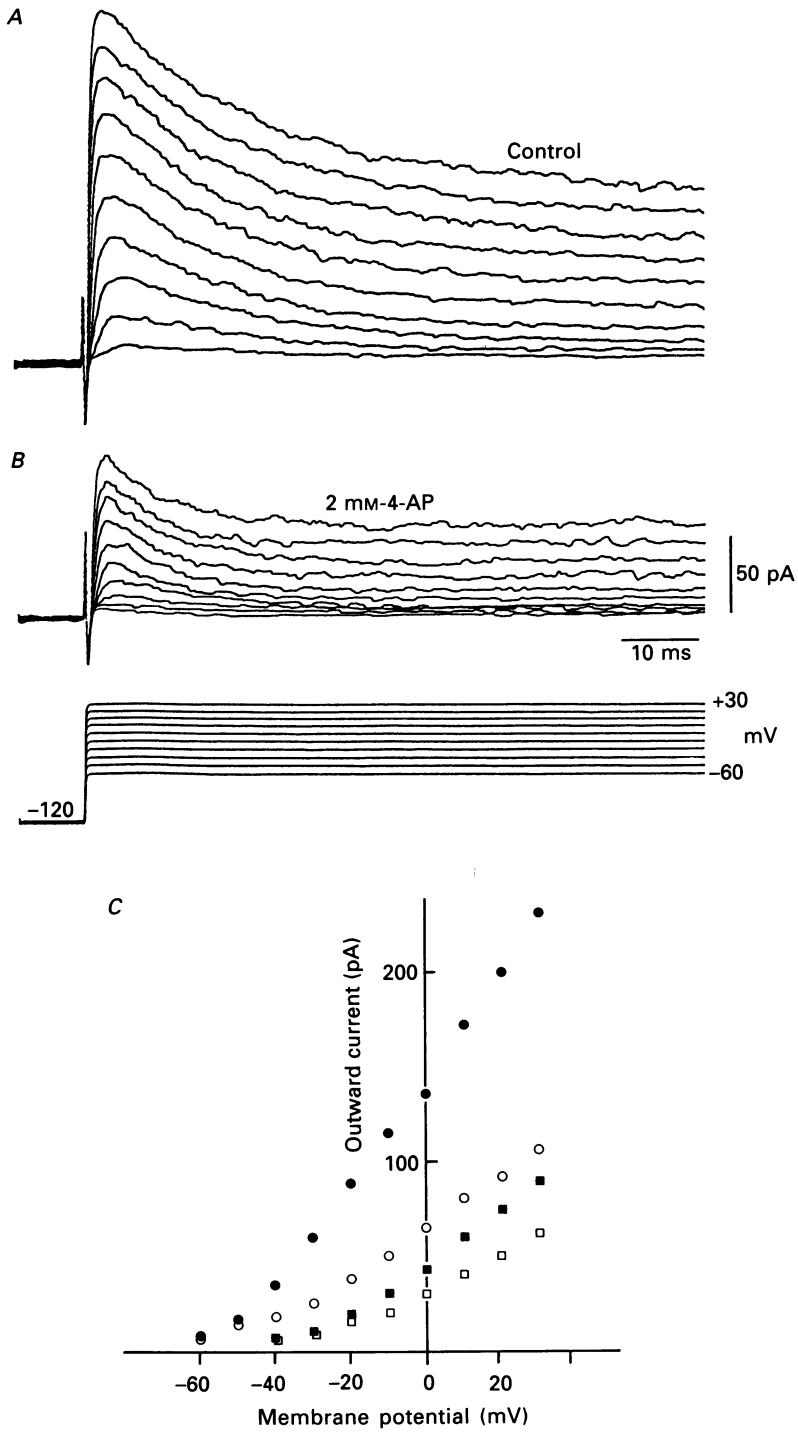

Fig. 6. For legend see facing page.

Specificity of tetraethylammonium and 4-aminopyridine on outward currents

Tetraethylammonium (TEA) and 4-aminopyridine (4-AP) have previously been described as relatively specific antagonists of the delayed rectifier current and transient outward current, respectively, in a number of preparations (Thompson, 1977; Hermann & Gorman, 1981; Stanfield, 1983). We therefore studied the effect of these agents on outward currents in cerebellar granule cells, in an attempt to separate the components which gave rise to the observed transient and late currents. Figure 5*A* shows a family of control currents evoked from −120 mV. The perfusion of 20 mM-TEA onto the cell produced a rapid reduction in the late current, and the early transient peak is relatively unaffected by this concentration of TEA. Figure 5*B* shows a family of outward currents obtained with TEA present in the bathing solution. The current–voltage relationship for peak transient and late outward currents, in control and TEA solutions, is plotted in Fig. 5*E*. TEA is clearly more effective at suppressing the late current. However, there is also a measurable reduction in the peak transient current, although this was always less than 20%. It is also apparent that a fraction of the late current persists in the presence of TEA (Fig. 5*B* and *E*). The effect of TEA was rapidly and fully reversible. Concentrations of TEA greater than 20 mM were not investigated.

Addition of 2 mM-4-AP to the external solution produced a decrease in the peak transient current, an effect which was also of rapid onset and reversal, generally within a few seconds of changing the perfusing solution. Figure 6 shows families of outward currents in control solution and in the presence of 2 mM-4-AP. The transient current was never completely abolished, even with higher concentrations (5–10 mM) of 4-AP. Current–voltage relationships for the peak and late outward current are plotted, for control and 4-AP solution, in Fig. 6*C*. The effect of 4-AP was clearly not specific for the transient current in these cells. The time constant of decay of the transient current was speeded by addition of 4-AP and may indicate that 4-AP exerts its effect by an open-channel blocking mechanism.

Development of ionic currents

It has been observed that voltage-dependent currents appear in a sequence which is specific for a given population of neurones (e.g. Spitzer, 1983). We investigated whether a developmental sequence of separate currents could be discerned for granule cells. Rather than study development as a function of days *in vitro*, cells were dispersed from rat cerebella at ages from E21 (21-day-old embryos) to P9 (9 days

Fig. 6. Effect of 2 mM-4-aminopyridine on outward currents. *A*, outward currents were evoked using an identical voltage protocol to that described in Fig. 5 (voltage records are shown in lower part of *B*). Transient outward currents of increasing amplitude were evoked by increasing voltage steps (from −120 mV to between −60 and +30 mV). Note that currents are displayed here on a faster time base than in Fig. 5. *B*, currents evoked 60 s after external solution containing 2 mM-4-AP had been perfused over the cells. The peak outward current is substantially reduced, and a clear reduction in outward current is also evident at later times. Calibration, 50 pA and 10 ms. *C*, current–voltage relationships for peak outward current in control (●) and 4-AP (○) solutions, and for late current in control (■) and 4-AP solutions (□).

after birth), and whole-cell recordings made between 3 and 48 h after plating the cells.

We detected inward sodium currents in granule cells taken from rats at all ages studied between E21 and P9, and have routinely obtained such recordings 16 h after plating. In a typical set of eighteen cells obtained from a P7 rat and studied 15–20 h after plating, eight had small (< 200 pA) inward currents, seven had large (> 200 pA) inward currents, and three had no inward current. It has been possible to record voltage-activated currents within 3 h of plating the cells, and we have recorded fast inward sodium currents (maximum $\simeq 200$ pA) in cells from E21 rats. Although we have found occasional granule cells with no active currents at all stages between E21 and P9, our observations contrast with those of Hockberger *et al.* (1987), who found no voltage-dependent conductances in granule cells during the first few days in culture. However, our observations are consistent with a progressive increase in current density with time in culture.

In some cells only a maintained outward current was observed, which had characteristics of the delayed rectifier type, in agreement with the observation that granule cells display small delayed outward currents first (Hockberger *et al.* 1987). Such a sequence has also been seen in developing rat sympathetic neurones (Nerbonne, Gurney & Raybury, 1986). In no case did we see inward sodium current or transient outward current in isolation, which might suggest that the expression of the channel proteins underlying these conductances begins at a similar time. Clearly, the study of development of ionic currents is complicated by the fact that granule cells are still dividing in the cerebellum at the ages used to obtain explants. Mitosis reaches a peak by post-natal day 7–8 (Altman, 1972). It is generally agreed that division of neurones ceases and all cells differentiate once they have been plated, regardless of their stage *in vivo*. Our observations suggest that a large proportion of granule cells have developed both inward and outward currents by P7, and it is clear that the ion channels underlying such currents are being expressed in many cells before the population peak for cell division.

DISCUSSION

Although the presence of a voltage-gated sodium current is readily shown in cerebellar granule cells, we have been unable to detect calcium currents in granule neurones at any age between E21 and P9, even under conditions which would make such currents particularly prominent. This finding agrees with the whole-cell experiments on cultured granule cells by Hockberger *et al.* (1987), who found that the calcium channel blocker cadmium had no apparent effect on inward current. It also verifies our own earlier experiments in which TTX abolished all inward current in these cells (Cull-Candy *et al.* 1986), although the observation contrasts with that of Hirano *et al.* (1986), who identified a calcium current in granule cells cultured from 7-day-old rats.

It is possible that the calcium current is labile in whole-cell recordings, or that it appears at later stages in development, as seen in spinal neurones (MacDermott & Westbrook, 1986). Also, it has been shown that sodium currents dominate in cerebellar Purkinje cells early in development, whereas the appearance of calcium

currents is correlated with the development of the main dendrite and dendritic growth cone in these cells (Llinás & Sugimori, 1978).

Separation of outward currents

The two voltage-activated outward currents found here, identified as the transient outward current and the late K^+ current, are similar to those present in many types of mammalian central neurones. As in other neurones, the transient outward current differs from the late current in two major respects: it inactivates rapidly after activation, and it displays a marked voltage-dependent resting inactivation. In descriptions of a transient outward current in molluscan neurones, it was observed that the current decayed essentially to zero for clamping potentials between approximately −46 and −30 mV (*Anisodoris*, Connor & Stevens, 1971; *Helix*, Neher, 1971), and it has since become widely accepted that the A-current decays to zero along a monoexponential time course.

We have attempted to separate the currents, with the particular aim of measuring the time course of decay of the transient current for comparison with other cells. We did not find any methods previously employed to be entirely satisfactory. A transient outward current has been observed in developing neurones from quail mesencephalic neural crest (Bader, Bertrand & Dupin, 1985). In these experiments, the transient current was not isolated, but instead the outward current at 12 s was taken as an asymptote, and two exponentials were fitted to the decay. In adult rat sympathetic neurones (Belluzzi, Sacchi & Wanke, 1985), transient outward currents which declined to zero were obtained by subtracting currents elicited by depolarization from holding potentials of −50 and −100 mV. Thus, the time course and amplitude of the late outward currents in sympathetic cells were unaffected by conditioning pre-pulses, whereas our results show that this procedure cannot be used in granule cells.

The outward currents described in adult guinea-pig hippocampal cells (Numann, Wadmann & Wong, 1987) in many respects resemble the present findings in rat cerebellar granule cells. In hippocampal cells an additional component of late outward current is evident on increasing the holding potential from −50 to −110 mV, and so a direct subtraction would not yield a transient current which decays to zero. Instead, separation was obtained by evoking families of currents from a holding potential of −100 mV, with and without a depolarizing pre-pulse, and subtracting the former from the latter.

Onset and removal of fast inactivation

Although we have not separated the components of outward current, our estimates of the time course of the fast decay of the transient component are comparable to other studies. Equally good single-exponential fits are obtained whether the asymptote is unconstrained, or set equivalent to the slowly decaying late current. The time constants of decay of the transient current varied between approximately 8 and 40 ms in cerebellar granule cells. At first sight this might seem a surprisingly wide range, but comparison with previous studies shows that such a finding is common (see Segal, Rogawski & Barker, 1984; Bader *et al.* 1985). Numann *et al.* (1987) found that the transient current in guinea-pig hippocampal neurones decayed

with a time constant of 20–40 ms at 22 °C, and they reported a weak voltage dependence of the falling phase. These findings are in good correspondence with the present results.

The steady-state inactivation curve fitted to the Boltzmann distribution gives constants for the potential at which 50 % of the channels are inactivated, V_h, and a slope factor for the steepness of the voltage dependence, V_c. These values allow convenient comparison with data from other cells. In rat sympathetic neurones (at 37 °C), $V_h = -78$ mV and the slope factor is 7·3 mV (Belluzzi *et al.* 1985), and in guinea-pig hippocampal neurones (at 22 °C), $V_h = -83$ mV and V_c is 7·5 mV. Similar estimates of $V_h = -72$ mV and $V_c = 7{\cdot}2$ mV were found here for cerebellar granule cells. It appears that V_h in the neuronal somata of molluscs (Connor & Stevens, 1971) and annelids (Johansen & Kleinhaus, 1986) is less negative than in mammalian neurones, which may represent a functional correlate of less-negative resting potentials in such cells. The time constant of 35 ms for removal of inactivation at −100 mV in the cerebellar granule cells also compares well with the estimate of 39 ms in hippocampal cells examined at the same potential (Numann *et al.* 1987). In both types of cell, the recovery was fitted well by a single exponential.

Our observation of a fairly wide range of time constants of inactivation raises the question of whether one is dealing with a homogeneous population of channels. Interestingly, two distinct forms of A-type potassium channel have recently been described in *Drosophila* (Solc, Zagotta & Aldrich, 1987). The two types have markedly different kinetics and voltage dependence of inactivation, clearly demonstrating multiple forms of transient outward current in a single species. The finding that multiple functional transient potassium channel components can be produced by alternative splicing from the product of a single gene locus in *Drosophila* reinforces the possibility of heterogeneity at the molecular level (Schwarz, Tempel, Papazian, Jan & Jan, 1988). Furthermore, the experiments reported here show that the A-conductance may make a contribution to the steady-state potassium current, as well as the transient current. Indeed, continued opening of A-channels at late times has recently been shown both in *Drosophila* myotubes (Solc *et al.* 1987) and in guinea-pig sensory neurones (Kasai, Kameyama, Yamaguchi & Fukuda, 1986).

Pharmacology of outward currents

In cerebellar granule neurones, TEA is clearly more effective on the late delayed rectifier-type outward current than on the transient outward current, and the effect is of rapid onset and wash-out. The TEA sensitivity of the late current supports the view that it corresponds to a delayed rectifier, but it should be noted that even at 20 mM-TEA, a component of the late current remains. Part of this may be a steady-state contribution from the channels underlying the transient current.

4-Aminopyridine is a selective blocker of the A-current in molluscan neurones (Thompson, 1977), although in other preparations its specificity clearly varies. We have found that 4-AP (at 2 mM) is only marginally selective for the peak transient current. Such a lack of specificity has also been noted for rat sympathetic neurones, where 4-AP was clearly non-specific at 1 mM (Belluzzi *et al.* 1985). It has also been reported that the amplitude of both the A-current and delayed current are suppressed by 4-AP (2 mM) in hippocampal neurones (Numann *et al.* 1987). It

therefore seems that 4-AP blocks voltage-activated K^+ currents in a non-specific manner in several types of mammalian neurone, although an effect of 4-AP on the late current is compatible with a steady-state contribution from the A-current.

Functional role of transient outward current

The transient outward potassium current, first described explicitly in the neuronal somata of various genera of mollusc (Connor & Stevens, 1971; Neher, 1971), appeared to be completely inactivated at normal resting potential. Quantitative analysis led to the proposal that I_A became available during the action potential after-hyperpolarization, and so I_A could regulate endogenous rhythymicity by affecting the rate of decay of the after-hyperpolarization. The A-current was viewed as conferring the function of 'encoder' to a cell, where the rate of action potential discharge reflects the degree of depolarization in a graded fashion.

In mammalian neurones, many of the functions adduced for the A-current concern the modulation of excitability. It has been shown that I_A dampens excitability in rat hippocampal pyramidal cells, and that when the current is suppressed by 4-AP, increased excitability results (Gustafsson, Galvan, Grafe & Wigström, 1982). Along the same lines, it has been proposed that in hippocampal cells, I_A functions to regulate threshold and discharge rate of action potentials, rather than properties of individual action potentials (Segal & Barker, 1984). Belluzzi *et al.* (1985) argue strongly that I_A underlies action potential repolarization in rat sympathetic neurones, although such a view would not necessarily exclude a role in the regulation of excitability. The A-current may also be involved in receptor-mediated events, and in longer-lasting changes in excitability. For example, in cultured hippocampal neurones, acetylcholine acts via a muscarinic receptor to raise excitability by inhibiting I_A (Nakajima, Nakajima, Leonard & Yamaguchi, 1986), while in dorsal raphe serotonergic neurones, I_A is regulated via α_1-adrenoreceptors (Aghajanian, 1985).

It is not possible, at present, to state conclusively what functional role the transient outward current plays in the normal physiology of the cerebellar granule cell. Clearly, the properties we have described are broadly similar to those found in other mammalian neurones, particularly guinea-pig hippocampal cells, and so the earlier possibilities apply equally to granule cells. It will be of particular interest to investigate the possibility of a functional modulation of excitability in granule cells by transmitter candidates.

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