Eur Biophys J (1991) 20:157–164

European Biophysics Journal

Voltage dependent calcium channels in cerebellar granule cell primary cultures

O. Moran, F. Lin, O. Zegarra-Moran, and M. Sciancalepore

Laboratorio di Neurofisiologia, Scuola Internazionale Superiore di Studi Avanzati, Via Beirut 2/4, I-34014 Trieste, Italy

Received February 13, 1991/Accepted in revised form May 7, 1991

Abstract. Voltage activated calcium channels were studied in rat cerebellar granule cells in primary culture. Macroscopic currents, carried by 20 m*M* Ba^{2+}, were measured in the whole-cell configuration. Slowly inactivating macroscopic currents, with a maximum value at a membrane potential around 5 mV, were recorded between the 1st and the 4th day in culture. These currents were completely blocked by 5 m*M* Co^{2+}, partially blocked by 10 μ*M* nifedipine, and increased by 2 to 5 μ*M* BAY K-8644. Two types of channels, in the presence of 80 m*M* Ba^{2+}, were identified by single channel recording in cell-attached patches. The first type, which was dihydropyridine agonist sensitive, had a conductance of 18 pS, a half activation potential of more than 10 mV and did not inactivate. This type of channel was the only type found during the first four days in culture, although it was also present up to the 11th day. The second type of channel was dihydropyridine insensitive, had a conductance of 10 pS, a half activation potential less than −15 mV, and displayed voltage dependent inactivation. This second type of channel was found in cells for more than four days in culture.

Key words: Calcium channels – Cerebellar granule cells – Patch clamp

Introduction

Primary cultures of cerebellar granule cells have the advantage of being highly homogeneous and surviving for a long time in vitro (up to 30 days). They maintain their excitability properties, as well as their metabolic and electrical responses to neurotransmitters. These neurons have voltage-activated sodium and potassium channels (Hockberger et al. 1987; Robello et al. 1989; Cull-Candy et al. 1989; Lin and Moran 1990; Galdzicki et al. 1990), and respond to exogenously applied excitatory or inhibitory neurotransmitters (Cull-Candy et al. 1988; Sciancalepore et al. 1989, 1990). Less information has been given about the voltage-dependent calcium currents in these cells. Evidence on the presence of calcium currents, based on fluorometric measurements of calcium influx, has been reported (Connor et al. 1987; Kingsbury and Balazs 1987). Although several authors have failed to detect calcium currents in cerebellar granule cells (Galdzicki et al. 1990; Hockberger et al. 1987; Cull-Candy et al. 1989; Jalonen et al. 1990), some brief reports describing the electrical measurements of calcium currents (Marchetti et al. 1990; Slesinger and Lansman 1990; Wojcik et al. 1990) have recently appeared.

We present here a patch-clamp study of calcium currents in rat cerebellar granule cells. We have recognized two types of voltage activated calcium channels: dihydropyridine (DHP)-sensitive and DHP-insensitive channels. We have found that the presence of each type of calcium channel in such cells depends on the time in culture.

Methods

Cell culture

Experiments were performed on primary cultures of cerebellar granule cells as previously described (Levi et al. 1984). Cells were enzymatically and mechanically dissociated from eight day old Wistar rats. The cells were seeded on poly-L-lysine treated plastic Petri dishes. Cytosine arabinoside (10 μ*M*) was added 20 h after plating to inhibit the mitosis of non-neuronal cells. Cells were used between the 2nd and the 11th days in culture (DIC).

Electrophysiological methods

Macroscopic membrane currents and single-channel currents were measured in the whole-cell and cell-attached configurations respectively (Hamill et al. 1981), using a standard patch-clamp amplifier (EPC-7, List).

Fire polished borosilicate glass pipettes (Hilgenberg), with resistance in the range of 4 to 6 *MΩ*, as measured in

Offprint requests to: O. Moran

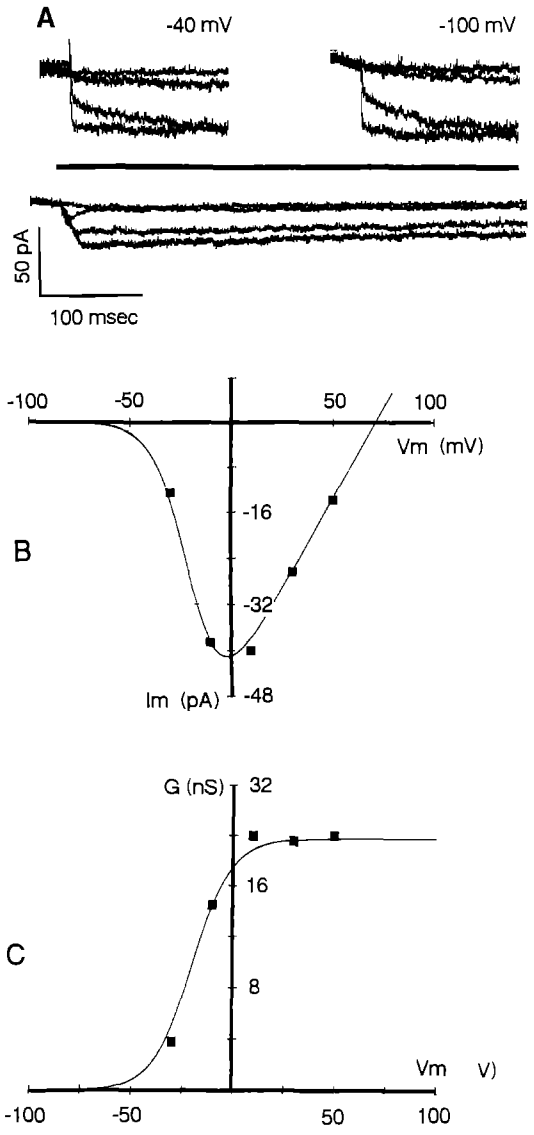

Fig. 1. **A** Calcium currents from whole-cell patch of rat cerebellar granule cell. The holding potential was −40 mV, and the test pulses were to V_m of −30, +10, +30 and +50 mV. The onset of the calcium currents recorded at a holding potential of −40 and −100 mV to the same test potentials as before are compared in the inset. Each trace is an average of 4 records. **B** Peak current I_p vs. voltage relationship. I_p is maximal (−43 pA) around −4 mV and the apparent reversal potential is about 71 mV. The continuous line was calculated from Eq. (1) (see text). **C** Voltage dependence of the peak calcium conductance G, estimated from I_p as described in the text. The continuous line shows the least square fit of the data to the expression given by Eq. (1). $G_{max} = 22.1$ nS, $V_{half} = -20$ mV, $k = 1$ and $a = -9.7$. Cells of 3 DIC

the intracellular/extracellular working solutions, were used for whole cell recording. The recording extracellular solution contained (in mM): NaCl 120, HEPES-NaOH 4.4, $BaCl_2$ 20, TEA 17.6 and TTX 1 μM. The pipette solution, dialyzing the intracellular compartment, was: CsF 80, CsCl 50, HEPES-NaOH 10, EGTA 5, $MgCl_2$ 2 and L-glucose 14. In both solutions the pH was adjusted to 7.4.

For single channel recording, Sylgard (Corning) fire polished pipettes of 10 to 12 $M\Omega$ were used. The recording solution used in the bath contained (in mM): K-Gluconate 120, HEPES-KOH 10 and EDTA 20. The pipette solution, in contact with the extracellular surface of the membrane patch, was: TEA 20, HEPES-KOH 10 and $BaCl_2$ 80. The pH of the solutions was adjusted to 7.4.

Stimulation and acquisition

The stimulation and data acquisition was performed with a 12 bit AD/DA converter (VR-ST, Instrutech), controlled by a microcomputer (ATARI, 1040ST). Holding potential was maintained at −100 to −40 mV. Single-channel or macroscopic membrane currents were evoked by voltage pulses, to a membrane potential of −50 to +50 mV and 160 to 450 ms duration. Stimulation pulses were given every 2 to 4 s. Before digital acquisition, the signal was low-pass filtered (Itaco 4302 or Krohn-Hite 3220) set at a cut-off frequency of 3 to 5 kHz. The current was sampled at 5 to 20 kHz. The acquired data was stored on a hard disk for later analysis.

In single-channel experiments, because of the high K^+ concentration in the bath solution, the cell membrane potential, V_r, was considered to be near to 0 mV. Therefore, the membrane patch potential, V_m, was estimated to be equal to minus the pipette potential V_{pip} ($V_m = V_r - V_{pip}$). All potentials will be referred to V_m, and the current will be expressed in terms of the channel current (negative current represents a cationic inward current). Most of the linear capacity transient component of the recorded current was analogically compensated. In whole-cell measurements, the holding potential was brought to −120 mV after each test pulse, and 4 pulses of 1/4 of test pulse amplitude were given (P/4 protocol, Armstrong and Bezanilla 1977). The P/4 responses were used to digitally subtract the uncompensated capacity transient and the leakage current. In single-channel experiments, records where channel activity was not elicited were appropriately scaled and used to subtract the leakage and uncompensated capacity currents. All experiments were performed at room temperature (24 to 25 °C).

Results

Single channel and whole membrane inward voltage dependent calcium channel currents carried by Ba^{2+} were recorded in rat cerebellar granule cells in culture. Long depolarizing pulses (160–450 ms) were given in a condition in which potassium channels were blocked by intracellular Cs^+ and/or extracellular TEA, and sodium channels were blocked by TTX in the bath or by removing Na^+ in the extracellular solution. Owing to the small current size, Ba^{2+} ions were preferred to Ca^{2+} as current carriers because of the larger permeability of the former in calcium channels (Reuter et al. 1985).

Whole-cell experiments

Macroscopic inward membrane currents were detected in the whole-cell configuration. A run down of the currents

was observed during the experiments. The currents decayed to about half of the initial value in about 7 min after establishing the whole-cell condition, and were completely abolished in about 20 min. The time course of the current decay became faster when the frequency of stimulation increased. Therefore, an interval of 2 to 4 s was considered optimal.

A family of currents evoked by test pulses at different potentials, V_m, are shown in Fig. 1 A. Each trace represents the average of 4 successive records. Currents peaked faster at increasing depolarizing pulses. Plots of the peak current, I_p, as a function of V_m (I-V curve), were constructed (Fig. 1 B). I_p was estimated from the least-square fit of experimental records with a third-order polynomial. Currents start to activate at test pulses near -30 mV. The maximum I_p observed in 21 different experiments was -34.2 ± 12.1 pA (mean $\pm$ standard deviation), obtained at 3.3 ± 5.7 mV. The potential at which the maximum I_p was obtained was independent of the holding potential in the range -100 to -40 mV (see inset on Fig. 1 A). When the holding potential was -40 mV, currents presented no inactivation characteristics at low V_m, and an incomplete and slow inactivation at V_m greater than 20 mV. When the holding potential was brought to -100 mV, a very slow inactivation component (time constant slower than 200 ms) was observed at test potentials as low as -10 mV in a few experiments (data not shown). The apparent reversal potential E_r was estimated from a linear fit to the last points of the I-V curve. A value of 67.1 ± 4.8 mV was obtained in 23 different cells.

Activation curves were constructed by plotting the peak conductance, G, against V_m, as shown in Fig. 1 C. Half of the maximum conductance was obtained at a V_m of -21.5 ± 11.1 mV ($n=23$). The activation curve was fitted using a Hodgkin and Huxley like model (Hodgkin and Huxley 1952), according to the equation:

$$m = \{1 - \exp[(V_m - V_{\text{half}})/a]\}^{-1} \quad (1)$$

where m is the probability of a single gating particle to be in the open state, V_{half} is the potential at which $m=0.5$ and a is the e-fold voltage dependence. The activation parameter m was estimated as

$$m^k = G/G_{\max} \quad (2)$$

where the exponent k is the number of activation particles per channel and $G_{\max}$ is the maximum conductance. The number of gating particles was found to be between 1 and 2, in analogy with that found in other preparations (Kokubun and Reuter 1984). Activation parameters estimated in 23 different cells yield V_{half} of -12.5 ± 8 mV, and e-fold voltage dependence a of -16 ± 6.4 mV.

Some pharmacological tests were performed in whole-cell experiments. The addition of 5 mM Co^{2+} to the bath solution completely abolished the currents, as is shown in Fig. 2 A. Superfusing the cells with 10 μM nifedipine reduced the currents by about 70%, as is shown in Fig. 2 B. No significant differences in the blocking effect of nifedipine was found on changing the holding potential from -100 mV to -40 mV. Currents became larger when the DHP calcium channel agonist BAY K-8644 was added to the extracellular solution, at a concentration of 2 to 5 μM

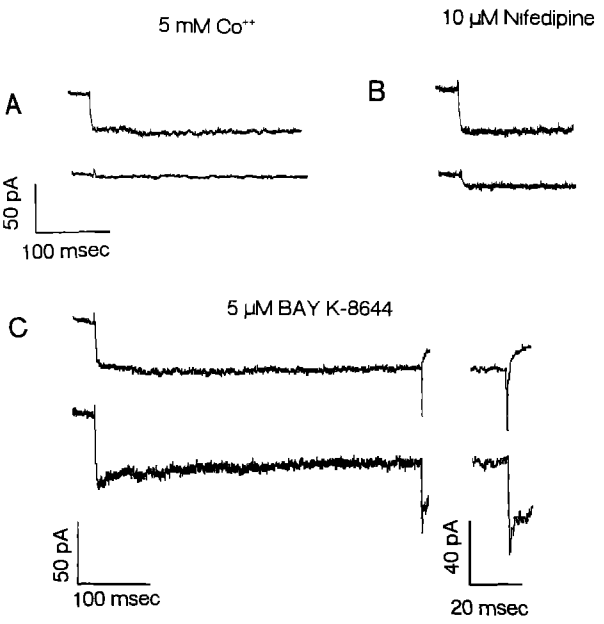

Fig. 2 A–C. Pharmacology of the calcium current in rat cerebellar granule cells of 3 DIC. **A** Addition of 5 mM Co^{2+} in the bath solution completely suppresses the calcium channel. **B** Nifedipine (10 μM) reduces the calcium current to about 30% of the control. **C** The extracellular addition of 5 μM BAY K-8644 increases the calcium current. An amplification of the tail currents shows a slower deactivation of the current at the end of the pulse produced by the DHP agonist. Holding potential was maintained at -80 mV, and the test pulse was to a V_m of $+10$ mV. Each trace is the average over 4 records

(Fig. 2 C). The increase of the current produced by BAY K-8644 was from 20% to 30% of the control, and was observed at any applied V_m. The tail current was prolonged by BAY K-8644, indicating a slower deactivation rate of calcium channels (Sanguinetti and Kaas 1986). The addition of this DHP calcium channel agonist also produced a shift of the maximum current in the I-V curve to the hyperpolarizing potentials (about -10 mV with 2 μM BAY K-8644). Unfortunately, a complete evaluation and recovery of the effects of the DHP agonists and antagonists in the whole-cell configuration was precluded by the rapid run-down of the currents.

We obtained homogeneous results in whole membrane current measurements from cells of 1 to 4 DIC. In later stages of development (more than 4 DIC) no reliable records were obtainable. This was because the neuritic processes growing introduced a space-clamp problem, deforming the shape of these small currents.

Single-channel experiments

Single channel records were obtained from cells of 1 to 11 DIC. Channels were recorded in about 30% of the cells tested. Two types of calcium channels were found in cell-attached experiments: DHP-sensitive and DHP-insensitive channels. Besides their sensitivity to the DHP agonists, these two types of channels displayed different conductances and voltage dependence in the activation

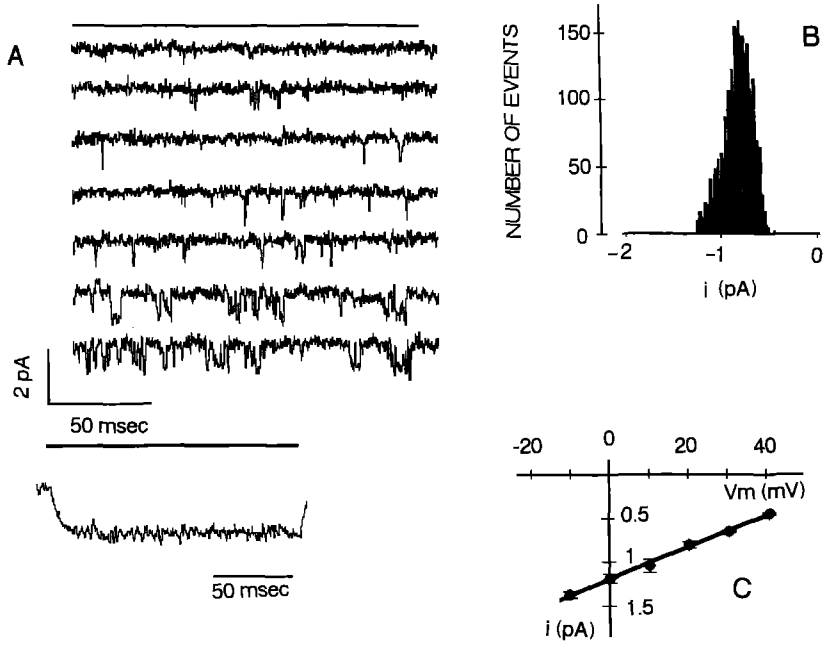

Fig. 3 A–C. DHP-sensitive calcium channels. **A** Successive single calcium channel records activated by a test pulse to 0 mV, from a holding potential of −80 mV. Although most of these records show opening events, channels activated in less than 20% of the test pulses at this potential (see also Fig. 4). An average of 164 single channel records, reconstructing the macroscopic current is shown in the inset. The duration of the test pulse is indicated by the solid bar. **B** Amplitude histogram constructed from 2484 single channel events evoked by a −10 mV test pulse. **C** The i-V relation of the single channel currents reveals a slope conductance of 18.5±0.5 pS, and an apparent reversal potential of 72 mV. Each point represents the mean current value obtained over, at least, 100 events. Bars are the standard deviations of the measurements. Cells of 4 DIC

and inactivation properties. The presence of each type of channel was also correlated with the time in culture.

DHP-sensitive channels

The first type of channel, DHP-sensitive channels, was recorded in cells of 1 to 4 DIC. These channels were also recorded in cells older than 4 DIC, although less frequently when the experiments were performed in the absence of BAY K-8644. When the first type of channel was recorded in cells older than 4 DIC, it was frequently found together with those of the second type (DHP-insensitive channels, see below). Nevertheless, when the patch pipette contained the DHP agonist (1 μM), the likelihood of finding the first type of channel in the patch, especially in cells of more than 4 DIC, was significantly increased.

The conductance of the DHP-sensitive channels was evaluated at different test potentials. In most cases, a well defined population of channels was found (Fig. 3 A), and no subconductance states were detected (Fig. 3 B). A slope conductance of 18.3±1.7 pS was estimated from the single channel current-voltage curve (i-V curve) obtained from 8 different experiments (see Fig. 3 C). The apparent E_r lies between 70 and 80 mV. The average of successive single channel records of DHP-sensitive channels clearly resembled the whole-cell current records (see inset to Fig. 3 A).

The DHP-sensitive channels were activated by voltage test pulses to V_m equal to or higher than −20 mV. Not all test pulses evoked channel activity. No events were recorded when the test V_m was less than −20 mV. The activity of the channels increased as a function of the pulse potential. It consisted of an increase of the open channel probability during the test pulse, as well as an increase of the probability to find an open channel during the test pulse (roughly evaluated as the percentage of pulses where channel opening events were present). The steady-state activation curve was constructed by fitting the overall open probability (estimated by the mean open channel probability during a test pulse times the total number of test pulses) recorded during a given number of test pulses (normally more than 20) against V_m. The single channel half activation potential, V_{half} (potential at which the overall open probability of a half of the maximum is achieved) was between +7 and +23 mV (Fig. 4 A). The maximum steepness of the voltage dependence yielded an e-fold increase of the overall open probability for 6.7±1 mV ($n=6$) change in voltage.

Dwell time distribution histograms were constructed for the open and shut states of the DHP-sensitive channels. The open time distribution was well fitted by a single exponential function, with a time constant of the order of 0.3 to 1 ms (Fig. 4 B). The shut time was fitted with a double exponential distribution, with a fast time constant of the order of 1 ms and a slow time constant of the order of 10 ms (Fig. 4 C). The voltage dependence of these channels can be also appreciated from the dependence of mean open time and the mean shut time on the test potential (Fig. 4 D).

The addition of DHP calcium channel agonist BAY K-8644 (1 μM) to the recording patch pipette caused several modifications in the overall open probability and in its voltage dependence. The presence of the DHP agonist increased the open channel probability during the test pulse, by increasing the mean open time, and decreasing the mean shut time (see Fig. 5 A). The probability to find a channel open in a given pulse is also increased by BAY K-8644 (Fig. 5 B and 5 C). Furthermore, the BAY K-8644 also changes the voltage dependence of the channels, shifting the V_{half} 10 to 20 mV to negative potentials (data not shown). This shift allows one to record single channel events at test V_m as low as −35 mV. The deactivation rate of channels was prolonged by the DHP agonist, allowing

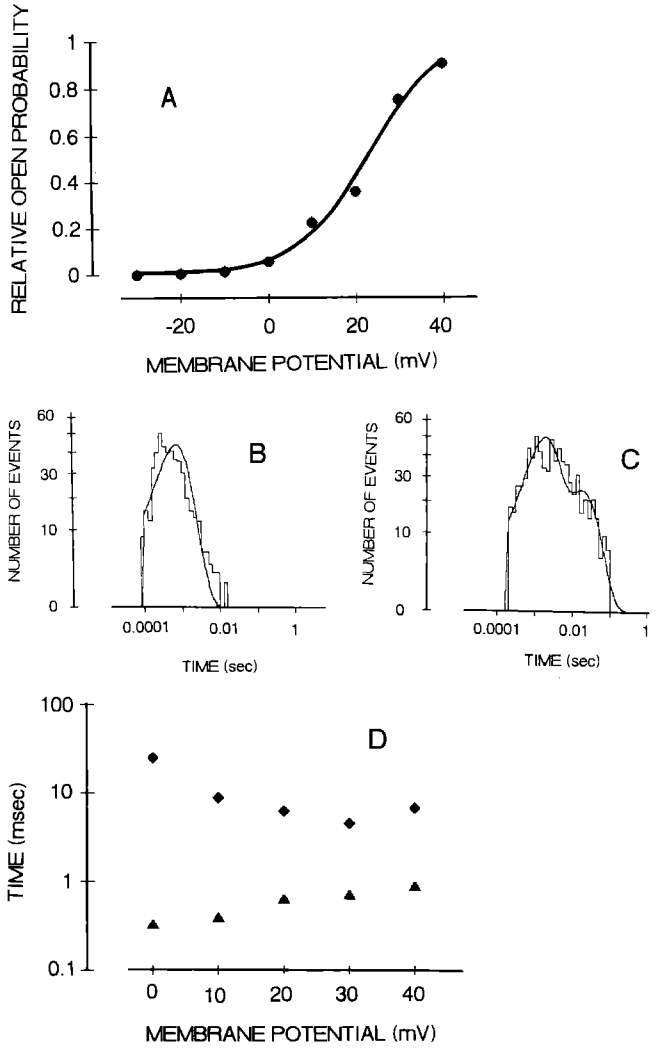

Fig. 4 A–D. Open probability and open and shut time of DHP-sensitive calcium channels. **A** The activation curve was normalized to the maximum overall open probability P_{max}. The smooth line was obtained by fitting the experimental overall open probability data P_0 against the membrane potential with the expression $P_0/P_{max} = [1-\exp[(V_m - V_{half})/a]\}^{-1}$, using the Simplex method. The overall probability was evaluated from 45 successive records at each test potential. The V_{half} was 23.6 mV and a is 6.1 mV. Open time distribution (**B**) and shut time distribution (**C**) evaluated from records obtained at a test potential of +20 mV from a holding potential of −80 mV. The histograms were fitted according to Sigworth and Sine (1987). The open time constant was 0.99 msec, and the shut time constants were 32 ms and 340 ms. 463 and 308 events were used to analyze the open and shut times respectively. **D** The mean open time (triangles) and the mean shut time (diamonds) are plotted as a function of the test potential. Each point represents the mean obtained over at least 200 events. Cells of 4 DIC

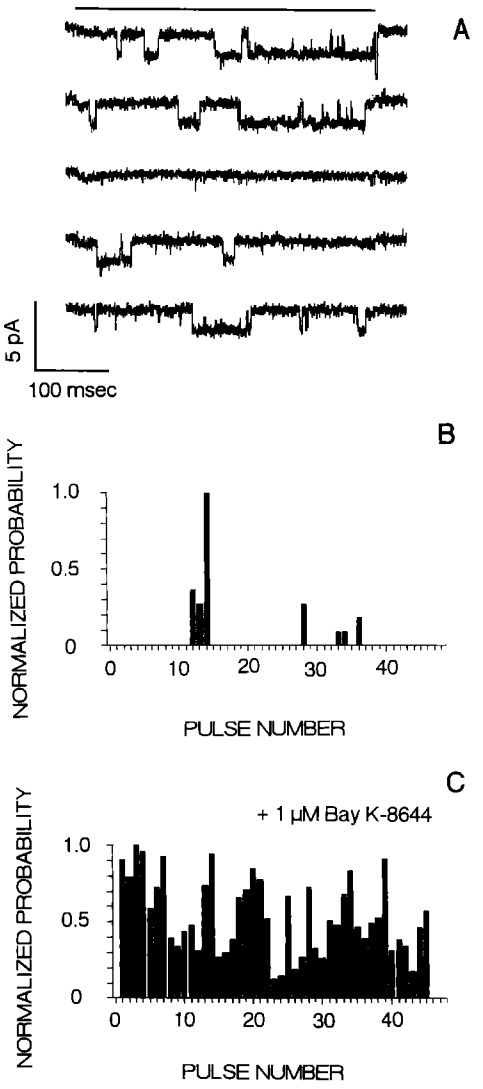

Fig. 5 A–C. Effect of BAY K-8644 on the calcium channel currents. Successive current traces of DHP-sensitive calcium channels, recorded with a patch pipette containing 1 μ*M* BAY K-8644 (**A**). Observe the longer open channel events, and the shorter close time events. Comparison of the time course of the open channel probability in successive test pulse estimated from patches made with a pipette containing the standard solution (**B**) and a pipette with 1 μ*M* BAY K-8644 (**C**). The holding potential was −40 mV and the test pulse was to −10 mV. The probability was normalized to the maximum in each experiment

us to record single channel events more than 25 ms after the end of the potential test pulse. No significant changes in the single channel conductance were found on addition of BAY K-8644.

DHP-insensitive channels

The second type of calcium channel found in rat cerebellar granule cells had a lower unitary conductance and was activated at lower V_m than the channels described so far. Channels of this second type displayed inactivation (Fig. 6 A). They were present in cells of more than 4 DIC, but were virtually absent in younger cells. In several experiments this type of channel was found together with the DHP-sensitive channels. In such cases it was relatively easy to distinguish between them by their different conductance, voltage dependence and, specially, their inactivation properties (see below). When BAY K-8644 was added to the recording patch pipette no changes similar to those described for the DHP-sensitive channels were found. Hence, the second type of channels was defined as DHP-insensitive calcium channels.

Amplitude histograms of single channel records of patches containing DHP-insensitive channels revealed the

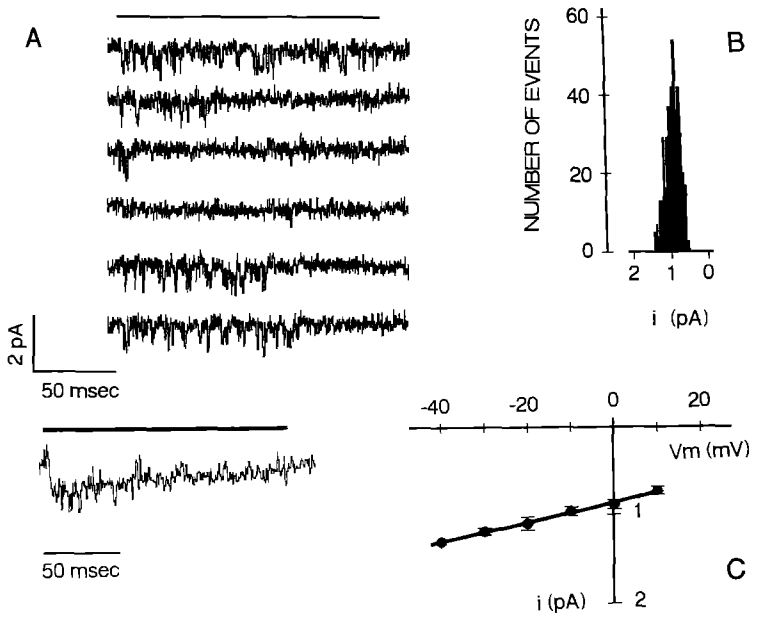

Fig. 6 A–C. DHP-insensitive calcium channels. **A** Single calcium channels records activated by a test pulse to 0 mV, from a holding potential of −80 mV. An average of 62 single channel records, reconstructing the macroscopic current is shown in the inset. The duration of the test pulse is indicated by the solid bar. **B** Amplitude histogram constructed from 2564 single channel events evoked by a −10 mV test pulse. **C** The *i-V* relation of the single channel currents reveals a slope conductance of 11.1 ± 1 pS, and an apparent reversal potential of 79.5 mV. Each point represents the mean current value obtained over, at least, 100 events. Cells of 6 DIC

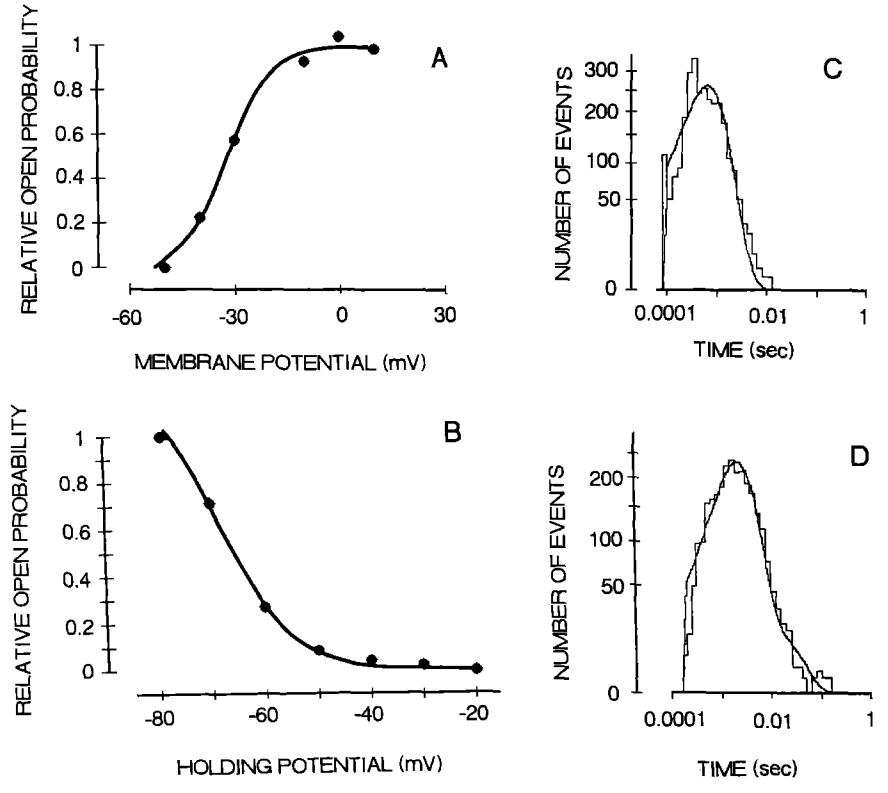

Fig. 7 A–D. Open probability and open and shut time of DHP-insensitive calcium channels. **A** The activation curve was normalized to the maximum overall open probability P_{max}. The smooth line was obtained by fitting the data as described in the legend of Fig. 4. The V_{half} was −31.8 mV and $a = 7.2$ mV. **B** Steady-state inactivation curve constructing fitting the normalized probability P_0 evaluated for a fixed test pulse at a V_m of −10 mV against the holding potential. The half inactivation was obtained at a potential of −65 mV and a *e*-fold decrease of P_0 is obtained by a change of 6 mV of the holding potential. Each point of the activation and inactivation curves represents a value estimated from at least 45 successive records. Open time distribution (**C**) and shut time distribution (**D**) evaluated from records obtained at a test potential of −80 mV. The open time constant was 0.74 ms, and the shut time constants were 2.9 ms and 85 ms 2684 and 1122 events were used to analyze the open and shut times respectively. The histograms were fitted according to Sigworth and Sine (1987)

presence of a homogeneous population, with no subconductance state (Fig. 6 B). A slope conductance was evaluated from the i-V curves obtained from 12 different experiments, yielding a value of 9.8 ± 1.5 pS (Fig. 6 C). The single channel conductance was invariant at test potentials between −50 and +30 mV. The apparent E_r was very similar to that estimated for the DHP-sensitive channels, with values of 70 to 80 mV.

Single channel currents evoked by test pulses at V_m of −40 mV were observed. The V_{half} of the DHP-insensitive channels was reached at −30 to −15 mV (Fig. 7 A). The steepness of the activation curve was similar to that of the DHP-sensitive channels, yielding an *e*-fold increment of probability of a potential change of about 7 mV. The DHP-insensitive channels inactivate during the test pulse, in contrast to the observation on DHP-sensitive channels. The inactivation is reflected in the clustering of single channel events at the beginning of the test pulse, and the low probability of finding them at the end of the pulse (see Fig. 6 A). The inactivation time constant, τ_i, was evaluated from the average of successive single channel records, as shown in the inset of Fig. 6 A. The value of τ_i

was a function of the test pulse potential, as well as of the holding potential. For a holding potential of −80 mV, the τ_i was estimated to be longer than 100 ms for test pulses at V_m lower than −20 mV and was faster than 45 ms when the V_m in the test pulses was increased above +10 mV. The overall open probability was strongly dependent on the holding potential. The steady-state voltage dependence of inactivation was estimated by plotting the overall open probability, evoked by a fixed test pulse at a V_m of −10 mV, against the holding potential between −80 mV and −20 mV (Fig. 7 B). The midpoint for inactivation was obtained with a holding potential between −65 and −55 mV.

The open time distribution of the DHP-insensitive channels was well fitted with a single exponential function, with a time constant of 0.7 to 0.8 ms (Fig. 7 C). The shut channel time histogram displayed a double exponential distribution, with a fast component of 1 ms to 4 ms, and a slow time component between 50 ms and 100 ms (Fig. 7 D). Both, mean open time and mean shut time were very slightly voltage dependent. As in the case of the DHP-sensitive channels, the DHP-insensitive channels were not always evoked by a test pulse. It follows that the voltage dependence of the overall open probability depends mainly on the probability of finding a pre-activated channel. When the channel is activated during a voltage test pulse, the open channel probability is little voltage dependent. This observation was confirmed with the fact that the number of pulses where events are present increased when the test potential was increased. These results suggest that the DHP-insensitive channels would have more than one closed state, and the transition between the open state and one of these shut states should be voltage independent.

Discussion

The macroscopic and microscopic properties of voltage dependent calcium channel currents carried by Ba^{2+} in rat cerebellar granule cells are described here. The density of calcium channels in these cells seems to be quite low, as compared with other channel classes studied in our laboratory using the same preparation, as potassium channels, sodium channels or excitatory amino acid activated channels (Galdzicki et al. 1990; Lin and Moran 1990; Sciancalepore et al. 1989, 1990; Lin et al. 1991). This is reflected in the relatively low probability for obtaining a patch with active channels, and on the small total current recorded. In the case of DHP-sensitive channels, a figure of 120 to 250 channels/cell could be estimated from the whole-cell current. These values would represent a density of 0.5 to 1.2 channels/μm^2, considering a cell input capacitance of 2.2 ± 0.4 pF/μm^2, and the empirical relation of 100 μm^2/pF (Lin and Moran 1990; Lin et al. 1991). This low channel density is in line with results reported in other cell preparations, as GH_3 pituitary tumor cells where a value of 1 channel/μm^2 was reported (Tsien 1983). Furthermore, the calcium channels (of either type described here) seem to be localized in clusters. This was deduced from the fact that multiple channel patches or silent patches were obtained when we tried to improve the active patch rate by increasing the patch pipette diameter.

The time course of the expression of calcium channels in the cerebellar granule cells in culture was followed by single channel recording. This task could not be completely accomplished by whole cell recording techniques, because of the technical problem created by the development of neuritic processes in the cells. Furthermore, owing to the small size of the cells (7 to 10 μm diameter), the dialysis of the intracellular content was quite fast (Pusch and Neher 1988), probably resulting in a wash out of cell components necessary for the normal channel functioning, as reflected by the rapid run down of the whole-cell currents. Probably an improvement of the whole-cell recording conditions can be achieved by adding nucleotides in the intracellular solution (Wojcik et al. 1990). Nevertheless, the information obtained from the whole cell experiment performed during the first four days in culture are still useful to confirm the findings of the single channel experiments.

The DHP-sensitive channels seem to be the only type of calcium channel present during the first 4 DIC. The single-channel records of this type of channel fit quite well with the whole-cell experiments: they show a voltage dependence, BAY K-8644 sensitivity and non-inactivating currents. Furthermore, the reconstruction of the macroscopic currents by averaging the single-channel records resembles the whole-cell results. A difference on the half activation potential estimated from the macroscopic currents and from the single channel currents was observed. This difference may be due to the different screening of the membrane surface by the different Ba^{2+} concentration used in these two experimental conditions. The properties of this type of channel are similar to those described as L-type calcium channels (Tsien 1983; Tsien et al. 1988; Fox et al. 1987) or high voltage activated calcium channels (Carbone and Lux 1987). When the cells become older, the DHP-sensitive channels become more sparse. DHP-sensitive channels can be more easily recorded in cells of more than 4 DIC by using the agonist BAY K-8644. This fact suggests their presence in the membrane of older cells, although probably in a sort of latent state, which can be modified to an active one by the DHP agonist.

In contrast, the DHP-insensitive calcium channels were found in cells of more than 4 DIC and were absent in younger cells. This type of channel has a lower activation threshold, smaller conductance and voltage dependent inactivating properties. Although the conductance and activation properties of these channels may be comparable with those of low voltage activated or T-type calcium channels (Carbone and Lux 1987; Droogmans and Nilius 1989), the kinetic and steady-state inactivation properties are different to that described for this type of channel, being more similar to N-type calcium channels (Carbone and Lux 1984; Nowycky et al. 1985; Fox et al. 1987, Bean 1989 a). This reflects the difficulties of classifying the different types of calcium channels in neuronal cells, as has already been advanced by several authors (see Tsien et al. 1988; Bean 1989 a, b; Swandulla et al. 1991). The density of these channels, roughly estimated from the

proportion of active patches, was higher compared to the DHP-sensitive channels in the older cells (in absence of the BAY K-8644). The number of successfully active patches in the first four DIC (containing DHP-sensitive channels) was higher than the number of good patches from the older cells (containing DHP-insensitive channels).

Finally, we can mention that the differential expression of calcium channels correlated with the time in culture could have a physiological meaning. It is interesting to observe that the appearance of DHP-insensitive channels and the loss of the DHP-sensitive channels in the granule cells between the third and the fourth DIC is coincident with the formation of synapses between the cells (see Hockberger et al. 1984). This fact suggests the possibility that the presence of different types of calcium channels may be related to specific voltage dependent calcium permeability requirements during development of the cells in vitro. Further studies of cerebellar slices on different stages of development are required to extend this conclusion to more physiological conditions.

Acknowledgements. M. S. was partially supported by the Fidia Research foundation.

Note. While this manuscript was under review Mori and co-workers (1991) reported a calcium channel expressed from rat brain cDNA, which is insentisive to DHPs and omega-conotoxin, and shares general structural features with DHP-sensitive calcium channels. This new type of channel, that is mainly expressed on cerebellar Purkinje and granule cells, could be related to the DHP-insensitive channel described here.

References

Armstrong CM, Bezanilla F (1977) Inactivation of the sodium channels. II Gating current experiments. J Gen Physiol 70:567–590

Bean BP (1989 a) Classes of calcium channels in vertebrate cells. Ann Rev Physiol 51:367–384

Bean BP (1989 b) More than one Ca^{2+} channel? TINS 12:128–130

Carbone E, Lux HD (1984) Low voltage-activated calcium conductance in embrionic chick sensory neurons. Biophys J 46:413–418

Carbone E, Lux HD (1987) Single low-voltage-activated calcium channels in chick and rat sensory neurones. J Physiol 386:571–601

Connor JA, Tseng H-Y, Hockberger PE (1987) Depolarization- and transmitter-induced changes in intracellular Ca^{2+} of rat cerebellar granule cells in explant cultures. J Neurosci 7:1384–1399

Cull-Candy SG, Howe JR, Ogden DC (1988) Noise and single channels activated by excitatory amino acids in rat cerebellar granule neurons. J Physiol 400:189–222

Cull-Candy SG, Marshal CG, Odgen D (1989) Voltage-activated membrane currents in rat cerebellar granule neurones. J Physiol 414:179–199

Droogmans G, Nilius B (1989) Kinetic properties of the cardiac T-type calcium channel in the guinea pig. J Physiol 414:627–650

Fox AP, Nowycky MC, Tsien RW (1987) Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurons. J Physiol 394:149–172

Galdzicki Z, Lin F, Moran O, Novelli A, Puia G, Sciancalepore M (1990) Development of voltage activated potassium currents in cerebellar granule cells in culture. Int J Neurosci 56:193–200

Hamill O, Marty A, Neher E, Sakmann B, Sigworth F (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100

Hockberger PE, Tseng H-Y, Connor JA (1987) Immunochemical and electrophysiological differentiation of rat cerebellar granule cells explant cultures. J Neurosci 7:1370–1383

Hodgkin AL, Huxley AF (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. J Physiol 117:500–544

Jalonen T, Johansson S, Holopainen I, Oja AA, Arhem P (1990) Single-channel and whole-cell currents in rat cerebellar granule cells. Brain Res 535:33–38

Kokubun S, Reuter H (1984) Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells. Proc Natl Acad Sci 81:4824–4827

Kingsbury A, Balazs R (1987) Effect of calcium agonists and antagonist on cerebellar granule cells. Eur J Pharmacol 140:275–283

Levi G, Aloisi M, Ciotti M, Gallo V (1984) Autoradiographic localization and depolarization-induced release of acidic amino acids in differentiating cerebellar granule cells cultures. Brain Res 290:77–86

Lin F, Moran O (1990) Voltage dependent sodium currents in cultured cerebellar granules. Biosci Rep 10:445–453

Lin F, Conti F, Moran O (1991) Competitive blockage of the sodium channel by intracellular magnesium ions in central mammalian neurones. Eur Biophys J 19:109–118

Marchetti C, Carignani C, Robello M (1990) A voltage dependent calcium current in cultured rat cerebellar granule cells. Proceedings of the IX Congresso della Società Italiana di Biofisica, Pura ed Applicata, p 185

Mori Y, Friedrich T, Kim MS, Mikami A, Nakai J, Ruth P, Bosse E, Hofmann F, Flockerzi V, Furiuchi T, Mikoshiba K, Imoto K, Tanabe T, Numa S (1991) Primary structure and functional expression from complementary DNA of a brain calcium channel. Nature 350:398–401

Nowycky MC, Fox AP, Tsien RW (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity. Nature 361:440–443

Pusch M, Neher E (1988) Rates of diffusional exchange between small cells and a measuring patch pipette. Pflügers Arch 411:204–211

Reuter H, Porzing H, Kokubun S, Prod'hom B (1985) 1,4-dihydropyridines as tools in the study of Ca^{2+} channels. TINS 8:396–400

Robello M, Carignani C, Marchetti C (1989) A transient voltage-dependent outward current in cultured cerebellar granules. Biosci Rep 9:451–457

Sanguinetti MC, Kaas RS (1986) Volgate-dependent modulation of Ca channel current in heart cells by BAY K-8644. J Gen Physiol 88:369–392

Sciancalepore M, Forti L, Moran O (1989) Changes of N-methyl-d-aspartate-activated channels of cerebellar granule cells with days in culture. Biochem Biophys Res Commun 165:481–487

Sciancalepore M, Galdzicki Z, Zheng X, Moran O (1990) Kainate activated single channel currents as revealed by domoic acid. Eur Biophys J 19:63–68

Sigworth FJ, Sine SM (1987) Data transformations for improved display and fitting of single-channel dwell time histograms. Biophys J 52:1047–1054

Slesinger PA, Lansman JB (1990) Just one type of Ca^{2+} channel underlies the macroscopic Ca^{2+} current in cerebellar granule cells. Biophys J 57:524a

Swandulla D, Carbone E, Lux HD (1991) Do calcium channel classification account for neuronal calcium channel activity? TINS 14:46–51

Tsien RW (1983) Calcium channels in excitable cell membranes. Ann Rev Physiol 45:341–358

Tsien RW, Lipscombe D, Madison DV, Bley KR, Fox AP (1988) Multiple types of calcium channels and their selective modulation. Trends Neurosci 11:431–443

Wojcik WJ, Travagli RA, Costa E, Bertolino M (1990) Baclophen inhibits with high affinity an L-type – like voltage-dependent calcium channel in cerebellar granule cell cultures. Neuropharmacology 29:969–972