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Maintained L-Type Ca^{2+} Channel Activity in Excised Patches of PTX-Treated Granule Cells of the Cerebellum

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Activity of high-threshold voltage activated neuronal Ca^{2+} channels, including dihydropyridine-sensitive (L-type) channels, rapidly disappears during cell dialysis in whole-cell recording conditions or after excision of a patch. To date, this phenomenom has been mainly related to phosphatase or protease activity. On the other hand, it has been suggested that Ca^{2+} channels may be regulated by G-proteins. Therefore, disruption of this regulatory pathway may also be involved directly or indirectly in the rundown process. Here, we show that treatment of cultured cerebellar granule cells with pertussis toxin (PTX) increases to 70% the probability for excising patches that display L-type Ca^{2+} channels activity in the inside-out recording configuration. Quantitative study indicates that, except a half decrease in the open probability, most features of the channel activity are retained after patch excision with minor modifications. The characteristics of the channel activity did not change with time during at least the first 9 min of the inside-out configuration. In addition, comparison of unitary currents recorded in the cell-attached configuration on treated and nontreated cells demonstrates that the PTX treatment slows the activation kinetics of the current and increases the duration of channel openings evoked at −20 mV but not at 0 mV depolarizing potential. These data suggest that L-type Ca^{2+} channel activity are under a tonic regulation of a PTX-sensitive mechanism, which is implied in the rundown process.

[Key words: L-type Ca^{2+} channel, rundown, inside-out patch, PTX treatment, granule cells, cerebellum]

Ca^{2+} channels are targets for multiple regulatory mechanisms, including phosphorylation and/or direct interactions with G-proteins. Disruption of these regulatory pathways after patch excision may explain the loss of activity (rundown process) generally observed with L-type voltage-dependent Ca^{2+} channels. In particular, phosphorylation/dephosphorylation of Ca^{2+} currents has been studied in cardiac and neuroendocrine cells where L-type Ca^{2+} channel activity disappears upon passing to an excised patch, and can be restored by supplying simultaneously ATP and protein kinase (Armstrong and Eckert, 1987; Ono and Fozzard, 1992). However, in neurons, experiments to directly phosphorylate L-type Ca^{2+} channels "out of the cell" have met with variable success. A whole sequence of events is probably required to maintain channel activity, which implies other reactions occur in addition to the phosphorylation step. G-proteins are good candidates to be involved in this sequence of events. Indeed, by homology to what is known for inwardly rectifying K^+ channels, a direct association of G-proteins with the Ca^{2+} channel has been proposed. Although its physiological relevance in intact cells remains controversial (Hartzell and Fischmeister, 1992), such a direct interaction has been suggested in muscle or heart cell L-type Ca^{2+}channels. The addition of activated Gs results in either a prolonged survival of these channel types in excised patches or an increase in their opening probability when incorporated in artificial lipid bilayers (see review in McDonald et al., 1994). In neurons, numerous studies of neurotransmitter-induced inhibition of N-type Ca^{2+} channels (see review in Tsien et al., 1988; Hille, 1994) have also suggested direct modulation of Ca^{2+} currents by G-proteins (Dolphin, 1991; Boland and Bean, 1994) with the involvment of either PTX-sensitive or -insensitive mechanisms (Hille, 1994). Concerning L-type neuronal currents, although in sympathetic neurons the membrane delimited pathway that regulates N-type channels does not affect L-type channels (Mathie et al., 1992), a direct regulation of L-current by G-protein has also been proposed (Dolphin and Scott, 1989), especially in cerebellar granule cells where a clear PTX-sensitive G-protein modulation of L-type channels has been evidenced (Haws et al., 1993; Chavis et al., 1994).

Therefore, in cases where rundown cannot be accounted for by phosphorylation/dephosphorylation, it can be envisaged that a disruption induced by patch excision of a direct G-protein regulation of Ca^{2+} channel activity may also be involved in the rundown process. However, this hypothesis requires that G-proteins intimately participate in normal channel activity, which implies its tonic regulation by G-proteins. Evidence for this interaction has been shown in a series of recent reports that discribed a PTX-induced upregulation of L-type channels in neurons, including cerebellar granule cells (Haws et al., 1993; Netzer et al.,1994; Chavis et al., 1995). In this line of thought, we looked for a PTX-sensitive mechanism in the survival of L-type channel activity in excised patches obtained from granule cells of the cerebellum. We show that PTX treatment of the cells dramatically increases the probability of recording a sustained Ca^{2+} channel activity over tens of minutes after patch excision.

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Materials and Methods

Cell culture. Rat cerebellar granule cells were mechanically dissociated from 5-d-old rats and plated in 35 mm culture dish (Corning, New York) coated with 500 μg/ml poly-L-ornithine (Sigma, St. Louis, MO). Cells were cultured in DMEM (GIBCO, Paisley, UK) supplemented by 25 mM KCl, 10% heat-inactivated horse serum (GIBCO), 50 μM insulin (Novo, DK), and 0.05 mg/ml gentamycin (Seromed, Berlin, Germany). Experiments were performed on granule cells grown for 5 to 8 d *in vitro* and morphologically identified by their small size, oval or round cell body and their bipolar neurites. Treated cells were incubated overnight with 1 μg/ml PTX (List Biological Laboratories, Campbell) before experiments.

Recording solutions. Bath solution contained in mM: K-gluconate 140, EGTA 10, $MgCl_2$ 1 and HEPES 10, adjusted to pH 7.4 with KOH. This solution was used to zero the membrane potential in cell-attached configuration. Dihydropyridines, Bay K 8644 (Research Biochemicals, Natick, MA) and nifedipine (Sigma), were dissolved in DMSO at a stock concentration of 10^{-2} M. When specified, nifedipine was directly added to the bath solution at 10^{-6} M final concentration. The pipette-filling solution contained in mM: $BaCl_2$ 105, KCl 7, HEPES 10, adjusted to pH 7.4 with Tris. In order to facilitate recordings of L-type Ca^{2+} channels 10^{-6} M Bay K 8644 was added to the pipette solution. Osmolarity of all solutions was between 290 and 300 mosmol·l^{-1}. All experiments were performed at room temperature.

Patch-clamp recordings. Single-channel recordings were made using cell-attached and inside-out configurations of the patch-clamp technique. Glass pipettes (TW 150F-6, WPI, Sarasota, FL) were coated with Sylgard elastomere (RTV 141, Rhone Poulenc, St. Fons, France), and fire polished to a final tip diameter of 1.5–3 μm (Pipette resistance: 2–4 MΩ). Potential differences between the recording pipette and the bath ground were set to zero prior to seal formation. Currents were recorded with an Axopatch 200 A amplifier (Axon Instruments, Foster City), low-pass filtered at 5 kHz cutoff frequency (−3 dB, four-pole Bessel filter) and sampled at 10 kHz using Clampex and Fetchex pClamp 6 software of Axon Instruments. Channel activity was evoked either by sustained depolarization of the patch to 0 mV or by step depolarizations from a holding potential of −60 mV, delivered every 5 sec. Simultaneous occurrence of channel openings suggested that patches typically contained two channels. Only a small minority of patches with apparent single-channel activity were recorded, and patches with obviously more than four channels were not considered.

Data analysis. Data were numerically low-pass filtered at 1 kHz cutoff frequency (−3 dB) with a digital Gaussian filter and analyzed using pClamp 6 (Axon Instruments) and in-house programs. When analyzing recordings obtained by depolarizing pulses, linear leak and capacitative currents were digitally subtracted using a template generated by fitting smooth functions to a sweep without channel activity. Channel openings and closures were detected when current crossed a discriminator level at 50% of the elementary current. As data were filtered at 1 kHz, only events of more than 400 μsec duration were considered. Amplitude of the currents was determined by fitting Gaussian distributions to amplitude histograms (bin width: 0.05 pA) constructed either from all digitalized points (in case of infrequent channel openings) or from idealized events determined by the 50% threshold method. Open probability (N.Po, number of channels in the patch × open probability) was calculated by adding the time spent open at each level and dividing by the duration of the analyzed period. In open-time analysis, patches containing more than one channel were not taken into consideration if simultaneous openings of the channels represented more than 15% of the total number of openings. Dwell open-time histograms were constructed using bins of 400 μsec width and neglecting the first one (0–400 μsec), distributions were fitted by sum of decaying exponentials. Histograms were fitted using a Levenberg-Marquardt least-square method. In figures, traces presenting channel activity have been low-pass filtered to 500 Hz for clarity sake, except when specified. Data are given as mean ± SD.

Results

Ca^{2+} channel activity was recorded in presence of Bay K 8644 (10^{-6} M) using 105 mM Ba^{2+} as the divalent ion charge carrier. Under these conditions, either sustained depolarizations to 0 mV or step depolarizations from −60 mV holding potential evoked-channel activity with L-type Ca^{2+} channel characteristics. In the cell-attached configuration, these channels were inhibited by the dihydropyridine (DHP) antagonist nifedipine (10^{-6} M, $n = 9$); their conductance was 28.9 ± 2.5 pS (mean ± SD, $n = 7$), and their activation threshold was around −40 mV ($n = 8$, Fig. 1). In nearly every patch, simultaneous openings in response to depolarization indicated that 2 to 4 L-type Ca^{2+} channels were present.

However, a few recordings displayed a clearly different type of channel activity, which was composed of very short openings evoked at depolarizing potentials higher than −20 mV. Such recordings were easily identified and systematically discarded from the analysis. In this way, we limited our study to the modifications of L-type Ca^{2+} channel activity induced by an over night PTX treatment of the cells. In particular, we were interested in the effect of this treatment on the rundown of channel activity that occurs after patch excision.

L-type Ca^{2+} channels from PTX-treated cells remain active after patch excision

Thirteen inside-out patches containing L-type Ca^{2+} channels were excised from PTX-treated cells in a normal bath medium (see experimental procedure). In eight cases, the open probability of the channels remained elevated after excision until patch rupture 21 ± 11 min later (range: 6 to 39 min; Fig. 2*A,B*). In contrast, only 2 out of the 15 patches excised from nontreated cells had channel activity up to patch rupture, 8 and 17 min after excision, respectively (Fig. 2*C*).

For the remaining PTX-treated and nontreated cells, the Ca^{2+} channel activity quickly disappeared in the inside-out configuration. No difference was observed between the activity survival time in the case of PTX-treated cells, 50 ± 30 sec (range: 6 to 102 sec, $n = 5$) and nontreated cells, 57 ± 58 sec (range: 7 to 212 sec, $n = 13$). Therefore, the significantly different proportion of patches in which no rundown did occur (2 × 2 contingency table, Fisher Exact Test, $p = 0.016$) indicates that PTX treatment of the cells dramatically increases the probability of excising patches with Ca^{2+} channels remaining active in the inside-out configuration. Patches in which channel open probability remained elevated after excision will be referred to below as active excised patches.

Characterization of L-type Ca^{2+} channel activity in inside-out configuration

Ca^{2+} channels recorded in the inside-out configuration retained their characteristic pharmacology of L-type channels. When nifedipine (10^{-6} M) was applied after several minutes of recording (11 and 8 min, respectively) on two active excised patches from PTX-treated cells, the DHP antagonist totally blocked the channel activity in each case. The same inhibition was observed on an active excised patch from a nontreated cell as well (Fig. 2*C*). Furthermore, an examination of the unitary channel conductance confirmed that these channels belong to the L-type Ca^{2+} current family. The value determined in active excised patches from PTX-treated cells was 28.7 ± 3.2 pS ($n = 5$) and was similar to the conductance characterized in the cell-attached configuration.

In addition, Figures 3 and 4 show that major features of L-type Ca^{2+} channel activity recorded from cerebellar granule cells are conserved in the inside-out configuration. These characteristics are: long openings in presence of Bay K 8644, openings of the channels on repolarization (Fig. 3), and the current activation kinetics with depolarizing potential (Fig. 4).

The quantitative study of the changes in channel activity with time after patch excision was performed on six out of the eight

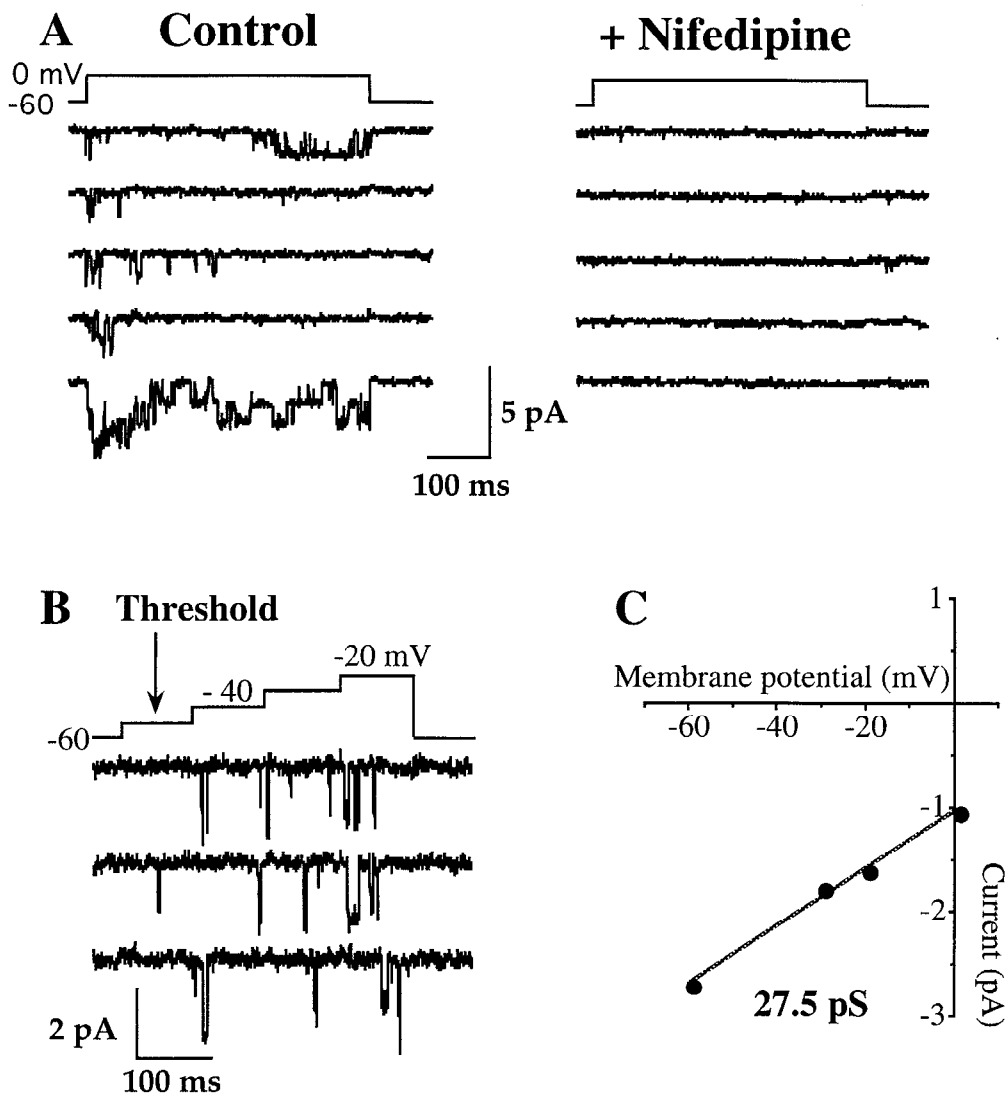

Figure 1. Typical examples of L-type Ca^{2+} channel activity recorded in cell-attached configuration (a single patch). *A,* Step depolarizations to 0 mV from a holding potential of −60 mV elicited activation of at least four L-type Ca^{2+} channels. The 10 successive traces show typical activity (*left*) and the blocking effect of 10^{-6} M nifedipine (*right*). *B,* Activation threshold of the channels was estimated using gradual depolarizations from −60 mV to −20 mV with 10 mV increments. Channel openings were already observed at −50, −40 mV which is the typical threshold of L-type Ca^{2+} channels recorded in presence of 10^{-6} M Bay K 8644. *C,* Current–voltage relationship was determined in the same patch. The calculated conductance is characteristic of L-type Ca^{2+} channels recorded using high concentration of Ba^{2+} as divalent ion charge carrier.

active excised patches from PTX-treated cells. Patches were maintained at 0 mV holding potential for 2–3 min in cell-attached configuration and for 9 min in the inside-out configuration. In each case, 3 min recordings provided enough data to accurately characterize channel activity. Therefore, evolution of the activity after patch excision was evaluated by comparing mean channel characteristics over successive 3 min periods. Analysis indicates that channel open probability, elementary current amplitude, and open dwell-time characteristics remained constant after patch excision (one-way ANOVA with repeated measurements, Fig. 5).

On the other hand, comparison of channel characteristics before and after patch excision shows that open probability was significantly reduced on passing to inside-out configuration. Although channels remained highly active after patch excision, their open probability over the 9 min of inside-out recording was only 42 ± 14% (n = 6, two-tailed paired t test, p = 0.001; Fig. 5*A*) of the probability observed in the previous 2–3 min of recording in cell-attached configuration.

In addition, open dwell times were also slightly but significantly different before and after patch excision. An increase was observed in the time constants of the two exponentials best fitting the open time distributions: from 1.70 ± 0.36 msec to 2.36 ± 0.79 msec and 6.79 ± 1.57 msec to 11.04 ± 2.42 msec when going from cell-attached to inside-out configuration respectively (n = 6; two-tailed paired t test, p = 0.03 and p = 0.003; Fig. 5*C,D*). The slower time constants indicate that both short and long openings of the channels were longer after patch excision. However, no significant difference was observed between the mean open time in cell-attached, 4.24 ± 0.88 msec, and inside-out configurations, 4.60 ± 0.36 msec (n = 6; Fig. 5*B*). A significantly higher proportion of short openings occurs after patch excision, which masks any effect on the mean open time of the longer duration openings. The modification of the proportion of short openings is indicated by the greater weight attached to the fast exponential fitting the open time distributions, 0.78 ± 0.11 as estimated in inside-out configuration, versus 0.60 ± 0.15 in cell-attached configuration (n = 6; two-tailed paired t test, p = 0.008; Fig. 5*D*).

Finally, no modification of the elementary current at 0 mV was found between the activity recorded in the cell-attached, −1.16 ± 0.09 pA, and the inside-out configurations, −1.19 ± 0.06 pA (n = 6; two-tailed paired t test).

In conclusion, many features of L-type Ca^{2+} channel activity from PTX-treated cells are retained after patch excision with minor modifications. Activity characteristics remain stable for at least 9 min. However, the open probability of the channels is reduced by approximately 60%.

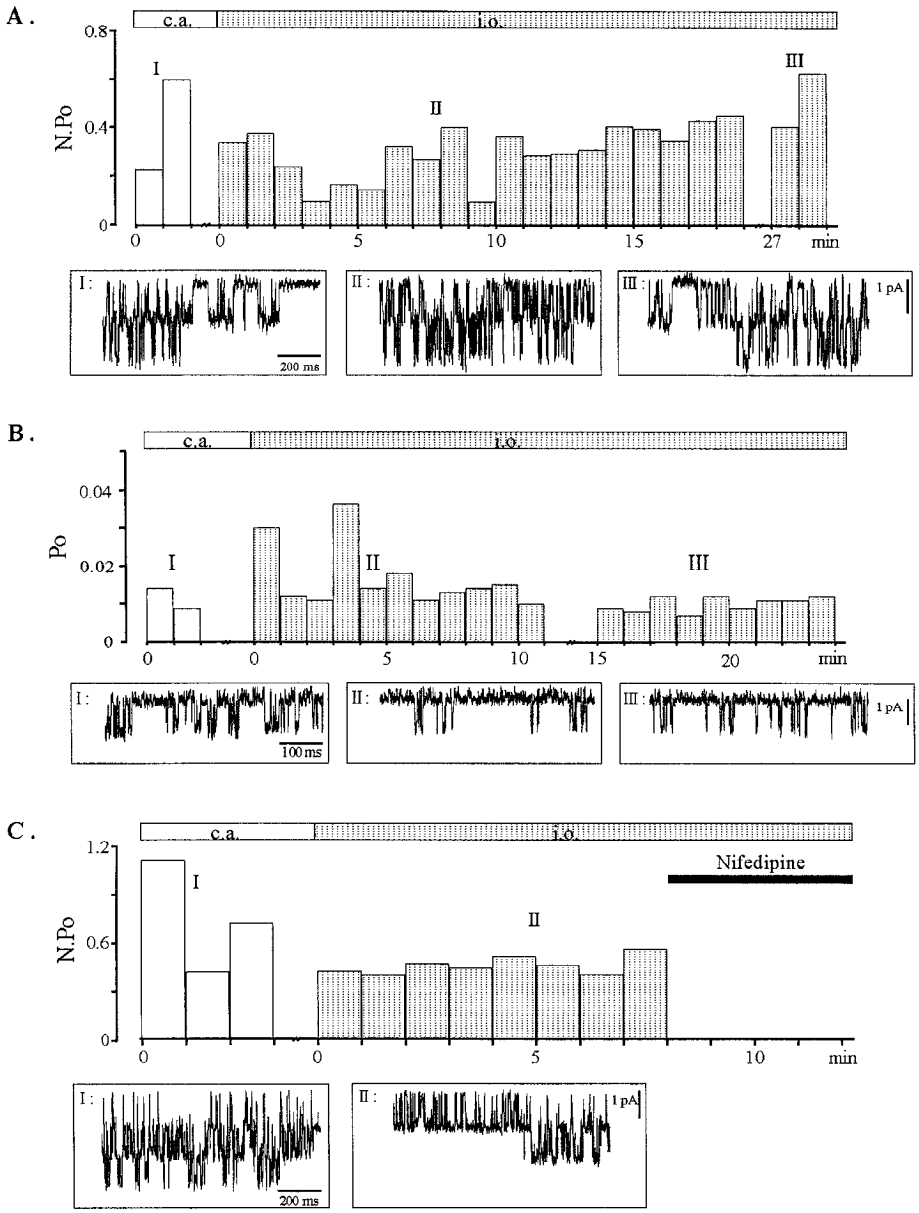

Figure 2. Open probability (*N Po*) of L-type Ca^{2+} channels recorded in cell-attached (*c.a.*) and inside-out (*i.o.*) configurations. N Po was calculated over periods of 1 min for the three patches (*A*, *B*, and *C*) maintained at 0 mV. *Insets* present examples of the channel activity recorded at different times as indicated by the numbers. *A* and *B*, Evolution of N Po of Ca^{2+} channels recorded on PTX-treated cells. Note that N Po remained elevated after patch excision until patch rupture. In *A* is typified the activity recorded in these conditions. By contrast, in *B* is presented the activity of the unique patch in which no simultaneous openings were observed, indicating that it might contain an isolated Ca^{2+} channel. Note that Po observed in this patch was also 10 times smaller. *C*, Evolution of N Po in a case where no rundown occurred in inside-out configuration although no PTX-treatment was applied. Only two such cases have been observed. Note that channel activity totally disappeared after application of 10^{-6} M of nifedipine as expected for L-type Ca^{2+} channels.

Comparison of L-type Ca^{2+} channel activity from PTX-treated and nontreated cells recorded in cell-attached configuration

In order to identify the underlying mechanism modified by the PTX treatment, characteristics of Ca^{2+} channel activity recorded in the cell-attached configuration on PTX-treated and nontreated cells were compared. We found that PTX treatment had no influence on the channel conductance and the elementary current at 0 mV. Respective values were 27.65 ± 2.27 pS (n = 10) and −1.09 ± 0.15 pA (n = 26) for the PTX-treated cells and 28.9 ± 2.5 pS (n = 7) and −1.06 ± 0.09 pA (n = 23) for the nontreated cells.

L-type Ca^{2+} channel activities from PTX-treated and nontreated cells are similar at 0 mV holding potential

To compare further the two cell populations, patches recorded for 2–3 min at 0 mV in cell-attached configuration were analyzed. Open-time characteristics were found to be similar for the channels of the two populations. Mean open time was 4.23 ± 1.45 msec (n = 15) in PTX-treated cells and 4.56 ± 1.83 msec (n = 12) in nontreated cells. In addition, further analysis of the open-time distributions did not reveal any difference. The time constants of the two exponentials best fitting the distributions were 1.70 ± 0.64 msec (n = 15) and 6.25 ± 2.32 msec (n = 14), and 1.96 ± 0.61 msec (n = 12) and 7.37 ± 2.04 msec (n = 9) for the PTX-treated and nontreated cells, respectively. The proportion of short openings were also equal: the weight attached to the fast exponential fitting the open-time distributions being 0.59 ± 0.19 (n = 15) and 0.63 ± 0.31 (n = 12) for the PTX-treated and nontreated cells, respectively. Finally, although open probability greatly differed from patch to patch, no difference was observed between mean probability for PTX-treated cells (0.176 ± 0.171, n = 20) and nontreated cells (0.165 ± 0.159, n = 16).

Differences between L-type Ca^{2+} channel activity evoked by step depolarization to −20 mV on PTX-treated and nontreated cells

We compared the activation kinetics of Ca^{2+} channels in PTX-treated and nontreated cells by adding successive individual cur-

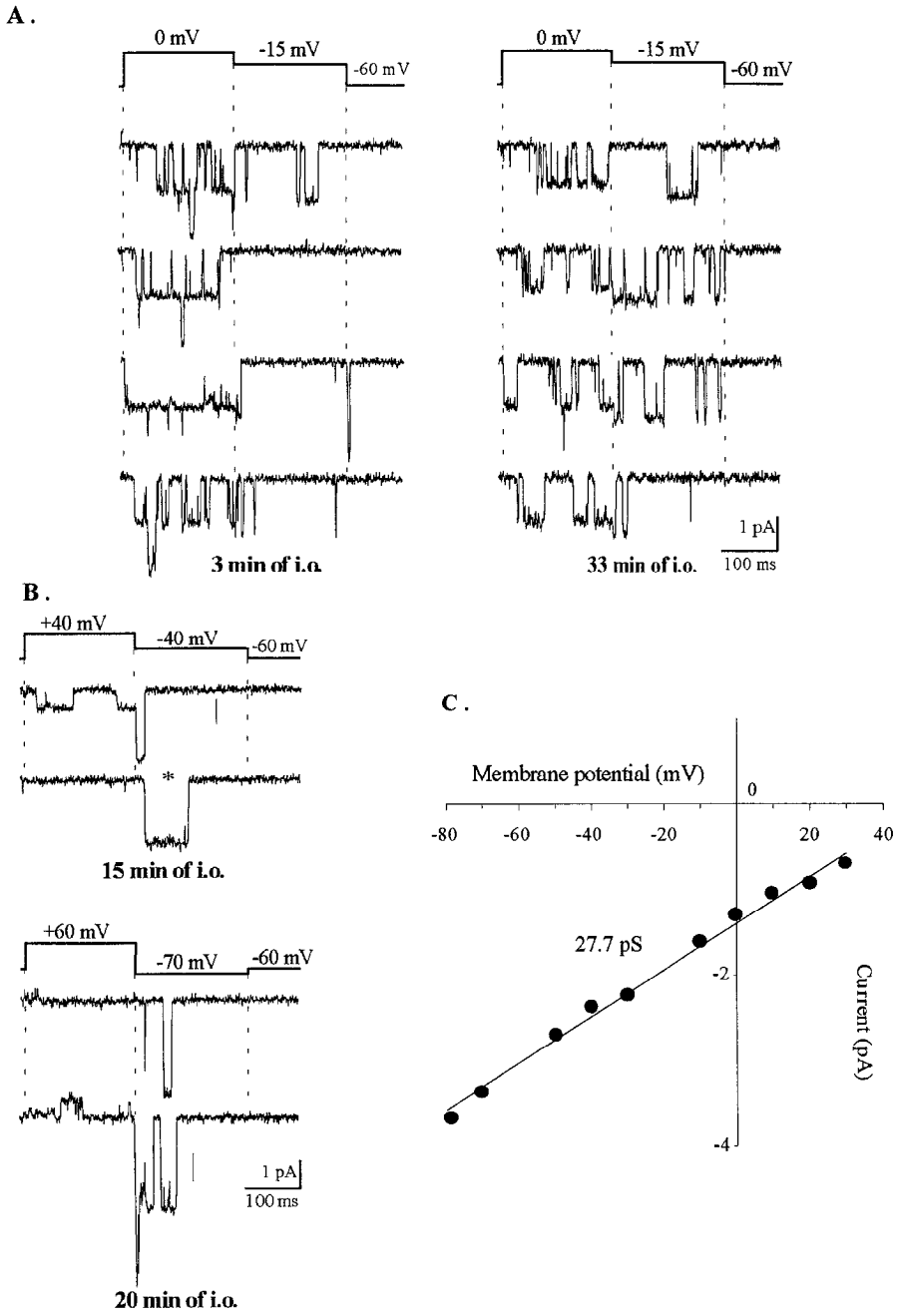

Figure 3. Representative traces of L-type Ca^{2+} channel activity recorded in inside-out (*i.o.*) configuration on PTX-treated cells (a single patch). *A*, After patch excision, Ca^{2+} channel activity was elicited up to patch rupture (39 min in the present case) by depolarizing protocol to 0 and −15 mV from a holding potential of −60 mV. Note the long openings that characterize recordings of L-type channel activity performed in the presence of 10^{-6} M Bay K 8644. *B*, Large events were observed on repolarization. Note openings on repolarization in the absence of any detectable activity during depolarization (*). *C*, Current–voltage relationship was calculated for elementary events recorded after patch excision. The conductance calculated is similar to that obtained in the cell-attached configuration (see Fig. 1).

rent traces, generated by step depolarizations to −20 mV from a holding potential of −60 mV, to obtain an ensemble average current. At this depolarizing potential, the activation kinetics of the averaged current were slow enough to allow reliable comparison. We found that the activation kinetics were significantly slower in PTX-treated than in nontreated cells. Average currents could be adequately fitted with mono-exponential functions whose time constants were: 69.76 ± 42.42 msec (n = 8) for the PTX-treated cells compared to 23.46 ± 13.57 msec (n = 7; unpaired t test, p = 0.016) for the nontreated cells (Fig. 6*A*).

In addition, further analysis of the activity evoked by step depolarisation to −20 mV revealed significant differences in the channel open-time characteristics. Mean open time observed on patches from PTX-treated cells was 4.23 ± 2.30 msec (n = 13) and only 2.67 ± 0.45 msec (n = 11, t test, p = 0.039) with nontreated cells. Concerning the open-time distributions, one exponential could often best fit the distribution in case of nontreated cells, but two exponentials were necessary for channel recordings from PTX-treated cells (Fig. 6*B*). Such a difference was clearly expressed in the weight attached to the fast exponential (PTX-treated cells: 0.68 ± 0.25, n = 15; nontreated cells: 0.94 ± 0.08, n = 11; unpaired t test, p = 0.003). However, the time constant of the fast exponential best fitting the open-time distributions did not differ between the two populations (PTX-treated cells: 1.37 ± 0.34 msec, n = 15; nontreated cells: 1.58 ± 0.18, n = 11). Therefore, it appears that PTX treatment of the cells facilitates long Ca^{2+} channel openings (time constant of the slow exponential best fitting the open time distribution: 7.69 ± 3.69 msec, n = 12) without modifying the short openings.

The fact that open-time modifications induced by PTX treatment could be demonstrated in activity elicited by step depolarizations to −20 mV but not by sustained depolarization at 0 mV

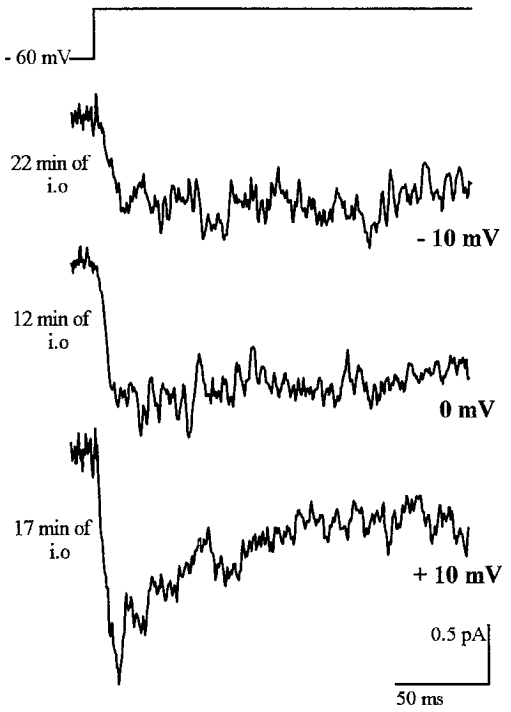

Figure 4. Average currents of L-type Ca^{2+} channels recorded on a PTX-treated cell in inside-out (*i.o.*) configuration. Each illustrated trace is the average of 60 individual current traces obtained by depolarizations to −10, 0, and +10 mV (top to bottom, respectively). On individual step recording, up to four simultaneous channel openings were observed (not shown). Note that the evolution of the current kinetics with the depolarizing potential is similar to that typically occurring in the cell-attached configuration.

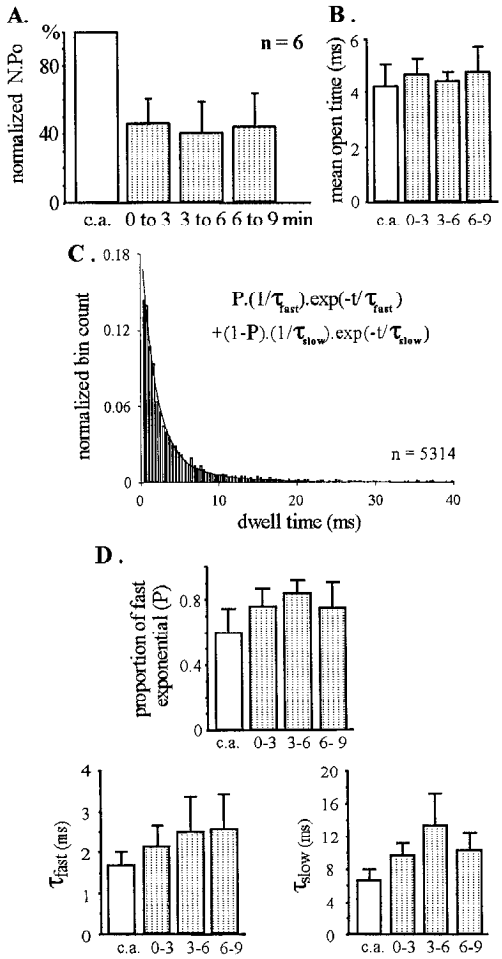

Figure 5. Mean characteristics of L-type Ca^{2+} channels recorded on PTX-treated cells in inside-out configuration. Histograms show characteristics of Ca^{2+} channel activity observed at a holding potential of 0 mV (six patches). Recordings were performed during 2–3 min in cell-attached configuration (*c.a.*) and during at least 9 min after excision of the patch. *A*, Mean (± SD) open probability (N Po) calculated per periods of 3 min following patch excision. For each patch, N Po was expressed as a percentage of the probability observed during 2–3 min recording of the channel activity in the cell-attached configuration. Note that following excision, Ca^{2+} channel N Po remained at 40–50% of that observed during cell-attached recording. *B*, Mean open time displayed by Ca^{2+} channels over periods of 3 min. No modification can be noticed between cell-attached and inside-out configurations. *C*, Example of open-time distribution calculated from 3 min recording of Ca^{2+} channel activity in inside-out configuration. The distribution could be adequately fitted by the sum of two exponential probability density functions, weighted with a proportion coefficient P. *D*, Mean values of the three parameters defining the open time distribution model: P, τfast and τslow (see expression in *C* and Experimental Procedure section). Note that excision of the patch induces a small but significant increase of each parameter.

(see above section), suggests that this effect may be potential dependent. However, the discrepancy could also be attributed to the difference in the protocols used, i.e., step depolarization versus sustained depolarization. Therefore, we checked that PTX treatment does not modify open-time characteristics of channel activity evoked by step depolarizations to 0 mV.

Values were similar to the ones previously calculated from recordings using sustained depolarizations at 0 mV. Mean open time, weight of the fast exponential best fitting the open-time distributions, time constants of the fast and slow exponentials were, respectively, 4.73 ± 1.45 msec (n = 7), 0.66 ± 0.28 (n = 7); 1.84 ± 0.68 msec (n = 7), 9.36 ± 3.33 msec (n = 6) for PTX-treated cells and 3.54 ± 1.16 msec (n = 7), 0.74 ± 0.20 (n = 7), 1.52 ± 0.27 msec (n = 7), 6.86 ± 1.62 msec (n = 6) for nontreated cells.

In conclusion, in cell-attached recordings, over night PTX treatment of granule cells of the cerebellum modifies activation kinetics of L-type Ca^{2+} channels and increases the probability of evoking long channel openings using small depolarizations. The main PTX effect is on inside-out recordings, since PTX treatment increases the probability to obtain maintained active channels in excised patches.

Discussion

In granule cells of the cerebellum, excision of a patch and exposure of the cytoplasmic face of the membrane to a saline solution leads within some 60 sec to the rundown of all Ca^{2+} channel activity. We have found that over night PTX treatment of the cells greatly reduces the rundown process. Under these conditions, Ca^{2+} channel activity recorded in the presence of Bay K 8644 is retained with its main characteristic over tens of minutes after patch excision in 70% of the cases. The effect has been characterized on the L-type activity present in the granule cells. In the following sections, we will successively discuss the characteristics of the Ca^{2+} channels studied and the possible mechanism implied in this PTX effect.

A large array of Ca^{2+} channel types is present on postnatal cerebellar granule cells grown in culture (Slesinger and Lansman, 1991; Ellinor et al., 1993; Forti and Pietrobon, 1993; Bossu

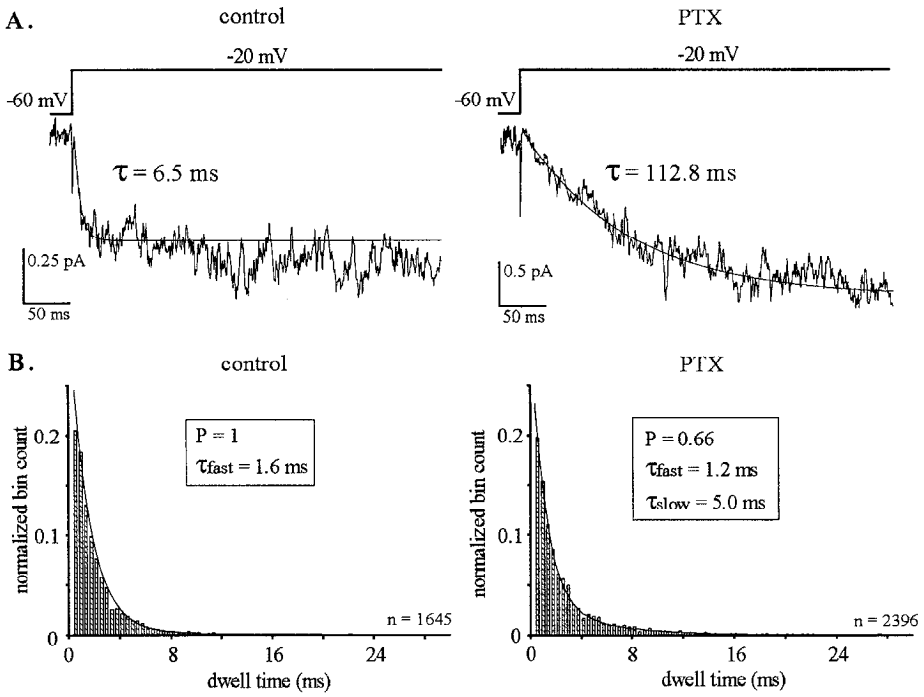

Figure 6. Difference between L-type channel activity evoked by step depolarizations to −20 mV on control and PTX-treated cells in cell-attached configuration. *A*, Difference in activation kinetics: each illustrated current trace is the average of 200 individual current traces obtained by depolarizations from −60 to −20 mV. Currents were fitted by a decaying exponential whose time constant (τ) reflects the activation kinetics of the current. The rise time of the average current is faster in control than in PTX-treated cells, as clearly expressed by difference in the τ value. The two traces represent extreme cases observed with nontreated and PTX-treated cells. *B*, Differences in open-time distributions: histograms present typical examples of open-time distribution observed in the case of control (*left*) and PTX-treated (*right*) cells. Note that in the former case, fitted probability density function was a single exponential, whereas two exponentials were necessary for the PTX-treated cell.

et al.,1994; Forti et al., 1994). In the present work, by imposing a holding membrane potential of −60 mV, most dihydropyridine insensitive channel activities were abolished, being largely inactivated at this potential (Forti et al., 1994). In fact, this condition yielded mainly silent patches unless 10^{-6} M Bay K 8644 was added to the bath or to the pipette solution. The dominant activity then recorded in cell-attached conditions was a nifedipine sensitive L-type activity. In the litterature, refined analysis of the L-type channels present in granule cells has unravelled various modalities of discharge, suggesting that different types of dihydropyridine-sensitive Ca^{2+} channels may be expressed in these neurones. In the present work, as channel activity was mainly elicited by maintained depolarization, one can assume that recordings of channels displaying fast inactivation (Slesinger and Lansman, 1991) or "anomalous gating" (low open probability, Forti and Pietrobon, 1993) were rare. Indeed, the conductance (29 pS) and open probability (above 0.2 in most cases) make the channels included in the present study close to the noninactivating L-type Ca^{2+} channels initially described by Pietrobon and Hess (1990) in cardiac cells and identified by Forti and Pietrobon (1993) in granule cells.

In this neuronal cell type, we observed that Ca^{2+} channel activity quickly disappears after patch excision in the great majority of cases, similar to that previously reported by Slesinger and Lansman, 1991. Although it is possible to excise a patch that remains active in inside-out configuration, this is very unprobable in untreated granule cells. Our main result is that PTX pretreatment of these cells dramatically increases this probability. However, the rundown process still occurs in 38% of recorded patches. The partial failure of the PTX treatment suggests either that an additional PTX-insensitive mechanism is also implied in the rundown process or that the over night treatment does not perform a complete ADP-ribosylation of Go or Gi proteins in every cell as it has been previously observed (Prézeau et al.,1994).

The PTX effect is likely to be related to the relieve of the PTX-sensitive pathways downmodulating L-type channel activity in granule cells. Indeed, metabotropic glutamate receptors have been shown to inhibit L-type channel activity via a PTX-sensitive mechanism (Chavis et al., 1994). The inhibitory pathway is not membrane delimited, but the authors failed to demonstrate the involvment of any second messenger. Therefore, they suggest that a direct block of the channels by Go- or Gi-like proteins would be a simple hypothesis to explain their results, if one assume that G-protein diffused to a certain distance from the receptor. In addition, a persistent block of L-type channels by a PTX-sensitive G-protein has also been described in granule cells (Haws et al., 1993). This last result is probably related to the runup of the Ca^{2+} current that occurs just after patch rupture on going to the whole-cell recording configuration in different cell types. Such increase in Ca^{2+} current was attributed to the dialysis of PTX-sensitive elements responsible for a tonic inhibition specifically exerted on L-type channels (Elhamdani et al., 1994; Netzer et al., 1994). We may hypothetize that in granule cells of the cerebellum, the disruption of these inhibition pathways by a PTX treatment would reveal a facilitated state of the Ca^{2+} channels, with the possible consequences of maintained activity on patch-excision. However, one might expect faster activation kinetics and an increased channel open probability after PTX treatment. On the contrary, we noticed a three times slower time constant for the current activation at −20 mV of treated cells, and the predicted increase in open probability was not observed (although such a tendency could have been masked by the large variance in the measured NPo). These data make difficult to superimpose the mechanisms underlying the relief of a tonic inhibition and the modifications observed in the channel gating.

The PTX effect described in our work may also be related to the phosphorylation/dephosphorylation processes that are considered to modulate Ca^{2+} channel activity. Indeed, it is generally assumed that phosphatases are responsible for rundown. In cardiac cells, activity after rundown is restored by MgATP plus PKA, and can be abolished by the addition of PKI (Ono and Fozzard, 1992). Further, a slower rundown occurs in the pres-

ence of the phosphatase inhibitor, okadaic acid. Although some data obtained in other preparations point to the possible generality of this mechanism (Amstrong and Eckert, 1987; Oyah and Sperelakis, 1989; Tewari and Simard, 1994), we have yet no evidence that it takes place in granule cells. However, it is worth noting that in these cells, elevation of the intracellular concentration of cAMP by preincubation with cell permeant analogs of cAMP has been reported to lead to a persistent Ca^{2+} channel activity after patch excision over a few minutes (Slesinger and Lansman, 1991; Lambert and Feltz, 1994). In addition, a distinct set of data suggests that it is rather proteolysis, which is at the origin of the rundown process. Indeed, calpastatin, a protease inhibitor, may be a necessary anti-rundown factor present in intact cells (Dufy et al., 1986; Kameyama et al., 1988; Romanin et al., 1991). However, an explanation linking the calpastatin effect to the calcineurin hypothesis of Chad and Eckert (1986) has once more introduced phosphatase activity as the underlying mechanism to rundown. The ubiquitous calcineurin becomes an active phosphatase by its conversion to an active calmodulin-independent form. This conversion would be induced by calpain once calpastatin inhibition is relieved, which systematically occurs on patch excision (Armstrong, 1989). In the present experiments, since the channel activity in excised patches was observed without providing ATP, the putative above mechanisms imply that PTX treatment acts by disrupting a phosphatase activity usually ripped off at the same time as the channel. Supporting this hypothesis, G-protein activation of protein phosphatase has been reported (Kitazawa et al., 1991; Pan et al, 1992) with the possibility that G-protein directly activates type 1 protein phosphatase (Bielefeldt and Jackson, 1994). Whether or not the PTX effect on the rundown phenomenom is acting via the phosphorylation/dephosphorylation pathways of Ca^{2+} channels remains to be carefully checked. It is worth noting that we observed that open probability after patch excision is only 40% of the one in the cell-attached configuration, and this decreased activity may be due to a partial dephosphorylation of the channels.

Alternatively to these mechanisms implying a direct or indirect regulation of Ca^{2+} channel activity by G-protein, many other PTX-sensitive pathways can be postulated to modify protein synthesis during the over night treatment with consequences on the rundown process. Indeed, Cullen et al. (1994) reported that the PTX-induced increase in glutamate release from granule cells can be explained by a PTX effect on L-type Ca^{2+} channels and is blocked by cycloheximide, suggesting that PTX acts on the synthesis of a protein associated with Ca^{2+} channels.

Finally, the PTX effect observed here might be explained by an action through Bay K 8644 binding. In cerebellar granule cells, Bay K 8644 alone does not prevent rundown to occur within a minute. However, since in GH3 cell Bay K 8644 markedly slows the rundown process (Armstrong and Eckert, 1987), one may wonder if the present effect could not reflect an increased affinity for Bay K 8644, resulting of the PTX action. Indeed, G-proteins have been proposed to modulate the dihydropyridine binding site (Dolphin and Scott, 1989). However, binding analysis rather suggest a reduced affinity for DHP agonist after PTX treatment (Bergamaschi et al., 1988). In order to clarify this point, a similar approach should be carried on granule cells in view of the many ways calcium channels are regulated in different cells.

Although the analysis detailed in the present work relates to L-type activity, the PTX-effect reported here may extend to the other high-threshold activated Ca^{2+} channels. PTX regulations have been extensively related to N-type activity (Hille, 1994), and indication of a PTX-sensitive pathway has also been found to be exerted by $GABA_B$ on P-type channels (Mintz and Bean, 1993). Therefore, there exists a possibility that a PTX-sensitive mechanism may be implied in the almost immediate extinction of all high-threshold voltage-activated Ca^{2+} channel activity consecutive to patch excision.

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