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Short communication

R- and L-type Ca^{2+} channels are insensitive to eliprodil in rat cultured cerebellar granule neurons

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Abstract

We have investigated, by using the whole-cell patch-clamp technique, the Ca^{2+} channel antagonist properties of eliprodil in cultured cerebellar granule cells which are known to express L-, N-, P- as well as Q- and R-type Ca^{2+} channels. Eliprodil maximally antagonized 50% of the voltage-dependent Ba^{2+} current with an IC_{50} of 4 μM. ω-Conotoxin-GVIA (3.2 μM) and ω-agatoxin-IVA (0.5 μM) blocked 28 and 43% of the current, respectively. When eliprodil (30 μM) was added to ω-conotoxin-GVIA or ω-agatoxin-IVA the magnitude of the maximal inhibition was identical to that obtained with eliprodil alone confirming a full blockade by eliprodil of N-, P- and Q-type Ca^{2+} channels. The L-type channel antagonist nimodipine (10 μM) blocked 24% of the current; this blockade was fully additive to that of eliprodil, indicating that the nimodipine-sensitive component of the current is eliprodil-insensitive. In the presence of eliprodil and nimodipine a residual Cd^{2+} sensitive current (25%), identified as the R-type current, remained unblocked. We conclude that in cerebellar granule neurons R- and L-type Ca^{2+} channels are insensitive to eliprodil. The nimodipine- sensitive channels present in cerebellar granule neurons may represent a neuronal subtype of L channels distinct from that (eliprodil-sensitive/nimodipine-sensitive) present in cortical or hippocampal neurons.

Keywords: Ca^{2+} channel; Cerebellum; Granule neuron; ω-Conotoxin-GVIA; ω-Agatoxin-IVA; Eliprodil

1. Introduction

Eliprodil is a neuroprotective compound, which has been shown to non-competitively antagonize the NR1A/NR2B NMDA receptor subtype by acting at the polyamine modulatory site (Scatton et al., 1994). This compound is also a potent antagonist of L-, N- and P-type neuronal Ca^{2+} channels in cultured or dissociated neurons as well as in transfected human embryonic kidney cells (HEK 293) (Church et al., 1994; Biton et al., 1994, 1995; Bath et al., 1996). This dual mechanism of action may explain its neuroprotective properties: the antagonism of the NR1A/NR2B subtype of NMDA receptors and the blockade of postsynaptic Ca^{2+} channels would prevent excessive neuronal Ca^{2+} overload during ischemia or trauma and the inhibition of N- and P-type Ca^{2+} channels at the presynaptic level would in addition limit the release of excitatory amino acid neurotransmitters.

Recent data have demonstrated that at least five subtypes of high voltage-activated Ca^{2+} channels exist in neurons which have been called L, N, P, Q and R subtypes (Nowicky et al., 1985; Mintz et al., 1992; Zhang et al., 1993). These different subtypes can be differentiated by using pharmacological tools such as organic compounds or peptidic toxins (Saccomano and Ahlijanian, 1994). The L-type Ca^{2+} channels are classically blocked by dihydropyridines, the N-type Ca^{2+} channels are selectively inhibited by ω-conotoxin-GVIA, the P and Q types are antagonized by low (20–50 nM) and high concentrations (100–200 nM) of ω-agatoxin-IVA, respectively (Randall and Tsien, 1995). The R subtype, which resembles the recently cloned Doe-1 Ca^{2+} channel, has been characterized in cerebellar granule neurons as the component of the Ca^{2+} current resistant to the above pharmacological agents (Ellinor et al., 1993; Zhang et al., 1993). In cultured cerebellar granule cells, 15–20% of the global Ba^{2+} current has been reported to flow through R-type Ca^{2+} channels while 35% of the global current has been identified as a Q-type current (Zhang et al., 1993; Randall and Tsien, 1995). The effects of eliprodil on Q- and R-type Ca^{2+} channels are presently unknown. In order to address this question, we have investigated the antagonistic properties

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of this compound on Ba^{2+} currents elicited in rat cultured cerebellar granule cells.

2. Materials and methods

2.1. Cell cultures

Primary cultures of rat cerebellar granular neurons were prepared from 8-day-old pups using a technique adapted from Vigé et al. (1993). Briefly, 300 μm cubes of cerebellum were trypsinized and the cells were dissociated by gentle trituration. Cells were resuspended in the following culture medium: basal Eagle's medium, 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamycin and seeded onto poly-D-lysine-coated glass coverslips (0.25×10^6 cells/coverslip) and placed in 12-well Corning dishes. Cells were incubated at 37°C in a humidified atmosphere, 5% CO_2 + 95% air. Cytosine β-D-arabinoside (1 μM) was added the day after seeding to prevent the replication of non-neuronal cells. The coverslips were transferred for patch-clamp experiments after 7–11 days in culture. Granular neurons were identified on the basis of their small size and round shape and most often appeared to be bipolar.

2.2. Electrophysiology

The chambers containing the cell preparation were placed on the stage of an inverted microscope (Olympus

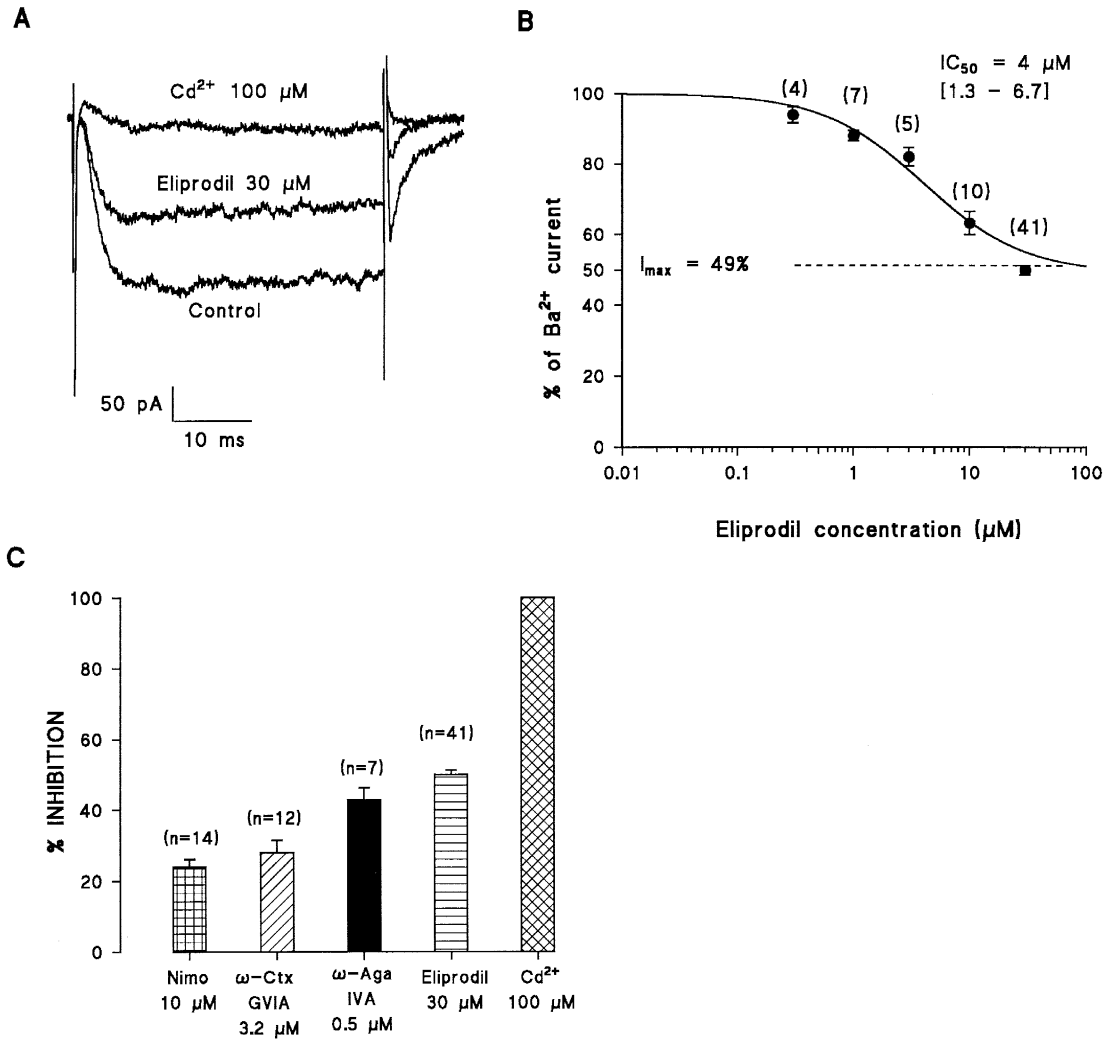

Fig. 1. Effect of eliprodil and reference compounds on the Ba^{2+} current in cultured granular neurons. (A) Time course of the whole-cell current in response to a step depolarization from −80 to 0 mV in the absence and in the presence of eliprodil. Zero current was obtained by application of 100 μM of Cd^{2+}. (B) Concentration dependence of the blockade by eliprodil of the whole-cell Ba^{2+} currents. Ba^{2+} currents were elicited in the same conditions as those used in (A) and measured at the end of the depolarizing pulse. Data from 4–41 granular neurons were averaged. The curve is the best fit of the data points obtained with the single-site model equation: $(100 - \min)(IC_{50}/(C + IC_{50})) + \min$, where C is the concentration of eliprodil. The corresponding IC_{50} value is given with a 95% confidence interval. (C) Comparison of the maximal inhibition produced by the selective Ca^{2+} antagonists nimodipine, ω-conotoxin-GVIA (ω-Ctx-GVIA), ω-agatoxin-IVA (ω-Aga-IVA) with that of eliprodil and Cd^{2+}. Measurements were done in the same conditions as in (B) and the number of cells is given in parentheses.

IMT2) equipped with Hoffman optics (Modulation Contrast, New York, NY, USA) and viewed at a total magnification of 400 ×. A polyethylene tubing (opening 500 μm) was approached within 3 mm of the cell under investigation and allowed a fast superfusion of solution (3–5 ml/min). For application of toxins, we used modified patch pipettes (opening 10–20 μm), connected to a polyethylene tubing containing the toxin solution and approached within less than 200 μm from the cell studied.

We used the whole-cell configuration of the patch-clamp technique. Pipettes were pulled from thick-walled borosilicate glass capillaries (Phymep, Paris, France) on a two-stages puller and had a resistance of 5–10 MΩ. Pipettes were approached from the cells with a 3D piezoelectric micromanipulator (Burleigh, PC1000). Whole-cell currents were recorded with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA) connected to a 386 DX personal computer driven by pClamp software (Axon

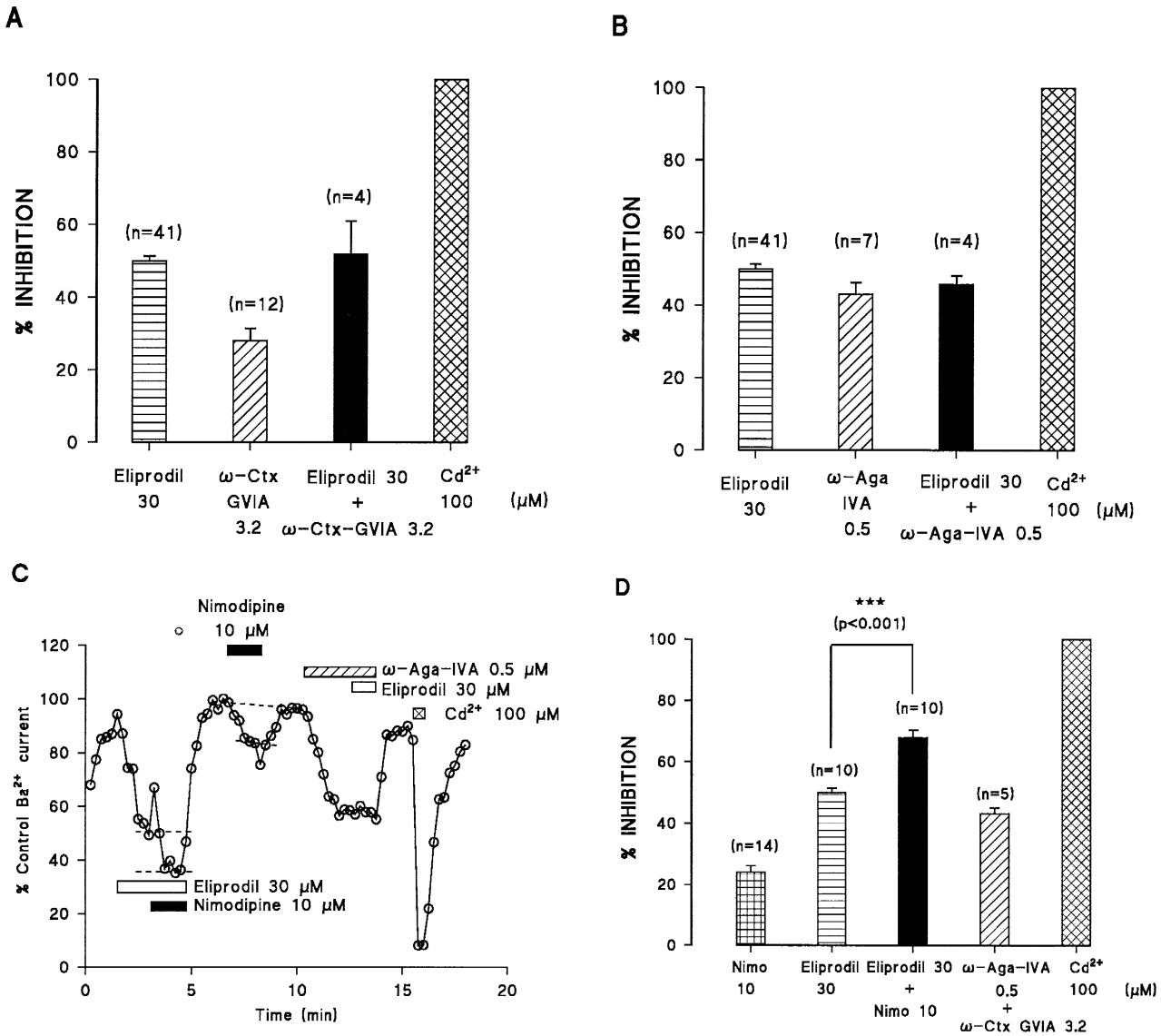

Fig. 2. Characterization of the eliprodil-sensitive and insensitive components of the Ba^{2+} current in cerebellar granular neurons. (A) Comparison of the maximal inhibition produced by eliprodil, ω-conotoxin-GVIA (ω-Ctx-GVIA), eliprodil + ω-Ctx-GVIA and Cd^{2+}. (B) Comparison of the maximal inhibition produced by eliprodil, ω-agatoxin-IVA (ω-Aga-IVA), eliprodil + ω-agatoxin-IVA and Cd^{2+}. (C) Relative current amplitude as a function of time measured at the end of a step depolarization from −80 mV to 0 mV. Thirty μM eliprodil was applied during the time shown by the open bar. A subsequent application of 10 μM nimodipine (solid bar) produced a further inhibitory effect, which was of a similar magnitude as that produced by application of 10 μM nimodipine alone (solid bar). In contrast, 30 μM eliprodil (open bars) added after 0.5 μM ω-agatoxin-IVA (hatched bar) did not produce any additional effect. Note the reversal of the ω-agatoxin-IVA inhibitory effect during washing. Zero current was obtained by the application of 100 μM Cd^{2+}. (D) Comparison of the maximal inhibition produced by nimodipine (Nimo), eliprodil, eliprodil + nimodipine, ω-agatoxin-IVA + ω-Ctx-GVIA, and Cd^{2+} 100 μM. Stars indicate statistical significance (Student *t*-test, paired data, $P < 0.001$). All the measurements were done in the same conditions as those used in (C), and in (A), (B) and (D), the number of cells is given in parentheses.

Instruments). For the measurement of voltage-sensitive Ca^{2+} currents, we used the P/N stimulation/leak subtraction protocol of the pClamp software.

Means are given with standard errors of the mean (S.E.M.). For concentration-response curves, the least-square fitting routine of the Fig.P software (Biosoft, Cambridge, UK) was used. Parameters providing the best fit are given with a 95% confidence interval.

2.3. Solutions and reagents

The standard extracellular solution (pH 7.4) contained (in mM): NaCl (147), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), HEPES/Tris-OH (10). The standard pipette solution (pH 7.2) contained (in mM): CsCl (140), $MgCl_2$ (1), $CaCl_2$ (1), EGTA (11), HEPES/Tris-OH (10), Na_2ATP (4). The free Ca^{2+} concentration was 10^{-8} M. For the measurement of voltage-gated Ca^{2+} currents, the solution (pH 7.4) contained (in mM): $BaCl_2$ (10), tetraethyl-ammonium chloride (144), $MgCl_2$ (2), CsCl (3), glucose (10), HEPES/Tris-OH (10).

The following chemicals were used: nimodipine (Bayer), eliprodil (Synthélabo Chemistry Dept.). These compounds were first diluted in dimethyl sulfoxide (DMSO) which had, in the final Ba^{2+}-containing solutions, a constant concentration of 0.08%. ω-Conotoxin-GVIA and ω-agatoxin-IVA (RBI, Alomone Labs) were diluted in the Ba^{2+}-containing solution.

3. Results

As shown in Fig. 1A, eliprodil at the maximal concentration of 30 μM inhibited 50% of the steady-state Ba^{2+} current resulting from a step depolarization applied from −80 to 0 mV (50 ms duration). This effect was reversible and the IC_{50} of eliprodil was of 4 ± 2.7 μM (Fig. 1B). The magnitude of the effect of eliprodil was greater than that obtained with 10 μM nimodipine (−24%) or 3.2 μM ω-conotoxin-GVIA (−28%) and was similar to that produced by the application of 0.5 μM of ω-agatoxin-IVA (−43%) (Fig. 1C). Surprisingly, the effect of the latter toxin was reversible, which contrasts with previous observations (Mintz et al., 1992) (Fig. 2C).

In another set of experiments and in order to characterize the nature of the eliprodil-sensitive and insensitive currents, we tested the effect of 30 μM eliprodil on the global Ba^{2+} current in the presence of 10 μM nimodipine, 3.2 μM ω-conotoxin-GVIA or 0.5 μM ω-agatoxin-IVA. As shown in Fig. 2A, the same magnitude of inhibition was obtained, whether eliprodil was applied in the presence or in the absence of ω-conotoxin-GVIA, suggesting that eliprodil blocked all the ω-conotoxin-GVIA-sensitive current. Similarly, eliprodil did not induce an additive effect when applied in the presence of ω-agatoxin-IVA (Fig. 2B). This latter result suggests that the component of the Ca^{2+} current inhibited by ω-agatoxin-IVA (P + Q component) overlapped with the one that is sensitive to ω-conotoxin-GVIA. This was further demonstrated with the lack of additivity observed when ω-conotoxin-GVIA was applied after ω-agatoxin-IVA (Fig. 2D). Finally, when 30 μM eliprodil were applied in the presence of 10 μM nimodipine, an additive effect was observed, suggesting that eliprodil and nimodipine block distinct current components (Fig. 2C,D). In the presence of eliprodil and nimodipine, 25% of the Cd^{2+}-sensitive current remained unblocked. Altogether, these results suggest that in this preparation (i) eliprodil is inactive on an L-type-nimodipine-sensitive component, (ii) eliprodil blocks N-, P- and Q-type Ca^{2+} channels, (iii) ω-agatoxin-IVA at 0.5 μM, a concentration which is supposed to block P- and Q-type Ca^{2+} channels, also blocks, at least partially, N-type Ca^{2+} channels, (iv) eliprodil does not block a current component which can be identified as the R type, given its insensitivity to eliprodil and nimodipine which, as shown above, block N-, P-, Q- and L-type Ca^{2+} channels.

4. Discussion

We have demonstrated in this study that eliprodil does not block L- and R-type Ca^{2+} channels in cultured cerebellar granule cells. Previous studies have shown that eliprodil, and to a lesser extent ifenprodil, are potent blockers of L-, N- and P-type Ca^{2+} channels in a number of preparations including recombinant channels (Church et al., 1994; Bath et al., 1996; Biton et al., 1994). The nimodipine-sensitive current represented about 25% of the total Cd^{2+}-sensitive Ba^{2+} current in cerebellar granule neurons. This finding is in agreement with recently published data in which the dihydropyridine-sensitive component of the global Ba^{2+} current in cerebellar granule cells was close to 20% (Pearson et al., 1995; Randall and Tsien, 1995). In the present study, eliprodil and nimodipine effects were clearly additive. Thus, L-type Ca^{2+} channels in cultured cerebellar granule neurons are insensitive to eliprodil, and may represent a subclass of L-type Ca^{2+} channels distinct from those present in other neuronal structures such as the cerebral cortex or hippocampus. This observation is consistent with recent results pointing to the peculiar pharmacological properties of L-type Ca^{2+} channels in cerebellar granule neurones. Unlike eliprodil, calcicludine, a venom peptide isolated from *Dendroaspis angusticeps*, has recently been shown to be a much more potent antagonist of L-type Ca^{2+} channels in rat cerebellar granule neurons ($IC_{50} = 0.2$ nM) than in rat dorsal root ganglion neurons ($IC_{50} = 60–80$ nM). Skeletal L-type Ca^{2+} channels were totally insensitive to this toxin. These results led to the conclusion that a subclass of L-type Ca^{2+} channels is present in some neurons, and particularly in cerebellar granule cells (Schweitz et al., 1994). Since three distinct genes and about nine splice variants encoding

dihydropyridine-sensitive L-type Ca^{2+} channels have been identified, one can speculate that a minor modification in the L-type Ca^{2+} channel α_1 subunit may account for the ineffectiveness of eliprodil in cerebellar granule neurons (Dunlap et al., 1995).

A recent study has suggested that in granule cells, ω-agatoxin-IVA is able to block almost completely L- and N-type Ca^{2+} channels in addition to P- and Q-type Ca^{2+} channels (Pearson et al., 1995). In another work, such a lack of selectivity of ω-agatoxin-IVA was not observed. However, the kinetics of washing of the toxin effect was unusually fast when used at high concentrations, reflecting probably the recovery from blockade of the Q current component (Randall and Tsien, 1995). Our results partially agree with those found in both studies. Like Pearson et al., we found that ω-agatoxin-IVA was not selective for P- and Q-type Ca^{2+} channels but unlike these authors, our data show that the current sensitive to this toxin is overlapping with N-type Ca^{2+} channels. On the other hand, and in agreement with the study of Randall and Tsien, we also found that the major part of the ω-agatoxin-IVA blockade was reversible on washing the toxin. Altogether, these data are consistent with our results and suggest that in cerebellar granule neurons, the pharmacology of high-voltage gated Ca^{2+} channels could be different from that usually found in other neuronal structures.

In the present study, we identified the Cd^{2+}-sensitive current, remaining after the application of 30 μM of eliprodil together with 10 μM of nimodipine, as being carried by R-type Ca^{2+} channels. This current would represent about 25% of the global Ba^{2+} current. This proportion is roughly equivalent to that which can be deduced from the blocking effect of nimodipine (25% inhibition) and that obtained with the association ω-agatoxin-IVA/ω-conotoxin-GVIA (43% inhibition). This finding is in good agreement with recent data dealing with the pharmacological dissection of the Ca^{2+} current in cerebellar granule cells, in which the R current was reported to amount to 15–20% of the global Ba^{2+} current (Zhang et al., 1993; Randall and Tsien, 1995).

The neuroprotective properties of eliprodil have been linked to its antagonistic properties on both NMDA receptors and high-voltage-activated Ca^{2+} channels (Scatton et al., 1994). The ineffectiveness of eliprodil at R-type Ca^{2+} channels indicates that this compound does not block all types of Ca^{2+} channels. Given the recent discovery of the R subtype and the absence of R-type-specific ligand, it is difficult to know the relative abundance and the exact physiological role of this channel. Nevertheless, since these channels display a rapid kinetics of inactivation, they probably are not of primary importance in the sustained Ca^{2+} entry occurring during brain hypoxia or trauma. The lack of activity of eliprodil on L-type Ca^{2+} channels in cerebellar granule cells was unexpected. Eliprodil, like calcicludine, could be a helpful tool to pharmacologically distinguish subtypes of dihydropyridine-sensitive Ca^{2+} channels. Combination of eliprodil with nimodipine could represent a very convenient way to isolate and study the R-type Ca^{2+} channels in cerebellar granule cells.

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