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Block of P-type Ca^{2+} channels by the NMDA receptor antagonist eliprodil in acutely dissociated rat Purkinje cells

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Abstract

The effect of eliprodil on P-type Ca^{2+} channels was investigated in acutely dissociated rat Purkinje neurons, by using the whole-cell patch-clamp technique. Eliprodil inhibited in a reversible manner the ω-agatoxin-IVA-sensitive Ba^{2+} current elicited by step depolarizations from a -80 mV holding voltage ($IC_{50} = 1.9$ μM). The Ba^{2+} current showed steady-state inactivation ($V_{1/2} = -61$ mV) which was shifted toward more positive values when the intracellular Ca^{2+} buffering was increased. In these conditions, the potency of eliprodil was decreased ($IC_{50} = 8.2$ μM), suggesting a modulation by intracellular Ca^{2+} of the eliprodil blockade. The potency of eliprodil was not modified at more depolarized holding potentials and was not dependent on the frequency at which the step-depolarizations were applied (0–0.2 Hz) indicating a lack of voltage and use dependence of the eliprodil blockade. When eliprodil was applied in the patch-pipette at a concentration which causes full block when applied externally, the Ba^{2+} current amplitude was not affected and external application of eliprodil was still efficacious, indicating an extracellular location of the binding site. Analysis of the time course of recovery from Ca^{2+} channel blockade obtained by concomitant application of eliprodil with Cd^{2+}, ω-agatoxin-IVA or fluspirilene, indicated that these latter compounds did not interact with eliprodil, suggesting that eliprodil acts at a different site. These results demonstrate that eliprodil blocks P-type Ca^{2+} channels in cerebellar Purkinje neurons and suggest that this property may contribute to its neuroprotective activity.

Keywords: Ca^{2+} channel; Whole-cell voltage-clamp; Dissociated Purkinje neuron; Inactivation; Eliprodil

1. Introduction

Eliprodil is a novel non-competitive NMDA receptor antagonist acting at the polyamine modulatory site (Scatton et al., 1994), which has recently been shown to also block L- and N-type voltage-dependent Ca^{2+} channels in cultured cortical neurons and in dissociated hippocampal neurons (Biton et al., 1994; Avenet et al., 1994). Since excess of Ca^{2+} entry is believed to be the major event leading to neuronal damage following ischaemia or trauma (Siesjö, 1990), the high efficacy of eliprodil in experimental models mimicking these pathologies might be explained by its dual ability to block postsynaptically the two major Ca^{2+} entry pathways, namely *N*-methyl-D-aspartate (NMDA)-gated and voltage-dependent Ca^{2+} channels (Scatton, 1994).

Although the different subtypes of Ca^{2+} channels have been localized in all neuronal elements (Lipscombe et al., 1988; Silver et al., 1990; Westenbroek et al., 1990; Mills et al., 1994), increasing evidence suggests that they are involved in different neuronal functions. L-type Ca^{2+} channels would play an important role in Ca^{2+} influx in depolarized neurons, thereby regulating intracellular Ca^{2+} concentration and a number of cellular processes such as long-term potentiation or modulation of neurite outgrowth (Westenbroek et al., 1990; Silver et al., 1990) while, N- and P-type Ca^{2+} channels would be implicated in transmitter release and exocytosis (Fossier et al., 1994). In favor of this hypothesis, recent data have shown that in nerve terminals, clustered N-type Ca^{2+} channels are tightly associated with syntaxin and presynaptic vesicles and are responsible for the exocytotic process via a mechanism which can be compared to Ca^{2+}-induced Ca^{2+} release

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in cardiac cells (Haydon et al., 1994; Sheng et al., 1994). Thus, antagonism of presynaptically located Ca^{2+} channels such as the N- and P-type Ca^{2+} channels would be expected to inhibit excessive excitatory amino acid transmitter release, thereby preventing the spread of neuronal excitation in ischaemic conditions. This strategy has recently been used to develop new neuroprotective compounds like the synthetic peptide SNX-111, a N-type Ca^{2+} channel antagonist analogue of ω-conotoxin-GVIA (Valentino et al., 1993). However, N-type Ca^{2+} channel antagonists do not block transmitter release in all brain areas. For example, in the hippocampus, which is known to be highly sensitive to ischaemia or hypoxia, P-type Ca^{2+} channels seem to play a predominant role in glutamate release (Luebke et al., 1993). Other Ca^{2+} channel types, most recently described such as Q-type and R-type may also be involved in transmitter release (Gaur et al., 1994; Wheeler et al., 1994).

The antagonism of presynaptic N-type Ca^{2+} channels previously demonstrated for eliprodil may play a part in its neuroprotective activity. Yet its effect on P-type Ca^{2+} channels is unknown. In order to address this question, we used acutely dissociated Purkinje neurons which are known to quite exclusively express P-type Ca^{2+} channels. We show here that eliprodil blocks P-type Ca^{2+} channels as potently as the N- and L-type Ca^{2+} channels. Part of these results was published in abstract form (Avenet et al., 1994).

2. Materials and methods

2.1. Cell preparation

Purkinje neurons were enzymatically dissociated from the cerebellum of 6–12 days old Sprague Dawley rats (Iffa Credo, l'Arbresle, France). Briefly, after anesthesia with ether, rats were decapitated, the cerebellum removed and sliced in 300 μm^3 cubes with a McIllwain tissue chopper. Blocks of tissue were washed 3 times and then trypsinized (trypsin 0.25 mg/ml; Sigma, type III) at room temperature in 10 ml of the standard external solution (see below) enriched in Mg^{2+} (2.6 mM). After 9–10 min of trypsinization under gentle mechanical agitation, 1 mg/ml of trypsin inhibitor (Sigma, type II-O) and 0.1 mg/ml of DNase (Sigma, type I) were added for 10 more min. The tissue cubes were then transferred in 10 ml standard external solution to which EGTA (2 mM) was added in order to chelate Ca^{2+}, washed 3 times in this solution and finally mechanically dissociated by gentle trituration with a fire-polished Pasteur pipette. The resulting cell suspension was diluted and transferred in the patch-clamp experimental chamber which consisted of a 30 mm diameter Petri dish in which a ring of silicon was casted to form a chamber of 800 μl. The cells were allowed to settle for 10 min before patch-clamp experiments in order to attach sufficiently to the plastic bottom of the dishes. Purkinje neurons were identified on the basis of their characteristic morphology, i.e. large 'eye-shaped' somata and small and single dendritic stump (Regan, 1991).

2.2. Electrophysiology

The chambers containing the cell preparation were placed on the stage of an inverted microscope (Olympus IMT2) equipped with Hoffman optics (Modulation Contrast, New York) 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 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) on a two stages puller and had a resistance of 5–10 MΩ. Pipettes were approached from the cells with a 3D hydraulic micromanipulator (Narishige, WR89). Whole-cell currents were recorded with an Axopatch 1D amplifier (Axon Instruments, Foster City, California) connected to a 386 DX personal computer driven by pClamp software (Axon Instruments). We used the P/N stimulation/leak subtraction protocol of the pClamp software.

Means are given with standard error of the mean (S.E.M.). For concentration-dependence 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). For the measurement of voltage-operated Ca^{2+} currents, the solution (pH 7.4) contained (in mM): $BaCl_2$ (5), tetraethyl-ammonium chloride (144), $MgCl_2$ (2), CsCl (3), glucose (10), 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 some experiments, EGTA was replaced by BAPTA (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), at the same concentration (11 mM). A high

Hepes/Tris-OH (100 mM) and EGTA (30 mM)-containing pipette solution was also used in some experiments.

The following chemicals were used: nimodipine (Bayer), fluspirilene (RBI), 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 (Latoxan) and ω-agatoxin-IVA (RBI, Alomone labs) were diluted in the Ba^{2+}-containing solution.

3. Results

3.1. Eliprodil block of P-type Ca^{2+} channels

Depolarization from a holding voltage of −80 to −0 mV elicited typical sustained Cd^{2+}-sensitive inward currents of large amplitude carried by Ba^{2+} ions. Eliprodil 0.1–30 μM reversibly antagonized the inward Ba^{2+} current (Fig. 1A,C) with fast onset and recovery (time constant of the recovery = 64.5 ± 6.3 s at 10 μM, six cells). The IC_{50} of the concentration-dependence

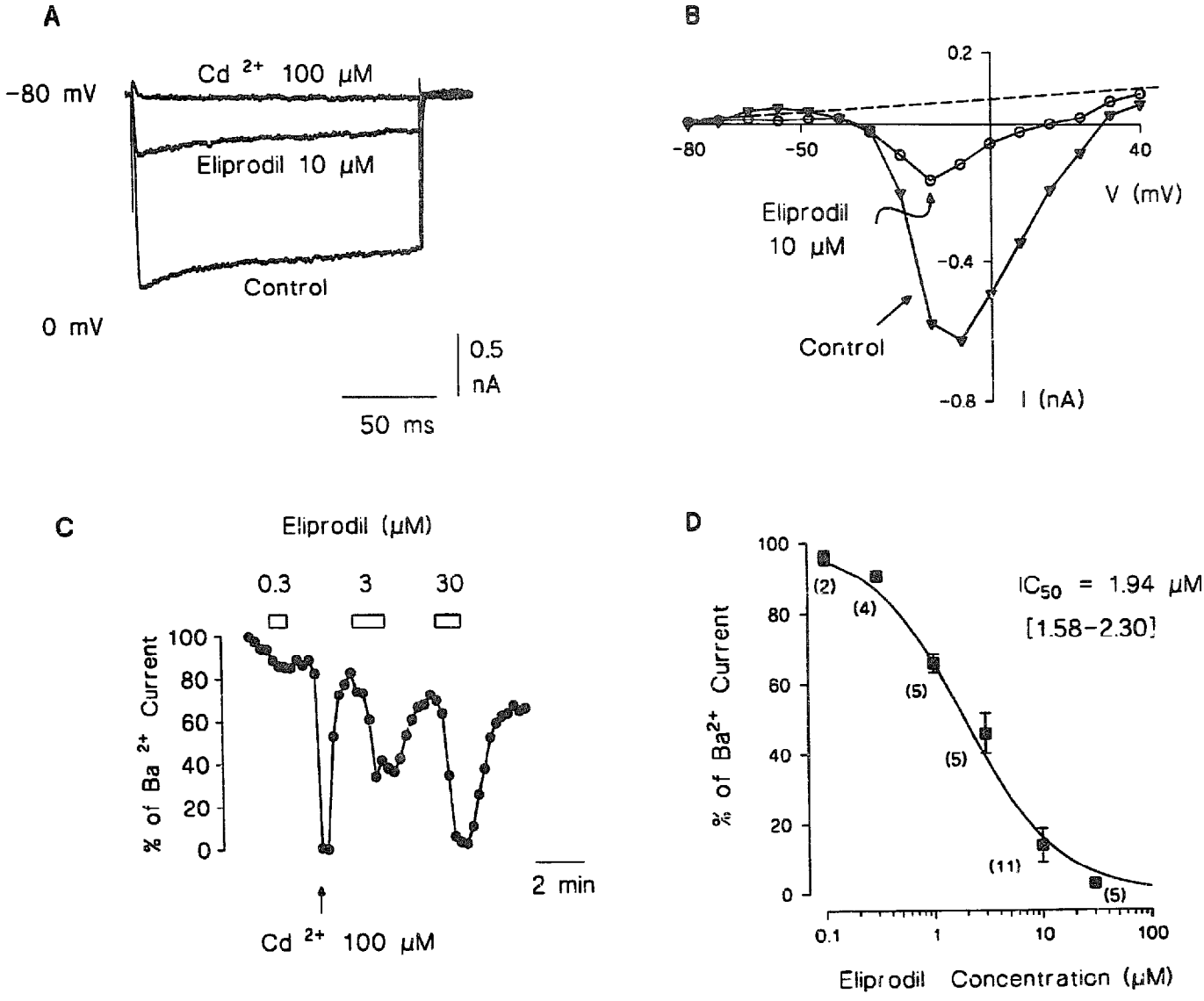

Fig. 1. Antagonism by eliprodil of Ba^{2+} currents in acutely dissociated Purkinje neurons. (A) Time course of the whole-cell current in response to a step depolarization from −80 mV to 0 mV in the absence and in the presence of 10 μM eliprodil. Zero current was obtained by application of 100 μM Cd^{2+}. (B) Effect of 10 μM eliprodil on the current-voltage relationship. Currents were elicited from a holding potential of −80 mV. The dashed line represents the leak current. (C) Relative current amplitude as a function of time measured at the end of the depolarizing pulse in the same conditions as those used in (A). Eliprodil was applied at the concentrations indicated during the time shown by the open bars. Cd^{2+} at 100 μM (arrow) produced a full and reversible block of the current. (D) Concentration dependence of the block by eliprodil of the whole cell Ba^{2+} currents. Voltage pulses were applied from a holding potential of −80 mV to 0 mV. Data from 2 to 11 Purkinje neurons were averaged. The curve is the best fit of the data points obtained with the single site model equation: $100 - ((C * 100)/(C + IC_{50}))$ where C is the concentration of eliprodil. The corresponding IC_{50} value is given with a 95% confidence interval.

relationship was 1.94 ± 0.36 μM ($P = 0.05$), a value very similar to that previously obtained for L- and N-type Ca^{2+} channels (1.5 μM in cultured cortical neurons) (Fig. 1D). The current-voltage (I-V) curve was not modified by eliprodil, the current peaking at a test potential of about −8 mV in the absence or in the presence of submaximal concentrations of eliprodil (Fig. 1B).

Most of the inward Ba^{2+} current was flowing through P-type Ca^{2+} channels since the selective P-type Ca^{2+} channel antagonist ω-agatoxin-IVA blocked $82\% \pm 5.3$ ($n = 5$) of the total current at 0.2 μM and $87\% \pm 1.9$ ($n = 7$) at 1 μM. The block by ω-agatoxin-IVA was irreversible after the wash of the toxin, unless short depolarizing pulses to +60 mV were applied (Fig. 2A). On the other hand, L- and N-type Ca^{2+} channels contributed for about 20–30% of the total inward current as demonstrated by the effect of 10 μM nimodipine and 3 μM ω-conotoxin-GVIA which inhibited $16 \pm 3.7\%$ ($n = 11$) and $18 \pm 2.5\%$ ($n = 6$) of the Ba^{2+} current, respectively (Fig. 2B). These results confirm that Purkinje neurons express mainly P-type Ca^{2+} channels, as shown previously (Mintz et al., 1992; Bindokas et al., 1993).

3.2. Voltage- and intracellular Ca^{2+} dependence of the eliprodil block

In order to study the voltage dependence of the eliprodil antagonism we first characterized the steady-state inactivation of the Ba^{2+} current by recording currents evoked by depolarization from different holding voltages. Fig. 3A shows that sustained depolarization of the Purkinje neurons led to a progressive decrease or run-down of the Ba^{2+} current. This slow voltage-dependent inactivation process was reversible with a similar time course, when the holding potential was clamped-back to −80 mV. Half steady-state inactivation ($V_{1/2}$) was obtained at a potential of -61 ± 1.4 mV ($P = 0.05$) and full inhibition occured at −40 mV (Fig. 3B). Changing intracellular buffering conditions by using BAPTA instead of EGTA or increasing the EGTA concentration to 30 mM and that of Hepes to 100 mM did not prevent the slow inactivation process but shifted the voltage dependence toward more positive values ($V_{1/2}$ values of −51 mV and −41 mV, respectively) (Fig. 3C,D).

Using a holding voltage of −60 mV, at which half of the current was still present, the potency of eliprodil ($IC_{50} = 3.4 \pm 0.65$ μM) was not significantly different from that measured at −80 mV, suggesting no voltage-dependent block of P-type Ca^{2+} channels by eliprodil (Fig. 4A,B). Interestingly, when the BAPTA buffer was used at a holding voltage of −80 mV, we observed a clear decrease in the eliprodil potency, the new IC_{50} being 8.25 ± 0.88 μM, suggesting a modulation by intracellular Ca^{2+} of eliprodil antagonism (Fig. 4C,D).

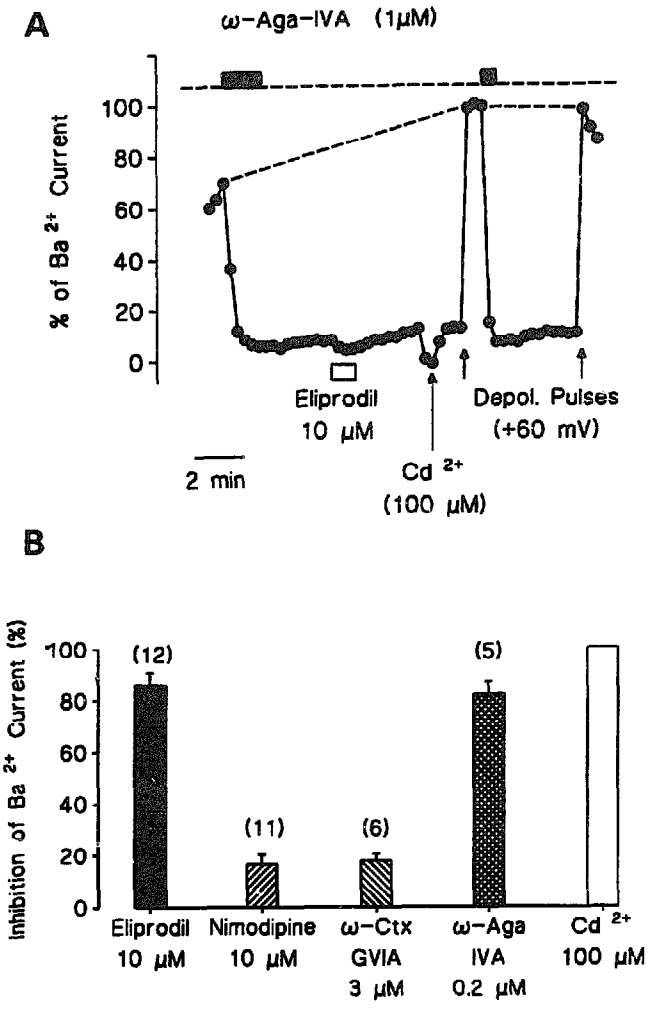

Fig. 2. Pharmacological characterization of Ca^{2+} channels in Purkinje neurons. (A) Relative current amplitude as a function of time measured at the end of a step depolarization from −80 mV to 0 mV. ω-Agatoxin-IVA (ω-Aga-IVA) was applied at 1 μM during the time indicated by the solid bars. 10 μM eliprodil were applied during the wash of the toxin (open box). Application of 100 μM Cd^{2+} (arrow) produced a full block of the current. Depolarizing pulses from −80 mV to +60 mV at the time indicated completly released the block by the toxin. (B) Comparison of the maximal inhibition produced by the selective Ca^{2+} channels inhibitors nimodipine, ω-conotoxin-GVIA (ω-Ctx-GVIA) and ω-agatoxin-IVA (ω-Aga-IVA) with that of eliprodil and Cd^{2+}. Measurements were done in the same conditions as those used in A and the number of cells is given in parentheses.

3.3. Lack of use dependence of eliprodil antagonism

Increasing the stimulation frequency did not affect the rate of onset of the block by eliprodil (not shown). Furthermore, when eliprodil was applied in the absence of depolarizing pulses, the blockade occurred with the same time course and magnitude as that observed when depolarizing pulses were applied at 0.2 Hz. Together, these data suggest that eliprodil can

block P-type Ca^{2+} channels in the resting state and that eliprodil-induced current inhibition shows no use dependence (Fig. 5A).

3.4. Location of eliprodil binding site

In order to investigate if eliprodil can reach its binding site when applied on the intracellular side of the cytoplasmic membrane, we included eliprodil at 10 μM in the standard pipette solution, a concentration at which an average 85% current block was observed when normally applied in the extracellular solution. In these conditions, the amplitude of the Ba^{2+} current, close to 2 nA, was in the range of that usually found. As shown in Fig. 5B the current did not decline even 7–8 min after the start of the whole-cell recording. Furthermore, subsequent application of 10 μM eliprodil in the external bath quickly decreased the current amplitude in a reversible manner. This result demonstrates that eliprodil does not bind to a site

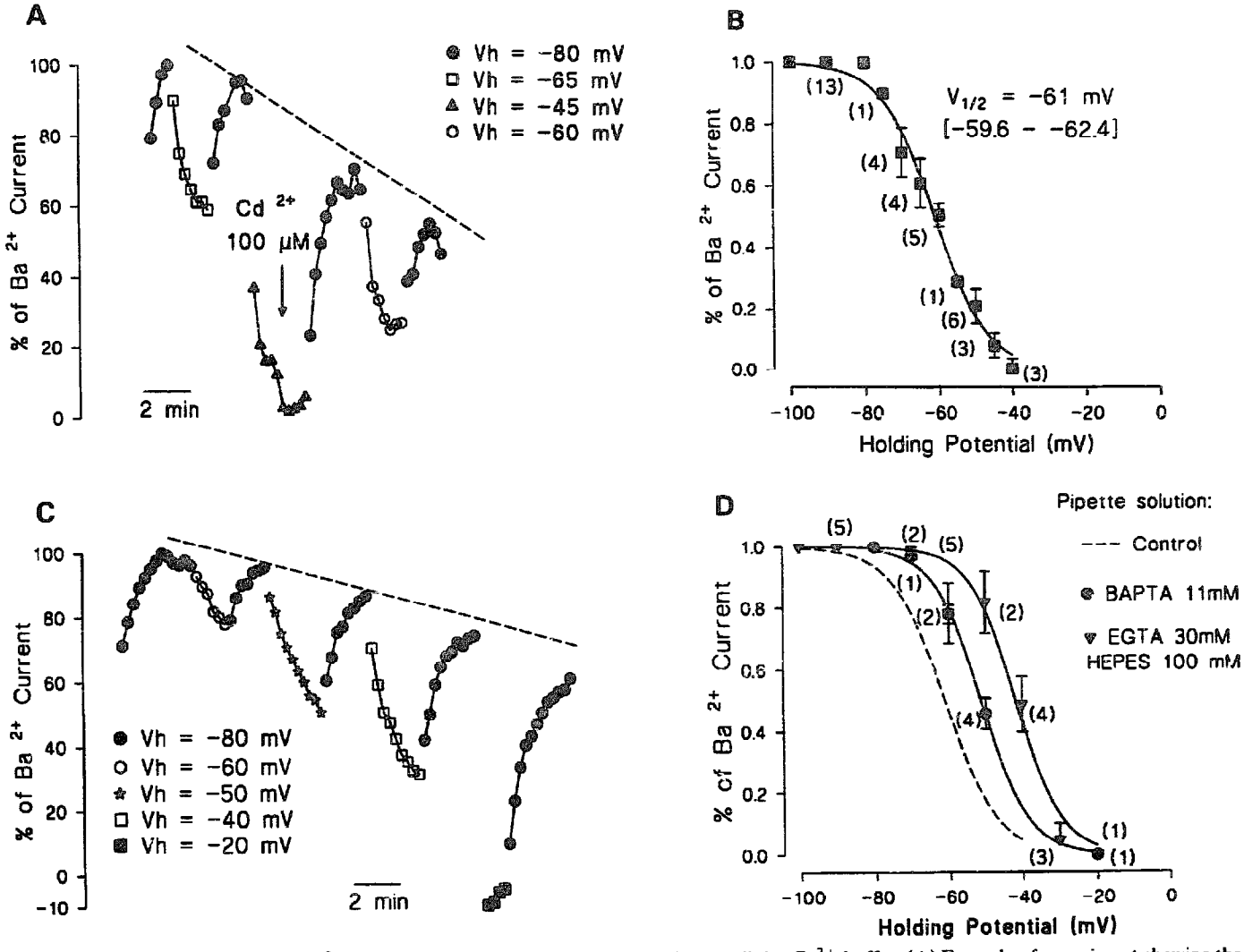

Fig. 3. Steady-state inactivation of Ba^{2+} currents in Purkinje neurons: effect of intracellular Ca^{2+} buffer. (A) Example of experiment showing the relative current amplitude as a function of time measured at the end of a step depolarization to 0 mV from a holding voltage of −80 mV (filled circles), −65 mV (open squares), −45 mV (filled triangles) or −60 mV (open circles). 100 μM Cd^{2+} were applied to obtain a full block of the current (arrow). The dashed line indicates the control current level (at −80 mV). The pipette was filled with the standard pipette solution. (B) Steady-state inactivation curve of the Ba^{2+} current measured in the standard conditions of panel A (standard pipette solution). The currents relative to that measured at −80 mV for each holding-voltage were obtained with the number of neurons indicated in parentheses and averaged. The continuous line is the best fit of the data points with the Boltzmann equation: $1/(1 + \exp((V - V_{1/2})/k))$ where V is the holding voltage, $V_{1/2}$ the half-inactivation voltage and k the slope factor. The corresponding $V_{1/2}$ and k values given with 95% confidence interval are -61 ± 1.4 mV and 7.11 ± 1.28, respectively. (C,D) Influence of the intracellular Ca^{2+} buffer on the steady-state inactivation curve of the Ba^{2+} current. (C) Same experiment as in A but with BAPTA 11 mM replacing EGTA 11 mM in the pipette solution. Example of experiment showing the relative current amplitude as a function of time measured at the end of a step depolarization to 0 mV from a holding voltage of −80 mV (filled circles), −60 mV (open circles), −50 mV (filled stars), −40 mV (open squares) and −20 mV (solid squares). (D) The modifications of the standard pipette solution are as indicated in the figure. The dashed curve is that obtained in control conditions (pannel B). The $V_{1/2}$ and k values, given with a 95% confidence interval, were -51 mV $\pm$ 1.2 and 6.45 ± 1.45 (filled circle) and -41 mV $\pm$ 3.9 and 6.23 ± 3.0 (filled triangles) and the number of cells is indicated in parentheses.

located on the inner side of the membrane, data which are consistent with its rather rapid kinetics of block.

The time course of recovery from channel blockade was characteristic of the antagonist used. Indeed, the recovery from Cd^{2+} block was typically faster (time constant = 16.2 ± 0.7 s; five cells) than that obtained with eliprodil (64.6 ± 6.3 s; six cells) whereas the recovery from blockade by fluspirilene, a diphenylbutylpiperidine which has been shown to be a potent blocker of P-type Ca^{2+} channels and not to interact with the ω-agatoxin-IVA binding site (Sah and Bean, 1994), was much slower (time constant > 200 s) than that of eliprodil. We used this property to investigate the possible interaction of eliprodil with the binding sites of Cd^{2+}, ω-agatoxin-IVA and fluspirilene.

In the experiment shown in Fig. 6A, the Ba^{2+} current was first blocked with Cd^{2+} and then, eliprodil was applied at a full blocking concentration. When both compounds were removed, the time course of recovery was identical to that obtained with eliprodil

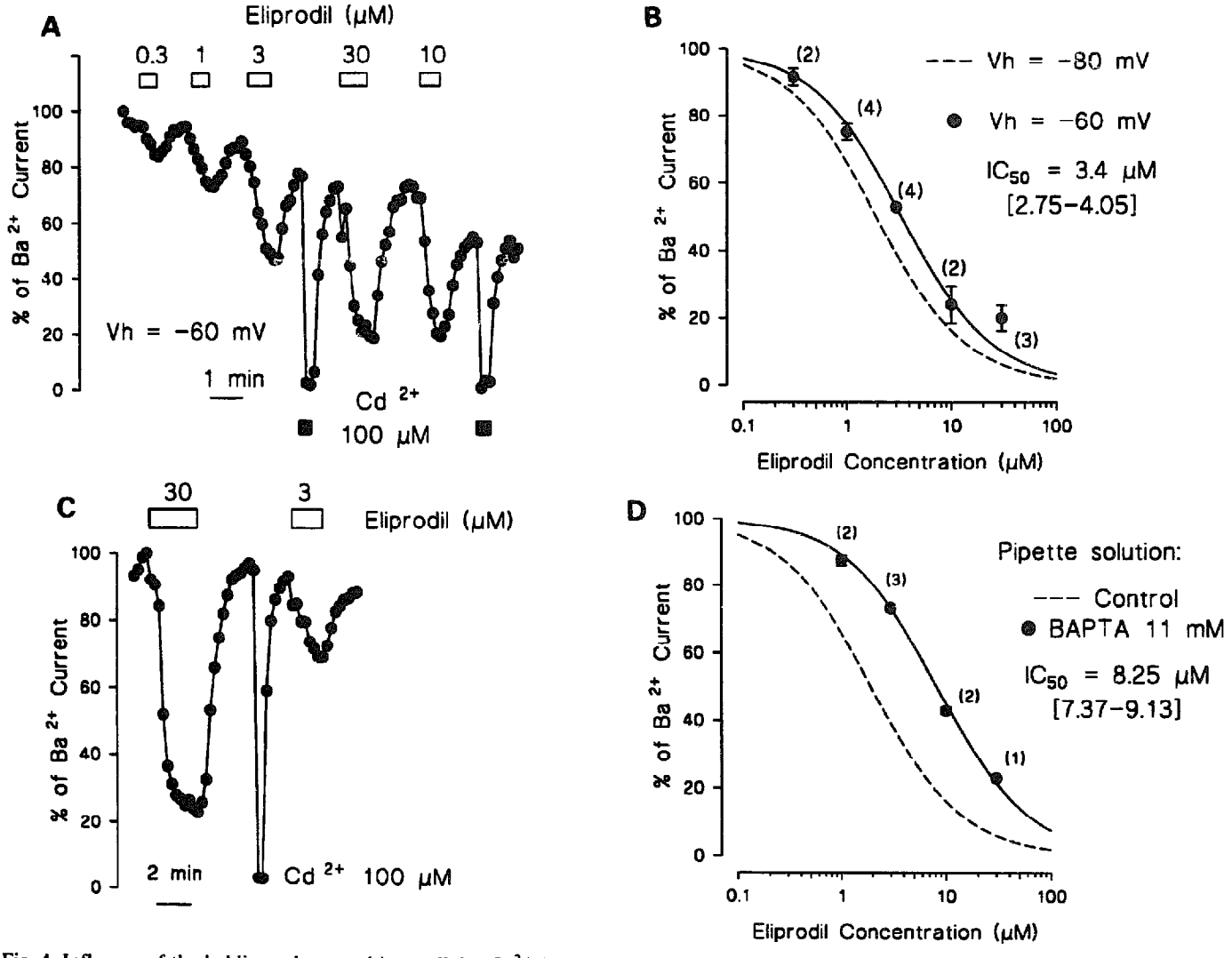

Fig. 4. Influence of the holding voltage and intracellular Ca^{2+} buffering conditions on the eliprodil concentration-dependence relationship. (A) Example of experiment showing the relative amplitude of the Ba^{2+} current as a function of time measured at the end of a depolarizing pulse from −60 mV to 0 mV. Eliprodil was applied at the concentrations indicated during the time shown by the open bars. 100 μM Cd^{2+} were added in order to evaluate the zero current (filled squares). (B) Concentration dependence of the block by eliprodil of the whole cell Ba^{2+} current performed using the conditions of panel A. Depolarizing pulses were applied from a holding voltage of −60 mV to 0 mV. Data were obtained from the number of neurons indicated in parentheses and averaged. The continuous line (filled circles) is the best fit of the data points with the single site model equation: $100 - ((C * 100)/(C + IC_{50}))$ where C is the concentration of eliprodil. The IC_{50} value is given with a 95% confidence interval. The dashed line represents the control concentration relationship of Fig. 1D. (C) Example of experiment showing the relative amplitude of the Ba^{2+} current as a function of time measured at the end of a depolarizing pulse from −80 mV to 0 mV. EGTA was replaced by BAPTA (11 mM) in the pipette solution. Eliprodil was applied at the concentrations indicated during the time shown by the open bars. (D) Concentration dependence of the block by eliprodil of the whole cell Ba^{2+} current when EGTA (11 mM) was replaced by BAPTA in the pipette solution (filled circles). Depolarizing pulses were applied from a holding voltage of −80 mV to 0 mV. The continuous line represents the fit of data points obtained as in panel B and the number of cells is indicated in parentheses. The dashed line represents the control concentration relationship of Fig. 1D.

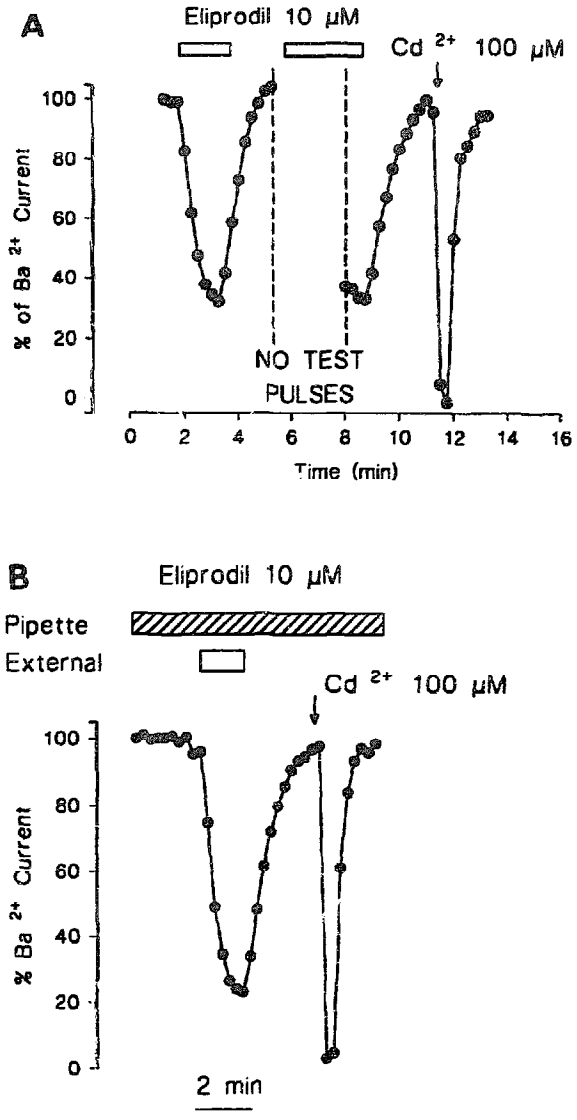

Fig. 5. Lack of use-dependent block of the Ba^{2+} current by eliprodil. Effect of intracellular application of 10 μM eliprodil. (A) Relative current amplitude as a function of time in response to depolarizing pulses to 0 mV from a holding potential of −80 mV. 10 μM eliprodil were applied extracellulary during the time shown by the open bars. During the time period indicated by the two vertical dashed lines the depolarizing pulses were stopped and eliprodil applied. Note that blockade of eliprodil occured in the absence of depolarizing pulses. (B) Relative current amplitude as a function of time measured at the end of a step depolarization from −80 mV to 0 mV in a cell in which 10 μM eliprodil were applied via the patch-pipette (hatched bar). Eliprodil was applied extracellularly during the time indicated by the open bar. In both panels, the maximum inhibition was obtained by application of 100 μM Cd^{2+} (arrows).

alone, indicating that Cd^{2+} did not prevent eliprodil from reaching its binding site. Similarly, we tested the interaction of eliprodil with fluspirilene. When 30 μM eliprodil were applied prior to fluspirilene (30 μM), recovery of the current at wash of both compounds followed the time course of that of fluspirilene, which was slowly reversible. This experiment indicates that eliprodil did not prevent the action of fluspirilene and therefore that the two compounds were probably act-

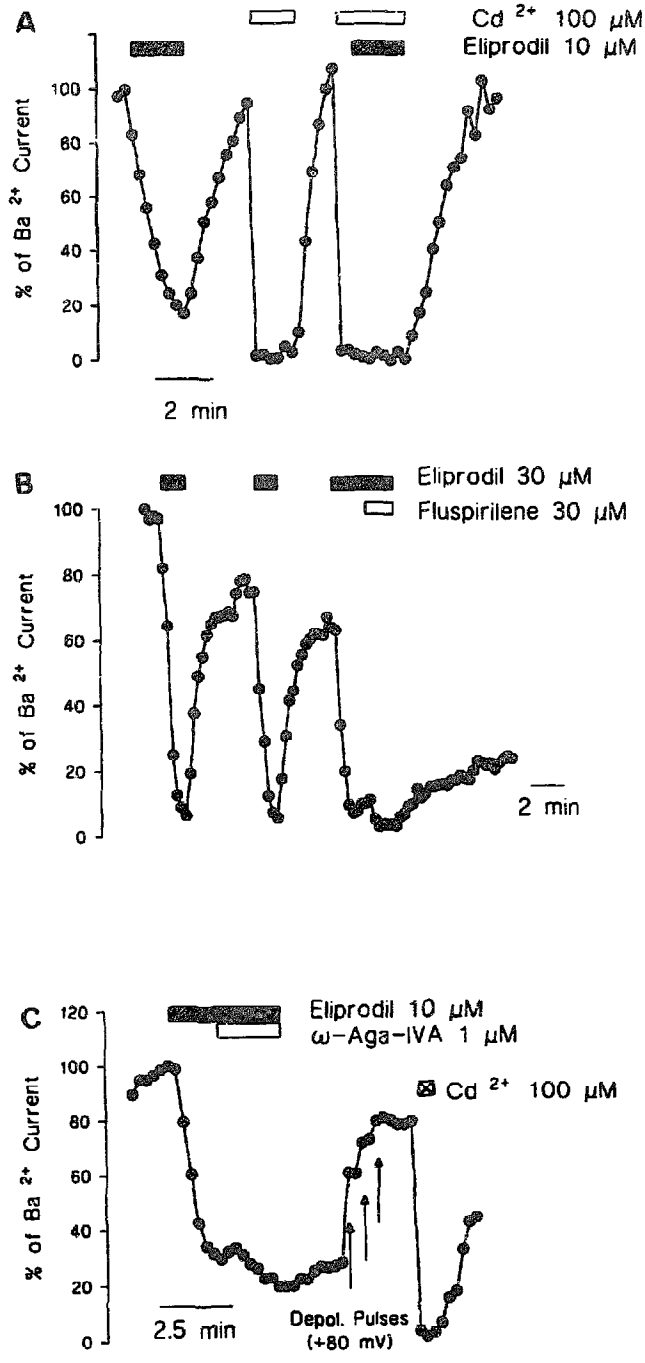

Fig. 6. Interaction of eliprodil with Cd^{2+}, fluspirilene and ω-agatoxin-IVA. In each panel, the relative current amplitude obtained by cell depolarization from −80 mV to 0 mV is plotted as a function of time and the total current evaluated by the block obtained with 100 μM Cd^{2+}. (A) Note that when 10 μM eliprodil (solid bars) were applied after a full block with 100 μM Cd^{2+} (open bars), the recovery of the current during the wash of both compounds followed a kinetic typical of that of eliprodil, slower than that observed during the wash of Cd^{2+} alone. (B) When 30 μM fluspirilene (open bar) were applied after 30 μM eliprodil (solid bars), the slow kinetic of the wash of both compounds was similar to that of fluspirilene (see text). (C) 10 μM eliprodil (solid bar) applied prior to perfusion of 1 μM ω-agatoxin-IVA (ω-Aga-IVA) (open bar) did not prevent the binding of the toxin as shown by the absence of recovery during the wash of both compounds until depolarizing pulses from −80 mV to +80 mV were applied (arrows).

ing at two different sites (Fig. 6B). In a last experiment, 10 μM eliprodil were applied prior to superfusion of 1 μM of ω-agatoxin-IVA. When both compounds were washed, the current did not recover, indicating that 10 μM eliprodil did not prevent ω-agatoxin-IVA to exert its blocking action (Fig. 6C). This was confirmed by the recovery of the current induced by the application of depolarizing pulses to +80 mV, which did not occur when depolarizing pulses were applied in the presence of 10 μM eliprodil (data not shown). This result strongly suggests that eliprodil and ω-agatoxin-IVA act at two different binding sites, an hypothesis consistent with the non-selective blocking properties of eliprodil.

4. Discussion

The major finding of this study is that the neuroprotective compound eliprodil fully and reversibly blocks P-type Ca^{2+} channels with a potency similar to that previously shown for the N- and L-type Ca^{2+} channels (Biton et al., 1994). A few compounds, such as fluspirilene or haloperidol, with IC_{50} values of 6 μM and 30 μM, respectively, have also been shown to block P-type Ca^{2+} channels in Purkinje neurons (Sah and Bean, 1994). Other compounds such as the piperidine derivative SB 201823-A ([4-[2-(3,4-dichlorophenoxy)ethyl]-*l*-pentylpiperidine hydrochloride]) and dextromethorphan (Benham et al., 1993; Netzer et al., 1993) were reported to nonselectively block neuronal high voltage-activated Ca^{2+} channels with IC_{50} values of 5 μM and 60 μM, respectively. Eliprodil, with an IC_{50} value of 1.9 μM for P-type Ca^{2+} channel blockade appears so far as the most potent antagonist among these compounds.

Most of the classical Ca^{2+} channel antagonists, such as dihydropyridine derivatives, produce a voltage-dependent block of Ca^{2+} channels (Wibo, 1989). In the case of Purkinje neurons, we could only study the influence of the voltage on the potency of eliprodil at a moderate depolarization level (−60 mV) since the Ba^{2+} current exhibited a strong steady-state inactivation (see below). The potency of eliprodil in antagonizing P-type Ca^{2+} channels was not stronger at a holding voltage of −60 mV compared to −80 mV. This is in contrast to its analogue ifenprodil which antagonized high voltage-activated Ca^{2+} channels in mouse cultured hippocampal neurons (Church et al., 1994), with a weak, but significant, voltage and frequency dependence. Smooth muscle cells have a rather low resting potential which makes them a good target for voltage-dependent Ca^{2+}-channel blockers like the dihydropyridine derivatives (Snedding et al., 1989). The lack of pressure effects observed at neuroprotective doses of eliprodil (Nowicki et al., 1994) may be explained by the absence of voltage dependence of its Ca^{2+} channel blockade, even if this compound would block peripheral L-type Ca^{2+} channels.

The steady-state inactivation of P-type Ca^{2+} channels observed in this study developed slowly after the onset of the holding voltage change, which may explain why it was not reported previously when a depolarizing prepulse protocol was used (Regan, 1991). A slow inactivation was also noticed recently for high voltage-activated Ca^{2+} currents in postnatal rat Purkinje and dorsal root ganglion neurons, but was absent in cardiac cells (Hockberger and Nam, 1994), which may indicate that this inactivation is a general property of neuronal high voltage-activated Ca^{2+} channels. According to our observations, this inactivation was, to some extent, dependent on the intracellular Ca^{2+} concentration since the voltage dependence was shifted toward depolarized values when a better buffering of intracellular Ca^{2+} concentration was achieved. A dependence of inactivation of P-type Ca^{2+} channels on intracellular Ca^{2+} concentration has been reported recently in synaptosomes (Tareilus et al., 1994).

Manipulation of intracellular buffering conditions by replacing EGTA with the faster buffer BAPTA (Adler et al., 1991), in addition to positively shifting the P-type Ca^{2+} channels steady-state inactivation curve, also lowered eliprodil potency. Both effects may be related to a change in surface charges at the intracellular side of the channel or to a direct Ca^{2+} or Ba^{2+} effect (Nargeot et al., 1994). Interestingly, this change at the intracellular side affected the binding of eliprodil extracellulary (see below). This property may confer to eliprodil an increased potency when the intracellular Ca^{2+} is enhanced, a situation which is believed to occur in ischaemic conditions. However, a direct effect of BAPTA, unrelated to Ca^{2+} chelation, cannot be completly excluded since BAPTA was shown to directly disrupt N-type Ca^{2+} channels regulation in sympathetic neurons (Shapiro et al., 1994).

Recent data have suggested that polyamines act as modulators of neuronal N- and L-type Ca^{2+} channels (Scott et al., 1993). The possibility exists, therefore, that eliprodil acts at homologous binding domains present in the NMDA receptor and Ca^{2+} channels. In support of this hypothesis, the blockade of Ca^{2+} channels in hippocampal pyramidal neurons by ifenprodil, a compound structurally related to eliprodil that also binds to the polyamine site of the NMDA receptor (Scatton et al., 1994) was modulated by spermine, suggesting a common site of action for spermine and ifenprodil. However, the action of ifenprodil was not affected by putrescine, another polyamine site agonist, indicating the possibility of multiple polyamine receptor subtypes or a partial overlap of the polyamine and ifenprodil (or eliprodil) recognition sites (Church et al., 1994).

Our study demonstrates that eliprodil does not interact with other P-type Ca^{2+} channel antagonists such as Cd^{2+}, ω-agatoxin-IVA, or fluspirilene. Thus, it is likely that the binding site for eliprodil which is shared by N-, L-, and P-type Ca^{2+} channels (since the potency of this compound is similar for these channel subtypes), is specific for this compound.

The N-type Ca^{2+} channel was the first Ca^{2+} channel subtype shown to play a predominant role in neurotransmitter release as demonstrated by the antagonism of transmitter release by ω-conotoxin-GVIA in a number of preparations (Dunlap et al., 1995). P-type Ca^{2+} channels seem also strongly involved since ω-agatoxin-IVA also inhibits neurotransmitter release and neurotransmission in neuronal preparations such as synaptosomes and hippocampal slices as well as in neuro-neuronal synapses (Uchitel et al., 1992; Dunlap et al., 1995; Fossier et al., 1994). In fact, as shown in these studies, both N- and P-type Ca^{2+} channels seem to be involved, particularly for glutamate release in the hippocampus, a brain area highly sensitive to ischaemia (Luebke et al., 1993; Yamamoto et al., 1994).

Recent data demonstrated that SNX-111, a synthetic ω-conotoxin peptide which selectively antagonizes N-type Ca^{2+} channels, was neuroprotective in several experimental models of cerebral ischaemia (Valentino et al., 1993; Yamada et al., 1994). To our knowledge, no data have provided evidence that selective P-type Ca^{2+} channel blockers could be neuroprotective in experimental in vivo models of hypoxia or ischaemia. It has been demonstrated that specific antagonists of P- and Q-type Ca^{2+} channels, but not ω-conotoxin-GVIA, reduce the release of excitatory amino acids and the frequency of epileptiform discharges in the rat cortical wedge (Robichaud et al., 1994). Moreover, antagonist compounds like dextromethorphan, SB 201823-A or flunarizine, which are non-selective blockers of high voltage-activated Ca^{2+} channels, exhibit neuroprotective properties in experimental in vivo models of hypoxia or ischaemia (Wauquier et al., 1989; Prince and Feeser, 1988; Benham et al., 1993). In contrast, specific antagonists of the L-type Ca^{2+} channels like nimodipine, nifedipine or nicardipine, which are ineffective in blocking neurotransmitter release (Dunlap et al., 1995; Bentué-Ferrer et al., 1993; Spedding et al., 1989), also failed to protect neurons from the consequences of ischaemia when administered after the ischaemic episode (Alps, 1992). Altogether, these data suggest that the inhibition of neurotransmitter release via a presynaptic blockade of N- and P-type Ca^{2+} channels may be a mechanism of neuroprotection of interest, especially in pathological situations where excessive neurotransmitter release occurs, as it is the case in delayed neuronal death following ischaemia.

In conclusion, our findings suggest that eliprodil could exert its neuroprotective activity through two mechanisms: presynaptically, it would inhibit excessive neurotransmitter release through an inhibition of N- and P-type Ca^{2+} channels. Postsynaptically, it would antagonize Ca^{2+} entry through NMDA receptors and voltage-activated Ca^{2+} channels, thereby inhibiting Ca^{2+} overload in neurons. Some results of the present study suggest that eliprodil inhibition of P-type Ca^{2+} channels could be modulated by intracellular Ca^{2+} concentration. This latter point, which needs to be further investigated, is of interest since eliprodil would be expected to be a more potent blocker of Ca^{2+} channels in damaged neuronal cells than in intact ones. This could explain the very large therapeutic index of this compound as compared to other neuroprotective agents (Nowicki et al., 1994; Scatton et al., 1994).

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