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On the inhibition of voltage activated calcium currents in rat cortical neurones by the neuroprotective agent 619C89

[1,2,4]A. Stefani, [3]A.H. Hainsworth, [1,2]F. Spadoni & [1,2]G. Bernardi

[1]Department Neuroscience, Università di Tor Vergata, Via di Tor Vergata 135, PtESud, 00133 Rome; [2]IRCCS S. Lucia; 00179 Rome, Italy and [3]School of Life Sciences, University of Greenwich, London SE18 6PF

1 The lamotrigine analogue 619C89, utilised to reduce postischaemic and posttraumatic neuronal injury, has been shown to inhibit sodium channels and cloned N-type calcium channels. To verify whether this neuroprotective agent also blocked native calcium channels, we have tested its action in cortical pyramidal neurones, acutely isolated from the adult rat brain.

2 619C89 inhibited more than 90% of the high voltage-activated calcium currents recorded in the whole-cell configuration. The response was relatively slow in onset (30–60 s), recovered incompletely (96%), but showed no consistent desensitization.

3 This inhibitory effect was not selective for any calcium channel subtype, being largely unaffected by ω-conotoxin-GVIA, ω-agatoxin-IVA, ω-conotoxin-MVIIC and dihydropyridine antagonists.

4 Saturating responses to 619C89 were detected for concentrations $\geqslant 50$ μM. Dose-response curves revealed that 619C89 have an approximately 8 μM binding site.

5 The effect of 619C89 was dependent on the divalent concentrations in that its potency was reduced on increase of the charge carrier up to 20 mM barium. Since the lamotrigine analogue shifted to the right the dose-dependence of the cadmium block, the 619C89-mediated inhibition of calcium currents seemed to rely on a direct interaction with the channel pore. Functional implications are discussed.

Keywords: Neuroprotective drugs; ischaemia; calcium conductance; whole-cell recordings; divalent ions

Introduction

Stroke as a result of cerebral ischaemia is the third leading cause of human death in developed countries. In addition, cerebral ischaemic events, if not fatal, usually produce major permanent disabilities as a consequence of immediate or delayed neurodegeneration. New understanding of the basic mechanisms underlying stroke-related events has recently emerged: excessive cellular firing discharge, exorbitant release of excitatory amino acids and improper influx of calcium (Ca^{2+}) ions are all implicated in the neurotoxic processes (Frandsen & Schousboe, 1993; Manev *et al*., 1989; Obrenovitch & Richards, 1995; Siesjo & Bengtsson, 1989; Urenjak & Obrenovitch, 1996). Newly developed compounds, aimed at reducing neuronal death during and following stroke by interference with voltage-gated inward currents, have been tested consistently in both animal models of ischaemia and human trials and some encouraging results have been reported (Leach & Swan, 1996; Meldrum *et al*., 1994; ONeill *et al*., 1997a,b; Smith & Meldrum, 1995).

The novel compound BW619C89 [4-amino-2-(4-methyl-1-piperazinyl)-5-(2,3,5,-trichlorophenyl) pyrimidine], from now on simply referred to as 619C89, exhibited potent neuroprotective properties in rat models of acute cerebral ischaemia where it reduced the volume of brain infarct when given either before or up to 1 h after middle cerebral artery (MCA) occlusion (Graham *et al*., 1994; Leach *et al*., 1993; Smith *et al*., 1993). In addition, it was proven effective in preventing neurodegeneration in post-traumatic models (Casanovas *et al*., 1996; Sun & Faden, 1995). Until recently, the neuroprotective capabilities of this compound have been attributed to the inhibition of voltage activated type II A sodium (Na^+) channels (Garthwaite *et al*., 1995; Xie *et al*., 1995; Xie & Garthwaite, 1996). The inhibition of tetrodotoxin-sensitive Na^+ channels is expected to dampen the repetitive cell firing as well as the synaptic glutamate release (Graham *et al*., 1993; Leach *et al*., 1986). However, that the neuroprotective abilities of this and other recently synthesized compounds depend upon the mere blockade of Na^+ channels has been consistently doubted in recent studies (MacDonald & Greenfield, 1997; Siniscalchi *et al*., 1996; Stefani *et al*., 1997a). 619C89, indeed, is a structural analogue of the anti-epileptic drug lamotrigine, which has been demonstrated to block not only Na^+ but also Ca^{2+} channels (Stefani *et al*., 1996a, 1997b; Wang *et al*., 1996); furthermore, 619C89 inhibited cloned N-type Ca^{2+} channels expressed in a human embryonic kidney (HEK)-derived cell line (McNaughton *et al*., 1997). The latter effect had an IC_{50} of about 16 μM, suggesting a higher potency than that described for Na^+ conductance.

The clinical utilization of 619C89 requires, in our opinion, a broad knowledge of its multiple effects on the different inward currents, which govern neuronal excitability. The tolerability of this agent, at first, would probably change if multiple Ca^{2+} channels were affected, instead of the mere N-type. Conversely, its effectiveness at low concentrations might imply a safe handling at concentrations lower than those suggested by previous studies. Finally, the prominence of each of its putative effect (suppression of Na^+ and/or Ca^{2+} channels, alteration of channel permeability) might strongly modify the cellular sensitivity to other neuroprotective agents (such as direct antagonists of postsynaptic glutamate receptors) being utilized in 'cocktail' therapies. We now have therefore examined the effects of 619C89 on native whole-cell Ca^{2+} currents in isolated neurones, freshly isolated from adult rat cortex. Preliminary data were presented in abstract form (Hainsworth *et al*., 1998).

[4]Author for correspondence.

Methods

Pyramidal neocortical neurones were dissociated from 50 male Wistar rats aged 1–2 months. Rats were anaesthetized by ether inhalation and killed by cervical dislocation. Briefly, as previously reported (Stefani *et al*., 1994, 1995) the sensori-motor neocortex was dissected under stereomicroscope from coronal slices 350–400 μM thick. Slices were incubated in a HEPES buffered Hank's balanced salt solution (HBSS), bubbled with 100% O_2 and warmed at 35°C. From 30–40 min later, one slice was transferred in HBSS media added by 1.5 mg ml^{-1} protease XIV. After 30–40 min of enzymatic treatment, the tissue was rinsed in HBSS and mechanically triturated. The cell suspension was then placed in a Petri dish mounted on the stage of an inverted microscope. Cells were allowed to settle for 10–12 min. Cortical neurones were chosen for recordings if presumed to be large pyramidal-shaped cells, usually with a typical apical process spared by the brief enzyme incubation time and capacitance not <8 pF.

Patch-clamp recordings in the whole-cell configuration (Hamill *et al*., 1981) were performed using pipettes (Corning 7052) pulled at a Flaming-Brown and fire-polished just prior to use. Pipette resistance ranged from 3–8 MOhms when filled by the internal solution consisting of (in mM): N-methyl-D-glucamine 185, HEPES 40, EGTA 11, Mg 4, phosphocreatine 20, ATP 2–4, GTP 0–0.2, leupeptin 0.2; pH was adjusted to 7.3 with phosphoric acid; the osmolarity was 275–280 mOsm/L. After obtaining the cell access, the neurone was usually bathed in a medium composed of (in mM): TEACl 165, $BaCl_2$ 2.5–5, CsCl 5, HEPES 10; pH was adjusted to 7.4 and the osmolarity to 300–305 with glucose. Only in a subset of recordings ($n=20$) NaCl 135–140 substituted TEA as the main cation (in this situation TTX 0.001 mM was added). Control as well as drug solutions were applied with a linear array of six, gravity-fed capillaries positioned within 500 μm of the patched neurone. This system allowed drugs to be applied and washed within seconds at well-defined concentrations (complete solution exchange may be achieved in less than 5 s). Recordings were made with an Axopatch 1D at room temperature (21–22°C). Series resistance compensation (70–80%) was routinely employed. Data were low-pass filtered (corner frequency = 5 kHz). For data acquisition and analysis pClamp 5.51 running on a PC Pentium was used. Barium (Ba^{2+}) currents were studied with voltage steps and ramps. Ramp speed (0.3–0.6 mV/ms) was chosen to maximise the agreement with the current/voltage relationship obtained with this method and the one derived from short (30 ms) step depolarization. Data are given as mean $\pm$ s.d. Student *t*-test was routinely applied (one-tail).

All compounds were obtained by Sigma. Nifedipine-containing solutions were light protected. 619C89 was synthesized in the University of Greenwich and was provided by Dr M.J. Leach.

Results

Voltage gated Ca^{2+} currents were recorded in the standard whole-cell configuration with Ba^{2+} as the current carrier. Voltage ramps from holding potential (Vh) = $-70/-60$ mV were usually applied (Figure 1A). This protocol activates high-voltage-activated (HVA) Ca^{2+} currents through different subtypes of Ca^{2+} channels, namely L, P/Q and N, whilst the contribution of low-voltage-activated conductance is negligible (Lorenzon & Foehring, 1995; Sayer *et al*., 1990; Stefani *et al*., 1996b). The neuroprotective compound 619C89 (0.1–100 μM) inhibited the whole cell Ca^{2+} current in all but one of the cells studied ($n=105$): a typical inhibition is shown in Figure 1A. Recovery from block was not always entirely complete (about 95% of the pre-drug value in Figure 1A). In this regard, Figure 1B presents six exemplary recoveries in different neurones; an average of these 'wash current' ($-92.4\% \pm 5.4$; $n=6$), if compared to six control recordings of analogous duration ($99.1\% \pm 2.85$, $n=6$; data not shown) showed modest significance ($P=0.064$). No consistent desensitization of the response was, however, observed.

As described by Figure 1C, 619C89 has a rapid initial action which progresses further to a steady state inhibition. A stable degree of inhibition is reached over about 30–60 s in the present experimental paradigm (ramp-activated currents at 0.2 Hz). In the depicted example (Figure 1C–D), full inhibition was reached with $\tau=49$ s (as the fitting of Figure 1D, which details the portion between arrows of Figure 1C, reveals). An average of the time constant of block, obtained in ten different cells ($n=10$), was calculated at 31.6 s (data not shown), well above commonly described Ca^{2+} current block in our preparation (Stefani *et al*., 1994, 1996b).

Block by 619C89 was dependent on command voltage, as in previous work on voltage dependent Na^+ channels (Xie & Garthwaite, 1996) and N-type Ca^{2+} channels (McNaughton *et al*., 1997). The fractional blocking efficacy of the drug (applied at a concentration of 10 μM) is greater at more positive ramp voltages (Figure 2C and F). The degree of block by a given concentration of the drug also increases when the cell is maintained at less negative holding voltages (not shown).

By studying 619C89-mediated inhibition with voltage commands instead of ramps (Figure 2) it also clearly emerges that, differently from standard Ca^{2+} channel modulators, known to interfere with G-protein-mediated mechanisms (Choi & Lovinger, 1996; Stefani *et al*., 1998), 619C89 did not slow activation kinetics (Figure 2A–B), nor was its effect decreased by the interposition of a depolarizing prepulse. This is clearly shown in Figure 2F and G, in which the drug-induced inhibition of 'facilitated' currents is even larger than the standard unfacilitated ones.

Blockade of the peak ramp-activated Ba^{2+} current by 619C89 yields a clear concentration-inhibition curve (Figure 3). Substantial inhibition (-13%) was described for concentrations as low as 0.5 μM. Saturating concentrations were at about 50 μM. A standard hyperbolic relation fitted to the inhibition data in the micromolar range has an IC_{50} of 7.4 μM and a Hill coefficient of 1.2 (Figure 3). As shown previously (Figures 1, 2 and 3), 50–100 μM 619C89 blocks up to 90% of the whole cell Ba^{2+} current. This finding implies that at high concentrations the drug is not selective for a particular Ca^{2+} channel type, but inhibits all (or the vast majority) of HVA Ca^{2+} channels. To address this aspect of the drug's pharmacology, we have utilized commonly used Ca^{2+} channel blockers.

As illustrated by Figure 4, none of the utilized antagonists succeeded in occluding the 619C89-induced inhibition. The experiment shown in Figure 4A and B clarifies that the difference of inhibition between control currents (A) and ω-CgTxGVIA-treated currents (B) is negligible (-81 vs -77%). Even the addition of P- as well as Q-type channel blockers (20 nM ω-AGA-IVA, putatively selective for P-like channels, and 2 μM ω-CgTxMVIIC) did not provide substantial antagonism (Figure 4C). In Figure 4D are presented the pooled observations performed with N-, P/Q- and L-type channel blockers.

The fractional inhibition to 50 μM 619C89 were: (i) 87.2% in control (± 6.9, $n=4$) and 85.9% after 2 μM ω-CgTxGVIA

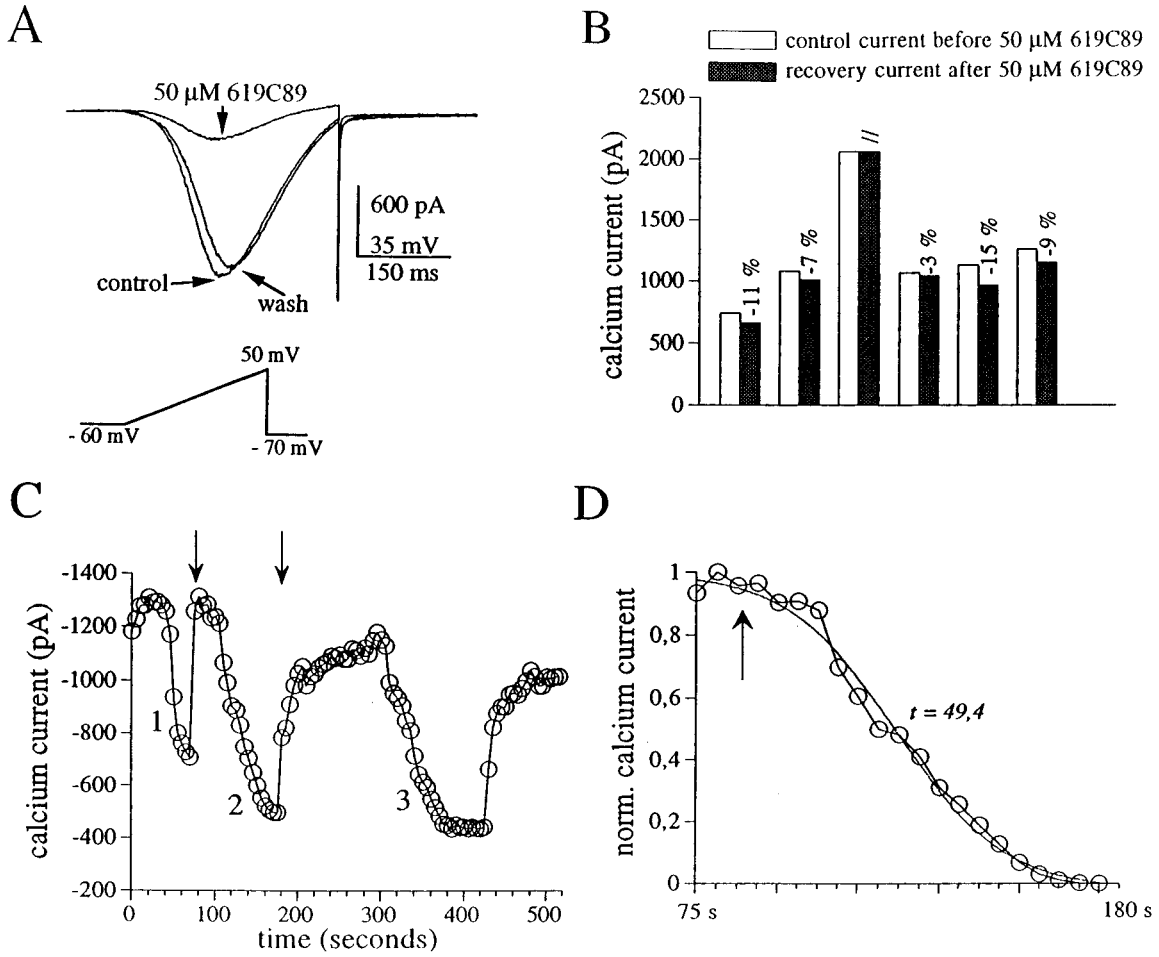

Figure 1 619C89 inhibits native Ca^{2+} currents in pyramidal neurones. (A) an exemplary inhibitory effect on ramp-activated currents. (B) six different 'recovery current', after 50 μM 619C89, in six cortical cells; the average wash is close to 93%. (C) in a different neuron, the current vs time histogram describes repeated application of 619C89. Noticeably, the response reached steady state rather slowly. The calculated time constant, in this experiment, was 49 s, as shown in D (detail of the current in C -between arrows-, after normalization). τ was estimated by the following eq.: $m3+((1-m3)/(1+\exp((m0-m1)/m2)))$.

($\pm$8; $n=4$; $P=0.146$); (ii) 87.5% in control ($\pm$6.1, $n=4$) and 83.8% in the combined presence of N/P/Q channel blockers ($\pm$9.5, $n=4$; $P=0.062$); (iii) 88.7% before the dihydropyridine (DHP) antagonist nimodipine ($\pm$2.5, $n=5$) and 89.7% in the presence of 5 μM nimodipine ($\pm$4.5, $n=5$; $P=0.38$). The possibility that our Ca^{2+} channel blockers were not working properly was ruled out by different findings: (a) the percentage block by each channel antagonist was in good agreement with our and others' previous findings (Choi & Lovinger, 1996; Lorenzon & Foehring, 1995; Stefani *et al*., 1996b); (b) the same stock of ω-CgTx was proven to be effective in that it largely prevented the mGluR-mediated modulation (data not shown, see, however, Stefani *et al*., 1998); (c) nifedipine, and not only nimodipine, was also tested (with similar results).

Cadmium ions (Cd^{2+}) are potent inhibitors of all HVA Ca^{2+} channel types and are believed to behave as 'open-channel' blockers, binding within the open pore lumen and preventing flux of permeative ions (Hille, 1984). The IC_{50} for this action is usually in the 200 nM$-$3 μM range (depending on the external concentrations of divalent). Here, a single binding equation fitted the concentration of 165 and 241 nM (with 5 mM Ba^{2+} as the charge carrier) and 2 μM (with 20 mM Ba^{2+}) (data not shown). The presence of 10 μM 619C89, however, modified the apparent potency of Cd^{2+}, shifting the fractional concentration-inhibition curve to the right, thus reducing the apparent IC_{50} of Cd^{2+} (the average of the three experiments is illustrated by Figure 5A). This suggests that the binding site for 619C89 (in the micromolar concentration range) either is or interacts with the binding site for Cd^{2+} ions. Thus, at concentrations above 1 μM, 619C89 may itself be an open-channel blocker.

This possibility is supported by the observation that 619C89 has a slightly lower blocking potency against currents recorded with 20 mM extracellular Ba^{2+} than it does against currents driven by 2.5 mM Ba^{2+} (Figure 5B). In particular, 10 μM 619C89 inhibited 61.1% current in 2.5 mM Ba^{2+} ($\pm$9.6; $n=6$) whilst the same drug concentration inhibited 32.3% current in 20 mM Ba^{2+} ($\pm$12.4; $n=6$; $P=0.003$).

Discussion

In recent years, we have tested to what extent old or new anticonvulsants may interfere with HVA Ca^{2+}-gated currents in rat cortical neurones (Calabresi *et al*., 1995, 1996; Stefani *et al*., 1995, 1996a,b); furthermore, we have hypothesised that such an effect may underlie the efficacy of these molecules in neuroprotective strategies (Stefani *et al*., 1997a).

The present report demonstrates that the neuroprotective agent 619C89 inhibits native HVA Ca^{2+} channels in rat

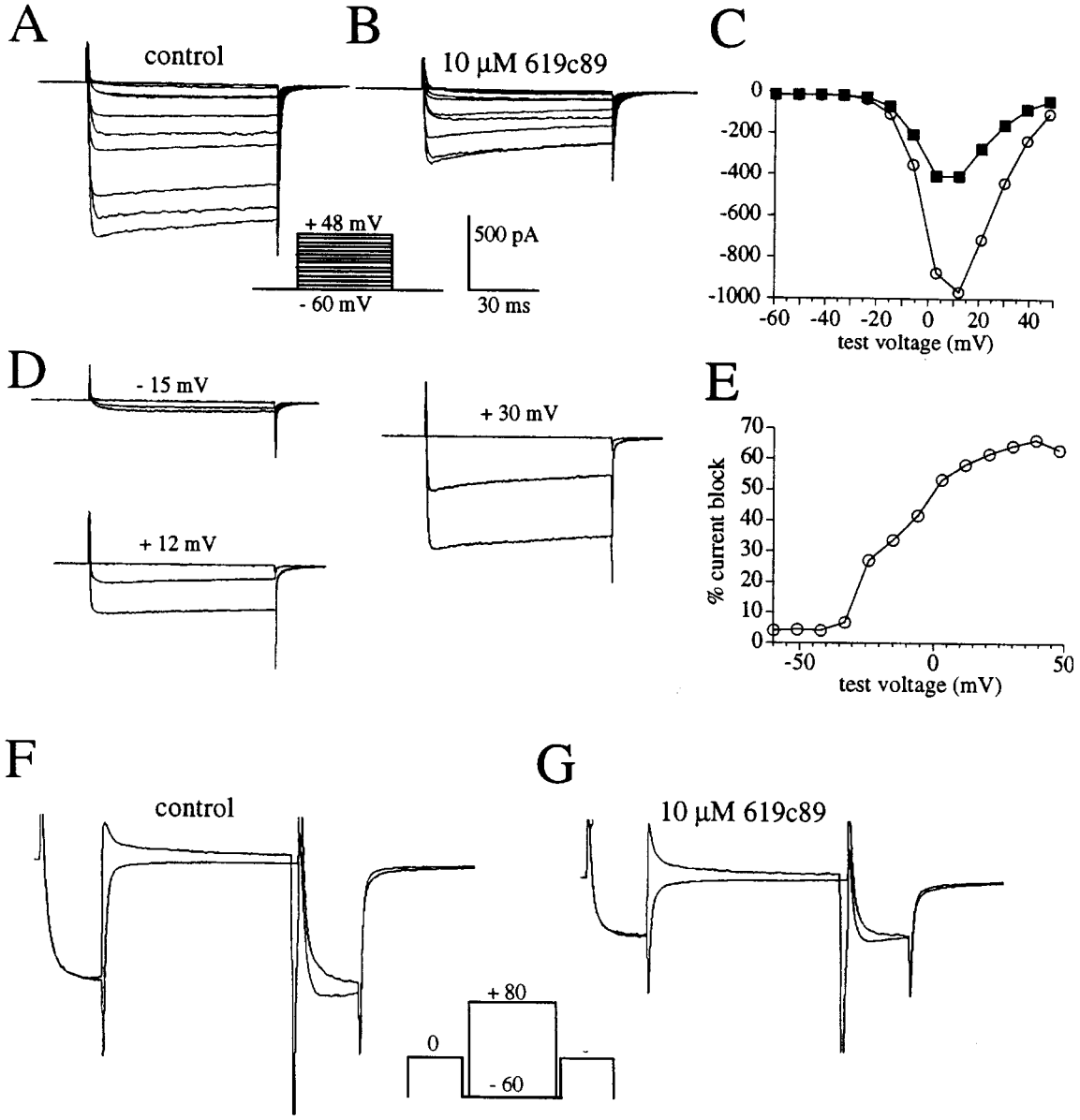

Figure 2 Increased voltages augment 619C89-induced inhibition at sub-saturating concentrations. (A–B) the large inhibition by 10 μM 619C89, when studied on test-activated currents, reveals the preferential block of not-inactivating portion. (C) the current vs test voltage relation (constructed by peak steady state currents in A and B) demonstrates that the 619C89 response is larger at positive potentials. (D) single raw traces from A and B are selectively shown, to further illustrate the voltage-dependence of the block. (E) the % current block by 619C89 is plotted against test potential. (F–G) no substantial reduction of drug-mediated inhibition is obtained by facilitation protocols.

cortical pyramidal neurones, with similar potency to that observed in cloned N-type channels (McNaughton *et al.*, 1997). As we observed, however, 619C89, at concentrations of 50 μM or above, blocked more than 90% of the HVA Ca^{2+} currents. Moreover, none of the selective blockers (nor a 'cocktail' of N- P- and Q-like channel blockers) succeeded in limiting substantially the drug response. Therefore, the drug is clearly not selective for N-type channels; instead, it inhibits all the whole-cell HVA Ca^{2+} current subtypes elicited in our preparation, which is composed of approximately 35% L-type, 25% N-type and 40% others (P, Q, R). Intriguingly, 619C89 blocks the total HVA Ca^{2+} current with a concentration-inhibition relation that appears to be a monotonic curve, suggesting that the binding site for 619C89 is common to all channel types present, with similar efficacy in all channels. The Hill coefficient for the hyperbolic curve of best fit of the data is close to unity (1.2) implying a 1:1 interaction between drug and channel molecules.

We have found that from a holding voltage of $-60/-70$ mV, the IC_{50} for blockade of the peak ramp-activated Ca^{2+} current by 619C89 is about 8 μM. This is close to the value obtained for cloned N-type channels (16 μM; McNaughton *et al.*, 1997) under similar conditions (Vh = -90 mV).

Furthermore, the drug's potency was similar to what described against both native neuronal Na^+ channels and cloned type IIA Na channels, as shown recently by Xie & Garthwaite (IC_{50} at -90 mV = 50 μM in Xie & Garthwaite, 1996). This finding supports the hypothesis that, indeed, the inhibition of HVA Ca^{2+} currents is one of the main mechanisms of action by which this agent exerts its

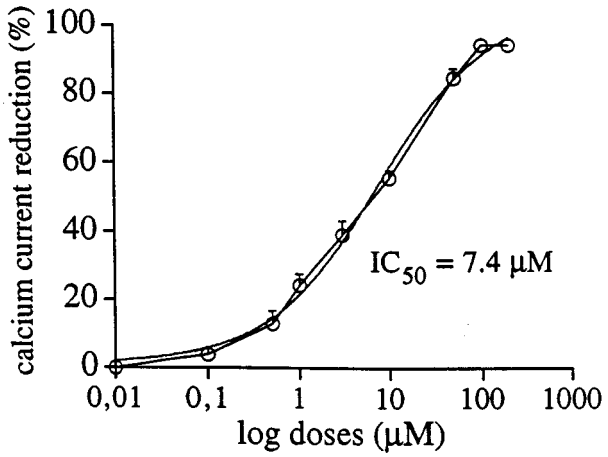

Figure 3 Dose-response of 619C89-mediated inhibition of cortical Ca^{2+} currents. The graph illustrates the % inhibition by different concentrations of 619C89. Note that substantial blockade is produced by concentrations as low as 0.5 μM. Each point represents the average of six experiments. Fitting was done with the following equation: m3 = (m0^m1/m0^m1 + m2^m1). IC_{50} = 7.4 μM.

neuroprotective efficacy. Differently from the fast occurring inhibition of Na^+ currents, the 619C89-induced blockade of Ca^{2+} currents develops quite slowly, reaching a steady state in about 40 s. This finding implies a sort of sequential, but independent, occurrence of Na^+ and Ca^{2+} current blockade *in vivo*, being the full inhibition of Ca^{2+} currents completed when the Na^+-driven excitability has been already dampened. Whether the slow kinetics of the Ca^{2+} current blockade derives from a complex interaction with the channel itself or is dependent upon a redistribution of intracellular Ca^{2+} stores, is hard to say. The latter hypothesis, however, seems unlikely, considering the constant inclusion, in our dialysing solution, of 11 mM EGTA.

On the other hand, we have provided evidence for competition between 619C89, Ca^{2+} and Ba^{2+} ions at a binding site on the Ca^{2+} channel, which in turn strongly suggests that 619C89 interacts directly with the pore lumen. The 619C89 molecule has two pKa values of 4.2 and 7.7, owing to protonatable nitrogen groups. At pH 7.4 roughly 66% of the molecules will be in monovalent cationic form, with a

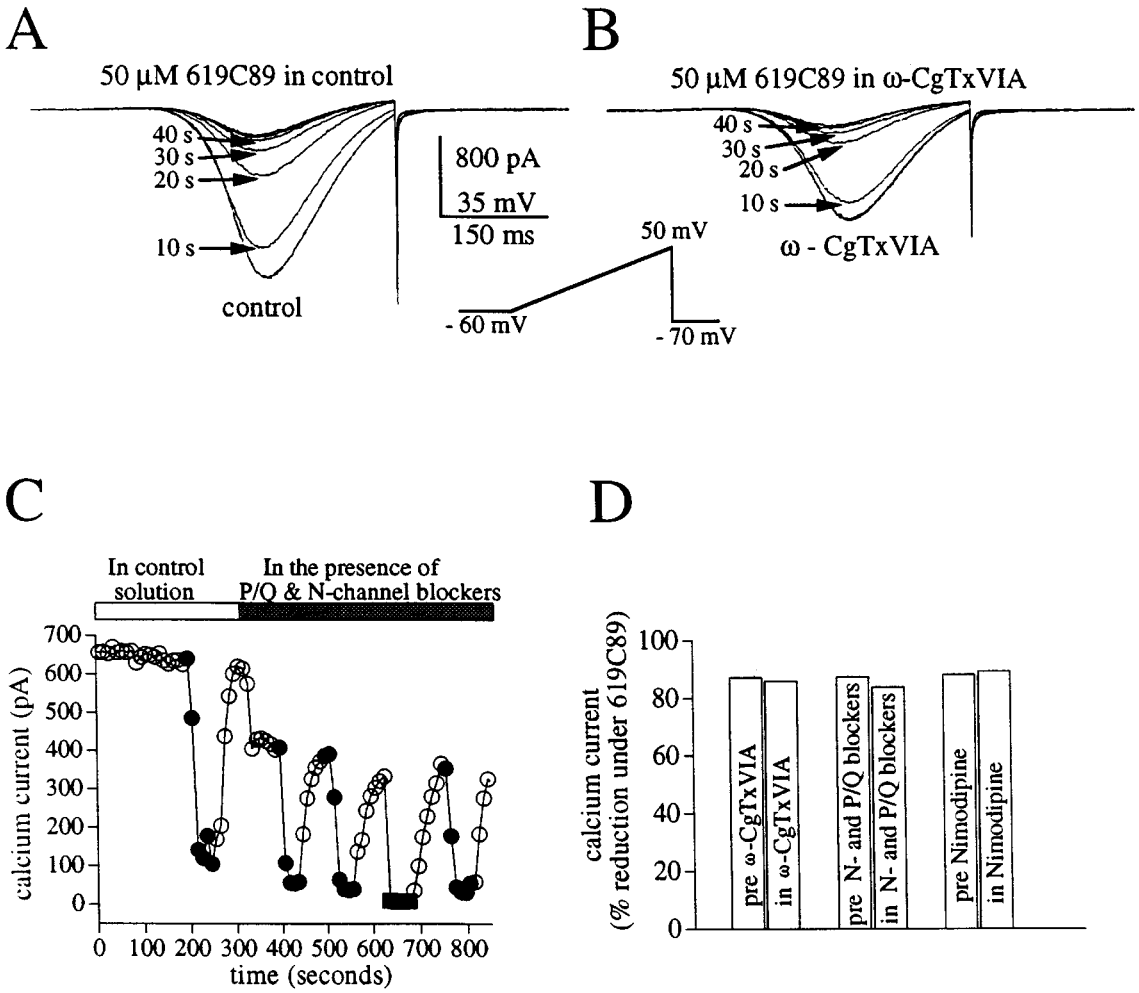

Figure 4 The inhibition by 619C89 is not sensitive to Ca^{2+} channel blockers. (A–B) shown are exemplary ramp-activated Ca^{2+} currents before (−81%, A) and after the addition of 2 μM ω-CgTxGVIA (−79%, B). Note the progressive increase of the drug-induced block which is complete in about 40 s (in both experimental conditions). (C) time-course of 619C89-mediated responses (filled round symbols) in the absence of any Ca^{2+} channel blocker (control) and in the presence of N-*plus*P-*plus* Q-like Ca^{2+} channel blockers. The combination of these Ca^{2+} channel blockers has dampened Ca^{2+} current by >50%; yet, the 619C89-mediated inhibition is not prevented. Note the brief Cd^{2+} application (filled squared symbols, instantaneous block). (D) cumulative histogram showing the inefficacy of any (or combination of) of the utilized blockers. Shown are the averaged inhibitions to 50 μM 619C89 before and after 2 μM ω-CgTx (n = 4, respectively −87.2% and −85.9%); before and after the combined application of blockers (n = 4, respectively −87.5% and 83.8%); before and after 5 μM nimodipine (n = 5, respectively −88.7% and −89.7%).

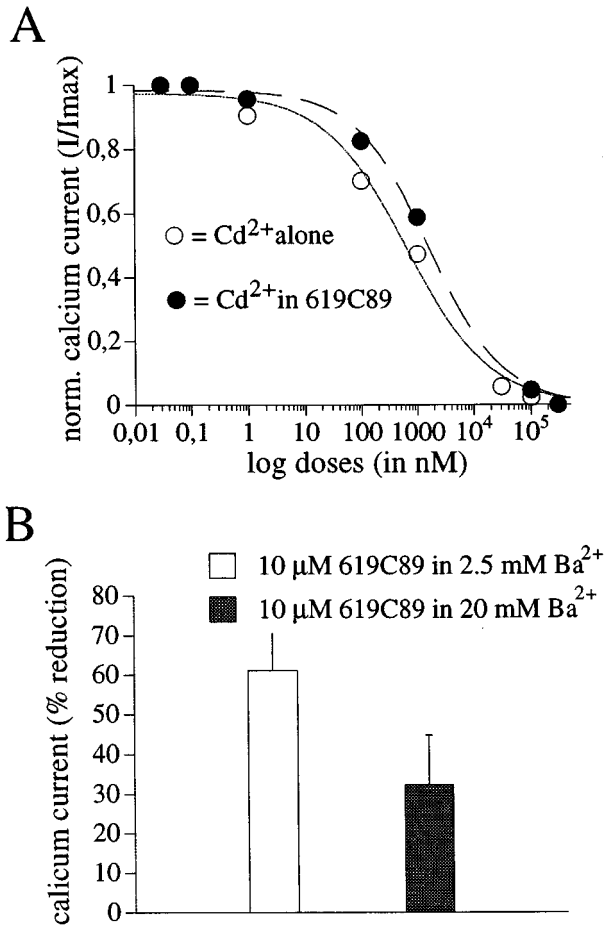

Figure 5 (A) the sensitivity to Cd^{2+} block is studied in absence (empty symbols) and in the presence (filled symbols) of 619C89. Data points represent the average of Ca^{2+} current in three experiments (see text). IC_{50} is shifted from 655–1505 nM by the lamotrigine analogue. (B) the histogram describes the % inhibition by 10 μM 619C89 in different concentrations of the charge carrier. Note the large difference in inhibition among 2.5 Ba^{2+} (−61.1%) and 20 mM Ba^{2+} (32.3%).

protonated amine group. The involvement of this group in the compound's blocking action remains to be tested.

'Mixed antagonists' with inhibitory potency against both voltage activated Na^+ channels and HVA Ca^{2+} channels are attracting increasing attention as potential neuroprotective therapies (Leach & Swan, 1996; ONeill *et al*., 1997a,b; Stefani *et al*., 1997a,b). Both blocking effects are likely to reduce neuronal Ca^{2+}entry, both pre- and post-synaptically, and also to reduce excessive glutamate release. When considering the pathogenesis of ischaemia, however, it is actually under scrutiny the real prominence of an abnormal glutamate transmission as an important factor of damage. Given the relevant role played by the reversal of the electrogenic, Na^+/Ca^{2+}-independent glutamate transporters and/or by the 'metabolic' source of glutamate, some studies have consistently doubted that the release of excitatory amino acids in the first phases of anoxia is largely dependent upon the activation of inward currents in the axon terminals (Hossman, 1994; Wahl *et al*., 1994; Urenjak & Obrenovitch, 1996). In other words, the initial emphasis on the so-called 'glutamate release blockers' (Graham *et al*., 1993; Meldrum *et al*., 1994) was largely disputed (Obrenovitch & Richards, 1995; Wahl *et al*., 1994; Urenjak & Obrenovitch, 1996). Nevertheless, the concomitant inhibition of Na^+ and Ca^{2+} currents at sites other than the axon terminals, namely somatodendritic sites, would finally limit neuronal excitability and thus reduce the energy demands of injured areas (Erecinska & Silver, 1989). Therefore, the exact impact of 619C89 or analogous molecules on the presynaptic mechanisms controlling glutamate release *in vivo* (Takahashi & Momiyama, 1993) may not *per se* represent the crucial parameter of efficacy.

It is worth considering that the clinical utilization of agents, such as 619C89, with putative multiple mechanisms of actions, might be biased by the occurrence of less predictable side effects. In particular, the potency of 619C89 in inhibiting HVA Ca^{2+} currents, together with the incompleteness of recovery (although almost negligible) raises the possibility that, *in vivo*, long-lasting suppression of 'physiological' neurotransmisison may take place. Interestingly, however, 619C89 showed consistent efficacy also at rather low concentrations (low micromolar), which in fact should be initially tested in clinical protocols designed for humans.

In conclusion, we find that the neuroprotective compound 619C89 is a potent, rapidly reversible inhibitor of all classes of native neuronal Ca^{2+} channels. Its half-maximal concentration is close to 10 μM, in keeping with previous studies and its binding site is or interacts with the channel lumen. This inhibitory action is likely to be an important factor in the compound's neuroprotective action.

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