Multiple Mechanisms Underlying the Neuroprotective Effects of Antiepileptic Drugs Against In Vitro Ischemia

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Background and Purpose—The possible neuroprotective effects of classic and new antiepileptic drugs on the electrophysiological changes induced by in vitro ischemia on striatal neurons were investigated. In particular, the aim of the study was to correlate the putative neuroprotective effects with the action of these drugs on fast sodium (Na+) and high-voltage–activated (HVA) calcium (Ca2+) currents.

Methods—Extracellular field potentials were recorded from rat corticostriatal brain-slice preparations. In vitro ischemia was delivered by switching to an artificial cerebrospinal fluid solution in which glucose and oxygen were omitted. Na+ and HVA Ca2+ currents were analyzed by whole-cell patch-clamp recordings from acutely isolated rat striatal neurons. Excitatory postsynaptic potential was measured following synaptic stimulation in corticostriatal slices by sharp intracellular microelectrodes.

Results—Neuroprotection against in vitro ischemia was observed in slices treated with carbamazepine (CBZ), valproic acid (VPA), and topiramate (TPM), whereas it was not achieved by using levetiracetam (LEV). Fast Na+ conductances were inhibited by CBZ and TPM, whereas VPA and LEV showed no effect. HVA Ca2+ conductances were reduced by CBZ, TPM, and LEV. VPA had no effect on this current. All antiepileptic drugs induced a small reduction of excitatory postsynaptic potential amplitude at concentrations higher than 100 μM without changes of paired-pulse facilitation.

Conclusions—The concomitant inhibition of fast Na+ and HVA Ca2+ conductances is critically important for the neuroprotection, whereas the presynaptic inhibition on glutamate transmission does not seem to play a major role. (Stroke. 2006;37:1319-1326.)

Key Words: antiepileptic drugs □ Ca2+ □ electrophysiology □ ischemia □ Na2+
Materials and Methods

Animals for Electrophysiological Recordings

Wistar rats, 4 to 6 weeks of age (Charles River, Calco (MI), Italy), were used for both extracellular and intracellular recordings in slices as well as for whole-cell patch-clamp recordings in acutely dissociated neurons, in accordance with European Communities Council Directive (86/609/EEC).

Extracellular and Intracellular Recordings

The composition of the Krebs’ solution for the slices was (in mmol/L) 126 NaCl, 2.5 KCl, 1.3 MgCl₂, 1.2 NaH₂PO₄, 2.4 CaCl₂, 10 glucose, 18 NaHCO₃. In the chamber, temperature was maintained at 34°C. Before the application of the in vitro ischemia, the pH of the extracellular solution was 7.4. In vitro ischemia was delivered by switching for 10 minutes to an artificial cerebrospinal fluid solution where sucrose replaced glucose, gassed with 95% N₂ and 5% CO₂.

Figure 1. AEDs show differential neuroprotective effects against in vitro ischemia in corticostriatal brain slice preparations. A,B,C,D, Time-course of the ischemia-induced changes of field potential amplitude in control condition and in the presence of 100 µmol/L CBZ, 300 µmol/L VPA, 100 µmol/L TPM, and 100 µmol/L LEV, respectively. E, Single experiments showing the changes of field potential amplitude after ischemia in control medium (upper traces) and in the presence of various AEDs (lower traces). For each experimental condition n=12.
Electrodes for extracellular recordings (15 to 20 mol/L \( \text{NaCl} \)) were filled with 2 mol/L \( \text{NaCl} \). An Axoclamp 2B amplifier (Axon Instruments) was used for extracellular recordings. The field potential amplitude was defined as the average of the amplitude from the peak of the early positivity to the peak negativity, and the amplitude from the peak negativity to peak late positivity. Quantitative data on modifications induced by ischemia are expressed as a percentage of the control values, the latter representing the mean of responses recorded during a stable period (15 to 20 minutes) before the ischemic phase.

Intracellular recordings from single striatal spiny neurons were obtained from corticostriatal slices. An Axoclamp 2B amplifier was used for conventional microelectrode recordings from brain slices. Intracellular sharp electrodes were filled with 2 mol/L \( \text{KCl} \) (30 to 60 mol/L \( \text{H}_{2}\text{O} \)). All AEDs were applied 15 minutes before the onset of the in vitro ischemia solution and maintained through all the experiment. The application of these drugs did not significantly alter per se the amplitude of the field potential amplitude. Values given in the text and in the figures are expressed as percent of control.

### Acutely Dissociated Neurons

Coronal slices from striatum were incubated in Hepes-buffered Hank's balanced salt solution (HBSS), bubbled with pure oxygen, before being prepared for recordings.\textsuperscript{14,15} Then, immediately before recordings a single striatal slice was incubated in HBSS media with 0.5-mg/mL protease XIV added. After 30 minutes the tissue was repeatedly washed in HBSS and mechanically triturated with a graded series of fire-polished Pasteur pipettes.\textsuperscript{14,15} The obtained supernatant was placed in a Petri dish mounted on the stage of an inverted microscope (Nikon Diaphot, Japan).

#### Whole-Cell Patch-Clamp Recordings

Current measurements were obtained by patch-clamp technique in conventional whole-cell patch-clamp technique.\textsuperscript{14,15} Patch-clamp recordings in the whole-cell configuration were performed using fire-polished pipettes (WPI PG52165-4) pulled at a Flaming-Brown.\textsuperscript{14,15} Pipette resistance ranged between 6 and 9 MOhm. Usually room temperature was used. Extracellular and dialyzing solutions were prepared in order to separate effectively Na\textsuperscript{+} or Ca\textsuperscript{2+} currents.\textsuperscript{14,15}

When Na\textsuperscript{+} and Ca\textsuperscript{2+} currents were investigated the internal solution composed respectively by (in mmol/L): N-methyl-D-glucamine 185, Hepes 40, ethylene glycol tetraacetic acid 11, MgCl\textsubscript{2} 4, CaCl\textsubscript{2} 0.2; (for Na\textsuperscript{+} currents) and by N-methyl-D-glucamine 185, Hepes 40, ethylene glycol tetraacetic acid 11, MgCl\textsubscript{2} 4, (for Ca\textsuperscript{2+} currents). Both the solutions finally added (in the working solution daily prepared), by phosphocreatine 20, ATP 2 to 3, GTP 0 to 0.2, leupeptin 0.2; the osmolarity was 264 to 270 mOsm/L (pH 7.3 adjusted by phosphoric acid). Na\textsuperscript{+} currents were recorded in presence of an external solution for Na\textsuperscript{+} currents consisted of (in mmol/L): TEA-Cl 100, Hepes 10, and BaCl\textsubscript{2} 5, MgCl\textsubscript{2} 1, NaCl 40, KCl 5, adjusted to pH 7.4 with NaOH (osmolarity 297 to 300 mOsm/L). Conversely, when HVA Ca\textsuperscript{2+} currents were examined, the neuron was usually bathed in a medium composed of (in mmol/L): TEA-Cl 165, CsCl\textsubscript{2} 5, Hepes-Na\textsuperscript{+} 10, and BaCl\textsubscript{2} 5 as the charge carrier; pH was adjusted to 7.35 to 7.45 and the osmolarity to ~300 mOsm/L. Recordings were made with an Axopatch 1D (Axon Instrument, USA). Series resistance compensation was routinely used (70% to 80%). Data were low-pass filtered (corner frequency=5 KHz). For data acquisition and analysis pClamp 9 running on PC was used.

Control and drug solutions were applied with a linear array of 6 gravity-fed capillaries positioned within 500 to 600 \textmu m of the patched neuron. Data analysis was performed off-line using Microcal Origin and Graphpad Prism softwares running on PC.

### Statistical Analysis

Values given in the text and in the figures are mean±SEM of changes in the respective cell populations. The evaluation of statistical difference was performed with 2-way ANOVA test for the different population and with Student’s t test, for means and SEM. No
more than 2 slices from the same animal were used. Moreover, each slice was used only for a single electrophysiological experiment.

**Drugs**

Nimodipine, ω-conotoxin GVIA (ω-CTX GVIA), ω-conotoxin MVIIC (ω-CTX MVIIC) and ω-agatoxin IVA (ω-ATX IVA) were from Alomone Labs (Israel). CBZ and VPA were from Sigma-Aldrich (Italy); LEV was from UCB-Pharma (Belgium); TPM was from Johnson & Johnson (USA).

**Results**

**Action of AEDs on Irreversible Loss of Field Potentials Induced by In Vitro Ischemia**

As previously reported, a period of in vitro ischemia (oxygen and glucose deprivation) lasting 10 minutes produced an irreversible loss of field potentials recorded in the striatum after the stimulation of glutamatergic fibers originating from cortical neurons. As shown in Figure 1A and 1E, during the incubation of the corticostriatal slices in 100 μmol/L CBZ the same period of in vitro ischemia induced only a transient suppression of the field potential that partially recovered after the replacement of the ischemic solution with control medium (n=12; P<0.0001 compared with control at 30 minutes after ischemia). Similar neuroprotective results were also detected in slices incubated in the presence of either 300 μmol/L VPA (n=12; P<0.0001 compared with control at 30 minutes after ischemia; Figure 1B and 1E) or 100 μmol/L TPM (n=12; P<0.0001 compared with control at 30 minutes after ischemia; Figure 1C and 1E). Conversely, the application of 100 μmol/L LEV did not cause the recovery of the field potential after the interruption of the ischemic period (n=12; P>0.05, compared with control; Figure 1D and 1E). The neuroprotective effects of CBZ, VPA, and TPM against in vitro ischemia were dose-related, and the EC50 was respectively 42 μmol/L, 221 μmol/L, and 45 μmol/L (n=12 for each drug and each concentration; Figure 2). The maximal neuroprotective effect was achieved by using 100 μmol/L CBZ, 300 μmol/L VPA, and 100 μmol/L TPM.

**Effect of AEDs on Voltage-Dependent Na⁺ Currents**

In order to address the possible cellular mechanisms underlying the differential neuroprotective profile of the various AEDs tested we analyzed their different effects on voltage-dependent fast Na⁺ currents. These experiments were performed by using whole-cell patch-clamp recording from isolated striatal neurons. Na⁺ currents were evoked by current steps ranging from the holding potential (−70 mV) to −20 mV. As shown in Figure 3, CBZ and TPM caused a significant inhibitory effect on Na⁺ currents with an IC50 of respectively 35 μmol/L and 40 μmol/L (n=11 for each drug and each concentration; P<0.001 for both drugs). The inhibitory effects of CBZ and TPM were reversible on drug
washout, at all the concentrations tested. Neither VPA nor LEV showed significant inhibitory effects on Na⁺/H1₁₀₀₁ currents (n₁₁; P₀.05 for both drugs).

Effect of AEDs on HVA Ca²⁺ Currents
By using whole-cell patch-clamp recording from isolated striatal neurons, we also investigated the action of AEDs on HVA Ca²⁺ currents. Barium currents were activated by ramps or step pulses.¹⁵ The holding potentials ranged from −70 mV to +40 mV for the ramps, and from −10 mV to +10 mV for the tests. Under these conditions, Ca²⁺ currents were dominated by HVA components.¹⁶ As shown in Figure 4, CBZ, TPM, and LEV significantly inhibited these currents (P₀.001 for all 3 drugs). The IC₅₀ was respectively 21 µmol/L, 10 µmol/L, and 22 µmol/L (n=12 for each drug and each concentration). The inhibitory effects of CBZ, TPM, and LEV were reversible on drug washout, at all the concentrations tested. VPA did not show significant inhibitory effects on Na⁺/H1₁₀₀₁ currents (n=11; P>0.05 for both drugs).

Distribution and Pharmacological Modulation of HVA Ca²⁺ Channels by AEDs
As shown in Figure 5A, we identified Ca²⁺ channel subtypes in putative striatal spiny neurons on the basis of their differential sensitivity to drugs and toxins: 5 µmol/L nifedipine (L-type, n=11), 1 µmol/L ω-CTX GVIA (N-type, n=13), 100 nM ω-ATX IVA (P-type, n=12), 100 nM ω-CTX MVIIC (Q-type, n=9). A cocktail of all these toxins revealed a resistant component of Ca²⁺ currents (R-type, n=10). We also analyzed the effect of 100 µmol/L CBZ, 100 µmol/L LEV, and 100 µmol/L TPM on the different subtypes of Ca²⁺ channels. Figure 5B shows that CBZ reduced both L- and Q-type Ca²⁺ currents. L-type Ca²⁺ currents were also inhibited by TPM. In addition, this AED reduced P-type Ca²⁺ currents. LEV did not affect L-type currents, whereas it reduced both N- and P-type currents.

Effects of AEDs on Excitatory Synaptic Potentials and Paired-Pulse Facilitation
Intracellular recordings were obtained from 40 electrophysiologically identified striatal spiny neurons. The main features of these cells have been previously described:¹³ resting membrane potential (−85±6 mV) and input resistance (39±9 mol/LΩ).

The four AEDs produced a small inhibitory effect on the amplitude of excitatory synaptic potentials (n=12 for each drug; P<0.05 at concentrations higher than 100 µmol/L; Figure 6A).

In order to investigate whether the small depression of excitatory postsynaptic potentials (EPSPs) was dependent on
pre- or postsynaptic sites of action,\textsuperscript{17} we measured synaptic responses to a pair of stimuli before and during the applications of these two AEDs. The depression of the EPSP amplitude induced by CBZ, VPA, TPM, and LEV was not associated with a significant increase in this ratio (n\(=\)10; \(P\textsuperscript{\text{\(>\)0.05}}\) for each drug; Figure 6B), ruling out a pure presynaptic site of action.

**Discussion**

The present study represents the first effort to analyze the mechanisms underlying the potential neuroprotective action of several AEDs by using a combined approach including the analysis of \(\text{Na}^+\) and \(\text{Ca}^{2+}\) currents as well as the measurement of glutamate-mediated synaptic potentials. Accordingly, the modulation of these electrophysiological events seems to play a role also in the mechanisms underlying the antiepileptic action of tested drugs.\textsuperscript{4} This electrophysiological analysis was performed in the striatum, a brain area selectively vulnerable to ischemia.\textsuperscript{2,3,6} Moreover, the selected neuronal subtype was the striatal GABAergic projecting spiny neuron. This cell, in fact, represents the large majority of striatal neurons (\(\approx95\%\)) and shows the highest sensitivity to energy deprivation among the various striatal neuronal subtypes.\textsuperscript{2,3,6,13}

In the present study we found that CBZ, VPA, and TPM exert neuroprotective effects, allowing a partial recovery of the field potential recorded from striatal slices toward control levels. Conversely, LEV did not show this neuroprotective effect.

Interestingly, whereas the neuroprotective effect of CBZ and TPM was achieved at concentrations ranging between 10 and 100 \(\mu\text{mol/L}\), the action of VPA was seen at higher concentrations. In fact, it is reasonable to assume that for both CBZ and TPM the concentrations able to rescue the field potential amplitude and to modulate \(\text{Na}^+\) and \(\text{HVA Ca}^{2+}\) conductances are comparable to the range of therapeutic cerebrospinal and free serum levels in epileptic patients.\textsuperscript{18,19}
This latter observation may have profound clinical implications suggesting that these 2 AEDs could be used as possible neuroprotective agents in patients without major adverse effects.

The concentrations of VPA required to rescue the field potential (300 μmol/L) were much higher than those reported to be effective and safe in these patients.20 Moreover, VPA, at all the tested doses, failed to affect both fast Na⁺ and HVA Ca2⁺ conductances in striatal spiny neurons.

It has been recently reported that VPA reduces brain damage and improves functional outcome in a transient focal cerebral ischemia model in rats by modulating caspase activity.21 Thus, we can argue that neuroprotective action of VPA requires repeated treatment and probably involves apoptotic rather than necrotic mechanisms.22,23

The lack of neuroprotective effect by LEV is rather surprising. It has been shown, in fact, that this drug reduces the ischemia-induced brain damage in vivo24 and is neuroprotective against kainate-induced toxicity.25 Moreover, the present study, as well as previous works,26,27 has shown that LEV reduces HVA Ca2⁺ currents, one of the major target of putative neuroprotective therapies.28–30 Conversely, we found that LEV, in line with a previous study,31 was unable to reduce fast Na⁺ currents. Moreover, we also found that whereas both TPM and CBZ significantly reduced L-type Ca2⁺ currents (in addition to P- and Q-type Ca2⁺ currents, respectively), LEV decreased N-type but not L-type Ca2⁺ currents. Thus, although striatal spiny neurons express a variety of HVA Ca2⁺ channels,32 it is possible that the neuroprotective effects of TPM and CBZ are mainly explained by the concomitant reduction of L-type Ca2⁺ currents and of fast Na⁺ currents. This hypothesis requires further investigation by using selective inhibitors of specific channel subtypes as it has been previously demonstrated in hippocampal slices.33 Unfortunately, most of toxins used as specific channels inhibitors such as tetrodotoxin and conotoxins cause per se dramatic inhibitory effects on the field potential amplitude that require a long time to be washed out. This experimental limitation hampers their use, at least in our experimental model.

None of the tested AEDs had a relevant inhibitory action on the corticostriatal glutamatergic transmission because the observed reductions of EPSP amplitude were very small. In addition, these changes in EPSP amplitude were not associated with an increase in paired-pulse facilitation suggesting that significant presynaptic changes of glutamate release were not occurring during the application of the various AEDs.9,14,17

TPM blocks fast Na⁺ and HVA Ca2⁺ ion conductances and antagonizes glutamate receptor at non-N-methyl-D-aspartate receptors.34 This drug also potentiates GABA transmission.35 This latter effect might also contribute to the neuroprotective action of TPM seen both in vitro (present study) and in vivo.35 However, in vitro experiments on neuroprotection against ischemia have shown that GABAergic AEDs have bell-shaped dose-response curve10 that has never been obtained in the presence of TPM. Thus, it is unlikely that an increase of the GABAergic transmission is the prominent mechanism for the neuroprotective effect of TPM.

Conclusion

Our experiments, as well as previous in vitro studies, seem to suggest that the concomitant inhibition of fast Na⁺ and HVA Ca2⁺ conductances could be critically important for the neuroprotection induced by classic and new AEDs against the acute effects of ischemia. We have previously shown that in vitro ischemia induces both short-term and long-term effects on the amplitude of excitatory synaptic potentials.3,7 The analysis of possible effects of AEDs on the ischemia-induced changes of excitatory synaptic transmission will be matter of future studies.

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References

18. Lindberger M, Tomson T, Larsson AM. Microdialysis sampling of carbamazepine, phenytoin and phenobarbital in subcutaneous extracellular fluid
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