Biochemical and Electrophysiological Studies on the Mechanism of Action of PNU-151774E, A Novel Antiepileptic Compound


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ABSTRACT

PNU-151774E [(S)-(+)-2-(4-(3-fluorobenzyloxy)benzylamino)propanamide methanesulfonate], a new anticonvulsant that displays a wide therapeutic window, has a potency comparable or superior to that of most classic anticonvulsants. PNU-151774E is chemically unrelated to current antiepileptics. In animal seizure models it possesses a broad spectrum of action. In the present study, the action mechanism of PNU-151774E has been investigated using electrophysiological and biochemical assays. Binding studies performed with rat brain membranes show that PNU-151774E has high affinity for binding site 2 of the sodium channel receptor, which is greater than that of phenytoin or lamotrigine (IC50, 8 μM versus 47 and 185 μM, respectively). PNU-151774E reduces sustained repetitive firing in a use-dependent manner without modifying the first action potential in hippocampal cultured neurons. In the same preparation PNU-151774E inhibits tetrodotoxin-sensitive fast sodium currents and high voltage-activated calcium currents under voltage-clamp conditions. These electrophysiological activities of PNU-151774E correlate with its ability to inhibit veratrine and KCl-induced glutamate release in rat hippocampal slices (IC50, 56.4 and 185.5 μM, respectively) and calcium inward currents in mouse cortical neurons. On the other hand, PNU-151774E does not affect whole-cell γ-aminobutyric acid- and glutamate-induced currents in cultured mouse cortical neurons. These results suggest that PNU-151774E exerts its anticonvulsant activity, at least in part, through inhibition of sodium and calcium channels, stabilizing neuronal membrane excitability and inhibiting transmitter release. The possible relevance of these pharmacological properties to its antiepileptic potential is discussed.

Seizures are characterized by synchronous firing of neuronal populations which involves an influx of sodium and calcium ions and the release of excitatory neurotransmitters. Even if the action mechanisms of classic and new antiepileptic drugs (AED) are not fully understood, a common link often appears to be the modulation of voltage-dependent sodium and calcium channels (Rogawski and Porter, 1990; Meldrum, 1996). Clinically used AEDs such as phenytoin, carbamazepine, and possibly valproate, decrease high-frequency repetitive firing of action potentials by voltage- and use-dependent inactivation of sodium channels. Among the new AEDs, lamotrigine has potent sodium channel-blocking properties (Lang et al., 1993; Zona and Avoli, 1997). Other newly developed compounds, such as remacemide, topiramate, felbamate, and gabapentin also exert some activity on voltage-sensitive sodium channels (Meldrum, 1996). Blockade of calcium currents contributes to the mechanism of action of many of the classic AEDs such as phenytoin (Twombly et al., 1988), ethosuximide (Gross et al., 1997), and barbiturates (Gross et al., 1997).

Inhibition of ion channels is functionally related to inhibition of neurotransmitter release, particularly of glutamate. Phenytoin, carbamazepine, and lamotrigine, for example, have all been shown to inhibit glutamate release (Woodbury, 1980; Waldmeier et al., 1996). Glutamate plays a key role in the generation of epileptic seizures. It has been demon-

ABBREVIATIONS: AED, antiepileptic drugs; Asp, aspartate; EEG, electroencephalography; GABA, γ-aminobutyric acid; PNU-151774E, [(S)-(+)-2-(4-(3-fluorobenzyloxy) benzylamino) propanamide, methanesulfonate]; MAO, monoamine oxidase; SRF, sustained repetitive firing; TTX, tetrodotoxin.
strated that glutamate concentrations correlate with seizure generation in many animal models and that glutamate is elevated in human epileptogenic foci (Leach et al., 1986). Inhibition of glutamate release might therefore contribute to anticonvulsant properties of a new compound.

PNU-151774E [[(S)-(+)-2-(4-(3-fluorobenzyloxy) benzylamino) propanamide methanesulfonate] is a broad spectrum anticonvulsant with potency comparable or superior to that of most classic anticonvulsant drugs in electrically and chemically induced seizure models. It possesses a wide protective index and a low potential to induce tolerance, locomotor, or cognitive side effects (Peverarelo et al., 1998; Fariello et al., 1998). Moreover, PNU-151774E reduces both the electroencephalographic (EEG) and behavioral component of the electrically induced limbic after-discharges in cynomolgus monkeys (Salvati et al., 1996). PNU-151774E is also active in rat models of medically intractable complex partial seizures (Maj et al., 1995, 1998). Although possessing a broader spectrum of action (Fariello et al., 1998), PNU-151774E is similar to phenytoin and lamotrigine in several models. Thus, use-dependent inhibition of neuronal sodium and calcium channels may underlie its anticonvulsant effect.

In this series of experiments, we have investigated the mechanisms of action of PNU-151774E electrophysiologically and biochemically. In particular, the following studies were carried out: 1) general receptor binding profile; 2) patch-clamp whole-cell recordings of spontaneous neuronal firing carried out: 1) general receptor binding profile; 2) patch-clamp whole-cell recording of sodium and calcium currents using rat hippocampal neuronal cultures; 3) patch-clamp whole-cell recording of γ-aminobutyric acid- (GABA) and glutamate-induced currents in mouse cortical cultured neurons; 4) veratrine- and KCl-induced glutamate release from rat hippocampal slices; and 5) fluorescent imaging of KCl and veratridine-evoked calcium transients into mouse cerebellar granule neurons.

The results of these studies suggest that PNU-151774E exerts its anticonvulsant activity, at least in part, through inhibition of sodium and calcium channels. These effects are consistent with an ability to stabilize neuronal membranes and inhibit transmitter release.

Materials and Methods

Experimental Animals

Two-month-old male Wistar rats (Charles River, Italy) weighing 200 to 250 g were sacrificed by decapitation, and the brains were quickly removed and regionally dissected for binding assay and glutamate release. Nineteen-day-old Wistar rat fetuses (Charles River, Calco, Italy) were used to prepare primary hippocampal neurons for patch-clamp experiments. Fifteen-day-old Swiss-Webster mouse fetuses and 8-day-old Swiss-Webster mice (Charles River, Wilmington, MA) were used in studies carried out by National Institutes of Health-National Institute of Neurological Disorders and Stroke for whole-cell patch-clamp electrophysiology and for fluorescent imaging, respectively. All animals were kept in a temperature- (21°C ± 1°C) and relative humidity- (60%) controlled room on a 12-h light/dark cycle (lights on between 6:00 AM and 6:00 PM) and allowed free access to water and food (standard diet from Agway Prolab used by National Institutes of Health-National Institute of Neurological Disorders and Stroke and Mucedola rodent type 4RF21 used by Pharmacia & Upjohn and University of Milan).

Membrane Preparation and Receptor-Binding Assay

Membranes were prepared from specific brain areas by repeated centrifugations. All binding assays were carried out according to established methods previously described in the literature. All assays were validated using appropriate reference standards. In each assay the IC₅₀ was calculated from displacement curves (LIGAND program) as detailed by Munson and Rodbard (1980). IC₅₀ values are the mean ± S.E.M. of at least three determinations obtained using at least eight concentrations of each test compound covering a 100,000-fold range.

Patch-Clamp Whole-Cell Electrophysiology

Rat Hippocampal Neurons. Hippocampal neurons were cultured from 19-day-old rat fetuses (Buchhalter and Dichter, 1991). After 6 to 12 days, membrane currents were recorded in vitro using standard whole-cell patch-clamp methods at room temperature (Axopatch-1D patch-clamp amplifier; Axon Instruments, Foster City, CA). The external bath solution consisted of 145 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 5 mM glucose, pH 7.3. The patch electrode solution for whole-cell configuration contained 130 mM potassium aspartate (KAsp), 10 mM NaCl, 1.3 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 1 mM EGTA, and 1 mM MgATP, pH 7.3. To record sodium currents, tetraethylammonium chloride and 4-aminopyridine were added to the external solution and KAsp substituted with CsAsp and CsF. To optimize calcium current recordings, external sodium was exchanged with choline, tetraethylammonium chloride and 4-aminopyridine were added, and the internal potassium was replaced by Tris and Tris phosphate buffer. PNU-151774E, phenytoin, and lamotrigine were dissolved in the external solutions at the concentrations indicated. Cells were superfused continuously, and test solutions were alternatively perfused in the recording chamber during current and action potential recordings. Current traces were stored for analysis using pClamp 5.1 software (Axon Instruments). Data are expressed as percentage of inhibition and are the means of four to five replications.

Mouse Cortical Neurons. Cortical neurons were cultured from 15-day-old mouse fetuses. Whole-cell recordings were obtained from cultured neurons after 13 to 16 days in vitro (Hamill et al., 1981) to identify direct interactions between PNU-151774E and ion channels gated by either glutamate or GABA. During the experiment, cells were perfused with an external salt solution containing 142 mM NaCl, 1.5 mM KCl, 1 mM CaCl₂, 10 mM HEPES, 10 mM glucose, 20 mM sucrose, 0.2 mM strychnine, and 0.3 mM tetrodotoxin; mOsmol 320, pH 7.4. Using GABA as the agonist, the external solution was supplemented with 1 mM MgCl₂ to block N-methyl-d-aspartate receptor channels. Using glutamate as the agonist, the external solution was supplemented with 1 μM glycine as co-agonist and 10 μM picrotoxin to block GABA A channels. The internal pipette solution consisted of 153 mM CsCl, 10 mM EGTA, 10 mM HEPES, and 4 mM MgCl₂; mOsmol 290, pH 7.4. Whole-cell currents were evoked by 1-s applications of an agonist (1 μM GABA or 10 μM glutamate) at −75 mV membrane potential (Axopatch 200A with 8-pole Bessel; Axon Instruments). The effect of PNU-151774E on agonist-evoked whole-cell currents was assessed by exposing cells to the simultaneous application of agonist plus 100 μM PNU-151774E. Statistical significance of the inhibitory effect of PNU-151774E on GABA and glutamate-induced responses was determined by Student's t test (n = 5–6). Recordings were computer digitized (1000 samples per s: Axotape, Axon Instruments).

Glutamate Release. After decapitation, rat hippocampi were rapidly removed and transferred into cold Krebs-bicarbonate buffer (composition: 122 mM NaCl, 3.1 mM KCl, 1.2 mM MgSO₄, 0.4 mM KH₂PO₄, 1.3 mM CaCl₂, 25 mM NaHCO₃, and 10 mM glucose; pH 7.4). Transverse slices (350-μm thick) were cut with a McIlwain tissue chopper and incubated in oxygenated buffer (O₂ 95%/CO₂ 5%) at 37°C for 60 to 90 min to allow functional recovery. After this period, the release of glutamate was determined by placing five slices in vials containing 1 ml of oxygenated buffer at 32°C for 15 min. Veratrine (10 μg/ml) and KCl (50 mM) were used for evaluating the inhibitory effects of the compounds on stimulated glutamate release. Aliquots of supernatant (300 μl) were removed after 15 min and
stored at −20°C until analysis. Glutamate was measured by HPLC with an electrochemical detector after a precolumn derivatization procedure with o-phthalaldehyde and β-mercaptoethanol (Donzanti and Yamamoto, 1988). IC_{50} values were calculated by regression analysis from at least five concentrations. Data were expressed as percentage of response to veratrine or KCl alone and are the means of six (PNU-151774E and lamotrigine) or three (other reference compounds) experiments.

**Fluorescent Imaging.** Granule cells were derived from the cerebellum of 8-day-old mice and were grown on 25-mm acar coverslips according to the method of Parks et al. (1991). Coverslips of cerebellar granule cells (6–9 days in vitro) were incubated for 30 to 60 min at 37°C in HEPES-balanced salt solution (composition: 135 mM NaCl, 4.2 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 0.34 mM Na₂HPO₄, 5.5 mM glucose, 4.2 mM HEPES, and 5.8 mM Na₂-HEPES) containing the calcium-sensitive fluorescent probe 5 μM indo-1 AM (Molecular Probes, Eugene, OR). Individual coverslips were then placed in a flow-through cell chamber attached to the stage of a Nikon Diaphot microscope and superfused (1 ml/min) continuously with warmed (37°C) normal HEPES-balanced buffer. All responses were corrected for background autofluorescence and quantitated according to White et al. (1992). Briefly, a field of representative cells (>50 cells/field) was selected from each coverslip and corrected for background fluorescence. The [Ca²⁺]_i was estimated from the indo-1 emission ratio (410 nm/480 nm). After obtaining several seconds of baseline recording (potassium outflow = 4.2 mM), cells were exposed for 60 s to 55 mM KCl or 10 μM veratrine. After a 5-min washout with buffer, cells were exposed to a second application of agonist alone, after which they were washed for 5 min. After this time, cells were challenged simultaneously with a solution containing KCl or veratrine and PNU-151774E for 60 s. Cells were then exposed to PNU-151774E for an additional 5 min and then to a second application of agonists plus PNU-151774E. Finally, cells were rechallenged with agonist alone. The results obtained from these studies are expressed as a percentage of the predrug control responses (total PNU-151774E for an additional 5 min and then to a second application of agonists plus PNU-151774E for 60 s. Cells were then exposed to PNU-151774E for an additional 5 min and then to a second application of agonists plus PNU-151774E. Finally, cells were rechallenged with agonist alone. The results obtained from these studies are expressed as a percentage of the predrug control responses (total area under the curve or peak height) obtained with agonist alone. Results from several coverslips (n = 4) were averaged and statistical significance was determined by Student’s t test.

**Drugs**

PNU-151774E methanesulfonate, [(S)-(+)-2-(4-(3-fluorobenzyl)oxo)benzylamino]propanamide, methanesulfonate (former FCE 26743; Department of Chemistry, Pharmacia & Upjohn S.p.A., Milan, Italy), phenytoin (Fluka AG, Buchs SG, Switzerland), lamotrigine isothionate, felbamate, gabapentin (Department of Medicinal Chemistry, Pharmacia & Upjohn S.p.A., synthesized using established procedures), carbamazepine, valproate sodium, riluzole, veratridine, and veratrine (Sigma Chemical Co., St. Louis, MO) were dissolved in distilled water unless otherwise specified. In studies carried out by National Institutes of Health-National Institute of Neurological Disorders and Stroke, PNU-151774E was dissolved in dimethyl sulfoxide and diluted to make a final concentration of 10, 30, or 100 μM. All experimental solutions were adjusted to pH 7.4 and to the appropriate osmolarity daily.

**Results**

**Receptor-Binding Profile on Rat Brain Receptors**

PNU-151774E showed significant affinity for binding site 2 of the sodium channel receptor without affecting site 1 (Table 1, demonstrated with the use of selective ligands (IC_{50} = 8.2 and >300 μM for 3H-batrachotoxin and 3H-saxitoxin, respectively) (Table 1 and Fig. 1). This affinity was higher than that of other anticonvulsants tested such as riluzole, phenytoin, carbamazepine, and lamotrigine. On the other hand, PNU-151774E exhibited negligible affinity (IC_{50} >100 μM) for several receptor types, but displaced [3H]pentazocine from σ1 binding sites with an IC_{50} of 0.019 μM. The selectivity between σ1 and σ2 was approximately 100-fold (σ2-IC_{50}=1.59 μM; Table 2).

**Patch-Clamp Whole-Cell Electrophysiology**

**Rat Hippocampal Neurons.** The effects of compounds on sustained repetitive firing (SRF) of action potentials were studied in cultured rat hippocampal neurons (n = 8 for each compound). To elicit repetitive firing, 600-msec depolarizing current pulses were used. The cell resting potential was maintained at −60 mV (±2 mV). Control cells exhibited SRF at 25 Hz. The addition of PNU-151774E to the superfusing solution, 100 μM, caused cessation of firing after three bursts. A similar effect was obtained with lamotrigine, although at a higher concentration (200 μM). Phenytoin caused a slow decline of firing rate and a progressive decrease of action potential amplitude at 300 μM (Fig. 2). The effect of all compounds tested on SRF was completely reversible.

In subsequent experiments, PNU-151774E was added at the concentration of 50 μM to the superfusing solution, and action potential bursts were elicited by 400-msec current injections every 5 s to assess a use-dependent mechanism. PNU-151774E reduced cell firing within 1 min of superfusion. After 5 min, the effect was stable, and only one action potential was produced by cell stimulation (Fig. 3). Neurons returned to control conditions within 1 min of washout.

Tetrodotoxin (TTX)-sensitive fast sodium and high voltage-activated calcium currents are the two main currents underlying the firing activity in hippocampal neurons. The relative blockade of these currents was therefore analyzed under voltage-clamp conditions in the presence of increasing concentrations of PNU-151774E, lamotrigine, and phenytoin (n = 10 for each compound).

TTX-sensitive fast sodium currents were elicited by a 20-ms depolarizing pulse at −10 mV. At this point, the currents reached maximal amplitude, having started from a preconditioning potential of −100 mV. The holding potential was clamped at −60 mV. All tested compounds reversibly suppressed the sodium currents in a concentration-dependent manner.

The inhibition of the currents by PNU-151774E was concentration-related from 10 to 200 μM. At a concentration of 100 μM, PNU-151774E produced a 30% inhibition of the current; higher concentrations of phenytoin and lamotrigine (300 and 200 μM, respectively) were needed to obtain comparable effects (Fig. 4).

The dihydropyridine-sensitive pathway of high-voltage-activated calcium currents was also investigated. The current was recorded after substitution of external sodium with choline and with cesium replacing potassium in the recording
The membrane potential was moved from a preconditioning potential of \(-290\) mV to a depolarizing step of \(+110\) mV. PNU-151774E inhibited the voltage-activated calcium current in a concentration-dependent way. An inhibition of approximately 40% was obtained at 100 \(\mu\)M. Phenytoin and lamotrigine inhibited calcium current by less than 20%, even at the highest concentrations tested, 600 and 400 \(\mu\)M, respectively (Fig. 5).

**Mouse Cortical Neurons.** As shown in Fig. 6, 100 \(\mu\)M PNU-151774E did not affect whole-cell peak currents evoked by the excitatory amino acid glutamate or the inhibitory neurotransmitter GABA.

### Glutamate Release Inhibition

PNU-151774E inhibited veratrine-induced glutamate release with an IC\(_{50}\) of 56 \(\mu\)M (Table 3 and Fig. 7). Phenytoin, carbamazepine, and lamotrigine showed similar inhibitory potency, whereas felbamate was less active. Valproate and gabapentin were inactive (IC\(_{50}\) > 1 mM). PNU-151774E was also able to inhibit KCl-induced glutamate release, although at a higher concentration (IC\(_{50}\), 185 \(\mu\)M). Lamotrigine was without significant effect (Fig. 7).

### Fluorescent Imaging

PNU-151774E produced a significant inhibition of both KCl- and veratridine-induced calcium inward transients. This effect was enhanced by continued exposure to PNU-151774E for 5 min. As shown in Fig. 8 and summarized in Table 4, the effect of 100 \(\mu\)M PNU-151774E was greater on veratridine-induced calcium inward transients. At this concentration, a complete block was observed; this was only

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**TABLE 2**

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>(^3)H-Ligand</th>
<th>Brain Region</th>
<th>(\text{IC}_{50} \pm \text{S.E.M.}) (\mu)M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma-1</td>
<td>Pentazocine</td>
<td>Striatum</td>
<td>0.019 ± 0.007</td>
</tr>
<tr>
<td>Sigma-2</td>
<td>DTG</td>
<td>Whole brain</td>
<td>1.59 ± 0.15</td>
</tr>
<tr>
<td>NMDA</td>
<td>CGS 19755</td>
<td>Cortex</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NMDA-gated channel</td>
<td>MK 801</td>
<td>Cortex</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Glycine-strychnine insensitive</td>
<td>Glycine</td>
<td>Cortex</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Quisqualate</td>
<td>AMPA</td>
<td>Cortex</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Kainate</td>
<td>Kainic acid</td>
<td>Cortex</td>
<td>&gt;100</td>
</tr>
<tr>
<td>GABA(_A)</td>
<td>GABA</td>
<td>Cerebellum</td>
<td>&gt;100</td>
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<tr>
<td>Benzodiazepine</td>
<td>Flunitrazepam</td>
<td>Cortex</td>
<td>&gt;100</td>
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<td>Striatum</td>
<td>&gt;100</td>
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<tr>
<td>Dopamine D-2</td>
<td>Spiroperidol</td>
<td>Striatum</td>
<td>&gt;100</td>
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**Fig. 1.** In vitro \(^3\)H-batrachotoxin binding in rat brain membranes: displacement curves by PNU-151774E and reference anticonvulsants.

**Fig. 2.** Typical tracing of depolarizing pulse-induced repetitive firing in rat hippocampal neurons in culture. Repetitive firing elicited with a 600-ms depolarizing current pulse in presence of control vehicle; PNU-151774E, 100 \(\mu\)M; phenytoin, 300 \(\mu\)M; and lamotrigine, 200 \(\mu\)M. Cell resting potential at \(-60\) mV (see Results for details).
partially reversible upon washout. The PNU-151774E effect was concentration-dependent between 10 and 100 μM.

Discussion

The mechanism of action of PNU-151774E has been investigated using electrophysiological and biochemical assays. Our results indicate that PNU-151774E directly inhibits neuronal voltage-activated sodium and calcium channels. This finding is supported by the data showing that PNU-151774E: 1) displaces \(^{3}H\)-batrachotoxin from binding site 2 of the sodium channel of rat brain membranes; 2) reduces cell firing in a use-dependent manner, by inhibiting voltage-activated sodium and calcium currents in rat hippocampal neuronal cultures; and 3) inhibits the effect of veratrine/veratridine and KCl on both glutamate release in rat hippocampal slices and calcium transients in mouse cortical neurons.

Membrane permeability to sodium influences neuronal cell firing, but there is experimental evidence that different classes of calcium channels (high- and low-voltage-sensitive calcium channels) are also relevant (McLean and Macdonald, 1983; Macdonald and Kelly, 1995; Ferroni et al., 1996). The present study shows that PNU-151774E is able to reduce cell firing in a use-dependent manner; this activity is more potent than that shown by phenytoin and lamotrigine. Literature data on the effect of phenytoin on neuronal firing are controversial. McLean and colleagues (1998) recently found that high concentrations of phenytoin (200 μM) are needed to reduce firing. This discrepancy was attributed to the different experimental conditions adopted in the two studies. Our data confirm the activity of phenytoin at high concentrations.

The effect of PNU-151774E on cell firing is likely the result of its inhibitory activity on sodium currents combined with its inhibitory effect on the calcium pathway underlying the firing activity.
In voltage-clamp experiments we studied the effect of PNU-151774E on high-voltage-sensitive calcium channels. It strongly blocked high-voltage-activated calcium currents, suggesting an inhibitory activity on both L- and N-type channels. To confirm this hypothesis, however, more detailed analysis on different calcium channel subtypes is needed. Under our experimental conditions the effect of PNU-151774E was greater than that of phenytoin and lamotrigine.

In addition to the modulation of voltage-gated ion channels, GABA and glutamate-gated channels are considered to be other molecular targets for AED effects (Rogawski and Porter, 1990; Dichter, 1994). PNU-151774E has no direct effect on whole-cell peak currents evoked by either GABA or glutamate, suggesting that the compound does not interact with GABA or glutamate-linked channels.

Electrophysiological results at sodium and high threshold
calcium channels correlate with neurochemical findings, wherein PNU-151774E was found to inhibit presynaptic neurotransmitter release induced by veratrine and KCl in rat hippocampal slices. Veratrine depolarizes neuronal membranes by opening sodium channels (Levi et al., 1980). Anticonvulsants with sodium channel-blocking properties such as lamotrigine, phenytoin, and carbamazepine reduce veratrine-induced glutamate release (Woodbury, 1980; Waldmeier et al., 1996). As shown in the present investigation, similar effects were found for PNU-151774E as well.

KCl-induced depolarization stimulates glutamate release, which is sodium-independent and depends on calcium influx via high threshold channels (Armstrong and Matteson, 1985). We found that PNU-151774E, but not lamotrigine, inhibited KCl-induced release.

These results are supported by calcium-imaging studies in mouse cerebellar cultured neurons: PNU-151774E significantly attenuates both veratridine- and KCl-evoked calcium transients. PNU-151774E was more effective against veratridine.

It is important to note that these electrophysiological and neurochemical effects of PNU-151774E are apparent at anticonvulsant concentrations. For example, we have previously shown that brain levels reach roughly 40 μM, 30 and 60 μM, and that these concentrations are sufficient to elicit anticonvulsant effects.

**Fig. 7.** Effect of PNU-151774E and lamotrigine on endogenous glutamate release from rat hippocampal slices in vitro. Slices were incubated with veratrine (10 μM) or KCl (50 mM) for 15 min in the presence of various concentrations of PNU-151774E and lamotrigine. Data are expressed as percentage of response to veratrine or KCl alone and are means ± S.E.M. of six experiments.

**Fig. 8.** Representative traces from the fluorescent-imaging studies. The first calcium influx transient represents the predrug control evoked by bath application of 55 mM KCl or 10 μM veratridine to populations of cultured mouse cerebellar granule cells. The effect of PNU-151774E on the evoked transients was tested following acute application and following a 5-min pretreatment. A final postdrug control response was evoked 5 min after cessation of drug treatment.
min after an oral dose of 10 mg/kg PNU-151774E in rats. These times correspond to the peak anticonvulsant effect observed in the maximal electroshock test (Fariello et al., 1998). This concentration approximates the concentration of PNU-151774E, which gives effective inhibition of excitatory amino acid release and reduction of SRF. Moreover, PNU-151774E has a preferable affinity for the brain where drug levels are approximately 10-fold higher than in plasma. This favorable pharmacokinetic profile provides an explanation for the finding that the compound is relatively free of cardiovascular side effects related to sodium and calcium channel blockade at anticonvulsant concentrations (C. Arrigoni, personal communication).

In the range of concentrations found in the brain, PNU-151774E has no affinity for noradrenergic, dopaminergic, serotoninergic, glutamatergic, and GABAergic receptors.

On the other hand, PNU-151774E displays considerable affinity for sigma-1 receptors. Although the functional role of sigma sites, in general, is not yet clear, they may be behaviorally involved in psychosis (Sagratella et al. 1991). PNU-151774E shows no effect on EEG or psychotomimetic effects at high doses in conscious cynomolgus monkeys, a species highly sensitive to sigma ligands. This lack of activity suggests that sigma-1 affinity is devoid of relevant functional consequences (Salvati et al. 1996).

PNU-151774E has been previously described as a potent and selective monoamine oxidase (MAO)-B inhibitor with pratically no effect on MAO-A (Strolin-Benedetti et al., 1994). The potential involvement of MAO-B inhibition in the antiepileptic activity is still unclear. However, high brain monoamine levels reduce seizure susceptibility (Mishra et al., 1993), and the synthesis and release of monoamines, in particular, of dopamine, is increased in seizure foci in the human brain, possibly as a defense response (Goldstein et al., 1988; Pintor et al., 1990). Interestingly, the MAO-B inhibitor, seleagine, is active in the kindling model of complex partial seizures (Löschner and Honack, 1995).

Moreover, the increased metabolism of catecholamines by MAO-B (primarily) and MAO-A (to a lesser extent) may contribute to oxidative stress under certain conditions.

Lipid peroxidation is believed to play an important pathogenic role in post-traumatic epilepsy, one of the leading causes of adult seizure onset (Willmore, 1990). This belief is based on studies of an animal model, whereby microinjection of ferric ions in the cortex of several animal species caused lipid peroxidation and induced delayed seizures (Triggs and Willmore, 1984; Singh and Pathak, 1990). The antioxidant vitamin E, but not the classic anticonvulsant phenytoin, has antiepileptic effects in this model and also prevents epileptogenesis (Willmore et al., 1986).

Therefore, the MAO-B inhibitory property might accrue to PNU-151774E's therapeutic potential, making this compound unique among classic and new AEDs and ideal for post-traumatic epilepsy as well as for prevention of postsurgical seizures.

MAO-B inhibition, in addition to the sodium and calcium inhibitory properties of PNU-151774E, might also extend its therapeutic potential to other neurodegenerative diseases (Choi, 1995). Indeed an MAO-B inhibitor, such as selegiline, has also been shown to exert neuroprotective effects in models of Parkinson’s disease (Olaban and Riederer, 1996).

In the model of kainate-induced status epilepticus and neurodegeneration, PNU-151774E actually strongly inhibited both seizures and neuronal cell loss (Maj et al., 1998).

In conclusion, our results show that PNU-151774E inhibits sodium and calcium currents and limits SRF, providing the biochemical mechanism for preventing the spreading of rapid neuronal discharges without interfering with normal action potentials. Whether MAO-B inhibition, potentially enhancing dopaminergic function, may contribute to its anticonvulsant properties deserves further investigation.

Acknowledgments
The skillful assistance of G. Marchi, D. Damiani, L. Catania, and L. Moses is gratefully acknowledged.

References
Lang DG, Wang CM and Cooper BR (1993) Lamotrigine, phenytoin and carbamaz-

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TABLE 4
Effect of PNU-151774E on KCl- and veratridine-calcium transients of mouse cerebellar granule neurons with or without preincubation (5 min)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>PNU-151774E µM</th>
<th>n</th>
<th>Control µM</th>
<th>PNU-151774E (without incubation) µM</th>
<th>PNU-151774E (with 5-min incubation) µM</th>
<th>Washout µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 mM KCl</td>
<td>100</td>
<td>4</td>
<td>100 ± 36</td>
<td>70 ± 6°</td>
<td>48 ± 3°</td>
<td>65 ± 4°</td>
</tr>
<tr>
<td>10 µM veratridine</td>
<td>100</td>
<td>4</td>
<td>100 ± 7</td>
<td>24 ± 6°</td>
<td>3 ± 1°</td>
<td>66 ± 2°</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>100 ± 3</td>
<td>79 ± 3°</td>
<td>39 ± 5°</td>
<td>107 ± 6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>100 ± 2</td>
<td>103 ± 2</td>
<td>106 ± 2</td>
<td>129 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Results represent the area under the curve (nanoles of calcium) and are expressed as percentage of predrug control response; n, number of coverslips studied. See text for details.

* Significantly different from control, P < .01.

Preclinical evaluation of PNU-151774E as a novel anticonvulsant.
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