Tolperisone-Type Drugs Inhibit Spinal Reflexes via Blockade of Voltage-Gated Sodium and Calcium Channels

Pál Kocsis, Sándor Farkas, László Fodor, Norbert Bilik, Márti Thán, Sándor Kolok, Anikó Gere, Mónika Csejtei, and István Tarnawa

Gedeon Richter Ltd., Pharmacology and Drug Safety Research, Budapest, Hungary

Received May 26, 2005; accepted August 25, 2005

ABSTRACT

The spinal reflex depressant mechanism of tolperisone and some of its structural analogs with central muscle relaxant action was investigated. Tolperisone (50–400 μM), eperisone, lanperisone, inaperisone, and silperisone (25–200 μM) dose dependently depressed the ventral root potential of isolated hemisected spinal cord of 6-day-old rats. The local anesthetic lidocaine (100–800 μM) produced qualitatively similar depression of spinal functions in the hemicord preparation, whereas its blocking effect on afferent nerve conduction was clearly stronger. In vivo, tolperisone and silperisone as well as lidocaine (10 mg/kg intravenously) depressed ventral root reflexes and excitability of motoneurons. However, in contrast with lidocaine, the muscle relaxant drugs seemed to have a more pronounced action on the synaptic responses than on the excitability of motoneurons. Whole-cell measurements in dorsal root ganglion cells revealed that tolperisone and silperisone depressed voltage-gated sodium channel conductance at concentrations that inhibited spinal reflexes. Results obtained with tolperisone and its analogs in the [3H]batrachotoxinin A 20α,β-benzoate binding in cortical neurons and in a fluorimetric membrane potential assay in cerebellar neurons further supported the view that blockade of sodium channels may be a major component of the action of tolperisone-type centrally acting muscle relaxant drugs. Furthermore, tolperisone, eperisone, and especially silperisone had a marked effect on voltage-gated calcium channels, whereas calcium currents were hardly influenced by lidocaine. These data suggest that tolperisone-type muscle relaxants exert their spinal reflex inhibitory action predominantly via a presynaptic inhibition of the transmitter release from the primary afferent endings via a combined action on voltage-gated sodium and calcium channels.

2-Methyl-1-(4-methylphenyl)-3-(1-piperidinyl)-1-propanone hydrochloride (tolperisone) is an old, centrally acting muscle relaxant drug that is mainly used for treating muscle spasticities of neurological origin and painful muscle spasms due to rheumatologic conditions. Besides being an effective antispastic agent (Pratzel et al., 1996; Dulin et al., 1998), tolperisone also has analgesic activity in rodents (Sakaue et al., 2004) and in humans (Svensson et al., 2003). It possesses relatively few side effects in humans (Dulin et al., 1998). Other propiophenone muscle relaxants include 1-(4-ethylphenyl)-2-methyl-3-(1-piperidinyl)-1-propanone hydrochloride (eperisone), which is also a registered drug (Bose, 1999), and (−)-2-(R)-methyl-3-(1-pyrrolidinyl)-1-(4-(trifluoromethyl)phenyl)propanone hydrochloride (lanperisone; Sakitama et al., 1997) and 1-(4-ethylphenyl)-2-methyl-3-(1-pyrrolidinyl)-1-propanone hydrochloride (inaperisone; Morikawa et al., 1992), two agents that had been tested in human phase III studies but not introduced into the clinical practice. 1-[(4-Fluorobenzyl)dimethylsilylmethyl]piperidine hydrochloride (silperisone), a sila analog of tolperisone (Farkas et al., 2005), has been shown in mice to have better separation between the desirable effects (i.e., reduction of abnormally increased muscle activity in various models) and anticipated undesirable effects (i.e., central nervous system depression and impairment of voluntary motor control) than currently available centrally acting muscle relaxant drugs. The development of this compound has been discontinued because of an unacceptable side effect found in the chronic toxicity studies. Only few reports dealing with the mechanism of action of tolperisone-like compounds have been published. Ono et al. (1984) have shown that tolperisone and eperisone exert a...
local anesthetic-like (membrane-stabilizing) action both on motoneurons and primary afferents in vivo as well as on peripheral nerves of rats in vitro. Tolperisone was found to inhibit action potential propagation on both A- and C-fibers of rat sciatic nerve (Quasthoff et al., 2003). The local anesthetic action of tolperisone and eperisone was implicated in their antinociceptive effects on acute pain in mice (Sakaue et al., 2004). Thus, the effect of tolperisone seems to be similar to that of lidocaine (lignocaine; \( \omega \)-diethylamino-2,6-dimethylanilide), which is known to inhibit voltage-dependent sodium currents. Indeed, molecular modeling studies revealed that tolperisone can bind to the same site to which lidocaine can bind (Fels, 1996). In a two-electrode voltage-clamp study in Xenopus oocytes, Quasthoff et al. (2003) found an inhibition of both Na\(_{\alpha 1.6}\) (tetrodotoxin (TTX)-sensitive) and Na\(_{\alpha 1.8}\) (TTX-resistant) recombinant sodium channels by tolperisone. Similar to lidocaine, both sioperisone (Düring and Koppenhöfer, 2001) and tolperisone (Hinck and Koppenhöfer, 2001) inhibited sodium currents of frog-isolated Ranvier nodes. The results of the latter study, however, suggest a significant involvement of potassium channels as well in the mediation of tolperisone’s action; therefore, the authors concluded that tolperisone cannot be regarded as having a lidocaine-like action, because the latter drug has negligible effect on potassium channels. Furthermore, voltage-clamp studies in snail neurons (Novales-Li et al., 1989) demonstrated an inhibition of voltage-dependent calcium currents by tolperisone and some of its analogs. The close chemical similarity of eperisone, lanperisone, and inaperisone (and to some extent sioperisone) to tolperisone suggests a similar mode of reflex inhibitory action.

For better understanding of the mechanism of suppressant action of tolperisone and its analogs on spinal reflexes, the effects of these agents on the spinal reflex machinery between the stimulated dorsal root and the ventral root conveying efferent discharges were studied in the rat spinal cord both in vitro and in vivo, in comparison with the local anesthetic lidocaine. The sodium channel blocking effects of these compounds were characterized in electrophysiological experiments on dorsal root ganglion (DRG) cells, in a functional assay using fluorescent membrane potential dyes, in cerebellar cultures, and in a radioligand binding assay using brain cortical synaptosomal preparation. Their effect on voltage-gated calcium channels was also analyzed.

**Materials and Methods**

Materials. Silperisone HCl, eperisone HCl, tolperisone HCl, inaperisone HCl, lanperisone HCl, and pipercuronium bromide were synthesized at Gedeon Richter Ltd. (Budapest, Hungary). Lidocaine was obtained from EGIS Pharmaceuticals (Budapest, Hungary). HEPES was purchased from Sigma-Aldrich (Budapest, Hungary). Aconitine was purchased from Fluka (Buchs, Switzerland). Scorpion venom (Leiurus quinquestriatus (Boston, MA)) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Tracheal cannula (Leiurus quinquestriatus from North Africa), bovine serum albumin, choline chloride, sucrose, and HEPES were purchased from Sigma-Aldrich (Budapest, Hungary). Aconitine was purchased from Fluka (Buchs, Switzerland). Salts and other chemicals for artificial cerebrospinal fluid (ACSF) and electrolyte-filling solutions were purchased from Sigma-Aldrich. Culture medium components were obtained from Invitrogen (Carlsbad, CA). The fluorimetric imaging plate reader membrane potential assay kit (blue) was purchased from Molecular Devices (Sunnyvale, CA).

For intravenous infusion, Rindex solution (68 mM NaCl, 3.5 mM KCl, 1.25 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), and 555 mM glucose) was purchased from Human Ltd. (Budapest, Hungary). The anesthetic solution for in vivo studies contained 0.25% \( \alpha \)-chloralose and 10% urethane (both from Sigma-Aldrich) dissolved in distilled water. The cannula for blood pressure monitoring was filled with saline containing 200 IU/ml heparin (Gedeon Richter Ltd.).

**Animals.** Wistar rats (bred at Gedeon Richter Ltd. or purchased from Toxicoop, Budapest, Hungary or Harlan, Indianapolis, IN) were used in all the studies. For the in vitro experiments, spinal cords and DRG cells were isolated from 6-day-old male rat pups. Male rats weighing 280 to 320 g were used for the in vivo experiments. The animal room was thermostated at 21 ± 1 °C and illuminated artificially from 6:00 AM to 6:00 PM. The rats had free access to food and water. All of the procedures conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals and were approved by the Institutional Ethical Committee.

**Isolated Hemisected Spinal Cord Preparation in Vitro.** Rat pups weighing 13 to 16 g were anesthetized with ether and then placed on crushed ice to cool down the spinal cord until the respiration of the animal stopped. The spinal cord was removed and hemisected along the midline. Hemicords were transferred into a storage chamber, and they were incubated at room temperature (23–26°C) in standard ACSF (124 mM NaCl, 3.5 mM KCl, 1.23 mM NaH\(_2\)PO\(_4\), 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 26 mM NaHCO\(_3\), and 10 mM glucose) bubbled with carbogen (95% \( O_2\) and 5% \( CO_2\)) for at least 30 min. One hemicord was placed into the recording chamber and perfused at 10 ml/min with ACSF at 26°C (regulated) if not stated otherwise. Glass suction electrodes were used both for stimulation and recording. Dorsal root stimulation-evoked ventral root potentials (DR-VRPs) were recorded from the L5 ventral root. The L5 dorsal root was stimulated with square-wave anodic current pulses (0.2 mA; 0.1 ms; supramaximal for all recorded potential components) at a frequency of 2 min\(^{-1}\). However, before and at appropriate times after drug applications, the input-output relationship (curve) was also determined using gradually increasing current intensities (0.01–0.2 mA). Evoked compound action potentials (afferent fiber potentials; AFPs) were recorded from the spinal cord surface with a glass suction electrode attached to the adjacent L4 dorsal root. In these experiments, a distance of at least 5 mm was kept between the site of stimulation and the dorsal root entry zone.

The first 200 ms of DR-VRP was analyzed. Responses were band-pass-filtered (0.02 Hz–10 kHz), amplified, and fed into a PC via an A-D converter (Digitdata 1200; Molecular Devices, Sunnyvale, CA; sampling rate, 10 kHz). A custom-made computer program (Stimulat) controlled both stimulation and data acquisition and performed the on-line data analysis. Evoked potentials were displayed and stored for later evaluation. Different components of the ventral root reflex response were separated according to their poststimulus latencies and durations. The drugs were added into the ACSF only when the measured parameters had become stable. Drug effects were determined when a steady-state inhibition had developed, which was dependent on the drug used (typically after 60–90 min of perfusion).

**Standard Surgery for the in Vivo Studies in Spinal Animals.** The method was essentially similar to that described previously in more detail (Parkas and Ono, 1995). Rats anesthetized with a mixture of chloralose (25 mg/kg i.p.) and urethane (1 g/kg i.p.) were used. The vagal nerves were severed, and the common carotid arteries were ligated bilaterally at the cervical region. Blood pressure was monitored via a cannula in the carotid artery. The femoral vein was also cannulated to allow intravenous injections. A tracheal cannula was inserted, and the animals were artificially ventilated throughout the experiment. The spinal cord was infiltrated with lignocaine (1%; 50 μl) and transected at the C1 level. The animals were fixed in a spinal stereotaxic frame, and a dorsal laminectomy was performed on vertebrae L1–L6. A pool was formed from the skin of the back and filled with warm paraffin oil. Rectal and oil pool temperatures were
maintained at 36 ± 0.2°C using two heating lamps. During the experiment, Rindex solution was infused (10–20 ml · kg⁻¹ · h⁻¹) to maintain the mean arterial blood pressure of spinal animals at around 60 mm Hg.

In Vivo Spinal Reflex Study. Ventrally and dorsal roots below L4 were cut bilaterally, L5 dorsal and ventral roots on both sides were isolated, and an ipsilateral pair of them was placed on bipolar silver wire hook electrodes. The dorsal root was stimulated by single impulses (stimulus strength, supramaximal voltage; pulse width, 0.05 ms; and frequency, 10/min). The first 10 ms of the ventral root reflex recorded using a differential amplifier was displayed, stored, and evaluated using the Stimulat software.

Study of Afferent Nerve Conduction in Vivo. In addition to the standard surgery, the right sciatic nerve was exposed in the femoral-popliteal region and placed on a bipolar silver wire electrode. A pool was formed from the skin of the back and of the leg and filled with warm paraffin oil. The sciatic nerve was stimulated by single square-wave impulses (stimulus strength, supramaximal for all A-fibers; i.e., 5–30 V; pulse width, 0.1 ms; and frequency, 10/min). The proximal end of L5 dorsal root transected at the dorsal root entry zone was placed on a silver wire bipolar hook electrode and crushed between the two hooks for monophasic recording of the arriving compound action potential of the afferent nerve fibers.

Motoneuron Excitability Test in Vivo. Excitability of the motoneuron soma and that of the primary afferent fibers was measured similarly to the technique described by Ono et al. (1979). Anaesthetized animals were paralyzed with picroxurinum bromide (100 μg/kg starting dose + 50 μg · kg⁻¹ · h⁻¹ in infusion). A tungsten microelecetrode, insulated with the exception of its tip, was inserted into the L5 motoneuron pool, which was stimulated by negative pulses (stimulus strength, 0.2–0.5 mA; pulse width, 0.05 ms; and frequency, 10/min). The compound action potential evoked by direct stimulation of motoneurons (MN; first peak) and the potential caused by (mono) synaptic activation of motoneurons (MS; second peak) were recorded from the L5 ventral root. The antidromic action potential, which reflects excitability of the primary afferent fibers (PAFs), was recorded from the L5 dorsal root. Tip position and stimulus strength were finely adjusted to yield similar amplitudes of MN and MS. Data acquisition and analysis in the in vivo studies were performed similarly to those described in the in vitro experiments, except that a higher (25 kHz) sampling rate was used.

Whole-Cell Current Measurements. DRG cells were acutely dissociated from rat DRG of 6-day-old male rats (Roy and Narahashi, 1992). Cells were plated on sterilized glass coverslips previously coated with poly-D-lysine. Cultures were kept in 2% serum-supplemented Dulbecco's modified Eagle's medium at 37°C in 5% CO₂. Inward currents were recorded by the whole-cell patch-clamp technique 1 day after the plating of the cells. Coverslips with the attached neurons were transferred to a recording chamber and constantly superfused with the extracellular solution (ES) at room temperature (22–25°C). The ES used in the sodium current experiments contained 70 mM NaCl, 70 mM choline chloride, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 5 mM HEPES, 5 mM HEPES-Na, 0.01 mM CdCl₂, and 20 mM glucose, pH 7.35. The IS for calcium current measurements contained 143 mM choline chloride, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 20 mM glucose. Patch electrodes (resistances, 1.5–2.5 MΩ) pulled from borosilicate capillary glass were filled with intracellular solution (IS). The composition of IS in the sodium current experiments was 130 mM CsF, 15 mM NaCl, 10 mM tetraethylammonium chloride, 0.1 mM CaCl₂, 2 mM MgCl₂, 2 mM ATP-γ-Na₂, 10 mM HEPES, and 1 mM EGTA, pH 7.25. The IS for calcium current measurements contained 110 mM CsCl, 4.5 mM MgCl₂, 9 mM HEPES, 9 mM EGTA, 4 mM ATP-γ-Na₂, 0.3 mM GTP, 14 mM creatine phosphate, and 50 U/ml creatine phosphokinase, unless indicated otherwise. Osmolarities of ES and IS were 310 and 290 mOsm, respectively. Sodium currents were evoked by 8-ms-long rectangular step depolarizations to 0 mV from different holding potentials at 10-s intervals. Calcium currents were elicited by 20-ms step pulses to 0 mV from a holding potential of −80 mV. An Axopatch 200A amplifier and the pClamp 8.0 software (Molecular Devices, Sunnyvale, CA) were used for recording and analysis. Capacitive transients were compensated; series resistance compensation was also always performed. Test compounds dissolved in the ES were applied onto the cells via multibarreled ejection pipettes controlled by electromagnetic valves. Currents were recorded from fast-kinetics tetrodotoxin-sensitive DRG cells in which application of TTX (0.3 μM) caused an at least 85% inhibition of the sodium current peak amplitude. The availability curves of the sodium current were fitted with the Boltzmann function: \[ I_{Na} = I_{Na,\text{max}} \times (1 + \exp[\frac{V_{t}}{V_{\text{H}}/2} - k_{H}]), \]
where \( I_{Na} \) is the peak current amplitude, \( I_{Na,\text{max}} \) is the maximum available sodium current (the upper asymptote of the fitted sigmoid curve), \( V_{t} \) is the applied holding potential, \( V_{\text{H}} \) is the holding potential at half-maximal availability, and \( k_{H} \) is the slope factor or width.

Fluorescent Membrane Potential Measurements. Average membrane potential in rat primary cerebellar cell cultures was monitored by fluorescence. In brief, primary cerebellar cell cultures were initiated from 4-day-old rats. After decapitation, the cerebellum was removed and incubated with 0.25% trypsin for 3 min. After a brief centrifugation (125g; 5 min), the cells were resuspended in culture medium (Dulbecco’s modified Eagle’s medium) containing 10% fetal bovine serum, 20 ng/ml nerve growth factor, 20 mM KCl, 2.5 μg/ml amphotericin B, 100 μM penicillin G, and 100 μg/ml streptomycin, and were plated onto poly-D-lysine-coated 96-well plates (1–2 × 10⁵ cells/well). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air and used for the measurement after 7 days in vitro. Membrane potential was monitored using the fluorometric imaging plate reader membrane potential assay kit (Molecular Devices) and FlexStation II (Molecular Devices), a plate reader fluorimeter with integrated eight-channel fluid addition capability. Cells were loaded with the fluorescent dye (100 μM/bottle) by incubating the plate for 20 to 60 min at 37°C. After loading, 50 μl of ACSF (control) or 50 μl of 4× concentrated test compound solution (dissolved in ACSF) was added to each well, and the plate was incubated at 37°C for an additional 10 min. Fluorescence measurements were carried out at 37°C. The dye was excited at 530 nm, and emission was sampled at 565 nm at 1.4-s intervals. After recording baseline for 15 s, 50 μl of 4× concentrated veratridine solution was added to the cells using the pipette of FlexStation, and fluorescence was monitored for an additional 105 s. This 2-min protocol was executed column by column on the whole plate. The concentration of veratridine (defined as EC₅₀) was determined on each experimental day by performing dose-response measurements with veratridine using cells from the same plating. Raw fluorescence data were expressed as ΔF/Δt values (fluorescence change normalized to baseline). The effects of blockers at various concentrations were quantified as percentage of inhibition of the control veratridine response.

[^H]BTX Binding. Rat cerebrocortical synaptosomes were prepared as described by Catterall et al. (1981) with minor modifications. In brief, the cortices of 7- to 8-week-old male rats were dissected and homogenized in 10 volumes of ice-cold sucrose buffer containing 5 mM K₂HPO₄, pH 7.4. The homogenate was centrifuged at 1000g (15 min; 4°C), and the resultant supernatant was retained and centrifuged at 20,000g (15 min; 4°C). Aliquots of the synaptosomal preparation were frozen on dry ice and stored at −80°C until use. Before use, aliquots were thawed at room temperature and centrifuged in a 10-fold volume of the same sucrose buffer (20,000g; 15 min; 4°C). The final pellet containing synaptosomes was resuspended in 4 volumes of Na⁺-free medium containing 130 mM choline chloride, 5.5 mM sucrose, 0.8 mM MgSO₄, 5.4 mM KCl, and 50 mM HEPES, pH 7.4.

Aliquots (100 μl) equal to approximately 6 to 8 mg/ml protein were used in[^H]BTX binding experiments. Binding assays were performed in the presence of 5.0 nM[^H]BTX, 1 μM tetrodotoxin, 4.0 μg of scorpion toxin, and various concentrations of the added drugs at 37°C for 60-min incubation time. Nonspecific binding was deter-
mired in the presence of 300 µM aconitine. The reaction was terminated by rapid filtration using a UniFilter-96 GF/B (PerkinElmer Life and Analytical Sciences). The filtration plates were washed five times with ice-cold wash buffer containing 5 mM HEPES, 130 mM choline chloride, 0.8 mM MgSO₄, 1.8 mM CaCl₂, and 0.01% bovine serum albumin. Radioactivity trapped on a 96-well filtration plate was measured by liquid scintillation spectrometry in 40 µl of Microscint 20 scintillation cocktail (PerkinElmer Life and Analytical Sciences) using a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences).

Statistics. Data are presented as mean ± S.E.M. For IC₅₀ determinations, sigmoidal fitting to parametric data were applied using Origin 6.0 (OriginLab Corp., Northampton, MA).

Results

Isolated Hemisected Spinal Cord in Vitro. A typical DR-VRP (Fig. 1A) consisted of a biphasic population spike (monosynaptic reflex, MSR; time to peak, 7.0 ± 0.4 ms) superimposed on the early phase of a tonic, long-lasting potential shift, reaching a maximum at 10 to 15 ms following the stimulation of the dorsal root. This latter potential basically represents population excitatory postsynaptic potential (EPSP) of motoneurons (Siarey et al., 1992). Nevertheless, small waves attributable to asynchronous firing of motoneurons were often discernibly superimposed on this tonic potential. When the stimulus intensity dependence of the evoked response was investigated (Fig. 1B, control curves), current intensities causing half-maximal activation of MSR, EPSP, and late EPSP were 0.05, 0.045, and 0.03 mA, respectively, whereas 0.2 mA was enough to maximally activate all components. This latter intensity, which was used in the experiments where drugs were tested, caused only negligible activation of C-fibers (not illustrated). Thus, C-fibers do not seem to participate in the generation of the first 180 ms of DR-VRP, including its highly NMDA antagonist-sensitive “tail part” (80–180 ms; Kocsis et al., 2003). This conclusion is in agreement with that of Thompson et al. (1992), who also found a predominant role of A-type afferent fibers in DR-VRP.

Increasing the stimulus intensity above supramaximal for Aδ did not result in further increase in any of these response components.

Tolperisone (50–400 µM), eperisone, lanperisone, inaperisone, and silperisone (25–200 µM) as well as lidocaine (200–800 µM) caused concentration-dependent depression of all studied components of DR-VRP (Fig. 2). As illustrated by the action of silperisone in a representative experiment (Fig. 1A), MSR and the tail of EPSP were the most sensitive parameters, whereas the early part of EPSP was apparently less attenuated. Apart from responses just above the threshold, the depression afforded by different doses of silperisone was not dependent on the stimulus strength. Namely, maximum responses of the input-output curves were attenuated, rather than a rightward shift of the curve (Fig. 1B). Hence, these depressant drug effects were unlikely to be the consequence of an elevated excitation threshold of primary afferent axons at the site of stimulation.

Tolperisone, silperisone, and lidocaine all dose dependently inhibited AFP in the concentration range that also depressed EPSP. To be able to make a precise comparison between drug effects on AFP and synaptic responses, we recorded the two activities in the same hemisected spinal cord preparation. To eliminate the interference caused by spiking of motoneurons (MSR), we recorded the EPSP-related component at an elevated temperature, at which excitability of motoneurons in the hemisected spinal cord preparation was depressed (Brooks et al., 1955). Elevation of the temperature of the bathing solution from 26 to 32 ± 1°C depressed MSR, allowing us to record the main component of the incoming AFP (Fig. 3A) and a nearly pure EPSP-related potential (Fig. 3B) simultaneously. Figure 3 shows that lidocaine was clearly more effective in blocking nerve conduction than EPSP, whereas silperisone had an opposite preference regarding the two potentials. Tolperisone was in between the two other drugs, having similar efficacies to block EPSP and AFP.

In Vivo Spinal Reflex Study. A typical ventral root reflex response to stimulation of the dorsal root and its attenuation by silperisone are shown in Fig. 4A. We differentiated three characteristic components of the ventral root reflex potential (as described previously in Farkas and Ono, 1995): monosynaptic, disynaptic, and polysynaptic reflex (MSR, DSR, and PSR, respectively; Fig. 4A). Silperisone (10 mg/kg i.v.) attenuated all of the three components with a
sensitivity order of components: MSR > DSR > PSR. Its action was longer lasting than that of tolperisone, eperisone, or lidocaine (about 40, 90, 85, and 70% recovery of MSR 1 h after i.v. injection, respectively; not illustrated). The profiles of tolperisone and eperisone (i.e., the relative sizes of effects on the three different ventral root reflex components) were practically identical. However, peak effects of tolperisone were a bit greater for all of the three components. The profile of silperisone was also similar to that of the above-mentioned two compounds. However, its peak effect on MSR was slightly weaker. The profile of lidocaine was substantially different, because it produced relatively weaker depressant effect on MSR compared with that on PSR (Fig. 4B).

Motoneuron Excitability Test in Vivo. The effects of silperisone, tolperisone, and lidocaine on excitability of motoneurons and primary afferents were studied in three to five animals. Results are shown in Fig. 5. Characteristic potentials recorded from the dorsal and ventral roots following intraspinal focal electrical stimulation of the area of the motoneuron pool are shown in Fig. 5A.

Silperisone (10 mg/kg i.v.) depressed MN (direct excitability) by 15%, whereas MS (the synaptic response) decreased by 68% on average (Fig. 5). It exerted no effect on excitability of PAFs. The onset of the effect of silperisone was relatively slow, and the inhibition reached its maximum in 15 min. Tolperisone (10 mg/kg i.v.) reached a maximum effect of 24, 80, and 8% inhibition of MN, MS, and PAF, respectively, in 6 to 8 min, whereas lidocaine (10 mg/kg i.v.) produced more marked depression of MN and PAF (30 and 14%, respectively; Fig. 5D) than did tolperisone or silperisone. On the other hand, its depressant effect on MS (67%) was less pronounced compared with tolperisone, and it was similar to that of silperisone. Although these differences may seem small, they were highly reproducible when the different compounds were administered successively in the same experiment.

Study of Afferent Nerve Conduction in Vivo. Possible contribution of a local anesthetic-type inhibition of afferent nerve conduction to the reflex inhibition following systemic

---

**Fig. 2.** Effects of different tolperisone-type centrally acting muscle relaxant drugs (tolperisone, TOLP; silperisone, SILP; eperisone, EPE; Lanperisone, LANP; and inaperisone, INAP) and lidocaine (LID) on DR-VRP components in vitro. IC₅₀ values of the drugs studied are based on inhibition of different reflex components: peak-to-peak amplitude of monosynaptic compound action potential (open columns), integral of EPSP (hatched columns), amplitude of EPSP (checked columns), and integral of late part of EPSP (80–180-ms poststimulus time; solid columns). All columns represent mean ± S.E.M. from four experiments.

**Fig. 3.** Effects of drugs on synaptic responses (EPSP) and on AFP. These experiments were performed at bath temperature of 32 ± 1°C to eliminate the monosynaptic reflex and thus to record a pure EPSP. The L5 dorsal root was stimulated (0.2 mA; 0.1 ms) A, afferent fiber potential recorded from the L4 dorsal root. It shows the action potentials of L5 dorsal root arriving at the spinal cord surface. B, population EPSP recorded from the L5 ventral root. C to E, dose-response curves of silperisone, tolperisone, and lidocaine, respectively. Data points represent the peak-to-peak amplitude of AFP (○) and baseline-to-peak amplitude of EPSP ( ]). Data are presented as mean ± S.E.M. from three experiments, each.
administration of silperisone, tolperisone, and lidocaine (10–10 mg/kg i.v.) was investigated. AFPs recorded from the L4 dorsal root following stimulation of the sciatic nerve reflected evoked action potentials of A-fibers (no synchronized C-fiber-mediated components could be recorded under our experimental conditions). Silperisone and tolperisone left AFP practically unchanged (2.7 ± 0.5 and 5.0 ± 1.2% inhibitions of the peak, respectively; n = 6). Lidocaine exerted slight but consistent inhibitory effect on AFP amplitude (10.7 ± 1.7%).

Effects on Voltage-Gated Sodium and Calcium Currents. Sodium currents recorded from medium-sized (25–35 μm) dorsal root ganglion cells had an average maximal peak current amplitude of 3.9 ± 0.4 nA (43 cells). Because in pilot experiments we found that the sodium channel inhibitory effect of tolperisone-like compounds is highly membrane potential-dependent, the concentration-response relationships were investigated at a membrane potential, where approximately half of the channels were in an inactivated state (V1/2), i.e., at −80 mV. Thus, to decrease the variability of drug sensitivities among DRG neurons, cells with V1/2 out of the range of −70 and −90 mV were not used for drug testing.

Tolperisone, silperisone, eperisone, and lidocaine inhibited peak sodium currents evoked by step depolarizations to 0 mV from a holding potential of −80 mV in a concentration-dependent manner (Fig. 6A). The inhibitory effect developed rapidly and was reversible. The IC50 values are listed in Table 1. Silperisone had a slightly higher potency to block sodium currents than the other three drugs.

The membrane potential dependence of the available current (sodium channel availability) was assessed by applying a voltage protocol in which membrane potential was held for 10 s at different voltages, increasing from −130 to −10 mV in 10-mV steps preceding the test pulses to 0 mV. In Fig. 6B, the effect of tolperisone (200 μM) on the steady-state inactivation curve is shown. The peak amplitude of the resultant current was normalized to the value of the maximum available current (recorded at −130 mV) and plotted against the membrane potential. Tolperisone (200 and 400 μM) as well as silperisone (80 and 160 μM) and lidocaine (200 and 400 μM) caused concentration-dependent shifts of the control inacti-


**Mechanism of Tolperisone-Type Drugs**

Sensitive calcium channels with an IC<sub>50</sub> of 218 nM inhibited both the \( \omega \)-conotoxin-sensitive current component and also most of the drug-resistant (R-type) current in DRG cells, because neither nitrrendipine (10 \( \mu \)M; \( n = 9 \)) nor \( \omega \)-agatoxin-IVA (0.1 \( \mu \)M; \( n = 9 \)) blocked the current significantly (5 \( \pm \) 1 and 4 \( \pm \) 1\% inhibition of calcium current amplitude, respectively), whereas \( \omega \)-conotoxin-GVIA, a selective N-type calcium channel blocker (3 \( \mu \)M), caused a 28 \( \pm \) 3\% inhibition (\( n = 4 \)). Silperisone (320 \( \mu \)M) inhibited both the \( \omega \)-conotoxin-sensitive current component and also most of the drug-resistant (R-type) current (Fig. 7A).

Silperisone concentration dependently inhibited voltage-sensitive calcium channels with an IC<sub>50</sub> of 218 \( \pm \) 22 \( \mu \)M (determined in eight cells). In another set of experiments, the patch pipette-filling solution lacked creatine phosphate, creatine phosphokinase, and GTP, agents that facilitate the phosphorylation of intracellular proteins. In these experiments, the blocking effect was more prominent (IC<sub>50</sub> = 65 \( \pm \) 9 \( \mu \)M; \( n = 8 \)). Tolperisone had an IC<sub>50</sub> of 1062 \( \mu \)M in the presence of creatine phosphate, creatine phosphokinase, and GTP; thus, it was ~5-fold less potent than silperisone. No IC<sub>50</sub> values were determined with eperisone and lidocaine; thus, the calcium channel-inhibiting efficacies of drugs were compared at 320 \( \mu \)M (Fig. 7B). At their IC<sub>50</sub> concentrations for sodium channel blockade, tolperisone, silperisone, eperisone, and lidocaine caused 22, 34, 36, and 7\% diminution of calcium currents, respectively.

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Current&lt;sup&gt;a&lt;/sup&gt;</th>
<th>[&lt;sup&gt;3&lt;/sup&gt;H]BTX Binding</th>
<th>VER</th>
<th>MSR</th>
<th>EPSP Integral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolperisone</td>
<td>198 ( \pm ) 21 (6)</td>
<td>40.9 ( \pm ) 2.5 (7)</td>
<td>58.3 ( \pm ) 2.4 (3)</td>
<td>62.7 ( \pm ) 9.4 (7)</td>
<td>130.7 ( \pm ) 10.4 (7)</td>
</tr>
<tr>
<td>Silperisone</td>
<td>111 ( \pm ) 18 (10)</td>
<td>7.2 ( \pm ) 0.8 (4)</td>
<td>14.2 ( \pm ) 0.6 (3)</td>
<td>55.9 ( \pm ) 1.5 (4)</td>
<td>72.5 ( \pm ) 13.8 (4)</td>
</tr>
<tr>
<td>Eperisone</td>
<td>250 ( \pm ) 95 (5)</td>
<td>21.7 ( \pm ) 2.5 (3)</td>
<td>37.1 ( \pm ) 1.8 (3)</td>
<td>43.3 ( \pm ) 5.8 (5)</td>
<td>76.0 ( \pm ) 9.4 (5)</td>
</tr>
<tr>
<td>Inaperisone</td>
<td>N.D.</td>
<td>34.8 ( \pm ) 3.0 (3)</td>
<td>41.8 ( \pm ) 3.5 (4)</td>
<td>29.6 ( \pm ) 3.8 (7)</td>
<td>59.7 ( \pm ) 7.4 (7)</td>
</tr>
<tr>
<td>Lanperisone</td>
<td>N.D.</td>
<td>13.7 (1)</td>
<td>31.2 ( \pm ) 2.9 (4)</td>
<td>33.0 ( \pm ) 3.1 (5)</td>
<td>114.7 ( \pm ) 15.1 (5)</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>297 ( \pm ) 86 (6)</td>
<td>132 ( \pm ) 9 (4)</td>
<td>60.6 ( \pm ) 6.2 (4)</td>
<td>186.2 ( \pm ) 17.6 (6)</td>
<td>198.5 ( \pm ) 42.8 (6)</td>
</tr>
</tbody>
</table>

N.D., not determined; VER, veratridine-evoked depolarization determined by microfluorimetry.

<sup>a</sup> Sodium currents were measured by whole-cell patch clamp.

**Discussion**

Tolperisone and its analogs as well as lidocaine suppressed the spinal segmental reflex activity both in vitro and in vivo. Various presynaptic and postsynaptic events may be considered as possible site(s) of this inhibitory action. Using an intraspinal stimulation protocol, we found that tolperisone and silperisone decreased motoneuronal (postsynaptic) excitability but to a lesser extent than the local anesthetic lido-
The compound action potential recorded from the dorsal root represents excitability of primary afferents. From the ventral root, two successive potentials could be recorded: the first spike was the consequence of direct electrical stimulation (nonsynaptic excitation of the motoneurons; MN), whereas the second spike was due to MS activation of motoneurons (Ono et al., 1984; Farkas and Ono, 1995).

Lidocaine was also the most effective among the three compounds in decreasing the excitability of primary afferent terminals and the conduction of afferent volley (AFP). These presynaptic events have a great impact on the quantity of transmitter substances (primarily glutamate) released from the terminals. A decreased transmitter release results in a depression of EPSP. To analyze precisely the relationship between depression of the afferent nerve conduction and synaptic transmission, dose-response studies were performed with simultaneous recording of AFP and DR-VRP. Although tolperisone, silperisone, and lidocaine all attenuated both AFP and EPSP, silperisone preferentially depressed EPSP, whereas lidocaine possessed a more pronounced depressant action on AFP. The profile of tolperisone was between that of the two other drugs, namely, it equally inhibited AFP and EPSP.

In summary, lidocaine had significantly greater depressant effects on the direct electrical excitability of motoneurons and primary afferents than silperisone, whereas their efficacies to inhibit the synaptic transmission were similar. The profile of tolperisone was in between that of the two other drugs.

Among the compounds studied, silperisone had the slowest rate of onset of effect (not shown). Since an apparently complete steady state usually could not be achieved with silperisone even within 90 min, this could cause slight under-estimation of its potency. As shown by Fig. 2, inaperisone was the most potent in attenuating both MSR and the early part (peak) of EPSP. However, it was equally potent to eperisone and silperisone in attenuating the “tail” and consequently the whole area under the curve of EPSP. Tolperisone and lanperisone were somewhat less potent than the above-mentioned drugs, but their patterns of actions were similar. Lidocaine was the least potent, especially in depressing the early part of EPSP. However, it was relatively potent in attenuating the tail (Fig. 2), which is in agreement with the results of Nagy and Woolf (1996) obtained in a similar model.

Thus, in spite of the fact that lidocaine had marked effects on AFP and excitability of presynaptic terminals, it was less efficient in depressing synaptic transmission compared with tolperisone and especially with silperisone. This suggests that the latter drugs may have additional inhibitory actions on EPSP generation.

In our in vitro model, EPSP could be abolished by coadministration of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid antagonist GYKI 52466 and the NMDA antagonist 2-amino-5-phosphonovalerate (Kocsis et al., 2003). However, according to our former (unpublished) studies, neither tolperisone nor silperisone had any effect on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or NMDA receptors, suggesting that the depressant effect on EPSP reflects a presynaptic action on the release of the excitatory transmitter rather than an action on postsynaptic glutamate receptors. Neither did the two drugs affect GABA-A receptors in binding experiments.

Voltage-gated sodium channels are regarded as one of the most important sites of action of lidocaine (Hille, 1977; Clare et al., 2000). The effect of lidocaine on sodium channels can explain both the depression of excitability and synaptic transmission observed at the spinal segmental level. Our present results clearly indicate that tolperisone and its analogs share this feature of lidocaine, which is in agreement with the findings of Ono et al. (1984), Hinck and Koppenhöfer (2001), Dürring and Koppenhöfer (2001), and Quasthoff et al. (2003). The lack of clear correlation between the blocking potencies of these drugs in the tests for sodium channels and those for depression of spinal reflex transmission in vitro (Table 1) may be related to differences between the compounds regarding the diffusibility inside the spinal tissue and/or to differences in activities on other channels.

Fig. 7. Effect of drugs on voltage-gated calcium channels in DRG cells. A, an experiment illustrating the effect of silperisone (SILP; 320 μM) on high voltage-activated calcium currents compared with that of specific blockers of various subtypes of calcium channels: nitrendipine (NIT; 10 μM; L-type-selective), ω-conotoxin-GVIA (CON; 3 μM; N-type-selective), and ω-agatoxin-IVA (AGA; 0.1 μM; P-type-selective). Peaks of current were plotted against time. B, inhibitory effect of different tolperisone type centrally acting muscle relaxant drugs (tolperisone, TOLP; silperisone, SILP; and eperisone, EPE) and lidocaine (LID) at 320 μM on calcium current peak amplitude. Columns represent mean ± S.E.M. in four to seven experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ΔV_H/2</th>
<th>I_H</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 Tolperisone</td>
<td>-19 ± 3 (7)</td>
<td>33 ± 12 (4)</td>
</tr>
<tr>
<td>400 Tolperisone</td>
<td>-22 ± 3 (6)</td>
<td>41 ± 9 (6)</td>
</tr>
<tr>
<td>80 Silperisone</td>
<td>-6 ± 1 (7)</td>
<td>13 ± 2 (6)</td>
</tr>
<tr>
<td>160 Silperisone</td>
<td>-8 ± 1 (7)</td>
<td>32 ± 7 (6)</td>
</tr>
<tr>
<td>200 Lidocaine</td>
<td>-13 ± 2 (7)</td>
<td>14 ± 4 (6)</td>
</tr>
<tr>
<td>400 Lidocaine</td>
<td>-17 ± 2 (9)</td>
<td>27 ± 7 (7)</td>
</tr>
</tbody>
</table>
The coupling between theafferent volley and the transmitter release can be modulated effectively by antagonists of certain subtypes of voltage-gated calcium channels as well (Burnashev and Rozov, 2005). Tolperisone slightly blocked these channels at sodium channel blocking concentrations. However, silperisone and eperisone were more potent in this regard, whereas lidocaine had only weak effect on calcium channels. This might underlie the stronger effect of silperisone on EPSP than on AFP compared with tolperisone. As suggested by the results with silperisone, under certain experimental conditions, which may affect the phosphorylation states of channel proteins, the potency of tolperisone-like drugs to inhibit calcium channels may be even higher. Therefore, we suggest that the effect of tolperisone and particularly silperisone and eperisone on Ca\(^{2+}\) channels contributes to their presynaptic action on transmitter release.

Regarding the significance of sodium channel blockade in the reflex inhibitory action of tolperisone-like compounds, it must be kept in mind that the resting membrane potential of large TTX-sensitive DRG neurons that are involved in the reflex inhibitory action of tolperisone-like compounds, it underlie the relative lack of motor side effects of tolperisone in clinical practice (Dulin et al., 1998) and the relative lack of impairment of normal motor control caused by tolperisone-like drugs, which was observed in conscious mice (Farkas et al., 2005). Although a majority of the present results were obtained in spinal preparations, we do not claim that the proposed mechanism of the muscle relaxant action of tolperisone-like drugs is restricted to the spinal cord level. Actually, the effects of these agents on central nervous system (cortical and cerebellar) preparations suggest that supraspinal mechanisms might also be affected.

Baclofen acting via GABA-B receptors, benzodiazepines potentiating GABA-A receptor-mediated primary afferent depolarization, and tizanidine stimulating \(\alpha\)2 receptors are centrally acting muscle relaxants that all act via an inhibition of transmitter release by a presynaptic action (Farkas et al., 1989; Delwaide and Pennisi, 1994). Our results indicate that a state-dependent blockade of sodium channels combined with an inhibition of calcium channels by tolperisone-like drugs offers another efficient way of treating spasticity disorders with less risk of motor side effects.

Acknowledgments

We thank Katalin Fekete, Katalin Oravecz, Anikó Kossár, Emília Borók, Piroska Fejes-Unghy, Gabriella Auth-Élès, Viktória Palcsó-Orosz, and Éva Csontos for excellent technical assistance, Dr. Péter Molnár for providing the Stimulat software, and Dr. József Nagy for providing the cell cultures.

References


Address correspondence to: Dr. Sandor Farkas, Pharmacology and Drug Safety Research, Gedeon Richter Ltd., H-1475, Budapest, 10, P.O. Box 27, Hungary. E-mail: s.farkas@richter.hu