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Title Page

Tolperisone-type drugs inhibit spinal reflexes via blockade of voltage gated sodium and calcium channels

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Running Title Page

Running title: Mechanism of tolperisone-type drugs

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Nonstandard abbreviations: AMPA, *R,S*-(±)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartic acid; MSR, monosynaptic reflex; DSR, disynaptic reflex; PSR, polysynaptic reflex; DRR, dorsal root reflex; DR-VRP, dorsal root stimulation evoked ventral root potential; AFP, afferent fiber potential; ACSF, artificial cerebro-spinal fluid; MN, motoneuron stimulation related compound action potential; MS, monosynaptically evoked action potential of motoneurons; PAF, compound action potential of primary afferent fibers; EPSP, excitatory postsynaptic potential; TTX, tetrodotoxin; [^{3}H]BTX, [^{3}H]Batrachotoxinin A 20-α-benzoate; DRG, dorsal root ganglion; ES, extracellular solution; IS, intracellular solution

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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, while 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 analogues in the [^{3}H]-BTX binding in cortical, 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, while 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.

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Introduction

Tolperisone (2-methyl-1-(4-methylphenyl)-3-(1-piperidinyl)-1-propanone hydrochloride) is an old centrally acting muscle relaxant (CMR) drug which 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) as well. It possesses relative 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), (-)-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 have not been introduced into the clinical practice. Silperisone (1-[(4-fluorobenzyl)dimethylsilylmethyl]piperidine hydrochloride), a sila analogue 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. CNS depression, impairment of voluntary motor control) than currently available centrally acting muscle relaxant drugs. The development of this compound has been discontinued due to 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

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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, ω-di ethylamino-2,6-dimethylacetanilide) 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 (Fels et al., 1996). In a two-electrode voltage clamp study in *Xenopus* oocytes Quasthoff and co-workers (2003) found an inhibition of both NaV1.6 (TTX sensitive) and NaV1.8 (TTX resistant) recombinant sodium channels by tolperisone. Similarly to lidocaine, both silperisone (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, since 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 analogues. The close chemical similarity of eperisone, lanperisone and inaperisone (and to some extent silperisone) to tolperisone suggests a similar mode of reflex inhibitory action.

For better understanding of the mechanism of suppressant action of tolperisone and its analogues 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.

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Methods

Materials. Silperisone HCl, eperisone HCl, tolperisone HCl, inaperisone HCl, lanperisone HCl and pipecuronium bromide were synthesized at Gedeon Richter Ltd. Lidocaine was obtained from EGIS Pharmaceuticals (Budapest, Hungary). N-methyl-D-aspartate (NMDA) was purchased from Sigma (St. Louis, MO), tetrodotoxin (TTX) from Latoxan (Valence, France). [^{3}H]-batrachotoxin ([^{3}H]-BTX; specific activity, 50 Ci/mmol) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA). Scorpion venom (*Leiurus quinquestriatus* from North Africa), bovine serum albumin, choline chloride, sucrose, 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 electrode filling solutions were purchased from Sigma. Culture medium components were obtained from Gibco (Gaithersburg, MD). The FLIPR Membrane Potential Assay Kit (Blue) was purchased from Molecular Devices Corp. (Sunnyvale, CA).

For intravenous infusion Rindex solution (containing in mM: NaCl-68, KCl-3.5, $CaCl_2$-1.25, $MgCl_2$-0.5, glucose-555) was purchased from Human Ltd. (Budapest, Hungary). The anesthetic solution for *in vivo* studies contained 0.25% α-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 (Richter).

Animals. Wistar rats (bred at Richter or purchased from Toxicoop or Harlan) 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 280-320 g were used for the *in vivo* experiments. The animal room was thermostated at 21±1 °C, and illuminated artificially from 6 a.m. to 6 p.m. The rats had free access to food and water. All procedures conformed to the guidelines of

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the NIH 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 13-16 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 were incubated at room temperature (23-26ºC) in standard artificial cerebro-spinal fluid (ACSF; composition in mM: NaCl 124, KCl 3.5, NaH_2PO_4 1.23, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 26, glucose 10) bubbled with carbogen (95% O_2 and 5% CO_2) for at least half an hour. 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-VRP) 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 (I-O curve) was also determined using gradually increasing current intensities (0.01-0.2 mA). Evoked compound action potentials (afferent fiber potentials; AFP) 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 (Digidata 1200; 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

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separated according to their post-stimulus 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 earlier in more detail (Farkas 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 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 lidocaine (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 ºC using two heating lamps. During the experiment Rindex solution was infused (10-20 $ml \cdot kg^{-1} \cdot h^{-1}$) in order to maintain the mean arterial blood pressure of spinal animals at around 60 mmHg.

***In Vivo* Spinal Reflex Study.** Ventral 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; frequency: 10/min). The first ten 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

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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; 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). Anesthetized animals were paralyzed with pipecuronium bromide (100 µg/kg starting dose + 50 $\mu g \cdot kg^{-1}h^{-1}$ in infusion). A tungsten microelectrode, insulated except 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; frequency: 10/min). The compound action potential evoked by direct stimulation of motoneurons (MN, first peak) and the one 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 (PAF), 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 at 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 dorsal root ganglia (DRG) of six-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 DMEM at 37 °C in 5% CO_2. Inward currents were recorded by the whole-cell patch clamp technique 1 day after plating of the cells. Coverslips with the attached neurons were transferred to a recording chamber and constantly superfused with the

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extracellular solution (ES) at room temperature (22-25 ºC). The ES used in the sodium current experiments contained (in mM): NaCl 70, choline cloride 70, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 5, HEPES-Na 5, $CdCl_2$ 0.01, glucose 20; pH=7.35. The ES for calcium current measurements contained choline cloride 143, $CaCl_2$ 5, $MgCl_2$ 1, HEPES 10, glucose 20. 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 (in mM): CsF 130, NaCl 15, tertraethylammonium chloride (TEA-Cl) 10, $CaCl_2$ 0.1, $MgCl_2$ 2, ATP-Na_2 2, HEPES 10, EGTA 1; pH=7.25. The IS for calcium current measurements contained: CsCl 110, $MgCl_2$ 4.5, HEPES 9, EGTA 9, ATP-Na_2 4, GTP 0.3, creatine phosphate 14, creatine phosphokinase 50 U/ml, 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-second 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 (Axon Instruments, Union City, 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 multi-barreled 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 percent inhibition of the sodium-current peak amplitude. The availability curves of the sodium current were fitted with the Boltzmann function: $I_{Na}=I_{Na,max}/\{1+\exp([V_H-V_{H1/2}]/k_H)\}$, where I_{Na} is the peak current amplitude, $I_{Na,max}$ is the maximum available sodium current (the upper asymptote of the fitted sigmoid curve), V_H is the applied holding potential, $V_{H1/2}$ is the holding potential at half-maximal availability, while k_H is the slope factor or width.

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Fluorescent Membrane Potential Measurements. Average membrane potential in rat primary cerebellar cell cultures was monitored by fluorimetry. Briefly, 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 (125×g, 5 min) the cells were re-suspended in culture medium (D-MEM) containing 10% foetal bovine serum, 20 ng/ml NGF, 20 mM KCl, 2.5 μg/ml amphotericin B, 100 U/ml penicillin G, 100 μg/ml streptomycin, and plated onto poly-D-lysine-coated 96-well plates (1-2 x 10^5 cells/well). Cultures were incubated at 37 ^oC in a humidified atmosphere of 5% CO_2/95% air and used for the measurement after 7 days *in vitro*. Membrane potential was monitored using the FLIPR membrane potential assay kit (Molecular Devices Corp., Sunnyvale, CA) and FlexStation II (Molecular Devices), a plate reader fluorimeter with integrated 8-channel fluid addition capability. Cells were loaded with the fluorescent dye (100 μl/well) by incubating the plate for 20-60 min at 37 ºC. After loading 50 μl ACSF (control) or 50 μl 4× concentrated test compound solution (dissolved in ACSF) was added to each well and the plate was incubated at 37 ºC for an additional 10 min. Fluorescence measurements were carried out at 37 ºC. The dye was excited at 530 nm, emission was sampled at 565 nm at 1.4-s intervals. After recording baseline for 15 s 50 μl 4× concentrated veratridine solution was added to the cells using the pipettor 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 EC_{80}) 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 $\Delta F/F$ values (fluorescence change normalized to baseline). The effects of blockers at various concentrations were quantified as percent inhibition of the control veratridine response.

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[^{3}H]-BTX Binding. Rat cerebrocortical synaptosomes were prepared as described by Catterall et al. (1981) with minor modifications. Briefly, the cortices of 7-8-week-old male rats were dissected and homogenized in 10 volumes of ice-cold sucrose buffer containing 5 mM K_2HPO_4, pH=7.4. The homogenate was centrifuged at 1000 g (15 min, 4 °C) and the resultant supernatant retained and centrifuged at 20000 g (15 min, 4 °C). Aliquots of the synaptosomal preparation were frozen on dry ice and stored at -80 °C until use. Prior to use, aliquots were thawed at room temperature and centrifuged in 10-fold volume of the same sucrose buffer (20000 g, 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 $MgSO_4$, 5.4 mM KCl and 50 mM HEPES (pH=7.4).

100-µl aliquots equal to approx. 6-8 mg/ml protein were used in [^{3}H]-BTX binding experiments. Binding assays were performed in the presence of 5.0 nM [^{3}H]-BTX, 1 µM tetrodotoxin, 4.0 µg scorpion toxin and various concentrations of the added drugs at 37 °C for 60 min incubation time. Non-specific binding was determined in the presence of 300 µM aconitine. The reaction was terminated by rapid filtration using a UniFilter-96, GF/B (PerkinElmer, Boston, USA). The filtration plates were washed 5 times with ice-cold wash buffer contained 5 mM HEPES, 130 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$ and 0.01% BSA. Radioactivity trapped on 96-well filtration plate was measured by liquid scintillation spectrometry in 40 µl Microscint 20 scintillation cocktail (PerkinElmer, Boston, USA) using a Topcount NXT microplate scintillation & luminescence counter (Packard, Meriden, USA).

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

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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 10-15 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 discernible 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, while 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 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 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 occurred (Fig. 1B). Hence, these depressant drug effects were unlikely 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. In order 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 is depressed (Brooks et al., 1955). Elevation of the temperature of the bathing solution to 31-33 °C depressed MSR allowing to record the main component of the incoming AFP (Fig. 3A) and a nearly pure EPSP related potential (Fig. 3B) simultaneously. Fig 3 shows that lidocaine was clearly more effective in blocking nerve conduction than EPSP, while 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 Figure 4A. We differentiated three characteristic components of the ventral root reflex (VRR) potential (as described previously in: Farkas and Ono, 1995): mono-, di- and polysynaptic reflex (MSR, DSR and PSR, respectively; Fig. 4A). Silperisone (10 mg/kg, iv.) attenuated all the three components with a sensitivity order of components: MSR>DSR>PSR. Its action was longer-lasting than those of tolperisone, eperisone or lidocaine (about 40, 90, 85 and 70% recovery of MSR 1 hour after iv. injection, respectively; not illustrated). The profiles of tolperisone and eperisone, i.e. the relative sizes of effects on the three different VRR components, were practically identical. However, peak effects of tolperisone were a bit greater for all the three components. The

profile of silperisone was also similar to that of the above two compounds. However, its peak effect on MSR was slightly weaker. The profile of lidocaine was substantially different, since 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 3-5 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 Figure 5A.

Silperisone (10 mg/kg i.v.) depressed MN (direct excitability) by 15%, while MS (the synaptic response) decreased by 68% in average (Fig. 5). It exerted no effect on excitability of primary afferents (PAF). The onset of the effect of silperisone was relatively slow, 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 6-8 min, while lidocaine (10 mg/kg i.v.) produced more marked depression of MN and PAF (30% and 14%, respectively; see Fig. 5D) than tolperisone or silperisone did. On the other hand its depressant effect on MS (67%) was less pronounced compared with tolperisone and 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 administration of silperisone, tolperisone and lidocaine (10-10 mg/kg iv.) 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). Since 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 ($V_{H1/2}$), i.e. at -80 mV. Thus, in order to decrease the variability of drug sensitivities among DRG neurons, cells with $V_{H1/2}$ 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 IC_{50} 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 mV to -10 mV at 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 resulted 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 inactivation curves ($V_{H1/2}$: -75.0±1.5 mV) towards the more hyperpolarized membrane potentials, but also decreased the maximum available current (Table 2).

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On the contrary, none of the drugs affected the voltage dependence of activation (I/V curve) of sodium currents (not illustrated).

In accordance with the data of Wu and Pan (2004) we found that L-type and P/Q-type calcium channels do not contribute considerably to the high-threshold voltage-activated calcium current in DRG cells, since neither nitrendipine (10 μM; n=4), nor ω-agatoxin-IVA (0.1 μM; n=9) blocked the current significantly (5±1% and 4±1% inhibition of calcium current amplitude, respectively), while ω-conotoxin-GVIA, a selective N-type calcium channel blocker (3 μM), caused a 28±3% inhibition (n=4). Silperisone (320 μM) inhibited both the ω-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_{50} of 218±22 μM (determined in 8 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_{50}=65±9 μM; n=8). Tolperisone had an IC_{50} of 1062 μM in the presence of creatine phosphate, creatine phosphokinase, and GTP, thus it was about five-fold less potent than silperisone. No IC_{50} values were determined with eperisone and lidocaine, thus the calcium channel inhibiting efficacies of drugs were compared at 320 μM (Fig. 7B). At their IC_{50} concentrations for sodium channel blockade tolperisone, silperisone, eperisone and lidocaine caused 22, 34, 36 and 7% diminution of calcium currents, respectively.

Effects on [^{3}H]-BTX Binding and Veratridine Induced Depolarization: All studied compounds inhibited [^{3}H]BTX binding in cortical synaptosomal preparation dose dependently. IC_{50}s are listed in Table 1. At similar concentrations, the same compounds also reduced membrane depolarization evoked by the sodium channel activator/inactivation inhibitor veratridine (Table 1). Similarly to the sodium current measurements lidocaine

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proved to be the least effective also in these assays. The results of the [^{3}H]BTX binding test are in good correlation with those of the veratridine depolarization assay, and the rank orders, with one exception, are in agreement with the orders found in the whole-cell current experiments. However, the potencies of the compounds in the former two assays are different from what was determined in the patch clamp experiments. The discrepancy may be related to the facts that the former assays apply veratridine and BTX, respectively, which may alter the sensitivity of sodium channels to this type of blocking agents. Furthermore, different parameters (peak current or steady-state responses) were measured in the different assays.

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 lidocaine did. 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), while the second one was due to monosynaptic (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 (first of all glutamate) released from the terminals. A decreased transmitter release results in a depression of EPSP. In order to analyze precisely the relationship between depression of the afferent nerve conduction and of the 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. Profile of tolperisone was between 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 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 underestimation 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 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 completely abolished by co-administration of the AMPA antagonist GYKI 52466 and the NMDA antagonist APV (Kocsis et al., 2003). However, according to our former (unpublished) studies neither tolperisone nor silperisone had any affect on AMPA 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 analogues share this feature of lidocaine, which is in agreement with the findings of Ono et al., 1984; Hinck and Koppenhöfer, 2001; Düring 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* (see Table 1) may be related to differences between the compounds regarding the diffusibility inside the spinal tissue and/or differences in activities on other channels.

The coupling between the afferent volley and the transmitter release can be modulated effectively by antagonists of certain subtypes of voltage gated calcium channels too (Burnashev and Rozov, 2005). Tolperisone slightly blocked these channels at sodium channel blocking concentrations. However silperisone and eperisone were more potent in this regard, while lidocaine had only weak effect on calcium channels. This might underlie the stronger effect of silperisone on EPSP than on AFP as 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 mediation of somatic spinal reflexes and regulation of the muscle tone at spinal segmental level, is usually between -60 and -70 mV (Caffrey et al., 1992; Liu et al., 2000; Xing et al., 2001). In this membrane potential range the steady-state inactivation curve of these sodium channels displays a steeply

changing phase. Thus even minor depolarization of the membrane results in the inactivation of a significant portion of sodium channels resulting in a weaker current (due to decreased channel availability). Under these conditions the incoming action potentials can trigger smaller calcium influx, resulting in decreased transmitter release from the primary afferent terminals. Such changes in the membrane potential occur during presynaptic inhibition in the spinal cord fibers under normal, physiological conditions due to the GABA-A mediated depolarization of the presynaptic primary afferent terminal.

Spinal presynaptic inhibition (for reviews see Schmidt, 1971 and Levy, 1980) is thought to be decreased in some states of spasticity yielding an imbalance between facilitatory and inhibitory influences on segmental spinal neuronal circuits involved in reflex transmission (Delwaide and Pennisi, 1994; Nielsen and Hultborn, 1993). Shifting the steady-state inactivation curve of sodium channels towards more negative potentials may compensate for the weakened physiological presynaptic inhibition and thus may be an efficient therapeutic strategy to restore the balance. The sodium channel blocking effect of tolperisone-like drugs in DRG cells is state dependent and results in a shift in the steady-state inactivation curve towards the hyperpolarized potentials besides some depression of the maximum current at high negative potentials. This feature is manifested as a preferential action on depolarized membranes. A state-dependent block of sodium channels is a common feature of several anticonvulsants, such as phenytoin, carbamazepine and lamotrigine (Clare et al., 2000). This means that these drugs bind preferentially to the inactivated and open states of the sodium channel alpha subunit (Wang et al., 2004). A sustained hyperexcitable and depolarized state of spinal neurons have been recently reported in a spasticity model (Bennett et al., 2003; Li et al., 2003, 2004a, 2004b) reminiscent of human spasticity disorders. Thus we propose that the state dependent action of tolperisone and its analogs on sodium channels may result in a selective inhibition of the function of those neurons that are involved in spasticity disorders.

This feature may underlie the relative lack of motor side-effects of tolperisone in the 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 the 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 (Delwaide and Pennisi, 1994; Farkas et al., 1989). 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.

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Footnotes

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Legends for Figures

Fig. 1. Effects of silperisone on dorsal root stimulation evoked ventral root potential (DR-VRP) components *in vitro*. A: Inhibition of DR-VRP by increasing doses of silperisone (50-200 μM) added to the perfusing medium. The insert shows the same potentials on an extended time-scale to allow the observation of the changes in the monosynaptic reflex. B: Effect of 50 (●), 100 (▲) and 200 μM (▼) silperisone, compared with control (■) on the stimulus-strength dependence of the peak to peak amplitude of monosynaptic compound action potential (MSR AMPL) and on the integral of EPSP (EPSP INT.). Data are presented as mean±S.E.M. from 4 experiments. Note that efficacy of silperisone was not dependent on the stimulus intensity, i.e. increasing the stimulus strength did not counteract the inhibition produced by silperisone.

Fig. 2. Effects of different tolperisone type centrally acting muscle relaxant drugs (tolperisone-TOLP, silperisone-SILP, eperisone-EPE, lanperisone-LANP, inaperisone-INAP) and lidocaine (LID) on dorsal root stimulation evoked ventral root potential (DR-VRP) components *in vitro*. IC_{50} values of the drugs studied are based on inhibition of different reflex components – peak-to-peak amplitude of monosynaptic compound action potential (hollow columns), integram of EPSP (hatched columns), amplitude of EPSP (checked columns), integral of late part of EPSP (80-180 ms post-stimulus time; solid columns). All columns represent mean±S.E.M. from 4 experiments.

Fig. 3. Effects of drugs on synaptic responses (EPSP) and on afferent fiber potential (AFP). These experiments were performed at bath temperature of 31-33 °C, in order 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-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 (□). Mean±S.E.M. from 3-3 experiments.

Fig. 4. Effect of drugs (tolperisone-TOLP, silperisone-SILP, eperisone-EPE, lidocaine-LID) on ventral root reflex evoked by dorsal root stimulation recoreded *in vivo* in spinal rats. A: representative averaged (n=10) potential before (left) and 10 min after (right) administration of silperisone (10 mg/kg i.v.). The reflex response consists of three components: the first, robust peak, the monosynaptic reflex (MSR); the smaller and somewhat more variable disynaptic reflex (DSR) and the longer-lasting polysynaptic reflex (PSR), which is hardly discernable from the baseline at this amplification. The arrows indicate the stimulation artifact. Calibration bars: 2 mV, 2 ms. B: Maximal inhibitory effects (within 30 minutes after drug administration) of drugs (10 mg/kg i.v.) on different reflex components: MSR (open columns), DSR (hatched columns), PSR (solid columns), as percentage of the control responses. All the data (B-D) are presented as mean ± S.E.M. from five experiments.

Fig. 5. Effect of drugs (tolperisone-TOLP, silperisone-SILP, lidocaine-LID) on direct electrical excitability of motoneurons (MN), their monosynaptic excitation (MS) and excitability of primary afferents (PAF). A: averaged potentials recorded from L5 ventral root (VR) and from L5 dorsal root (DR), before (left) and 6 minutes after (right) administration of silperisone (10 mg/kg i.v.). The arrows indicate the stimulation artifact. Silperisone had negligible effect on PAF, and little effect on MN, but strongly inhibited MS. Calibration bars: VR: 1 mV, 2 ms; DR: 5 mV, 2 ms. B: Comparison of the effects of tolperisone (n=3),

silperisone (n=5) and lidocaine (n=3), each at 10 mg/kg i.v. on PAF (open columns), MN (hatched columns) and MS (solid columns). Data are presented as mean±S.E.M.

Fig. 6. Effect of tolperisone on voltage gated sodium channels in DRG cells. A: Current traces from bottom to top were recorded in the presence of 0, 40, 80, 160, 320, and 640 μM tolperisone, in a representative experiment. The applied voltage command protocol is shown above the traces. B: Effect of tolperisone on the steady-state inactivation characteristics. Tolperisone (200 μM;▼) shifted the control (■) inactivation curve and decreased the maximum current at high negative potentials. The normalized curve (●) indicates a pronounced parallel leftward shift in the current availability curve of the control curve. Data are mean±S.E.M. from seven cells.

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, eperisone-EPE) and lidocaine (LID) at 320 μM on calcium current peak amplitude. Columns represent mean±S.E.M. in 4-7 experiments.

Tables

Table 1.
IC_{50} values (μM; mean ± S.E.M.) of various tolperisone-type muscle relaxant drugs and lidocaine in *in vitro* assays. The number of observations is shown in parenthesis.

Compound	CURRENT	[^{3}H]BTX	VER	MSR	EPSP
tolperisone	198±21 (6)	40.9±2.5 (7)	58.3±2.4 (3)	62.7±9.4 (7)	130.7±10.4 (7)
silperisone	111±18 (10)	7.2±0.8 (4)	14.2±0.6 (3)	55.9±1.5 (4)	72.5±13.8 (4)
eperisone	250±93 (5)	21.7±2.5 (3)	37.1±1.8 (3)	43.3±5.8 (5)	76.0±9.4 (5)
inaperisone	n.d.	34.6±3.0 (3)	41.8±3.5 (4)	29.6±3.8 (7)	59.7±7.4 (7)
lanperisone	n.d.	13.7 (1)	31.2±2.9 (4)	33.0±3.1 (5)	114.7±15.1 (5)
lidocaine	297±86 (6)	132±9 (4)	60.6±6.2 (4)	186.2±17.6 (6)	198.5±42.8 (6)

n.d.: not determined
[^{3}H]BTX: [^{3}H]BTX binding
VER: veratridine evoked depolarization determined by microfluorimetry
CURRENT: sodium currents measured by whole-cell patch clamp
MSR: monosynaptic reflex amplitude
EPSP: EPSP integral

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Table 2.
Effects of tolperisone, silperisone and lidocaine on steady-state inactivation of voltage gated sodium channels of DRG cells. $\Delta V_{H1/2}$ is the shift of the half-maximal voltage for inactivation caused by the given drug, I_H is the percentage inhibition of current peak amplitude at a holding potential of -130 mV. The number of observations is shown in parenthesis.

Compound	$\Delta V_{H1/2}$ (mV)	I_H (%)
200 μM tolperisone	-19±3 (7)	33±12 (4)
400 μM tolperisone	-22±3 (6)	41±9 (6)
80 μM silperisone	-6±1 (7)	13±2 (6)
160 μM silperisone	-8±1 (7)	32±7 (6)
200 μM lidocaine	-13±2 (7)	14±4 (6)
400 μM lidocaine	-17±2 (9)	27±7 (7)

-

Fig 1

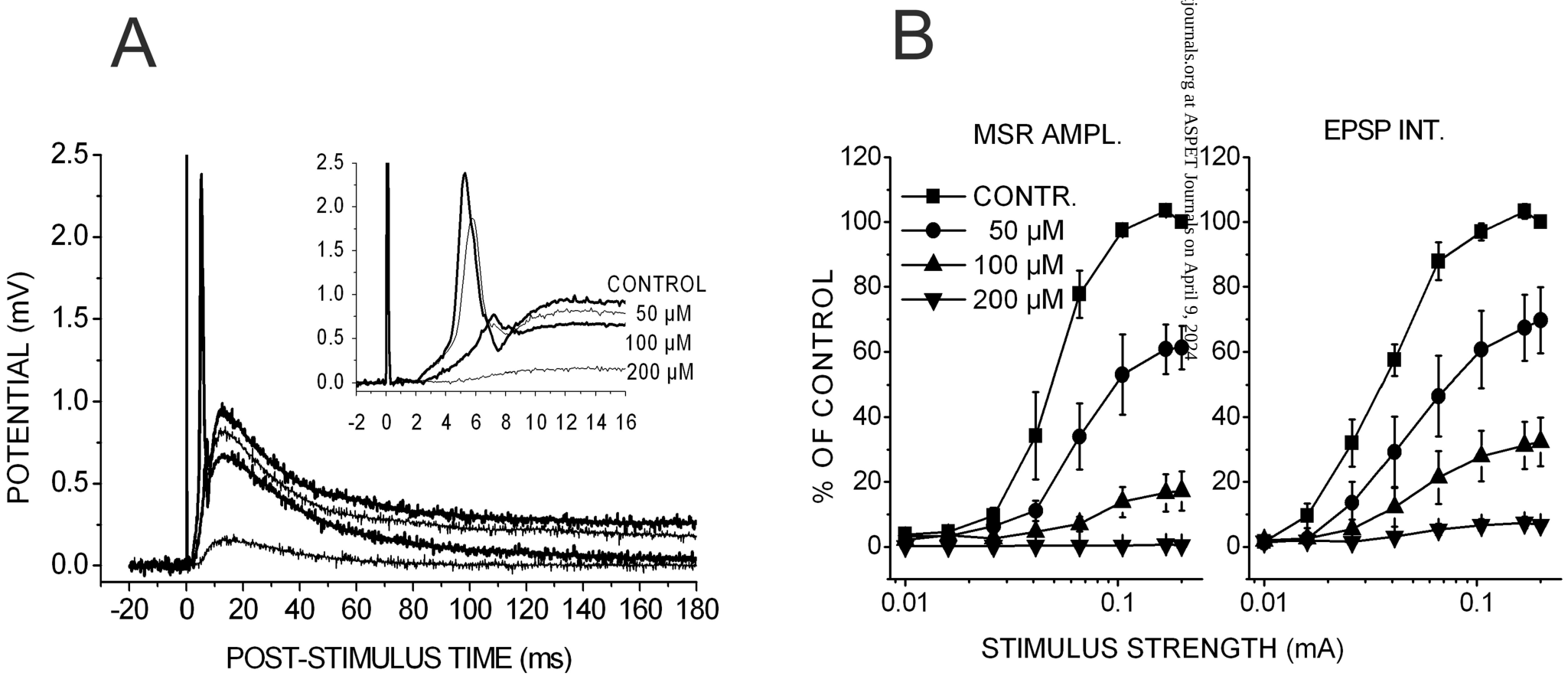


Fig 2

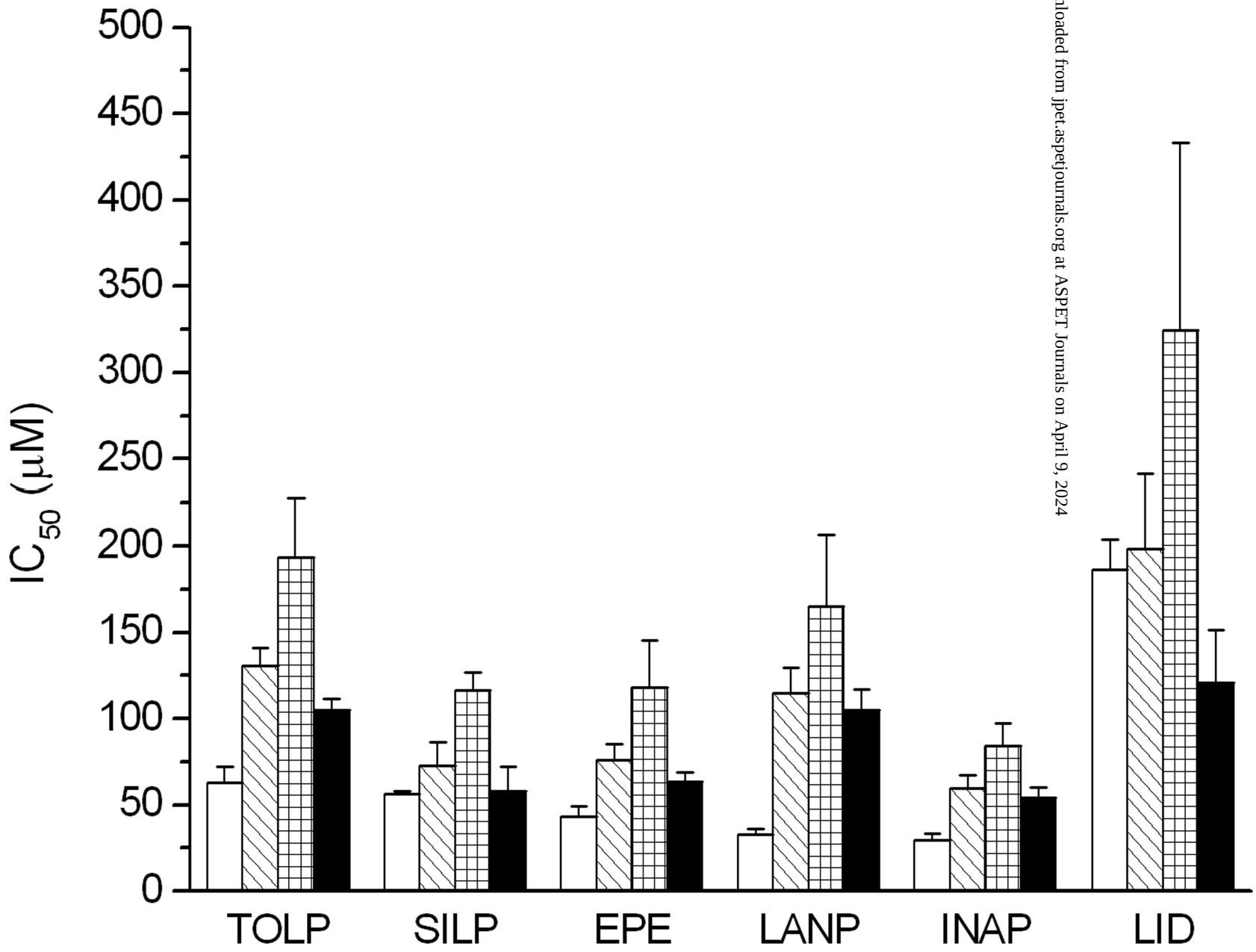


Fig 3

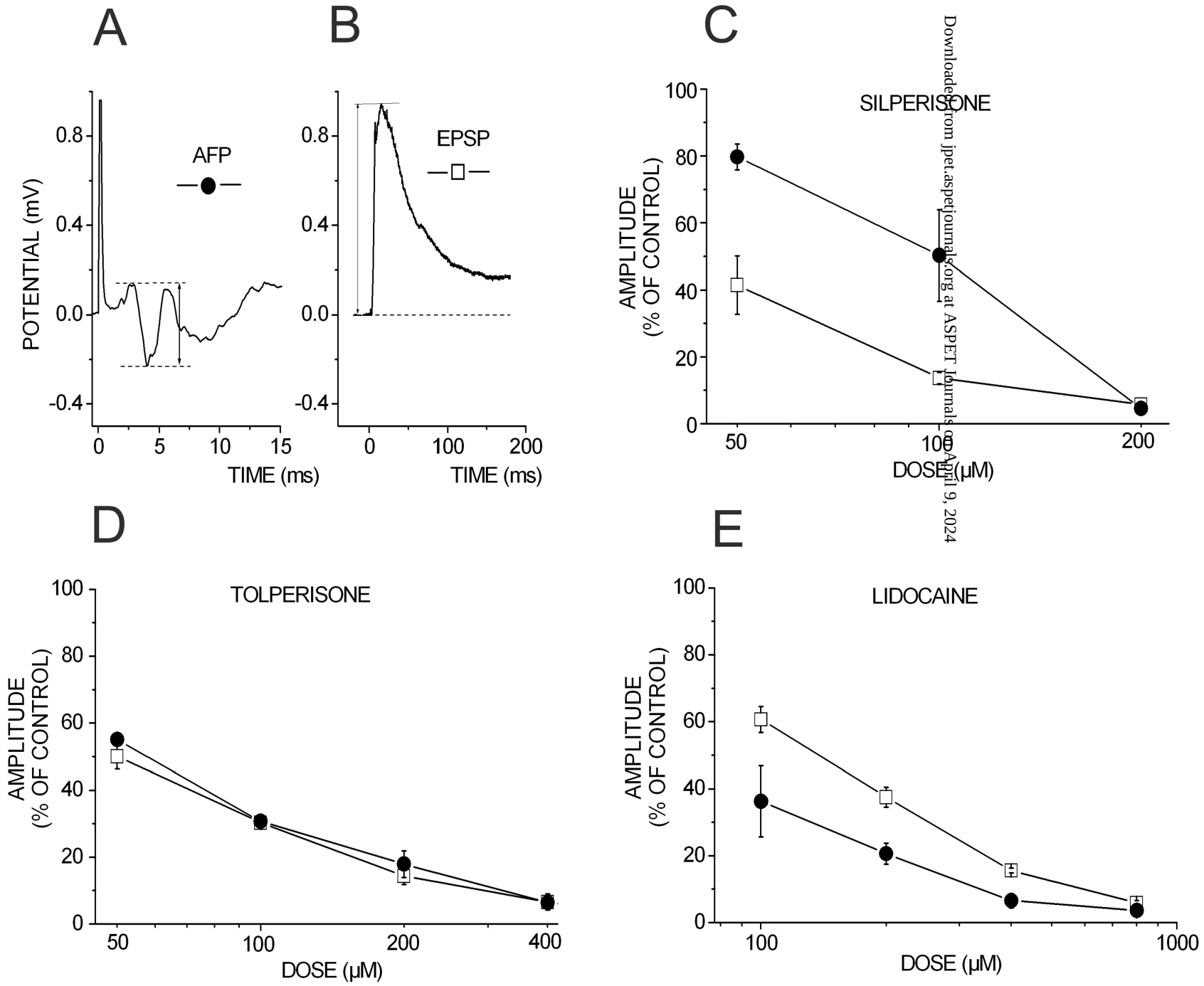


Fig 4

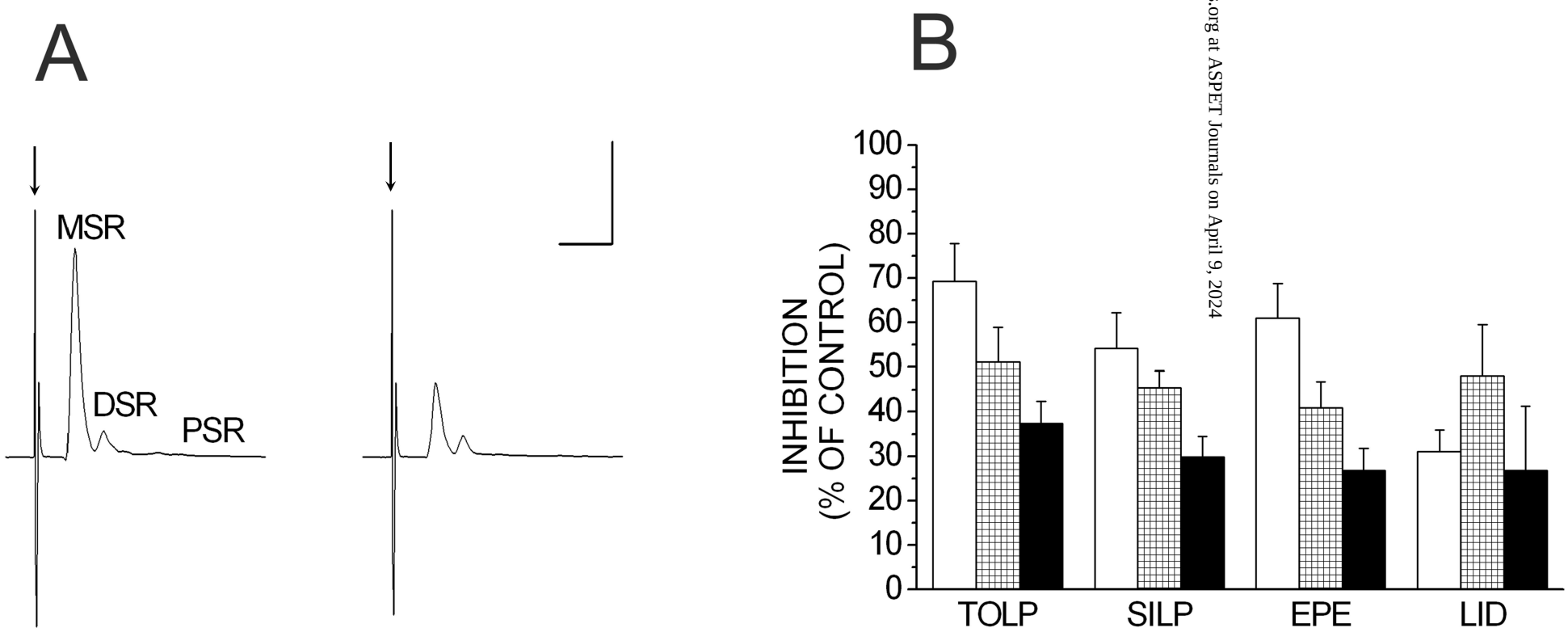
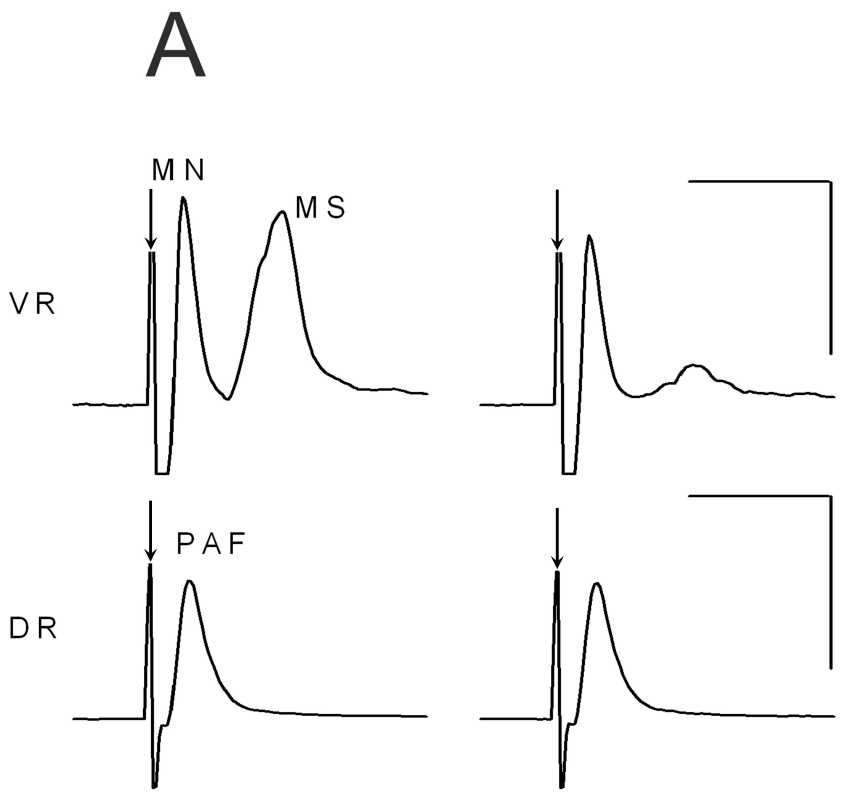


Fig 5



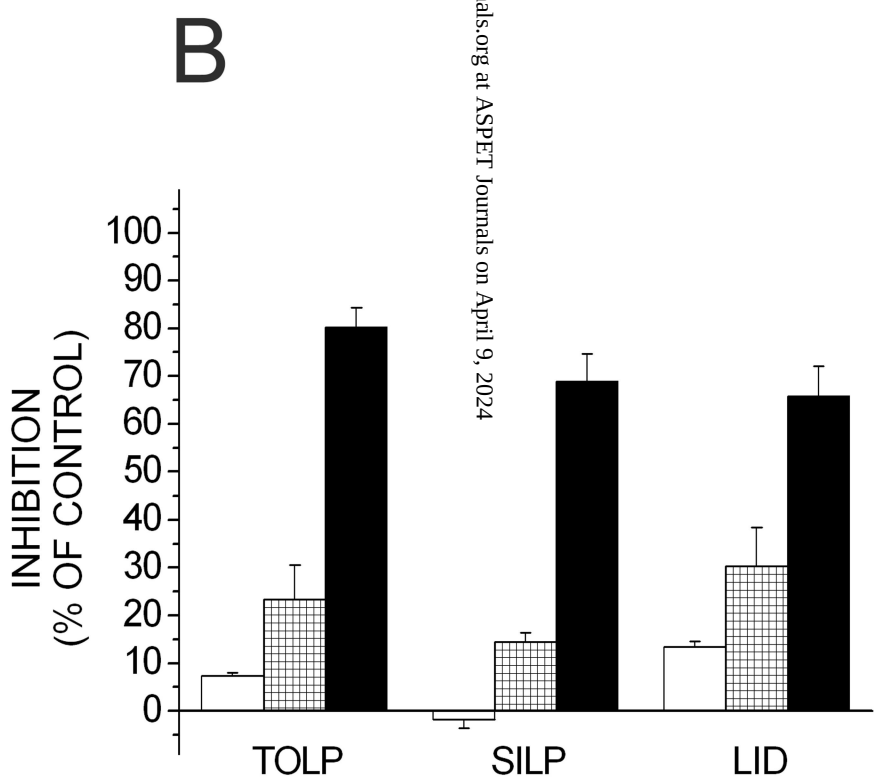
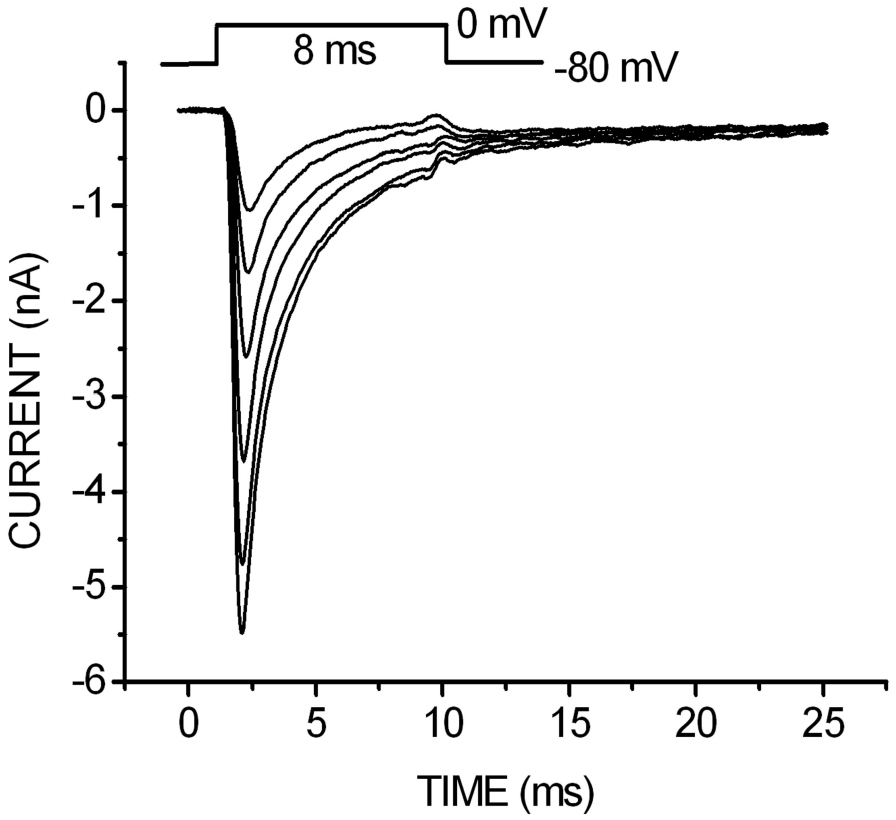


Fig 6



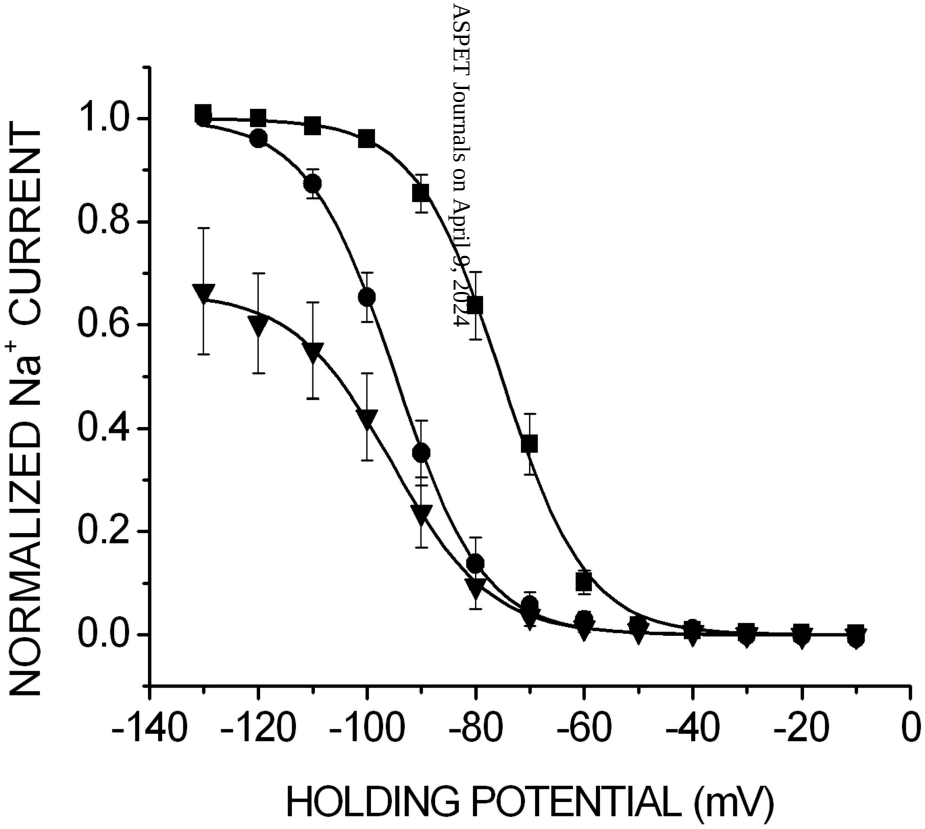


Fig 7