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SHORT COMMUNICATIONS

Inhibition of spinal noradrenaline uptake in rats by the centrally acting analgesic tramadol

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Abstract—Tramadol is a centrally acting analgesic with low affinity to opioid receptors. A further mode of action is inhibition of noradrenaline uptake as measured in standard assays. Since tramadol shows antinociception at the spinal site, it was to be tested whether uptake blockade could be verified in spinal tissue. Therefore, synaptosomes and slices had to be prepared from the dorsal half of the spinal cord and the uptake of [^{3}H]noradrenaline into synaptosomes to be characterized. The uptake was linear for at least 3 min. The apparent K_m was 0.16 μM and V_{max} was 7.9 pmol/min/mg protein. Tramadol inhibited the uptake competitively as analysed with Dixon plots with a K_i of 0.6 μM. Uptake inhibition was effected in order of potency by (+)-oxaprotiline > nisoxetine > (−)-tramadol > (−)-oxaprotiline = tramadol > (+)-tramadol. Slices were preincubated with [^{3}H]noradrenaline then superfused and stimulated electrically. Nisoxetine, tramadol and its (−)-enantiomer enhanced mainly the stimulation-evoked overflow indicating uptake inhibition without releasing effects. Experiments with inclusion of the noradrenaline uptake inhibitor desipramine provided evidence that tramadol interfered with the noradrenaline transporter. The results show that spinal synaptosomes and slices are valid preparations to study local noradrenaline uptake and release. Tramadol enhances extraneuronal noradrenaline levels in the spinal cord by competitive interference with the noradrenaline uptake mechanism.

Key words: spinal cord; synaptosomes; noradrenaline; uptake; release; tramadol

Tramadol, (1*RS*; 2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, is a centrally acting analgesic with a five to 10 times lower antinociceptive potency as compared to morphine [1]. Its affinity to the opioid receptor in the micromolar range is, however, low by comparison [2, 3]. A further mode of action of tramadol has been identified as inhibition of noradrenaline uptake [3–7], which may contribute to the antinociception *in vivo* since effects were blocked by the purported α_2-adrenergic antagonist yohimbine [3, 6] and by yohimbine or idazoxane [8]. The noradrenergic antinociceptive effects were, in part, observed at the spinal site after intrathecal injection of tramadol [3, 6]; the *in vitro* data as regards noradrenaline uptake inhibition were obtained in standard assays using various tissues from the supraspinal part of the central nervous system [3–7]. Since most primary sensory afferent axons terminate in the dorsal horn of the spinal cord (for review see Ref. 9), modulation of sensory inputs by noradrenaline derived from the descending inhibitory pathways occurs most likely at this site. Therefore we used tissue from the dorsal part of the spinal cord to look for the modulation of the noradrenergic transmission by tramadol. Noradrenaline release from slices of this region has been characterized previously [10] but synaptosomes derived from that part have been used rarely for the demonstration of noradrenaline uptake (e.g. Ref. 11); therefore we investigated the characteristics of noradrenaline uptake in our preparation in more detail.

Materials and Methods

Experimental procedure. Male Sprague–Dawley SPF rats, weighing 290–430 g (Lippische Versuchstierzucht, Extertal 1, F.R.G.) were killed by decapitation. The vertebral column was excised as described by Yaksh and Harty [12] and the spinal cord was ejected by hydraulic pressure. After chilling, the meninges were removed from the cord and the length of the cord was corrected to contain segments from Th 10-L 2. The dorsal half of the spinal cord was dissected using a steel blade and a glass support.

Accumulation of [^{3}H]*noradrenaline in synaptosomes.* A crude synaptosomal pellet was prepared according to Gray and Whittaker [13]. Briefly, the tissue was homogenized in ice-cold 0.32 M sucrose (100 mg tissue/1 mL) in a glass homogenizer with a teflon pestle using five full up and down strokes at 840 rpm. The homogenate was centrifuged at 4° for 10 min at 1000 *g*. Subsequent centrifugation of the supernatant at 17,000 *g* for 55 min yielded a pellet (P_2) which was resuspended in 0.32 M glucose (about 0.5 mL/100 mg original weight). Incubations were run in triplicate. Synaptosomal suspension (50 μL) containing approximately 200 μg protein [14] was added to 850 μL incubation medium containing the drug to be tested and preincubated for 5 min at 37° under an atmosphere of 5% CO_2 in O_2. Accumulation was started by addition of [^{3}H]noradrenaline to yield a concentration of 0.1 μM in a final volume of 1 mL. Accumulation was stopped after 60 sec, if not stated otherwise, by addition of 8 mL ice-cold incubation medium, immediately followed by filtration through Whatman GF/B filters. Filters were washed with 5 mL incubation medium, then extracted with Ready Protein® (Beckman) for at least 15 hr and counted for radioactivity. The incubation medium contained (mM): NaCl 119, KCl 3.9, $CaCl_2$ 0.51, $MgSO_4$ 0.65, Na_2HPO_4 15.6, NaH_2PO_4 3.4, glucose 10, ascorbic acid 0.57 and iproniazid 0.0156. The pH was adjusted to 7.4 by addition of NaOH (23°).

Values were corrected for accumulation of tritium at 0°. IC_{50} values were calculated by nonlinear adaptation of the data to the law of mass action and K_i values were obtained by use of the Cheng–Prusoff equation [15]. K_m and V_{max} values were estimated by linear regression of the reciprocals of the [^{3}H]noradrenaline concentrations and the measured uptake velocities [16]. In kinetic experiments, K_i values were obtained by calculation of the intersection of the linear regression lines of the data transformed according to Ref. 17.

Release of [^{3}H]*noradrenaline from slices.* The tissue was cut transversely with a McIlwain tissue chopper at a thickness of 0.4 mm. Slices were preincubated with 0.1 μM [^{3}H]noradrenaline at 37° for 30 min during stimulation with electrical pulses applied at 0.1 Hz, 24 mA and 2 msec width. After preincubation, slices were transferred to superfusion chambers equipped with platinum plate electrodes and

superfused for 105 min at a rate of 1 mL/min at 37°. Five minute samples were collected starting 50 min after the onset of stimulation. The slices were stimulated electrically with rectangular pulses (3 Hz, 24 mA and 2 msec width) for two periods of 2 min each after 60 and 90 min of superfusion (S_1, S_2). At the end of the experiment, slices were solubilized in 0.5 mL Soluene 350® (Packard). The radioactivity in superfusates and slices was measured after addition of Ultima Gold® (Packard). The incubation and superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03; the medium was saturated with 5% CO_2 in O_2. The pH was adjusted to 7.4 by the addition of NaOH. Drugs were added 15 min before S_2 or were present throughout superfusion when indicated.

The fractional rate of tritium outflow in release experiments was calculated by dividing the tritium content in the superfusate by the tritium content in the slice at the start of the respective collection period. The stimulation-evoked overflow of tritium was calculated by subtraction of the estimated basal outflow and was expressed as a percentage of the tissue tritium content at the start of stimulation.

Means ± SEM are given throughout. Differences between means were tested for significance by the Mann–Whitney U-test.

Pretreatment with DSP – 4. In some experiments on [^{3}H]noradrenaline accumulation, rats were injected i.p. with the serotonin uptake blocker, zimeldine, 10 mg/kg, 45 min prior to the i.p. injection of the noradrenergic neurotoxin, DSP-4, 63 mg/kg. The experiments were performed 7 days after the treatment.

Substances used in the experiments. Levo-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 40.0–52.3 Ci/mmol (New England Nuclear, Boston, MA, U.S.A.); desipramine HCl, iproniazid phosphate, l-noradrenaline HCl (the Sigma Chemical Co., St Louis, MO, U.S.A.); DSP-4 (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine HCl, RBI, Natick, MA, U.S.A.); tramadol HCl, (+)-tramadol HCl, (−)-tramadol HCl (Grünenthal, F.R.G.); zimeldine di-HCl (Astra). (+)- and (−)-oxaprotiline was kindly provided by Ciba-Geigy (Basle, Switzerland) and nisoxetine HCl was synthesized by Dr E. Graudums (Grünenthal, F.R.G.). All drugs were dissolved in water. In accumulation experiments, specific activity of [^{3}H]noradrenaline was reduced with unlabelled noradrenaline to about 5 Ci/mmol.

Results

Accumulation of [^{3}H]noradrenaline in spinal synaptosomes. Preliminary experiments with incubation times up to 9 min showed linear uptake of [^{3}H]noradrenaline in the crude synaptosomal preparation for up to 3 min (not shown). Therefore the chosen incubation period of 1 min was within the linear range. Kinetic analysis of two experiments run in triplicate performed according to [16] showed an apparent K_m of 0.16 μM, and V_{max} was 7.9 pmol/min/mg protein.

Effects of DSP-4 pretreatment on [^{3}H]noradrenaline accumulation. Rats were pretreated with the noradrenergic neurotoxin DSP-4 as described in Materials and Methods. Synaptosomes were prepared 7 days later, and the accumulation of [^{3}H]noradrenaline was allowed to proceed for 60 sec. Synaptosomes from control animals accumulated 4.5 ± 0.8 pmol [^{3}H]noradrenaline/min/mg protein (N = 4) while synaptosomes from treated animals accumulated 0.41 ± 0.06 pmol/min/mg protein (N = 3), i.e., less than 10% of controls.

Inhibition of [^{3}H]noradrenaline accumulation. Synaptosomes were incubated with [^{3}H]noradrenaline either in the presence or absence of drugs. Accumulation of drugs under control conditions (no drug present) was 4.9 ± 0.5 pmol [^{3}H]noradrenaline/min/mg protein (N = 18). The accumulation of radioactivity was inhibited by all drugs tested (Fig. 1). The rank order of potency was (three independent experiments per substance; K_i values calculated according to Ref. 15): (+)oxaprotiline (K_i 0.38 ± 0.08 nM) > nisoxetine (K_i 1.10 ± 0.25 nM) > (−)-tramadol (K_i 0.28 ± 0.06 μM) > (−)-oxaprotiline (K_i 0.52 ± 0.08 μM) =tramadol (K_i 0.63 ± 0.17 μM) > (+)-tramadol (K_i 4.49 ± 0.25 μM). Therefore, potency of enantiomers differed by a factor of about 1400 for oxaprotiline and 16 for tramadol.

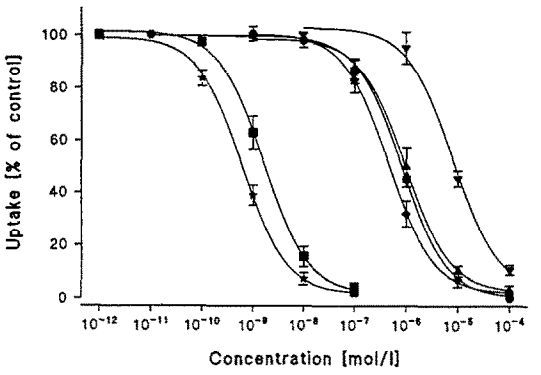

Fig. 1. Effect of various drugs on the accumulation of tritium in spinal cord synaptosomes incubated with 0.1 μM [^{3}H]noradrenaline. The accumulation in the presence of (+)-oxaprotiline (★), (−)-oxaprotiline (●), nisoxetine (■), tramadol (▲), (−)-tramadol (◆) and (+)-tramadol (▼) is expressed as a percentage of control. Each data point represents the mean ±SEM of three independent experiments.

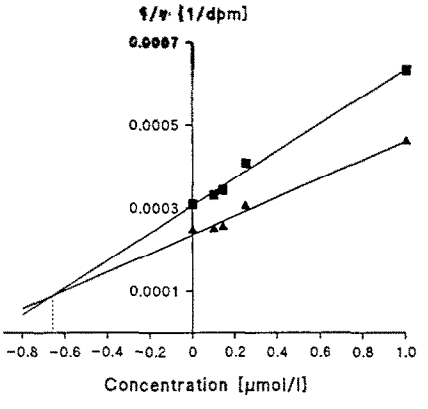

Fig. 2. Inhibition by tramadol of the accumulation of tritium in spinal cord synaptosomes incubated with either 0.1 (■) or 0.2 μM (▲) [^{3}H]noradrenaline. Representation of the data according to Ref. 17.

The mode of interaction of tramadol with the [^{3}H]-noradrenaline carrier was investigated using Dixon plot analysis [17]. Tramadol inhibited the accumulation of tritium in synaptosomes from the spinal cord in a competitive manner shown by the intersection of the regression lines above the x-axis (Fig. 2). Calculation of the inhibitor constant K_i by linear regression analysis yielded a value of 0.66 μM; this value was confirmed in a

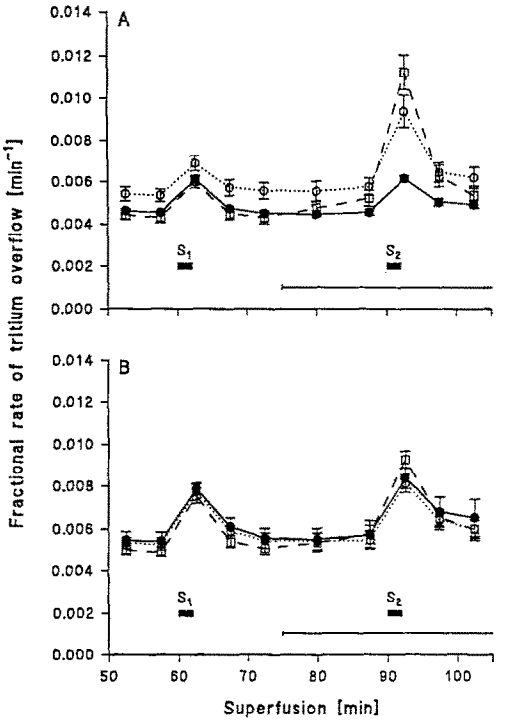

Fig. 3. Effects of tramadol on the outflow of tritium from rat spinal cord slices preincubated with [^{3}H]noradrenaline. After preincubation, slices were superfused and stimulated electrically twice (S_1, S_2). (A) The superfusion medium contained no drug. (●): controls, N = 14; (○); tramadol, 10 μM, was added 15 min before S_2, N = 5; (□) tramadol, 100 μM, was added 15 min before S_2 as indicated by the horizontal bar, N = 6. (B) The superfusion medium contained 1 μM desipramine. (●): controls, N = 6; (○): tramadol, 10 μM, was added 15 min before S_2, N = 6; (□): tramadol, 100 μM, was added 15 min before S_2 as indicated by the horizontal bar, N = 6.

further experiment which yielded a value of 0.61 μM (not shown). So the values obtained with the kinetic analysis were very similar to those obtained in the inhibition experiments which were calculated by means of the Cheng–Prusoff equation.

Effects on tritium overflow from slices preincubated with [^{3}H]*noradrenaline.* Slices from the dorsal half of the spinal cord were preincubated with [^{3}H]noradrenaline then superfused with drug-free medium and stimulated electrically for two periods of two min each after 60 and 90 min of superfusion. Stimulation at S_1 elicited an overflow of 0.78 ± 0.03% of the tritium in the tissue (N = 94). When no drug was added at S_2, the stimulation-evoked overflow was of about the same magnitude (Fig. 3) and the ratio S_2/S_1 remained near unity. Addition of tramadol had little influence on the basal outflow of tritium but enhanced the evoked overflow in a concentration-related manner (Fig. 3A); a significant enhancement by 27% of the stimulation-evoked overflow was already observed at the concentration of 1 μM (N = 7, not shown) and at 10 μM, the evoked overflow almost doubled. When experiments were repeated with superfusion medium containing 1 μM desipramine, stimulation at S_1 elicited an overflow of 2.19 ± 0.12% of the tritium in the tissue (N = 23). When no further drug was added at S_2, stimulation evoked an about equal amount of tritium in the overflow (Fig. 3B) resulting in a ratio S_2/S_1 near unity. Addition of tramadol 1 (not shown) and 10 μM had no significant effect on the evoked overflow while 100 μM enhanced it by about 38% ($P < 0.05$, Fig. 3B). So effects by tramadol were substantially attenuated when uptake sites were already blocked by desipramine.

A further set of experiments compared the effects of the enantiomers with the uptake inhibitor nisoxetine. The ratio S_2/S_1 of controls was 1.06 ± 0.27 (N = 16). The (−)-enantiomer enhanced it to 1.31 ± 0.07 at 1 μM (N = 6; $P < 0.05$) and 2.03 ± 2.03 ± 0.15 at 10 μM (N = 6; $P < 0.05$). The (+)-enantiomer effected ratios of 1.07 ± 0.04 at 1 μM (N = 6) and 1.21 ± 0.04 at 10 μM (N = 6) which did not differ significantly from those of controls. The reference compound nisoxetine enhanced the ratio to 1.40 ± 0.16 at 1 nM (N = 7) and 2.28 ± 0.27 (N = 7; $P < 0.05$ and was thus at least three orders of magnitude more potent than the (−)-enantiomer.

Discussion

Measurement of noradrenaline uptake in spinal cord synaptosomes is not a commonly used model, and has not been characterized in detail (e.g. Ref. 11). Although uptake sites could be labelled, preparation of functional synaptosomes failed in tissue like the cerebellum [18]. Therefore, we investigated our crude synaptosomal preparation from the dorsal, the sensory part of the spinal cord, more closely. In order to approach true uptake, i.e. unidirectional transmitter flow, only the initial linear rate of accumulation should be measured [19]. This precondition was met in our experimental set-up. In addition, deamination was blocked by iproniazid to assure accumulation of authentic [^{3}H]noradrenaline. Transmitter accumulation may occur in other than their own terminals, e.g. noradrenaline is readily taken up in dopaminergic terminals although with lower affinity ($K_m \sim 2$ μM) [20]. The specific noradrenergic neurotoxin, DSP-4, which reduced spinal noradrenaline levels by about 80% [21, 22] and which had been shown to inhibit [^{3}H]noradrenaline uptake in and subsequent release from spinal slices [10], was used to eliminate the majority of functional noradrenergic axon terminals. The results show that in our preparation [^{3}H]noradrenaline is mainly accumulated in these noradrenergic terminals. The small remaining accumulation of noradrenaline, less than 10% compared to controls, is probably due to incomplete destruction of terminals by DSP-4 since noradrenaline levels were still measured after treatment [21, 22]. The K_m for [^{3}H]-noradrenaline uptake (0.16 μM) found in the present investigation complies with that described for the high affinity transport system in adrenergic neurones [20]. The preparation showed an even slightly higher affinity for noradrenaline uptake than a corresponding preparation of hypothalamic axon terminals (0.32 ± 0.06 μM, N = 4; unpublished results). The uptake capacity was in the same range as that observed previously with hypothalamic synaptosomes (7.9 pmol/min/mg protein vs 16.2 pmol/2 min/mg protein). Thus our synaptosomal preparation represents a valid model for the study of uptake inhibition.

The substances used for reference inhibited the noradrenaline uptake in a concentration related manner. Nisoxetine showed a similar potency as reported for the inhibition of accumulation in occipital cortex synaptosomes (K_i 1.3 nM) [23] and oxaprotiline enantiomers were slightly more potent than reported for the inhibition of uptake into synaptosomes from rat midbrain-diencephalon (IC_{50} of 3.6 nM for the (+)- and 3 μM for the (−)-enantiomer) [24]. Tramadol and the enantiomers were slightly more potent in spinal synaptosomes than reported for supraspinal preparations [3, 4, 7]. In addition, the kinetic analysis according to Ref. 17 shows, that tramadol inhibits the noradrenaline transporter in a competitive manner.

The stereoselectivity of noradrenaline uptake inhibition

has long been known [25]. One of the highly stereoselective drugs is oxaprotiline where the activity resides in the (+)-enantiomer with a selectivity of three orders of magnitude [24] which was also observed in our spinal preparation with an even slightly higher selectivity than reported. In comparison, stereoselectivity by one order of magnitude as previously observed with the tramadol enantiomers in hypothalamic synaptosomes [7] and found with our spinal preparation appears to be moderate yet significant. Serum levels of tramadol which produce antinociception in mice range from 0.8 to 10.8 μM from threshold to maximum effective dose, respectively [26]. Therefore inhibition of noradrenaline uptake *in vivo* seems to be mainly due to the (−)-enantiomer of tramadol.

Inhibition of neurotransmitter accumulation measured as uptake in nerve terminals may be caused by induction of neurotransmitter release [25, 27]. This effect would be apparent in concomitant release experiments as enhancement of the basal release rate. In our release experiments, however, little influence at the highest concentration used, 100 μM, could be observed but prominent enhancement of the stimulation-evoked release occurred. This observation confirms that the effects of nisoxetine, tramadol and its (−)-enantiomer were probably due to true uptake blockade. The interference of tramadol with the noradrenaline transporter was further substantiated by those release experiments where uptake sites were blocked with the noradrenaline uptake blocker desipramine. Under these conditions there was an almost complete lack of effects by tramadol, indicating that its site of action had been eliminated.

In conclusion, our results demonstrate that synaptosomes and slices prepared from the dorsal spinal cord can be used to study local noradrenaline uptake and release. The centrally acting analgesic tramadol enhances the extraneuronal noradrenaline concentration also at the level of the spinal cord by interference with the neuronal noradrenaline uptake mechanism. In addition, it could be shown that the uptake inhibition is competitive in nature.

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Departments of Pharmacology and †Biochemical Pharmacology
Grünenthal GmbH
52078 Aachen
Germany

WOLFGANG REIMANN*
HAGEN-HEINRICH HENNIES†

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* Corresponding author: Department of Pharmacology, Grünenthal GmbH, Zieglerstraße 6, 52078 Aachen, Germany. Tel. (241) 5692495; FAX (241) 5692455.

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