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Tapentadol inhibits calcitonin gene-related peptide release from rat brainstem *in vitro*

Maria Cristina Greco, Lucia Lisi, Diego Currò, Pierluigi Navarra*, Giuseppe Tringali

Institute of Pharmacology, Catholic University Medical School, Rome, Italy

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ABSTRACT

We have previously developed an *in vitro* model of rat brainstem explants. The latter release sizable amounts of calcitonin gene-related peptide (CGRP); basal release can be stimulated by such secretagogues as high KCl concentrations, veratridine or capsaicine. In this paradigm we investigated the activity of the analgesic agent tapentadol; the effects of tapentadol were compared to those of a classical opioid receptor agonist, morphine, and the selective noradrenaline reuptake inhibitor reboxetine. Morphine inhibited basal CGRP release, with statistical significance from 1 nM onward and maximal (−44%) inhibition at 100 μM. Morphine also inhibited K^+-stimulated peptide release, with a significant effect from 1 μM and maximal (−39%) decrease at 100 μM, but failed to inhibit release stimulated by 10 μM capsaicin. At variance, reboxetine had no effect on baseline CGRP outflow, but was able to inhibit both K^+-stimulated [significant inhibition from 1 μM onward and maximal (−37%) decrease at 100 μM], and capsaicin-stimulated release [significant effect from 1 μM and maximal (−31%) decrease at 100 μM]. Likewise, tapentadol had no effect on baseline CGRP release up to 100 μM, but decreased secretion stimulated by 56 mM KCl or capsaicin, with significant effects from 0.1 and 1 μM respectively; maximal inhibition over 56 mM KCl and capsaicin stimuli was −29% and −31%, respectively. Naloxone antagonized the effect of morphine, but not those of reboxetine and tapentadol, on K^+-stimulated CGRP secretion. In conclusion the present study provides consistent pharmacological evidence that tapentadol acts as a noradrenaline reuptake inhibitor agent in this experimental model.

1. Introduction

Calcitonin gene-related peptide (CGRP) is thought as a major neurotransmitter of trigeminal signaling, thereby playing a pivotal role in headache pathophysiology, migraine in particular [20]. The facial and cranial structures (from facial skin to cerebral vessels of the pia and dura mater) are densely innervated by trigeminal neurons whose cell bodies are located within the trigeminal ganglia and project their axonal nerve terminals throughout the entire rostrocaudal extent of spinal trigeminal nucleus [15]. CGRP and, to a lesser extent, substance P immunoreactivities are densely localized within the central projections of trigeminal primary neurons [15].

The release of CGRP from isolated rat brainstem, which contains the spinal trigeminal nuclei, might therefore represent a useful *in vitro* model to investigate the function of synaptic junctions between primary and secondary neurons along the pain neurotransmission pathways. We have recently developed and validated an *in vitro* model involving the static incubation of acute rat brainstem explants and the measurement of CGRP immunoreactivity released in the incubation medium. The whole rat brainstem releases sizable amounts of CGRP; peptide secretion is increased in a concentration-dependent manner by two nonspecific depolarizing agents: veratridine, acting primarily through the opening of Na^+ channels, and 56 mM KCl solutions, which elicit direct Ca^{2+} ion influx within the cells. Moreover, release in strongly increased by capsaicin, a specific stimulus acting *via* binding and activation of the vanilloid receptor. In this experimental model, brainstems remain viable and functional during the time frame of the experiments, as assessed by the lactate dehydrogenase assay for cellular toxicity. We have successfully used this model to investigate the effects of both endogenous peptides involved in the control of nociception, and analgesic agents [4,18].

In the present study, we used this model to investigate the effects of novel analgesic agent, tapentadol [19], on CGRP release. Tapentadol displays a dual mechanism of action: it acts both as

* Corresponding author at: Institute of Pharmacology, Catholic University Medical School, Largo Francesco Vito 1, 00168 Rome, Italy. Tel.: +39 06 3015 4253; fax: +39 06 233 235 103.
E-mail addresses: maricrigre@hotmail.it (M.C. Greco), lucia.lisi@rm.unicatt.it (L. Lisi), dcurro@rm.unicatt.it (D. Currò), pnavarra@rm.unicatt.it (P. Navarra), gtringali@rm.unicatt.it (G. Tringali).

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a μ-receptor agonist (MOR) and a noradrenaline (NA) reuptake inhibitor (NRI) [19]. Each of these mechanisms may potentially interfere with CGRP release from trigeminal nerve terminals at the brainstem level. Thus, in order to obtain a pharmacological characterization of tapentadol activity in this model, we also investigated the effects of a paradigmatic MOR agonist, morphine, and those of a selective NRI, reboxetine [21].

2. Materials and methods

2.1. Animals

Male Wistar rats, weighting 200–300 g, were used. They were kept four per cage and maintained at a temperature of 23 ± 1.5 °C, with a relative humidity of 65 ± 2%. The animals were exposed to 12 h of light (06.00 am–06.00 pm) followed by 12 h of dark, and they had free access to food and water. The use of animals for this experimental work has been approved by the Ethics Committee of the Catholic University Medical School, and subsequently by the Italian Ministry of Health (licensed authorization to G. Tringali). All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Experimental procedures

On the day of experiment the animals were decapitated and the brains were rapidly removed. After removal of the cerebellum, brainstem was dissected within the following limits: coronal sections approximately from −9 to −15 mm posterior to bregma, 4 mm lateral to bregma (Paxinos' The Rat Brain in Stereotaxic Coordinates), with a depth of about 2–3 mm. This central nervous system area contains the second order neurons of the nucleus of the spinal tract of the trigeminal nerve (Sp5; Sp5C; Sp5I and Sp5O), where CGRP is released from C-fibers arising from trigeminal ganglia. Total dissection time was <2 min from decapitation.

The brainstem were incubated in 24-well plates (one brainstem per well), at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% O_2 in 300-μl incubation medium, Minimum Essential Medium (MEM) with Earle's salts (Gibco, Milan, Italy), supplemented with 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 60 μg/ml ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA) and 20 IU/ml aprotinin (Sigma Chemical Co.), pH 7.4. After 1 h pre-incubation, the explants were subjected to a 1-h control incubation in plain medium to assess basal CGRP release. This was followed by a second 1-h incubation, replacing plain medium with medium containing test substances or, for the control group, medium alone. In experiments with naloxone, the latter was added to the medium 30 min before the other test drugs. Whenever KCl was used, MEM was replaced by a medium consisting of 56 mM KCl and 67 mM NaCl, with the same concentration of the other ions as found in MEM.

In time-course experiments, 1-h pre-incubation periods were carried out in the presence of 56 mM KCl or 10 μM capsaicin. Thereafter, 30-min, 60-min and 90-min incubations were carried out with KCl or capsaicin given alone or in the presence of tapentadol 10 μM, according to a parallel-groups experimental design.

Viability of tissues exposed to the above conditions was assessed by measuring lactate dehydrogenase (LDH) activity in tissue homogenates and incubation media with a CytoTox 96® Assay (Promega, Madison, WI, USA). Tissue was considered viable if LDH levels into the incubation medium were less than 40% of total LDH activity. In no case LDH levels were exceeding the toxicity threshold limit [data expressed as the percentages of total LDH activity, the means ± standard deviation of 3 determinations. Controls 9 ± 3; tapentadol 0.1 mM 11.5 ± 2.5; 56 mM KCl 10 ± 3.5; KCl + tapentadol as above 7.8 ± 1; capsaicin 10 μM 6 ± 0.5; capsaicin + tapentadol as above 6 ± 1].

Medium samples were stored at −35 °C until assayed for CGRP immunoreactivity. No prior extraction form incubation media was required.

2.3. CGRP radioimmunoassay

CGRP release was measured by a radioimmunoassay (RIA) technique validated in our laboratory and previously described in detail [5].

2.4. Chemicals

Tapentadol was a kind gift by Grünenthal GmbH (Aachen, Germany). Morphine sulfate was purchased from commercial source (Oramorph®) as oral solution (10 mg/5 mL). Naloxone, Reboxetine and Capsaicin were purchased from Sigma (Sigma Chemicals Co., St. Louis, MO, USA). All study drugs were dissolved in sterile water for injection, except for capsaicin that was dissolved in 100% ethanol, to obtain 10 mM stock solutions. Further dilutions were made in the incubation medium. All drugs tested did not interfere with the CGRP assay.

2.5. Statistical analysis

All data are expressed as CGRP ratio, unless otherwise stated. The latter was calculated dividing the amount of CGRP released in the second 1-h incubation period by the amount released in the previous 1-h period. Expression of data as ratio minimizes CGRP variations among different tissues explants. In time-course experiments, data were expressed as pg CGRP/mg of wet tissue.

Each experiment was repeated at least two times, according to a randomized block design. A preliminary two-way ANOVA for the factors "time" and "treatment" was carried out; if no significant differences were found between experiments conducted in different days with the same study design, integrated pooled analysis of the data was justified, and all results were presented as the mean ± SEM of (n) replicates per experimental group. Data were subsequently analyzed by *post hoc* Newman–Keuls for comparisons between group means, or Student's *t* test when appropriate, using a PrismTM computer program (GraphPad, San Diego, CA, USA). Differences were considered statistically significant if $p < 0.05$.

3. Results

The effects of test drugs were investigated on baseline CGRP release, as well as on release stimulated by a nonspecific calcium-dependent and a specific receptor-operated stimulus, 56 mM KCl solutions and capsaicin respectively.

Morphine was used as the paradigm of MOR agonist agent. Morphine produced a concentration-dependent decrease on baseline CGRP release, which was statistically significant from 1 nM onward and caused a maximal −44% inhibition at 100 μM (Fig. 1A). Likewise, morphine was able to inhibit K^+-stimulated CGRP release, with a significant effect from 1 μM and maximal −39% decrease at 100 μM (Fig. 1B). In this system, capsaicin induced a concentration dependent increase in CGRP release, with +26%, +162% and +1015% increases at 1 nM, 0.1 μM and 10 μM capsaicin respectively. Morphine given in the range of doses 0.01–100 μM was able to fully counteract the increase induced by 1 nM capsaicin, but had no inhibitory effects on capsaicin 0.1 μM (data not shown) and even, to the concentration of 0.01 and 1 μM, tended to increase the stimulus by capsaicin 10 μM although not in a statistically significant manner (Fig. 1C).