Journal of the Neurological Sciences 380 (2017) 205–211

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Effects of naftopidil on inhibitory transmission in substantia gelatinosa neurons of the rat spinal dorsal horn *in vitro*

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ARTICLE INFO

Article history:
Received 23 February 2017
Received in revised form 7 July 2017
Accepted 22 July 2017
Available online 27 July 2017

Keywords:
Alpha-1A/D adrenoceptor
IPSC
Naftopidil
Patch-clamp recording
Substantia gelatinosa

ABSTRACT

Objective: Naftopidil is used clinically for the treatment of voiding disorders in benign prostatic hyperplasia. Previous *in vivo* experiments in which naftopidil was applied intrathecally abolished rhythmic bladder contraction, suggesting that naftopidil might inhibit a voiding reflex through interaction with spinal dorsal horn neurons. Here we aimed to clarify the mechanism of action of naftopidil on dorsal horn neurons.
Methods: Whole-cell patch-clamp recordings were performed using substantia gelatinosa neurons of adult rat spinal cord slices. Miniature or evoked inhibitor and excitatory postsynaptic currents (IPSCs and EPSCs, respectively) were analyzed.
Results: Bath-applied naftopidil increased the frequency but not the amplitude of miniature IPSCs (mIPSCs) in 38% of neurons tested; in contrast, the effect of naftopidil on miniature EPSCs (mEPSCs) were mild and observed in only 2 out of 19 neurons. Naftopidil enhanced the amplitude of both GABAergic and glycinergic evoked-IPSCs (eIPSCs) that were elicited by focal stimuli in the presence of either the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), or the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV).
Conclusions: Although naftopidil was developed as an alpha-1 adrenoceptor antagonist, our previous spinal cord slice experiments showed that the activation of an alpha-1 adrenoceptor in substantia gelatinosa increases the frequency of mIPSCs. This result suggested that, under our conditions, naftopidil may interact with a receptor(s) other than an alpha-1 adrenoceptor in the spinal dorsal horn. The present results suggested that naftopidil enhances the release of GABA and glycine by activating inhibitory interneuron terminals in the spinal dorsal horn *via* a receptor other than an alpha-1 adrenoceptor, thereby modulating sensory transmission in the substantia gelatinosa.

1. Introduction

Naftopidil, a phenylpiperazine derivative, is an alpha-1D/A adrenoceptor antagonist that is a useful drug for lower urinary tract symptoms in patients with benign prostatic hyperplasia [1–3]. The currently accepted mechanism for the action of naftopidil is a relaxation of prostate smooth muscle *via* blocking of alpha-1 adrenoceptors, which reduces the pressure in the prostatic urethra [4]. These investigations on the peripheral mechanisms of naftopidil have been supplemented by several studies showing that the intrathecal injection of naftopidil, but not of another alpha-1 adrenoceptor antagonist, tamsulosin, decreases the frequency of bladder contraction [5]. These results suggested that naftopidil may act on the spinal cord as well as on prostatic smooth muscle [6–8]. Micturition is controlled by a reflex arc *via* the spinal cord, and the alpha-1D adrenoceptor has been shown to be widely distributed in the spinal cord [9]. Moreover, alpha-1D knockout (KO) mice have been reported to have increased voiding volume and decreased frequency of voiding *versus* normal mice, indicating that the spinal cord might be one of the possible sites of action of naftopidil [10,11]. However, the mechanisms of action of naftopidil on the spinal cord are not clear. Our previous studies using spinal cord slice preparations demonstrated that the activation of alpha-1 adrenoceptors in substantia gelatinosa (lamina II of the dorsal horn (Rexed laminae)) causes an increase in the release of both GABA and glycine inhibitory transmitters from spinal interneuron terminals [12,13]. Therefore, our previous *in vitro* studies are inconsistent with relevant *in vivo* experiments [12–14]. In the present study, we performed electrophysiological

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http://dx.doi.org/10.1016/j.jns.2017.07.030
0022-510X/

analysis of the effect of naftopidil on inhibitory synaptic transmission in substantia gelatinosa of spinal cord slice preparations obtained from L6 or S1 spinal cord. The substantia gelatinosa is known to play a pivotal role in sensory synaptic transmission [15–20]; the lower lumber and sacral spinal cord, which receive sensory inputs from pelvic organs, might control the output of the autonomic efferents [21,22]. Thus, the present study focused on the effects of naftopidil on inhibitory synaptic transmission of substantia gelatinosa neurons in the lower spinal cord.

2. Materials and methods

All experiments were performed in accordance with the "Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences" of the Physiological Society of Japan and were approved by the local Animal Experiment Committee of the Kumamoto Health Science University (Japan). All efforts were made to minimize animal suffering and the number of animals used for the studies.

2.1. Spinal cord slice preparation

The methods for obtaining slices of the adult rat spinal cord and for blind patch-clamp recordings from substantia gelatinosa neurons have been described in detail elsewhere [23–26]. Briefly, male adult Sprague-Dawley rats (6–7 weeks old) were deeply anaesthetized with urethane (1.2 $g \cdot Kg^{-1}$, i.p.), and lumbosacral laminectomy was then performed. The lumbosacral spinal cord was removed and placed in a pre-oxygenated Krebs solution at 1–3 °C. Immediately after removal of the spinal cord, the rats were given an overdose of urethane and were then sacrificed by exsanguination. After removal of the dura mater, all ventral and dorsal roots were cut and the pia-arachnoid membrane was then removed. The spinal cord was placed in a shallow groove formed in an agar block and glued to the bottom of the microslicer stage with cyanoacrylate adhesive. The spinal cord was immersed in cold Krebs solution and a 500-μm-thick transverse slice was cut. The slice was placed on a nylon mesh in the recording chamber and then perfused at a rate of 15–20 ml/min with Krebs solution saturated with 95% O_2 and 5% CO_2, at 36 ± 1 °C. The Krebs solution contained (in mM): NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11 (pH 7.4).

2.2. Patch-clamp recordings from substantia gelatinosa neurons

Under a dissecting microscope with transmitted illumination, the substantia gelatinosa was clearly discernible as a relatively translucent band across the dorsal horn. However, the contour of individual substantia gelatinosa neurons cannot be visualized under these conditions; therefore, gigaohm sealing was performed blindly from the center of the substantia gelatinosa under visual control.

Whole-cell patch-clamp recordings were made from substantia gelatinosa neurons using a patch-pipette with a resistance of 8–15 MΩ [23,24,26]. The pipette solution contained either (in mM): Cs_2SO_4 110, tetraethylammonium (TEA) 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, and ATP-Mg 5 (pH 7.2) for analysis of inhibitory postsynaptic currents (IPSCs); or K-gluconate solution, consisting of K-gluconate 135, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, and Mg-ATP 5 (pH 7.4) for the analysis of excitatory postsynaptic currents (EPSCs). With the cesium pipette solution, a holding potential was easily depolarized to 0 mV, since the K^+ channels were blocked by cesium and TEA. To evoke monosynaptically, IPSCs were generated as described previously [11,27,28] in the presence of the non-NMDA-receptor antagonist CNQX (20 μM), and the NMDA-receptor antagonist APV (50 μM). These eIPSCs were generated at a frequency of 0.2 Hz using a focal monopolar silver electrode (50-μm diameter) that was insulated, except for the tip, and was located within 150 μm of the recorded neurons. Miniature IPSCs (mIPSCs) were analyzed in the presence of tetrodotoxin (TTX) (0.5 μM). Signals were acquired with a patch-clamp amplifier (Axopatch 200B, Molecular Devices, Union City, CA, USA). The data were digitized with an analog-to-digital converter (digidata 1321A, Molecular Devices), and stored and analyzed with a personal computer using the pCLAMP data acquisition program (version 8.2, Molecular Devices). The recordings were made in voltage-clamp mode at a holding membrane potential of either −70 or 0 mV to isolate EPSCs and IPSCs, respectively [26]. The frequencies and amplitudes of mIPSCs were measured automatically with MiniAnalysis software (ver. 6.0.3, Synaptosoft, Decatur, GA, USA). All spontaneous EPSC and IPSC (sEPSC and sIPSC) events were detected and analyzed using Mini Analysis software; detection criteria for the events included a 5 pA event threshold, fast rise time, and a decay curve that approximated an exponential decay [29].

2.3. Drug application

1-[4-(2-methoxyphenyl) piperaznyl]-3-(1-naphthyloxy) propan-2-ol (naftopidil) (PubChem CID: 4418) (Asahi Kasei Pharma Co., Tokyo) was dissolved in dimethyl sulfoxide (DMSO) (PubChem CID: 679) (Wako, Osaka, Japan) (1%) with Krebs solution. The other drugs were dissolved in Krebs solution and applied by perfusion *via* a three-way stopcock without changes in the perfusion rate or temperature. The time necessary for the solution to flow from the stopcock to the surface of the spinal cord slice was approximately 15 s. The other drugs used in this study were TTX (PubChem CID: 6324668) (Wako), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (PubChem CID: 3721046) (Sigma, St. Louis, MO, USA), DL-2-amino-5-phosphonovaleric acid (APV) (PubChem CID: 135342) (Sigma), strychnine (PubChem CID: 441071) (Sigma), and bicuculline (PubChem CID: 10237) (Sigma), DMSO (Wako).

2.4. Statistical analysis

All data are expressed as mean ± S.E.M. Statistical significance was determined as $P < 0.05$ using a paired two tailed *t*-test. Cumulative probability plots were constructed for mIPSC amplitude and frequency, and were compared under different experimental conditions using the Kolmogorov-Smirnov test. In all cases, n refers to the number of neurons studied.

3. Results

Whole-cell patch-clamp recordings were made from a total of 143 substantia gelatinosa neurons. Recordings could be obtained from slices for >10 h, and stable recordings were made from single substantia gelatinosa neurons for up to 3 h. All of the neurons analyzed in the present study had membrane potentials that were less than (*i.e.*, more negative than) −55 mV. After establishing a whole-cell configuration, sEPSCs were observed at a holding potential of −70 mV in all substantia gelatinosa neurons tested; the associated data are covered in the last part of this section.

3.1. Effect of naftopidil on mIPSCs in spinal substantia gelatinosa neurons

To examine the effects of naftopidil on inhibitory synaptic transmission, mIPSCs were recorded at the membrane potential of 0 mV. No excitatory synaptic responses were observed at this holding potential

Table 1
Effects of naftopidil on miniature inhibitory postsynaptic currents (mIPSCs) of rat substantia gelatinosa neurons.

mIPSCs	Control	Naftopidil 30 μM	Control	Naftopidil 100 μM
Frequency (Hz)	4.5 ± 1.6	12.4 ± 2.2	6.8 ± 1.7	22.4 ± 5.5
Amplitude (pA)	14.1 ± 1.4	15.8 ± 2.1	12.9 ± 1.6	14.1 ± 1.9
n	40		5	

Values are shown as means ± SEM. n, number of neurons tested.