Biochemical and Biophysical Research Communications 447 (2014) 205–209

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Excitatory $GABA_A$ receptor in autonomic pelvic ganglion neurons innervating bladder

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ARTICLE INFO

Article history:
Received 8 March 2014
Available online 3 April 2014

Keywords:
$GABA_A$ receptor
Autonomic neuron
Bladder
Major pelvic ganglion neurons

ABSTRACT

Major pelvic ganglia (MPG) are relay centers for autonomic reflexes such as micturition and penile erection. MPG innervate the urogenital system, including bladder. γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system, and may also play an important role in some peripheral autonomic ganglia, including MPG. However, the electrophysiological properties and function of $GABA_A$ receptor in MPG neurons innervating bladder remain unknown. This study examined the electrophysiological properties and functional roles of $GABA_A$ receptors in bladder-innervating neurons identified by retrograde DiI tracing. Neurons innervating bladder showed previously established parasympathetic properties, including small membrane capacitance, lack of T-type Ca^{2+} channel expression, and tyrosine-hydroxylase immunoreactivity. $GABA_A$ receptors were functionally expressed in bladder innervating neurons, but $GABA_C$ receptors were not. GABA elicited strong depolarization followed by increase of intracellular Ca^{2+} in neurons innervating bladder, supporting the hypothesis GABA may play an important role in bladder function. These results provide useful information about the autonomic function of bladder in physiological and pathological conditions.

1. Introduction

The neurons of rat major pelvic ganglia (MPG) provide autonomic innervation to urogenital systems, including urinary bladder, external genitalia, and lower bowel. The MPG is comprised of a mixture of sympathetic and parasympathetic neurons, and plays an important physiological role in a variety of autonomic reflexes, including micturition and penile erection [1]. Various transmitters such as acetylcholine, norepinephrine, and serotonin are involved in autonomic regulation of the lower urinary tract [2]. Disorders of the central nervous system and peripheral organs may involve lower urinary tract dysfunctions, including overactive bladder syndrome or detrusor overactivity. Several transmitters are pharmacological targets for the development of drugs that control micturition disorders [3].

In the central nervous system, γ-aminobutyric acid (GABA) is typically an inhibitory neurotransmitter [4]. There are three types of GABA receptors: (1) ionotropic $GABA_A$ and (2) $GABA_C$, and (3) metabotropic $GABA_B$ [4]. GABA-inhibited bladder contractions are evoked by stimulation of preganglionic nerve fibers [5,6]. Thus, GABA may play an important role in lower urinary tract function. Further, during intracellular recording in rat MPG, GABA induced biphasic responses, early depolarization, late depolarization, and hyperpolarization which were mediated by $GABA_A$ and $GABA_C$ receptors, respectively [7]. MPG provides autonomic innervation to diverse urogenital organs and the lower bowel. In addition, we previously reported $GABA_A$ receptors were expressed mostly in tyrosine hydroxylase (TH)-positive sympathetic neurons, as well as in a specific subset of non-adrenergic, non-cholinergic (NANC) neurons of rat MPG [8]. However, innervation-specific expression of GABA receptors and their functional role in pelvic ganglia have yet to be fully characterized. Therefore, this study examined the functional expression and role of ionotropic GABA receptors in MPG neurons innervating urinary bladder. $GABA_A$ receptors, not $GABA_C$ receptors, were found to be functionally expressed in MPG neurons innervating bladder, which mediated depolarization followed by intracellular Ca^{2+} increase.

2. Materials and methods

2.1. DiI labeling and isolation of MPG neurons

Adult male Sprague–Dawley rats (200–300 g) were anesthetized with pentobarbital sodium (50 mg/kg *i.p.*). A small incision

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http://dx.doi.org/10.1016/j.bbrc.2014.03.136
0006-291X/

was made into the abdominal wall. DiI (Molecular Probes, Invitrogen, USA), a retrograde axonal tracer, was injected into the wall of the urinary bladder in order to label postganglionic neurons in the MPG. A 10 μL Hamilton syringe with a 28-gauge needle was used to make three to six injections (total volume 20–30 μL) into the dorsal surface of the bladder wall, while avoiding injection of the bladder lumen, as described previously [9,10]. One week after tracer injection, the rats were again anesthetized. MGP neurons were isolated using enzymatic dissociation, as previously described [11]. Isolated MPG neurons were plated onto 12-mm coverglass coated with poly-L-lysine and maintained with minimal essential medium (MEM) containing 10% fetal bovine serum and 1% penicillin–streptomycin (all from Life Technologies, Grand Island, NY, USA), in a humidified 95% air −5% CO_2 incubator at 37 °C. Current recordings were performed within 6 h after plating. Animal use and care procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Wonju College of Medicine.

2.2. Patch clamp analysis

Bladder MPG neuron ion currents were recorded using whole-cell ruptured patch clamp methods, as previously described [12,13]. Patch electrodes were fabricated from a borosilicate glass capillary (BF150-117-15, Sutter Instrument, San Rafael, CA, USA) using a P-97 Flaming Brown micropipette puller (Sutter Instrument). Electrodes were coated with Sylgard 184 (Dow Corning, Midland, MI, USA) and fire-polished on a microforge (Narishige, Tokyo, Japan). Electrode tip resistance was 2–3 MΩ, when filled with the solution described above. An Ag/AgCl wire was used to ground the bath. Cell membrane capacitance and series resistance were compensated for electronically (>80%) using a patch clamp amplifier (EPC-9, Heka Electronik, Lambrecht, Germany). Voltage protocol generation and data acquisition were performed using Pulse/Pulsefit (v8.50) software (Heka Elektronik, Lambrecht, Germany). Current traces were low-pass filtered using a 4-pole 2.5 kHz Bessel filter then digitally stored for later analysis.

To measure membrane potential, current-clamp recordings were performed with the gramicidin-perforated whole cell configuration patch-clamp technique using an EPC-9 amplifier and Pulse/Pulsefit (v8.50) software. A stock solution of gramicidin D was prepared at 50 mg/ml in dimethylsulfoxide and diluted in the pipette solution to a final concentration of 50 μg/ml before use. All electrophysiological recordings were performed at room temperature (~20–24 °C).

2.3. Solution and drugs

Pipettes used to record $GABA_A$ current contained (in mM) 20 KCl, 115 K-aspartate (potassium aspartate), 10 HEPES, 10 ethylene glycol bis (2-aminoethyl ether)-N, N,N′,N′-tetraacetic acid (EGTA), 2.5 $CaPO_4$, 5 MgATP, and 0.1 Na_2-GTP (pH 7.2). The bath solution contained normal physiological salts (PSS) in mM : 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, and 10 glucose (pH 7.4). To isolate Ca^{2+} currents, the pipette and bath solution contained (in mM) 120 *N*-methyl-D-glucamine methanesulfonate (MS), 20 tetraethylammonium (TEA) MS, 20 HCl, 11 EGTA, 1 CaCl2, 10 HEPES, 4 Mg-ATP, 0.3 Na_2-GTP, and 14 creatine phosphate (pH 7.2), and 145 TEA-MS, 10 HEPES, 10 CaCl2, 15 glucose, and 0.00001 tetradodoxin (pH 7.4), respectively.

For current-clamp recordings, patch pipettes were filled with a solution containing (in mM) 140 KCl, 5 EGTA, 10 HEPES, 0.5 $CaCl_2$, and 5 NaCl (pH 7.2). A normal PSS was used for the bath solution.

Drugs used in the experiments were purchased as follows: Collagenase type D and trypsin from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA), mucimol, Bicucculin, and TPMPA from Tocris (Cookson Inc., Bristol, UK), DNAse type I and all other media from Sigma (St. Louis, MO, USA). For stock solutions (10 mM to 1 M), all drugs were dissolved in distilled water.

2.4. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the Lambda DG-4 (Sutter Instruments, Novato, CA, USA) fluorescence measurement system. MPG neurons were placed on glass coverslips and loaded with fura-2/AM in normal PSS in darkness for 1 h at room temperature. After tracer loading, neurons were washed and transferred to the perfusion chamber of a fluorescence microscope. Fura-2 signals were obtained by alternating excitation at 340 or 380 nm, and detection of emission at 510 nm. Data were analyzed using MetaFluor (Sutter Instrument) software.

2.5. Immunohistochemistry

Immunohistochemistry on MPG was described previously [8]. Briefly, rats were anesthetized with ketamine, then MPGs were removed under a surgical microscope. MPGs were immersed in 4% paraformaldehye (0.1 M phosphate buffer, pH 7.4) for 2 h, washed using phosphate buffered saline, followed by cryoprotected overnight storage in 30% sucrose solution. Cryosections (10 μM thick) were made from whole MPG, and every sixth section was mounted on the same slide coated with Vectabond™ (Vector Labs, USA). Sections were treated with 5% normal sera (Vector labs) for 1 h and then incubated overnight in a humidified chamber with rabbit anti-tyrosine hydroxylase (Chemicon; 1:500) antibody at 4 °C. Double labeled images of DiI and thyrosine hydroxylase were captured with a DXM 1200CCD camera (Nikon, Japan) attached to a light microscope (Optiphot, Nikon).

2.6. Data analysis

Data were presented as mean ± SEM. Groups were compared using a two-tailed unpaired Student's *t*-test. A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used for multiple comparisons. Statistical significance was defined as $p < 0.05$ for a single comparison and $p < 0.01$ for multiple comparisons.

3. Results

3.1. Characteristics of MPG neurons innervating bladder

Injection of DiI into the bladder wall of the adult rat resulted in retrograde labeling of MPG neurons (Fig. 1A). Male rat MPG is a large, unique autonomic ganglion containing both sympathetic and parasympathetic neurons. DiI-labeled neurons were mostly TH-negative (Fig. 1A). However, some DiI-labeled neurons were co-localized with TH (Fig. 1A, arrow). Electrophysiological properties of DiI-positive single MPG neurons were examined. DiI-positive cells were identified under a fluorescent microscope (Fig. 1B). MPG neuron cell types were recognized by their electrophysiological properties, including cell membrane capacitance and T-type Ca^{2+} channels [14,15]. A voltage ramp from −100 mV to +60 mV evoked Ca^{2+} channels. T-type Ca^{2+} currents were detected as a prominent hump at low-voltage range (−50 mV to −20 mV) (Fig. 1C). T-type Ca^{2+} currents were detected in DiI-negative neurons but not in DiI-positive neurons (Fig. 1C). Mean membrane capacitance of DiI-positive neurons was 26.1 ± 9.7 pF. Neuron firing patterns were classified as tonic or phasic according to their rate of accommodation during depolarizing current injection. In addition, rebound action potential spikes after anodal break in