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Phosphodiesterase types 3 and 4 regulate the phasic contraction of neonatal rat bladder smooth myocytes via distinct mechanisms

Kui Zhai [a,b,c], Yan Chang [a], Bin Wei [a], Qinghua Liu [d], Véronique Leblais [b,c], Rodolphe Fischmeister [b,c,*], Guangju Ji [a,**]

[a] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China
[b] Inserm UMR-S 769, LabEx LERMIT, F-92296 Châtenay-Malabry, France
[c] Université Paris-Sud, Faculté de Pharmacie, F-92296 Châtenay-Malabry, France
[d] Institute for Medical Biology, College of Life Sciences, South-Central University for Nationalities, Wuhan, China

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ABSTRACT

Activation of the cyclic AMP (cAMP) pathway reduces bladder contractility. However, the role of phosphodiesterase (PDE) families in regulating this function is poorly understood. Here, we compared the contractile function of the cAMP hydrolyzing PDEs in neonatal rat bladder smooth myocytes. RT-PCR and Western blotting analysis revealed that several isoforms of PDE1–4 were expressed in neonatal rat bladder. While 8-methoxymethyl-3-isobutyl-1-methylxanthine (a PDE1 inhibitor) and BAY-60-7550 (a PDE2 inhibitor) had no effect on the carbachol-enhanced phasic contractions of bladder strips, cilostamide (Cil, a PDE3 inhibitor) and Ro-20-1724 (Ro, a PDE4 inhibitor) significantly reduced these contractions. This inhibitory effect of Ro was blunted by the PKA inhibitor H-89, while the inhibitory effect of Cil was strongly attenuated by the PKG inhibitor KT 5823. Application of Ro in single bladder smooth myocytes resulted in an increase in Ca^{2+} spark frequency but a decrease both in Ca^{2+} transients and in sarcoplasmic reticulum (SR) Ca^{2+} content. In contrast, Cil had no effect on these events. Furthermore, Ro-induced inhibition of the phasic contractions was significantly blocked by ryanodine and iberiotoxin. Taken together, PDE3 and PDE4 are the main PDE isoforms in maintaining the phasic contractions of bladder smooth myocytes, with PDE4 being functionally more active than PDE3. However, their roles are mediated through different mechanisms.

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1. Introduction

The urinary bladder is a hollow smooth muscle organ that stores and expels urine. One of the most important features of this muscle is its ability to generate considerable electrical activities and phasic contractions which have been observed in whole isolated bladder [1,2], multicellular detrusor preparations [3,4], and even isolated cells [5,6]. These spontaneous bladder activities change markedly with age [1]. During the first 2–3 weeks of life, they are characterized by high-amplitude low-frequency spontaneous contractions [3,7]. Later, they turn into low-amplitude high-frequency pattern characteristic of normal adult rat [7]. It is hypothesized that the spontaneous activities are initiated at the bladder neck region [7] or near the bladder dome [8] and propagated throughout the whole detrusor muscle [8] by gap junctions [9]. The spontaneous activities in neonatal rat bladder are likely to be myogenic, as they occur in the absence of nerve stimulation and are not inhibited by tetrodotoxin [7]. It has been established that these spontaneous phasic contractions are highly organized and modulated by different pathways, positively through muscarinic receptor activation [3,10] and negatively through the nitric oxide (NO)–cGMP–protein kinase G (PKG) pathway [3].

An increase in cyclic nucleotides, cGMP and cAMP, reduces the contractile tone of several types of smooth muscles, including vascular [11], gastric [12] and bladder smooth muscles [13,14]. The levels of these cyclic nucleotides are tightly controlled by their synthesis through adenylyl cyclases (ACs) and guanylyl cyclases (GCs), and their degradation through phosphodiesterases (PDEs). PDEs have been classified into 11 subfamilies according to their amino acid sequence, catalytic

Abbreviations: 007, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer; BAY, 2-(3,4-dimethoxybenzyl)-7-{(1R)-1-[(1R)-1-hydroxyethyl]-4-phenylbutyl}-5-methyllimidazo[5,1-f][1,2,4]triazin-4(3H)-one (BAY-60-7550); BK channels, large conductance Ca^{2+}-activated K^+ channels; Cil, N-cyclohexyl-N-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butyramide (cilostamide); H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide 2HCl hydrate; IBTX, iberiotoxin; MIMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; PLB, phospholamban; Ro, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-20-1724); SNAP, S-nitroso-N-acetylpenicillamine.

* Correspondence to: R. Fischmeister, UMR-S 769, LabEx LERMIT, Faculté de Pharmacie, Université Paris-Sud, 5, rue J.-B. Clément, 92296 Châtenay-Malabry, France. Tel.: +33 146835906; fax: +33 146835475.
** Correspondence to: G. Ji, National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, China. Tel.: +86 10 64889873; fax: +86 10 64846720.
E-mail addresses: rodolphe.fischmeister@inserm.fr (R. Fischmeister), gj28@ibp.ac.cn (G. Ji).

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characteristics, substrate preference and regulatory properties [15]. Several cAMP-PDEs are known to play a significant role in the contractile tone of urinary bladder smooth muscle. PDE1, a Ca^{2+}/calmodulin-stimulated PDE, has been shown to regulate the contractions of human [16,17], guinea pig [17] and rat [18] bladder smooth muscles. PDE2, a cGMP-stimulated PDE, PDE3, a cGMP-inhibited PDE, and PDE4, a cAMP-specific PDE, were reported to control the tonic contraction of rabbit and rat urinary bladder tissues [18,19]. PDE4 was also described as a regulator of phasic contractions in human bladder [20]. Moreover, PDE4 inhibition was shown to effectively suppress detrusor overactivity in rats with bladder outlet obstruction [21]. Recently, a series of publications from Petkov's group indicated that large conductance Ca^{2+}-activated K^+ channels (BK channels) are involved in the effects induced by 3-isobutyl-1-methylxanthine (IBMX, a non selective PDE inhibitor) and 8-methoxymethyl-3-isobutyl-1-methylxanthine (MIMX, a selective PDE1 inhibitor) in human and guinea pig bladder smooth muscles [14,17,22]. However, the underlying mechanisms involved in the effects mediated by other PDEs are still largely undefined in bladder smooth muscle.

In this study, we specially focused on cAMP-PDEs (PDE1–4) in neonatal rat urinary bladder smooth muscle. Our first aim was to characterize the expression pattern of PDE1–4 by RT-PCR/Western blotting and to evaluate the contractile function of these four isoforms in neonatal rat bladder. The second aim was to explore the underlying mechanisms of PDE3 and PDE4 in controlling the phasic contractions of neonatal rat bladder by classical pharmacological method combined with Ca^{2+} imaging.

2. Materials and methods

2.1. Animals and tissues

All animal procedures described in this study were performed according to the Guidance for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and with approval from the Institute of Biophysics Committee on Animal Care. Neonatal Sprague–Dawley rats (female and male, 10 days old) were purchased from Vital River Laboratories (Beijing, China). Neonatal rats were anesthetized by 5% chloral hydrate and the whole bladder was removed, placed into cold Tyrode solution composed of (mM): 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4). After the cleaning of the fat tissues, the bladder was cut into two longitudinal pieces by using fine dissecting scissors along the axis from the neck to the fundus.

2.2. Pharmacological reagents

Cilostamide (Cil) and KT 5823 were from Tocris Bioscience (Bristol, UK), MIMX and Ro-20-1724 (Ro) from Calbiochem (Merck Chemicals Ltd, Nottingham, UK), BAY-60-7550 (BAY) from Cayman Chemical (Bertin Pharma, Montigny-le-Bretonneux, France), and 007 from Biolog Life Science Institute (Bremen, Germany). All other reagents were from Sigma-Aldrich unless mentioned. Drug concentrations were established according to their pharmacological properties (affinity and selectivity) as described in the literature.

2.3. Reverse transcriptional polymerase chain reaction (RT-PCR)

Total RNA was prepared from neonatal rat bladder tissues using the TRIzol RNA purification system (Invitrogen). According to the manufacturer's instructions (Promega), cDNA was generated from mRNA (2 μg) using the M-MLV reverse transcriptase (Promega). PCR was carried out on the generated cDNA (same quantity for each PDE isoform) using the primer sequences shown in Table 1. PCR conditions were set up as follows: 94 °C for 2 min, then the following three steps for 30, 34, or 40 cycles (Table 1): denaturation at 94 °C for 30 s, annealing at indicated temperatures for 30 s, elongation at 72 °C for 60 s, and a final step of cooling at 4 °C. PCR products were run on a 1.7% agarose gel and visualized under UV light using ethidium bromide staining. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a housekeeping control.

2.4. Western blotting analysis

Western blotting analysis was performed as previously described [23] with minor modifications. Frozen whole bladder tissues were homogenized in SDS-PAGE sample loading buffer containing 50 mM Tris–HCl, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue. The samples were heated at 90 °C for 5 min, centrifuged to remove insoluble materials, and resolved on 10% SDS gel. The resolved proteins were transferred to PVDF membrane (Millipore) at 300 mA for 45 min. The membranes were blocked for 1 h with Tris-buffered saline-Tween 20 (TBST) containing 10% BSA at room temperature and then were incubated overnight with anti-PDE2A IgG (1:500; PD2A-101AP, FabGennix, Frisco, TX, USA), anti-PDE3A IgG (1:500; sc 11834, Santa Cruz Biotechnology, CA, USA), anti-PDE3B IgG (1:500; sc 20793, Santa Cruz Biotechnology), anti-PDE4A IgG (1:500; PD4-112AP, FabGennix), anti-PDE4B IgG (1:500; PD4-201AP, FabGennix), anti-PDE4D IgG (1:500; PD4-401AP, FabGennix), anti-phospholamban (1:2000; ab2865, Abcam), or anti-phospho S16 phospholamban (1:1000; ab15000, Abcam) at 4 °C. After washing and incubating with the secondary antibodies, final detection was performed using enhanced chemiluminescence detection solutions 1 and 2 (1:1) (ECL, Millipore).

2.5. Contractile measurement of neonatal rat bladder strips in vitro

Contractile measurement of neonatal rat bladder strips was performed as previously reported with minor modifications [3]. The bladder strips (with intact mucosa) were tied up and mounted in a classical isolated organ bath (coupled the model BL-420F acquisition system, Chengdu TME Technology Co., Ltd, Sichuan, China) to measure isometric tension. The bladder strips were allowed to equilibrate for 1.5 h before drug testing. After equilibration, 100 nM carbachol was applied to enhance the amplitude and frequency of phasic contractions, without significantly changing the baseline tension (Supplementary Fig. S1), as previously described [3]. The effects of PDE inhibitors were studied on the carbachol-enhanced phasic contraction in neonatal rat bladder strips. To characterize the signaling pathway involved in their effects, bladder strips were pretreated for 8–10 min before the addition of the PDE inhibitors in the presence of H-89 (a PKA inhibitor), ryanodine [a ryanodine receptor (RyR) antagonist], KT-5823 (a PKG inhibitor) or iberiotoxin (IBTX, a BK channel inhibitor). In experiments performed in the presence of pharmacological reagents diluted in dimethylsulfoxide (DMSO), control bladder strips were treated in the presence of the equivalent concentration of DMSO (<0.1%).

The mean amplitude and frequency of carbachol-enhanced phasic contractions were measured during 5 min before or 5 min after application of the drugs (PDE inhibitors or DMSO). The effect of these drugs on both parameters was expressed in percentage of the amplitude and frequency of carbachol-enhanced phasic contractions measured before drug application. Baseline tone was not appreciably changed by various treatments and therefore was not subjected to detailed analysis.

2.6. Measurement of Ca^{2+} fluorescence

The fibrosal and mucosal layers of the bladder were removed and the remaining smooth muscle layer was cut into pieces. Ca^{2+} imaging was performed either on isolated smooth muscle cells or on single smooth muscle cells in intact tissue. Single neonatal rat bladder smooth muscle cells were prepared as previously described with minor modifications [24]. The bladder pieces were first incubated in Ca^{2+}-free

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