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

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ABSTRACT

Activation of the cyclic AMP (cAMP) pathway reduces the 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 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. Taking 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.

Key words: PDE; Ca^{2+} sparks; BK channels; bladder smooth myocytes; neonatal rat.

Highlights

- mRNAs and proteins of several PDEs isoforms are expressed in neonatal bladder;
- both PDE3 and PDE4 regulate the carbachol-enhanced phasic contractions of neonatal rat bladder strips, PDE4 being functionally more active than PDE3;
- Ca^{2+} sparks and BK channels are involved in the effects of PDE4 inhibition but not PDE3 inhibition;
- Ca^{2+} transients and intracellular SR Ca^{2+} content are significantly reduced by PDE4 inhibition but not by PDE3 inhibition;
- the effects of PDE3 inhibition are mediated via a PKG-dependent pathway whereas the effects of PDE4 inhibition are mediated via a PKA-dependent pathway.

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-methylimidazo[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.

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 receptors 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 muscle [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 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 muscle. 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 tissue [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 muscle [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 CaCl₂, 1.0 MgCl₂, 10 glucose, 10 HEPES, (pH 7.4). After the cleaning of the fat tissues, the bladder was cut into two longitudinal pieces by using a 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 isoforms) using the primers 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 to 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 solution 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 hour 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 (Supplementay 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 PDEs inhibitors in the presence of H-89 (a PKA inhibitor), ryanodine [a ryanodine receptor (RyR) antagonist], KT-5823 (a PKG inhibitor) or 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 Tyrode solution supplemented with 2 mg/ml papain, 1 mg/ml dithiothreitol, and 1 mg/ml BSA for 20 min at 37°C, and then in low Ca^{2+} (0.1 mM)-Tyrode solution supplemented with 1 mg/ml collagenase H, 1 mg/ml dithiothreitol, and 1 mg/ml BSA for further 20 min at 37°C. The tissue pieces were washed twice in Ca^{2+} -free Tyrode solution and triturated with wide-pore Pasteur pipette. Cells were concentrated and kept in the low Ca^{2+} (0.1 mM)-Tyrode solution supplemented with 1 mg/ml BSA solution at 4°C. For Ca^{2+} imaging, isolated bladder smooth myocytes were plated on glass coverslips, incubated with 5 μ M Fluo-4 AM (Molecular Probes) for 15 min at room temperature and further perfused with normal Tyrode solution for 20 min at room temperature. The protocol for intact tissue imaging was conducted according to the method developed by Ji et al. [25]. The detrusor muscle strips were gently digested in 0.5 mg/ml collagenase type II with 1 mg/ml BSA for 5 min at 32°C and

transferred to warm Tyrode solution to stop digestion. Strips were then incubated with 20 μM Fluo-4 AM for 1 hour by slow shaking at room temperature in dark. The strips were transferred to a recording chamber with the serosal surface on the bottom, fixed with a Kevlar fiber retaining clip, and perfused with warm Tyrode solution for 40 min at room temperature before Ca^{2+} imaging. Ca^{2+} -transients of single cells in intact tissues were recorded in the presence of 100 nM carbachol to mimic the conditions of contraction measurements.

Images were captured using the 40x oil immersion objective of an inverted microscope (Leica) connected to a software-controlled (Las AF, Leica) cooled charge coupled camera (Leica SP5 confocal microscope). X-Y images were collected every 573 ms for 2 min, and X-t images were obtained with line scan at an interval of 1.43 ms for 10 s. X-t images were processed and analyzed using MATLAB 7.1 software (MathWorks) and ImageJ (Scioncorp). Kinetic data were calculated by fits of the data using custom software written in MATLAB that fit fluorescence data to a six-parameter equation (F_0 , start time, rise time, peak F/F_0 , half-time of decay, and final offset using a nonlinear least squares fitting routine) [26]. Rise times indicate the period of time from 10 to 90% of the event fitted from baseline to peak F/F_0 . Ca^{2+} propagation velocity was determined by individually calculating the time required to traverse a distance of at least 20 μm in the calibrated images. X-Y images were analyzed using Leica Las AF software and fluorescence profile was constructed and transferred to Excel. In all X-Y images, a mean baseline fluorescence intensity (F_0) was obtained by averaging fluorescence value of the continuous 20 images without Ca^{2+} transient activity. The amplitude and frequency of Ca^{2+} transients of each cell were calculated before and after drug application. The effects of drugs on amplitude or frequency were expressed as percentage of control. Kinetic parameters

of Ca^{2+} signal (t_{max} : time to peak, $t_{1/2\text{on}}$: time to half-peak, $t_{1/2\text{off}}$: time from the peak to obtain half recovery) were determined using Microsoft Excel software.

2.7. Data analysis

Data were represented as mean $\pm$ SEM of n strips in organ bath experiments, n cells in Ca^{2+} imaging recordings or N rats in RT-PCR/western blotting experiments. Significant differences were determined by Student's t -test. Data from more than two groups were compared by one-way, repeated measures ANOVA and significant differences between groups were determined by the Student-Newman-Keuls (SNK) test for paired comparisons. Only results with values of $P < 0.05$ were considered significant.

3. Results

3.1. Expression and function of PDEs in neonatal rat bladder

To determine the expression pattern of cAMP-PDEs (PDE1-4) in neonatal rat bladder, RT-PCR and western blotting analysis were carried out. As shown in Fig. 1A-B, among PDE1 isoforms, only PDE1A mRNA was slightly detected while PDE1B and PDE1C mRNAs were absent in all samples tested. PDE2A gene, the only identified gene for PDE2, was expressed. Using a PDE2A polyclonal antibody, we observed two bands of PDE2A in neonatal rat bladder, at approximately 60 and 100 kDa (Fig. 1D). The 60 kDa band likely corresponds to proteolytic fragments of PDE2A protein, as previously reported [27]. Both PDE3A and PDE3B mRNAs were observed (Fig. 1A), PDE3A expression being higher than that of PDE3B (Fig. 1B). Moreover, PDE3A and PDE3B proteins were also expressed in neonatal rat bladder (Fig. 1D). Using a PDE3A polyclonal antibody, we detected two bands, at approximately 65 and 100 kDa, the latter one being consistent with the reported size of PDE3A protein [28]. Likewise, using a PDE3B polyclonal antibody, two bands were detected at approximately 72 and 135 kDa, but the former was unidentified (Fig. 1D). The mRNAs of the four PDE4 isoforms were detected, with the following rank order: PDE4A>PDE4D≈PDE4B>PDE4C. To further analyze the expression of PDE4A, PDE4B, and PDE4D transcripts, RT-PCR with specific primers (Table 1) were performed. Three PDE4A transcripts (PDE4A1, PDE4A6, and PDE4A?), 4 PDE4B transcripts (PDE4B1, PDE4B2, PDE4B3, and PDE4B4), and all PDE4D except PDE4D4 transcripts were detected in neonatal rat bladder (Fig. 1C). Using 3 polyclonal antibodies targeted to PDE4A, PDE4B or PDE4D, we detected 3 PDE4A bands [two consistent with

PDE4A1 (76 kDa) [29] and PDE4A6 (87 kDa) [30], and the upper one consistent with the uncharacterized variant PDE4Ax [29]], 3 bands which are consistent with 4 variants of PDE4B protein [PDE4B4 (62 kDa) [31], PDE4B2 (78 kDa) [31], and PDE4B1/3 (107/100 kDa) [31]] and 5 potential variants of PDE4D protein [PDE4D5/7 (100 kDa) [32] and PDE4D3/8/9 (90 kDa) [32]]. Altogether, our results indicate that mRNAs and proteins of several PDE families and isoforms are expressed in neonatal rat bladder.

Next, we evaluated the function of PDE1-4 in neonatal rat bladder by using selective PDE inhibitors [33]. MIMX (10 μ M) was used to block PDE1 activity. It has been shown to interfere with PDE1 catalytic site with a low micromolar affinity and to exhibit selectivity over other PDEs of 30 to 50-fold [34-37]. As shown in Fig. 2, MIMX had no significant effect on the amplitude (Fig. 2E) and frequency (Fig. 2F) of carbachol-enhanced phasic contractions. BAY-60-7550 (BAY) is a selective PDE2 inhibitor characterized by a low IC_{50} of 5 nM [38]. However, 100 nM BAY did not significantly change the amplitude (Fig. 2E) and frequency (Fig. 2F) of carbachol-enhanced phasic contractions. In contrast, cilostamide (Cil, 1 μ M), a selective PDE3 inhibitor with an IC_{50} of 5-27 nM [39-41], decreased the amplitude of carbachol-enhanced phasic contractions by $38.8 \pm 4.8\%$ ($P < 0.001$) without changing their frequency (Fig. 2B and 2E-F). Ro-20-1724 (Ro, 5 μ M), a selective PDE4 inhibitor with an IC_{50} of ~ 1 μ M [36, 39, 42], elicited a more pronounced effect, reducing both the amplitude and frequency of carbachol-enhanced phasic contractions by $65.1 \pm 10.2\%$ ($P < 0.001$) and $43.9 \pm 5.5\%$ ($P < 0.001$), respectively (Fig. 2C and 2E-F). The inhibitory effect of Ro was significantly higher than that observed with Cil (Fig. 2E-F), suggesting that PDE4 is much more important in controlling the neonatal rat bladder contractility. Furthermore, when Cil and Ro were applied concomitantly,

the carbachol-enhanced phasic contractions were abolished (Fig. 2D). These results indicate that in neonatal rat bladder, PDE3 and PDE4 are the main PDE isoforms regulating carbachol-enhanced phasic contractions, with PDE4 being functionally more active than PDE3.

3.2. Ca^{2+} sparks and BK channels activation are involved in the effects of Ro but not Cil

As frequency modulation of Ca^{2+} sparks is known to be involved in regulating smooth muscle tone [43, 44], we determined whether it participated in PDE3/4 mediated effects. Firstly, we evaluated the effect of PDE inhibition on spontaneous Ca^{2+} sparks in neonatal rat bladder isolated smooth myocytes. As shown in Fig. 3, the PDE3 inhibitor Cil (1 μM) did not modify the frequency and the kinetic parameters of Ca^{2+} sparks (Fig. 3A-E). In contrast, PDE4 inhibition induced by Ro (5 μM) markedly altered Ca^{2+} sparks properties: Ro increased their frequency by about 1.8 fold ($P < 0.05$) (Fig. 3H), and reduced both the rise time (Fig. 3I) and the half time decay (Fig. 3J) by 22.4% and 20.9%, respectively ($P < 0.05$), without modifying their amplitude (Fig. 3G). Secondly, we tested the hypothesis that part of the reduction of neonatal rat bladder phasic contractions is through increasing Ca^{2+} spark frequency. Consistently, an inhibitor of Ca^{2+} sparks, ryanodine, significantly rescued the Ro-induced inhibitory effects on carbachol-enhanced phasic contractions by decreasing its inhibitory effect on contraction amplitude by about 2-fold (Fig. 3L) and abolishing its effect on contraction frequency (Fig. 3L).

To investigate the involvement of BK channels in the contractile function of PDE3 and PDE4, we used iberiotoxin (IBTX, 30 nM), a selective inhibitor of these channels [45]. As shown in Fig. 4, application of IBTX had no effect on carbachol-enhanced phasic contractions measured in the absence or presence of Cil. By contrast, IBTX greatly attenuated the Ro-

induced inhibitory effects on carbachol-enhanced phasic contractions by decreasing its inhibitory effect on contraction amplitude (Fig. 4C) and abolishing its effect on contraction frequency (Fig. 4C). Taken together, our results indicate that an increase in Ca^{2+} sparks frequency and an activation of BK channels are involved in the relaxing effect of PDE4 inhibition on neonatal bladder phasic contractions but not in the effect of PDE3.

3.3. Effects of PDE inhibition on Ca^{2+} transient and sarcoplasmic reticulum (SR) content

Previous study indicated that action potentials and correlated calcium transients are fundamental mechanisms in generating phasic contraction of bladder smooth muscle [5, 46]. Therefore, we determined the effects of PDE inhibition on Ca^{2+} transients. Based on the previous method [25], carbachol-enhanced Ca^{2+} transients of single cells were recorded in intact tissue by laser scanning confocal microscope. In the presence of 1 μM Cil, the amplitude of carbachol-enhanced Ca^{2+} transients was slightly but not significantly decreased by $19.9 \pm 7.0\%$ ($n=20$ cells), whereas their frequency was unchanged (Fig. 5C). In contrast, in the presence of 5 μM Ro, the average peak of carbachol-enhanced Ca^{2+} fluorescence was reduced from 1.33 ± 0.01 to 1.10 ± 0.01 (23 cells of 5 tissues, $P<0.001$) and the frequency was decreased 2-fold (from 15.6 ± 2.1 to 7.3 ± 1.0 Hz, $P<0.001$) (Fig. 5C). These results suggested that PDE4 but not PDE3 controls these Ca^{2+} transients. Since Ca^{2+} release from SR is considered to be a major source of Ca^{2+} transients, the effect of PDE inhibition on SR Ca^{2+} content was examined by application of 10 mM caffeine on single cells isolated from neonatal bladder. As shown in Fig. 5D, caffeine elicited a typical Ca^{2+} transient whose amplitude and kinetics parameters were not altered by the PDE3 inhibitor Cil. However, PDE4 inhibition induced by 5 μM Ro decreased

the amplitude of caffeine-induced Ca^{2+} transients by 33.2% ($P < 0.05$ versus vehicle, $n = 31$ cells; Fig. 5E) and also shortened the half-time decay of these transients by 26.2% ($P < 0.05$ versus vehicle; Fig. 5F). Furthermore, we also found that phospholamban (PLB) phosphorylation at Ser16 (a PKA site) is increased under PDE4 inhibition but not PDE3 inhibition (Fig. 5G-H).

3.4. PDE3 and PDE4 regulate the phasic contraction of neonatal rat bladder smooth myocytes through different signaling pathways

We further explored the signaling pathways involved in the PDE3- and PDE4-mediated regulation of neonatal rat bladder phasic contractions. As PDE3 and PDE4 are cAMP-degrading PDEs, the blockade of these PDEs should induce an increase in cAMP concentration which may activate protein kinase A (PKA). Thus, to test the role of PKA in the functional effects of PDE3 and PDE4 inhibitors, the PKA inhibitor H-89 was used [47]. As shown in Fig. 6A-C, H-89 (100 nM) did not significantly modify the carbachol-enhanced contractions, even in the presence of Cil. However, the inhibitory effect of Ro on the amplitude and frequency of carbachol-enhanced contractions was strongly attenuated by H-89 (Fig. 6C). This suggests that PKA participates in the PDE4 inhibitor- but not in the PDE3 inhibitor-mediated effect on neonatal rat bladder contractile function.

Another target of cAMP is the guanine nucleotide exchange protein Epac (Exchange protein activated by cAMP) which has recently been reported to mediate smooth muscle relaxation [48]. As shown in Fig. 6D-E, the selective Epac activator 007 (30 μM) had no significant effect on carbachol-enhanced phasic contractions, suggesting that Epac is not involved in the phasic contractions of neonatal rat bladder.

As PDE3 also hydrolyzes cGMP, we examined the role of PKG in mediating the functional inhibitory effect of PDE3 inhibition, by using a selective PKG inhibitor, KT 5823 (1 μ M) [49]. As shown in Fig. 7A-B, application of KT 5823 did not alter carbachol-enhanced phasic contractions, but greatly attenuated the Cil mediated-inhibition of the amplitude of contractions by about 70% ($P<0.01$). We also observed that the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP, 100 μ M) markedly reduced the amplitude of carbachol-enhanced contractions by 48% ($P<0.001$) without changing their frequency (Fig. 7C-D). This suggests that PKG participates in the inhibitory effect of the PDE3 inhibitor on neonatal rat bladder contractile function.

4. Discussion

In the present study, we established for the first time the expression profile of cAMP-PDEs (PDE1-4) in neonatal rat bladder, with the following rank of order: PDE3A, PDE4A>PDE2A, PDE3B, PDE4B, PDE4D>PDE1A. To date, 21 different PDE4 isoforms, encoded by 4 separate genes (PDE4A, B, C, and D) through alternative messenger RNA splicing and the use of different promoters, have been identified in rat tissues [51]. We clearly identified several PDE4 isoforms in neonatal rat bladder, including 3 PDE4A transcripts (PDE4A1, PDE4A6, and PDE4A? which might in fact correspond to part of PDE4A1), 4 PDE4B transcripts (PDE4B1, PDE4B2, PDE4B3, and PDE4B4), and all PDE4D except PDE4D4 transcripts.

It has been previously shown that PDE1 plays an important role in controlling the tonic [16, 18, 19] as well as the phasic [17] contractions of rat, guinea pig, or human urinary bladder

smooth muscle, making PDE1 a potential target for treating human bladder diseases such as overactive bladder [52, 53]. Here, we found that PDE1 was not involved in the regulation of phasic contractions of neonatal rat bladder. These apparent discrepancies might be explained by species differences in PDE1 expression. Consistently, we observed that among PDE1 isoforms, only PDE1A mRNA was slightly detected while PDE1B and PDE1C mRNAs were absent in neonatal rat bladder, whereas in human bladder, PDE1C mRNA appears to be predominant [50]. The lack of contractile effect of PDE1 inhibition in neonatal rat bladder might also be related to the prominent role of other PDEs, as recently reported in vascular smooth muscle cells [41]. This hypothesis deserves further investigation. Similarly, although PDE2A is expressed in neonatal rat bladder, inhibition of PDE2 with BAY [at a concentration of 100 nM, i.e. >20-fold its IC₅₀ [38] which should fully block PDE2 activity], did not alter its phasic contractions. A possible hypothesis for this is that PDE2 expression is localized in a subcellular compartment which is distinct from the contractile compartment. Alternatively, PDE2 might be expressed in cells other than smooth muscle cells in bladder. Taken together, our data indicate that PDE1 and PDE2 are not major PDEs in controlling neonatal rat bladder contractility. In contrast, the carbachol-enhanced spontaneous contractions were significantly reduced by Cil and Ro, and even abolished by co-application of the two inhibitors. This strongly suggests that both PDE3 and PDE4 are critical in controlling neonatal rat bladder contractility, and that PDE3 and PDE4 act in a synergistic manner. Therefore, co-application of PDE3 and PDE4 inhibitors, or a dual PDE3/4 inhibitor, might provide a novel therapy to effectively treat human bladder disorders as suggested by a previous study [14].

We demonstrated that PDE3 and PDE4 exert their regulatory role through different

mechanisms, involving an increase in Ca^{2+} spark frequency only in the case of PDE4 inhibition. Two main pathways have been described in the literature to account for an increase in Ca^{2+} spark frequency in smooth muscle cells: the first one involves phosphorylation of RyRs through a cAMP-PKA pathway [43, 54]; the second one involves PLB phosphorylation which leads to an increase in SERCA activity, SR Ca^{2+} load [55] and hence Ca^{2+} spark activity. However, the correlation between Ca^{2+} spark activity and SR content was questioned by recent findings. Guo et al. [56] showed that Ca^{2+} spark frequency decreases and sparks terminate earlier in permeabilized ventricular myocytes despite an SR Ca^{2+} load increase, suggesting spark local control following single RyR opening. In this study, we demonstrated that Ca^{2+} sparks frequency was significantly increased by Ro in single neonatal bladder smooth myocytes (Fig. 3H). Thus, the effects of Ro on Ca^{2+} sparks was mainly attributed to the enhanced phosphorylation of RyRs, similar to what was found in cardiac myocytes, where PDE4D3 is part of the RyR2 complex in SR membrane and controls cAMP-PKA-mediated phosphorylation of RyR2 [57]. However, Ro (but not Cil) also increased phosphorylation of PLB at Ser16 (Fig. 5G-H). While this normally leads to an increased SR Ca^{2+} content, this was not the case here (Fig. 5D) because Ro also abolished Ca^{2+} transients (Fig. 5B-C). The latter was most likely due to activation of BK channels leading to hyperpolarization of the membrane and inhibition of Ca^{2+} entry through voltage-dependent Ca^{2+} channels.

It is well established that BK channels can be directly activated by Ca^{2+} sparks [44, 58]. Furthermore, BK channels have been shown to be the physiological effector of a number of endogenous and exogenous relaxant agents in human and guinea pig bladder smooth muscle [14, 17, 22, 59, 60]. Indeed, a common view is that BK channel activation leads to membrane

hyperpolarization and decreases Ca^{2+} entry through voltage-dependent Ca^{2+} channels to promote relaxation. Moreover, expression of BK channels has been confirmed in neonatal rat bladder [1]. In our study, we demonstrated the role of BK channels in the PDE4-mediated pathway regulating neonatal rat bladder contractility, as pharmacological inhibition of these channels by IBTX markedly reversed the inhibitory effect of Ro on carbachol-enhanced spontaneous contractions. Additionally, we observed that Ro largely reduced Ca^{2+} transients in these smooth muscle cells. Finally, we demonstrated that the canonical cAMP-PKA signaling pathway is recruited under PDE4 inhibition to control carbachol-enhanced phasic contractions in neonatal rat bladder. Hashitani *et al.* reported that forskolin, an adenylate cyclase activator, suppressed the contraction by decreasing Ca^{2+} sensitivity without affecting Ca^{2+} transients in guinea pig bladder smooth muscle [46]. This is not contradictory with our results but supports the idea that multiple mechanisms contribute to cyclic nucleotide-induced relaxation in smooth muscle cells [11].

We also showed that the PDE3-mediated control of neonatal rat bladder contractility was independent of the PKA/ Ca^{2+} spark/BK channel/ Ca^{2+} transient cascade described for PDE4. By contrast, we found that PKG was required for the inhibitory effect of Cil on carbachol-enhanced phasic spontaneous contractions in neonatal rat bladder. The role of a NO-cGMP-PKG pathway in this PDE3-mediated effect is further supported by our results showing that the NO donor, SNAP, exerted similar inhibitory effect on the phasic contractions of neonatal rat bladder and also by previously published data obtained in the same tissue [3].

It is important to take into consideration that we performed all contractile experiments on strips with an intact mucosa. This raises the question of the contribution of non-smooth

muscle cells in the contractile effect of PDE inhibitors, especially of the mucosal cells in the urothelium which is known to affect spontaneous contractility of smooth muscle cells [61]. However, a subset of experiments, namely the measurement of Ca^{2+} spark activity, was performed on isolated smooth muscle cells. As the effect of Ro was still present under these experimental conditions, it clearly indicates that the PDE4-related cascade is present and functional in neonatal rat bladder smooth muscle cells. Concerning the PDE3-mediated effect, this is less clear as PDE3 inhibition exerted no effect on the Ca^{2+} signaling, even in intact bladder for Ca^{2+} transient measurements. However, in preliminary experiments, we observed that the inhibitory effect of Cil on carbachol-enhanced phasic contraction was preserved in neonatal rat bladder strips in which the inner mucosa was gently removed (data not shown). This should be confirmed by additional experiments, but suggests that the urothelium is not necessary for the PDE3 effect.

5. Conclusion

In summary, our results underline the importance of PDE 3 and 4 subtypes in controlling the carbachol-enhanced phasic contraction of neonatal rat bladder smooth myocytes. We found that PDE3 and PDE4 exert their regulatory role through different signaling pathways, PDE3 involving PKG activation while PDE4 involving PKA activation (Fig. 8). In brief, PDE3 blockade activates PKG and inhibits the phasic contractions through unknown mechanisms. PDE4 blockade induces PKA activation which leads to an increase in Ca^{2+} spark frequency, and thus an increase in BK channel opening followed by the reduction of Ca^{2+} transient and finally the inhibition of phasic contractions of neonatal rat bladder smooth myocytes. Our results give

a novel insight into the action mechanisms of PDE and would be of potential significance in the usage of these inhibitors on treating human bladder disorders.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1: Primer sequences used for the expression of different PDE isoforms.

Target gene	Forward primers (5'→3')	Reverse primers (5'→3')	Fragment size (bp)	Cycles	Annealing temperature
PDE1A	ccactttgtgatcggaagtc	ttctgctgaatgatgtccacc	323	34	55°C
PDE1B	cagggtgacaaggaggcagag	gacatctggttggtgatgcc	344	34	55°C
PDE1C	tctcaaaggatgactggagg	gcttctctgtcacctgtc	256	34	55°C
PDE2A	cctcctgtgacctctctgacc	tgaactgtgggacacctgg	294	34	55°C
PDE3A	tcacagggccttaactacac	ggagcaagaattggtttgtcc	339	34	55°C
PDE3B	cctcaggcagttttatacaatg	tgcttcttcacatccctgtc	387	34	55°C
PDE4A	gtggagaagtctcaggtggg	tggaaactgtcaggcaggg	212	34	55°C
PDE4A?	ctgcgctctccaaccagttccta	gttctcggtgcgcctgctgga	429	30	60°C
PDE4A1	gccaacacctaccttacctg	cacggttcagcatccttt	135	40	55°C
PDE4A2	ccccaaggagatgaccaa	actgtcgcagcactgacg	226	40	60°C
PDE4A3	attcggagtatgaagacttgc	gagacatgggctgggact	218	40	55°C
PDE4A5	ccgatccgcatccagcaa	gatggcggtggcccatttt	218	40	60°C
PDE4A6	caacatgcttgggaccgacctg	tcgaacgctgcggagactgg	412	40	65°C
PDE4B	tagaagataacaggaactgg	gcaatgtctatgtcagtctc	247	34	58°C
PDE4B1	gcaaacagcagacatctcca	ccttgccctttcactttgtc	236	40	58°C
PDE4B2	gtcttgtccgggtcagtggt	gcttccctctctttgctt	165	40	58°C
PDE4B3	gccaggctttgcttactgtc	gccaaagtgttcctctgtc	209	40	58°C
PDE4B4	ggcgacactcgtggatat	gctgacagagcggtaggt	467	40	60°C
PDE4B5	tctgtatcttgggggttacatca	tctccatgcgtttctctgtg	445	40	58°C
PDE4C	acgtggcgtaccacaacagc	taccgcgaggtgatggttctc	242	34	55°C
PDE4D	ggataatggaggagtcttcc	cgattgtcctccaaagtgtcc	295	34	55°C
PDE4D1	ctttccctgtctcttcgcaga	gtgagctcccgattaagcatc	163	40	56°C
PDE4D2/1	atatgaaggagcagccctcatg	ccagaccgactcatttcagaga	221/307	40	56°C
PDE4D3	gcatgtgaataccttcccttt	tgcacaaagtgttcttgag	138	40	56°C
PDE4D4	cacggagcgctacctgtact	tgcacaaagtgttcttgag	219	40	56°C
PDE4D5	tgaccaggaaaatggctcag	cattgtccacatcgaacatgta	294	40	56°C
PDE4D6	ccacacctgaatggctttg	ggtgagctcccgattaagca	185	40	56°C
PDE4D7	gcctctgagtggattacagtttc	cagtcgagctcgcggatgt	354	40	56°C
PDE4D8	tggatcttagccctcgttt	tgcacaaagtgttcttgag	209	40	56°C
PDE4D9	tatgaagccgagatccaggt	tgcacaaagtgttcttgag	150	40	56°C
GAPDH	caagttcaacggcacagtcaag	gcaccagtggatgcagggat	477	34	55°C

Figures and legends

Fig. 1.

Expression pattern of PDE1-4 in neonatal rat bladder. (A-C) RT-PCR was performed on mRNA extracted from whole bladder tissue in the presence (+) or absence (-) of primers for GAPDH, PDE1A, PDE1B, PDE1C, PDE2A, PDE3A, PDE3B, PDE4A, PDE4A?, PDE4A1, PDE4A2, PDE4A3, PDE4A5, PDE4A6, PDE4B, PDE4B1, PDE4B2, PDE4B3, PDE4B4, PDE4B5, PDE4C, PDE4D, PDE4D1, PDE4D2, PDE4D3, PDE4D4, PDE4D5, PDE4D6, PDE4D7, PDE4D8, and PDE4D9. PCR products were resolved by electrophoresis on a 1.7% agarose gel. *A* and *C* show the representative gels stained with ethidium bromide. Position of molecular weight markers is indicated in base pairs (Bp). The histogram in *B* shows the densitometric ratios of PDE isoform / GAPDH bands, expressed as means $\pm$ SEM of 4 neonatal rat bladders. (D) Western blotting analysis was carried out on proteins extracted from whole bladder tissue isolated from 2 different neonatal rats. Representative blots for PDE2A, PDE3A, PDE3B, PDE4A, PDE4B, and PDE4D are shown. Position of molecular weight markers is indicated in kDa.

Fig. 2.

Effect of PDE inhibition on the phasic contractions of neonatal rat bladder smooth muscle. (A-D) Original traces of carbachol-enhanced phasic contractions in neonatal rat bladder strips before and after the application of DMSO (<0.1%, A), the PDE3 inhibitor Cil (1 μ M, B), the PDE4 inhibitor Ro (5 μ M, C), or Cil+Ro (D). The arrows indicate the times of drugs application.

(E-F) Summary data showing the average effect of DMSO (vehicle) and different PDE inhibitors (10 μ M MIMX as PDE1 inhibitor, 100 nM BAY as PDE2 inhibitor, Cil or Ro) on the carbachol-enhanced phasic contractions amplitude (E) and frequency (F). Data are expressed as mean $\pm$ SEM of n independent bladder strips as indicated in brackets. *** P <0.001 *versus* vehicle; ## P <0.01 and ### P <0.001 as indicated.

Fig. 3.

Role of Ca^{2+} sparks in the effect of PDE3 and PDE4 inhibition in neonatal rat bladder smooth myocytes. (A-J) Spontaneous Ca^{2+} sparks of single smooth myocytes isolated from neonatal rat bladder were recorded and analyzed. A and F show typical line-scan images and corresponding fluorescence profiles of spontaneous Ca^{2+} sparks before and after the application of 1 μ M Cil (A) or 5 μ M Ro (F). Histograms represent the mean data for F/F_0 , frequency, rise time and half time to decay in the absence (DMSO) or presence of Cil ($n = 13$ cells; B-E) and Ro ($n = 9$ cells. G-J). (K) Original trace of carbachol-enhanced phasic contractions in a neonatal rat bladder strip in the presence of 10 μ M Ryanodine (Rya) and 5 μ M Ro. (L) Summary data showing the average effect of DMSO (vehicle), Rya, Ro, and Rya+Ro on the carbachol-enhanced phasic contractions amplitude and frequency. Data are shown as mean $\pm$ SEM of n independent bladder strips, as indicated. * P <0.05 and *** P <0.001 *versus* vehicle; ## P <0.01 and ### P <0.001 as indicated.

Fig. 4.

Role of BK channels in the effect of PDE3 and PDE4 inhibition on the phasic contractions of

neonatal rat bladder smooth muscle. (A-B) Original traces of carbachol-enhanced phasic contractions in neonatal rat bladder strips in the presence of 30 nM IBTX and 1 μ M Cil (A) or IBTX and 5 μ M Ro (B). The arrows indicate the times of drug application. (C) Summary data showing the average effects of DMSO (vehicle), IBTX, Cil, Ro, IBTX+Cil and IBTX+Ro on the carbachol-enhanced phasic contractions amplitude and frequency. Data are mean $\pm$ SEM of n independent bladder strips, as indicated. * P <0.05 and *** P <0.001 *versus* vehicle; ## P <0.01 and ### P <0.001 as indicated; NS shows no significant difference.

Fig. 5.

Effect of PDE3 and PDE4 inhibition on Ca^{2+} transients, intracellular SR Ca^{2+} content, and phosphorylation of PLB in neonatal rat bladder. (A-C) Effect of Cil and Ro on Ca^{2+} transients in a single smooth myocyte in intact bladder tissue. A and B show representative traces of Ca^{2+} transients measured before and after the application of 1 μ M Cil (A) or 5 μ M Ro (B). Drug application is indicated by arrow. Dynamic parameters of Ca^{2+} transients (amplitude and frequency) are expressed in % of that obtained with vehicle (C). Data are mean $\pm$ SEM of $n = 20$ cells from 4 strips (Cil) and $n = 23$ cells from 5 strips (Ro). (D-F) Effect of Cil and Ro on caffeine-induced Ca^{2+} transients of single smooth myocytes isolated from neonatal bladder. D shows representative fluorescence profiles induced by 10 mM caffeine in the presence DMSO, Cil or Ro. Histograms represent the mean $\pm$ SEM for F/F_0 (E), $t_{\max}$ (F), $t_{1/2\text{on}}$ (F) and $t_{1/2\text{off}}$ (F) of Ca^{2+} transients induced by caffeine in the presence of DMSO ($n = 31$ cells), Cil ($n = 21$ cells) or Ro ($n = 23$ cells). (G-H) Effects of Cil and Ro on phosphorylation of PLB in neonatal rat bladder tissues. The representative blot is shown (G) and at least 3 separate experiments were

performed from 4 different rats, giving similar results. P-PLB/PLB ratio was quantified and expressed as mean $\pm$ SEM. *P<0.05 and ***P<0.001 *versus* vehicle.

Fig. 6.

Role of PKA in the PDE-mediated control of contractility of neonatal rat bladder. (A-B) Original traces of carbachol-enhanced phasic contractions in neonatal rat bladder strips in the presence of the PKA inhibitor H-89 (100 nM) and 1 μ M Cil (A) or H-89 and 5 μ M Ro (B). The arrows indicate the times of drug application. (C) Summary data showing the average effect of DMSO (vehicle), H-89, Cil, H-89+Cil, Ro and H-89+Ro on the amplitude and frequency of the carbachol-enhanced phasic contractions in neonatal rat bladder strips. (D) Representative trace of carbachol-enhanced phasic contractions in a neonatal rat bladder strip before and after application of the Epac activator (007, 30 μ M). (E) Summary data showing the average effect of 007 on the amplitude and frequency of the carbachol-enhanced phasic contractions. Data are shown as mean $\pm$ SEM of *n* independent bladder strips, as indicated. *P<0.05, **P<0.01 and ***P<0.001 *versus* vehicle; #P<0.05 as indicated; NS shows no significant difference.

Fig. 7.

Role of PKG in the PDE3-mediated control of contractility of neonatal rat bladder. (A) Original trace of carbachol-enhanced phasic contractions in a neonatal rat bladder strip in the presence of the PKG inhibitor KT 5823 (1 μ M) and 1 μ M Cil. The arrows indicate the times of drug application. (B) Summary data showing the average effect of DMSO (vehicle), KT 5823, Cil and KT 5823+Cil on the carbachol-enhanced phasic contractions amplitude and frequency. (C)

Representative trace of carbachol-enhanced phasic contractions in a neonatal rat bladder strip before and after application of the NO donor SNAP (100 μ M). (D) Summary data showing the average effect of SNAP on carbachol-enhanced phasic contractions amplitude and frequency. Data are shown as mean $\pm$ SEM of n independent bladder strips, as indicated. * $P < 0.05$ and *** $P < 0.001$ *versus* vehicle; ## $P < 0.01$ as indicated.

Fig. 8.

Schematic diagram of the signaling pathways mediating PDE3 and PDE4 function in neonatal rat bladder smooth myocytes. The inhibitory effects of Cil and Ro on phasic contractions are mediated by different mechanisms. In brief, PDE3 blockade by Cil elicits PKG activation which inhibits the phasic contractions through unknown mechanisms. PDE4 blockade by Ro induces PKA activation which leads to an increase in Ca^{2+} spark frequency, and thus an increase in BK channels opening followed by the reduction of Ca^{2+} transient and finally the inhibition of phasic contractions of neonatal rat bladder smooth myocytes.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8