Urol Res (2001) 29: 388–392
DOI 10.1007/s00240-001-0221-6

ORIGINAL PAPER

Yuhong Qiu · Patricia Kraft · Elizabeth C. Craig
Xiulin Liu · Donna Haynes-Johnson

Identification and functional study of phosphodiesterases in rat urinary bladder

Received: 18 May 2001 / Accepted: 20 September 2001 / Published online: 31 October 2001
© Springer-Verlag 2001

Abstract Cyclic nucleotides are important secondary messengers involved in modulating the contractility of various smooth muscles. Phosphodiesterases (PDE) play important roles in this process by modulating the levels of cyclic nucleotides and their duration of action. This study was designed to identify and characterize the PDE isoenzymes in rat urinary bladder and to evaluate their roles in regulating bladder smooth muscle tone. The involvement of cAMP and cGMP pathways in this process was also assessed. The studies were carried out with tissues from male and female rats and no significant sex-related difference was found in the results. Utilizing the unique pharmacological properties of different isoenzymes, PDE1, 2, 3, 4, and 5 were identified in rat bladder. Organ bath experiments showed that forskolin was most potent in relaxing pre-contracted rat bladder strips while sodium nitroprusside was moderately effective, suggesting the relaxation was mainly mediated by the cAMP pathway and that the cGMP pathway is moderately involved. For PDE inhibitors, the non-specific inhibitor papaverine was most effective in relaxing pre-contracted bladder strips. Among isoenzyme-selective inhibitors, vinpocetine, EHNA, and sildenafil induced more relaxation than milrinone and rolipram.

Keywords Phosphodiesterase · Urinary bladder · Cyclic nucleotides

Introduction

Cyclic nucleotides are important secondary messengers that are synthesized by their prospective cyclases and degraded by various phosphodiesterases (PDEs). PDEs play critical roles in modulating the levels of cyclic nucleotides and their duration of action insides the cell via their ability to hydrolyze these compounds. Currently, 21 PDE genes have been cloned and are classified into 11 families (PDE1–11) according to their sequence homology, and their biochemical and pharmacological properties [2, 5, 12, 19]. The effectiveness of PDE inhibitors in controlling the contractility of corpus cavernosal smooth muscle [4], vascular smooth muscle [9], and tracheal smooth muscle[13, 14] has been well documented, yet the PDE isoenzymes and their role in modulating bladder smooth muscle tone is still under intensive study. To date, the PDE isoenzymes in the bladder have only been studied in humans [17] and pigs [15]. It has been reported that bladder relaxation is mainly mediated by the cAMP pathway in humans [16], pigs [18], and rabbits [8]. The involvement of the cGMP pathway is not as well established and varies between species [1]. In this study, we identified PDE isoenzymes from male and female rat urinary bladder. In addition, the effects of various PDE inhibitors, as well as adenylate cyclase and guanylyl cyclase stimulators on the relaxation of pre-contracted male and female rat bladder strips were evaluated using the organ bath technique.

Y. Qiu (✉) · P. Kraft · E.C. Craig · X. Liu · D. Haynes-Johnson
Reproductive Therapeutics,
R.W. Johnson Pharmaceutical Research Institute,
1000 Route 202 South, Raritan, NJ 08869, USA
E-mail: yqiu@prius.jnj.com
Tel.: +1-908-7045512
Fax: +1-908-5266469

Materials and methods

Materials

All the chemicals were purchased from Sigma except where indicated. Sildenafil was extracted from Viagra tablets.

Tissue preparation

Mature male and female Wistar rat (250–300 g) were killed with carbon dioxide. The bladder was excised and the bladder body was separated from the neck. Fat and connective tissues were removed.

Isolation of PDEs

Enzyme isolation was carried out as described previously [10]. Briefly, tissues were homogenized, centrifuged and loaded on a

1 ml Mono-Q column pre-equilibrated with column buffer (20 mM HEPES, pH 7.2, 1 mM EDTA, 0.5 mM PMSF). After the unbound proteins were washed out with 5 ml of column buffer, the enzymes were eluted with a linear gradient of 100–600 mM NaCl in the same buffer and 1 ml fractions were collected.

Phosphodiesterase assay

PDE activities in the elution fractions were determined as described previously [10]. The data presented here are representative of at least two enzyme preparations from tissues of each sex. For every study, each reaction was performed in duplicate. The PDE activity was expressed as pmoles of cyclic nucleotide hydrolyzed per minute per milliliter of enzyme preparation. When inhibitors were used, they were dissolved in either water or DMSO. The final concentration of DMSO in the reaction mixture did not exceed 5% (v/v), and the same amount of DMSO was added separately as a solvent control when necessary. In the inhibitor studies, the enzyme concentration was adjusted such that the maximum hydrolysis of the initial total substrate did not exceed 15%, and the rate of product formation increased in a linear fashion with time and enzyme concentration.

Organ bath experiments

The organ bath experiments were carried out as described previously [10]. Longitudinal strips (2 mm×8 mm) of rat bladder were prepared. The strips were allowed to recover for 30 min in the organ chamber without any stretch, and then were stretched incrementally (0.5 g tension/stretch) in order to reach the optimal resting isometric tension. After every two to three stretches, the tissue was contracted with 1 μM carbachol. If the amplitude of this contraction was within 10% of the previous contraction, the optimal resting tension was reached. The strips were allowed to recover for 30 min with frequent washes. To study the ability of the compounds to induce relaxation, the strips were pre-contracted with 1 μM carbacol. To eliminate any prostaglandin mediated effects, 1 mM of indomethacine was added. When the contraction was stabilized, an accumulative dosage curve was constructed for each compound. The following stock solutions were prepared: Sodium nitroprusside (SNP) at 100 mM in saline; milrinone and rolipram at 100 mM in DMSO; forskolin and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) at 30 mM in DMSO; papaverine, sildenafil, and vinpocetine at 10 mM in saline, DMSO and EtOH respectively. All of the compounds were further diluted in saline. The stabilized contraction induced by carbacol minus baseline was defined as 100% tension. The relaxation was expressed as percentage tension.

Results

Characterization of PDE isoenzymes

The PDE isoenzymes were isolated from the urinary bladders of male and female rats through an anionic exchange column and the elution fraction was assayed for its cAMP and cGMP hydrolysing activity. PDE isoenzyme selective inhibitors were employed to characterize the identity of the isoenzymes. Similar PDE activity profiles were obtained from both sexes. No significant difference was observed between sexes and these will thus be referred to jointly.

The bladder homogenates were loaded on a Mono-Q column and eluted with a linear NaCl gradient. When the elution fractions were assayed using cGMP as the substrate, two major peaks were observed (Fig. 1; P1, P2). A potent PDE5 selective inhibitor, sildenafil, inhibited most of the P1 activity (Fig. 1), suggesting that the cGMP hydrolysing activity of P1 was largely contributed by PDE5. The majority of the P2 activity was inhibited by EHNA (Fig. 1), indicating that PDE2 was responsible for the majority of the activity in this peak. There were two recognizable peaks that overlapped extensively when cAMP was used as the substrate (Fig. 2; P1, P2). Rolipram, a PDE4 selective inhibitor, inhibited about two thirds of the activity in P2 and P3, suggesting PDE4 was the major contributor for cAMP hydrolysing activity (Fig. 2). In the presence of milrinone, a selective PDE3 inhibitor, roughly one third of the activity in P2 and P3 was suppressed, indicating there was some PDE3 in these two peaks. PDE2 and PDE3 can utilize both cAMP and cGMP as substrates. Their abilities to hydrolyze cAMP are affected by cGMP in opposite directions. cGMP inhibits PDE3 activity while it stimulates PDE2 activity. When cAMP hydrolysing activity was assayed in the presence of cGMP, P2 activity was stimulated while P3 activity was inhibited (Fig. 2), confirming the presence of PDE2 in P2 and PDE3 in P3. Since there was more PDE2 activity in P2, it masked the presence of PDE3. PDE1 is unique among the PDE

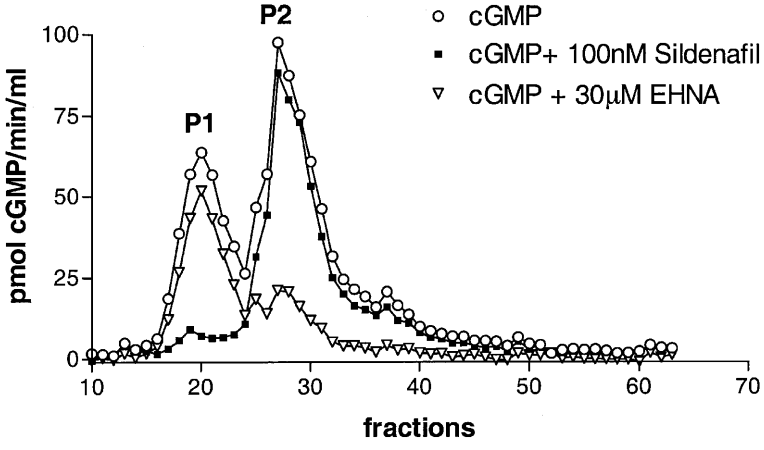

Fig. 1 Representative elution profile of cGMP PDE activity from male rat bladder. cGMP PDE activity was assayed in the presence and absence of sildenafil or EHNA. Sildenafil is a PDE5 specific inhibitor with reported IC_{50} of 3.9 nM [3]. EHNA [(erythro-9-(2-hydroxy-3-nonyl) adenine] is a PDE2 specific inhibitor with a reported IC_{50} of 1.0 μM [7]

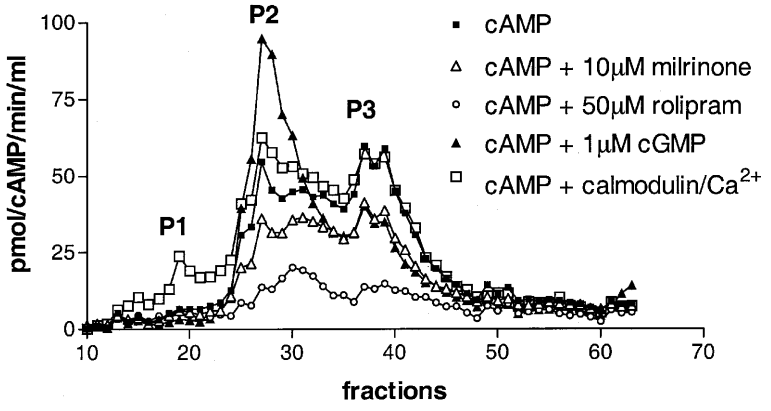

Fig. 2 The same elution fractions in Fig. 1 were assayed for cAMP PDE activity in the presence and absence of cGMP, or milrinone, or rolipram, or 200 μM Ca^{2+} and 40 units of calmodulin. Milrinone is a PDE3 specific inhibitor with a reported IC_{50} of 0.3 μM [6]. Rolipram is a PDE4 specific inhibitor with a reported IC_{50} of 2.0 μM [11]

isoenzymes in that its activity can be stimulated by calcium/calmodulin. To investigate whether PDE1 was present in the elution, a cAMP PDE assay was carried out in the presence of calcium/calmodulin. A small new peak appeared (Fig. 2; P1), suggesting the presence of PDE1 in the preparation.

Effects of various PDE inhibitors and cyclase stimulators on the relaxation of pre-contracted bladder strips

To evaluate the functional role of each PDE in relaxing rat bladder muscle, the ability of PDE inhibitors to relax male and female bladder muscle strips pre-contracted by carbachol were tested in organ bath experiments. In addition, an adenyl cyclase activator (forskolin) and a guanylyl cyclase activator (SNP) were also used to assess the involvement of cAMP and cGMP pathways in the relaxation process. The results are summarized in Fig. 3 and Table 1. Forskolin was the most potent relaxant, followed by the non-specific PDE inhibitor, papaverine. Among the selective PDE inhibitors, vinpocetine (PDE1 inhibitor), EHNA and sildenafil were more effective than milrinone, rolipram. SNP induced moderate relaxation. No significant difference was observed between male and female bladder strips.

Discussion

cAMP and cGMP are important secondary messengers that control many physiological processes including smooth muscle contractility. PDEs modulate intracellular cyclic nucleotide levels via their ability to hydrolyse cyclic nucleotides, and therefore, are critical components in controlling smooth muscle tone. In this report, we have identified the PDE isoenzymes in rat urinary bladder. In addition, organ bath experiments have been conducted to evaluate the effects of various PDE inhibitors and the adenyl and guanylyl cyclase activators on the relaxation of pre-contracted bladder strips. Since there are significant differences in the physiology and the pathology of male and female urinary tracts, male and female bladder tissues were compared in all the experiments. However, no significant differences were observed between males and females.

PDE1, 2, 3, 4 and 5 were identified in rat bladder. PDE1, 2 and 3 hydrolyze both cAMP and cGMP. PDE4 is cAMP specific while PDE5 is cGMP specific. The activities were eluted in three peaks (P1, P2, and P3). cGMP activity was mainly detected in P1 and P2, while cAMP activity was found in P2 and P3. Selective PDE inhibitors were employed to characterize the activities in the elution. P1 contained mostly the cGMP hydrolyzing activity that was contributed by PDE5. P3 contained mainly the cAMP hydrolysing activity that was contributed by PDE3 and 4. On the other hand, P2 had considerable amount of cAMP and cGMP hydrolyzing activities, contributed from PDE2, 3 and 4. Thus, it appeared that PDE3 and 4 activities were found in both P2 and P3. The PDE4 gene family is the largest one among all the PDE families. It consists of four different genes, each of which encodes multiple splicing variants. PDE3 also consists of products from two different genes. It is possible that similar pharmacological activities found in P2 and P3 represent different isoforms of PDE3 and PDE4, or different charged forms of the same isoform or even proteolytic fragments of the same isoform. Although the amount of PDE1 detected in rat bladder was small, it was definitely present. PDE6 is a retina specific PDE and thus was not pursued further in this study. Although the existence of PDE7–11 was not determined due to the lack of specific inhibitors, judging from the extent of activity inhibited by the known inhibitors, it is not likely that significant amounts of these are present in rat bladder. PDE1–5 have also been identified from human and porcine detrusor [15, 17]. One observable difference is that considerable amount of PDE1 has been reported in human and porcine bladder while our results indicated there was a very limited amount of PDE1 in rat bladder. This probably reflects a species difference.

Forskolin is a potent activator of adenylate cyclase. It completely relaxed bladder strip pre-contracted by carbachol, suggesting that cAMP accumulation was very effective in this relaxation process. This observation is in agreement with previous reports that the relaxation of

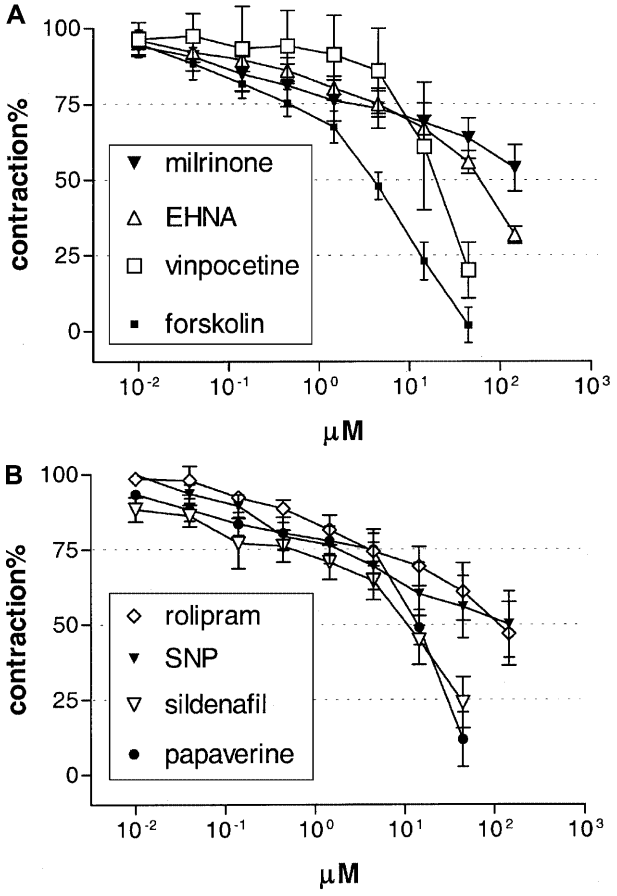

Fig. 3A, B Effects of PDE inhibitors and adenylate and guanylyl cyclase activators on the relaxation of pre-contracted male rat bladder strips. The above baseline tension induced by carbachol was defined as 100%. Relaxation was expressed as mean percentage tension ± SD at each dose. Four to 12 strips from 2–4 preparations were tested for each inhibitor. **A** EHNA, milrinone, vinpocetine, and forskolin; **B** SNP, Rolipram, sildenafil, and papaverine

Table 1 The effects of PDE inhibitors, adenylate cyclase and guanylyl cyclase activators on relaxation of pre-contracted rat bladder strips. Complete relaxation down to baseline was defined as 100%. E_{max}% represents maximal relaxation achieved at highest concentration tested for each compound. Each value represents the mean ± SD for 4–12 tissue strips from 2–4 preparations

Compound	Maximum concentration (μM)	E_{max}(%) (mean ± SD)	EC_{50} (μM)
vinpocetine	44	79.93 ± 9.79	21.13
EHNA	144	68.31 ± 2.84	30.12
milrinone	144	49.09 ± 7.73	ND
rolipram	144	53.14 ± 10.66	ND
sildenafil	44	76.02 ± 8.49	12.74
papaverine	44	88.23 ± 9.08	14.3
forskolin	44	98.03 ± 5.82	5.83
SNP	144	50.03 ± 11.10	ND

bladder strips is mainly mediated by the cAMP pathway in species including rabbits [8], humans [16], and pigs [18]. The involvement of NO induced cGMP accumulation in bladder relaxation is not very consistent among different reports in the literature [1]. It seems that the degree of relaxation varies depending on the species examined, the way NO is delivered, and the reagents used for the pre-contraction of bladder strips. Generally, minimal to moderate relaxation was observed. NO is the natural activator for soluble guanylyl cyclase. In this study, SNP was used as the NO donor. It induced about 50% relaxation in bladder strips, indicating that the NO mediated cGMP pathway played a moderate role in rat bladder relaxation. This is different from the results in the human [16] and porcine [18] bladder where the cGMP pathway has very limited involvement.

Of the PDE inhibitors studied, the non-specific PDE inhibitor, papaverine, induced the most relaxation (88%), confirming the importance of cyclic nucleotide accumulation in urinary bladder relaxation. Both cAMP and cGMP concentration can be elevated by papaverine. Vinpocetine, sildenafil and EHNA were the next effective relaxants, elicited 70–80% relaxation. Milrinone and rolipram were less effective, generating about 50% relaxation. Although cAMP accumulation was very effective in inducing relaxation and most of the cAMP hydrolyzing activities were contributed by PDE3 and PDE4, the inhibitors of these two PDEs exhibited the least effect. There are at least two possible explanations. First, in isolated tissue strips, the basal activity of adenylate cyclase may be very low, as is also the case for the cAMP turn over rate. Blockage of cAMP hydrolysis alone is insufficient to elevate the intracellular cAMP to a critical level. Second, the solubility of milrinone and rolipram may limit their accessibility to the cells. There might be a cellular compartmentalization issue in addition.

Although vinpocetine has been found to be the most effective among selective PDE inhibitors in relaxing human and porcine detrusor smooth muscle [16, 18], its effectiveness in relaxing rat bladder strips was still surprising given the small amount of PDE1 present in rat bladder. It is possible that compartmentalization played a significant role here. Sildenafil is a potent PDE5 inhibitor with an IC_{50} of 3.9 nM in the enzyme assay. PDE5 inhibition increases cGMP levels, which can also be achieved by SNP stimulation of soluble guanylyl cyclase. Yet sildenafil was much more effective in inducing relaxation in rat bladder strips than SNP. Part of the explanation might be the cross inhibition of sildenafil to other PDEs. Sildenafil has an IC_{50} of 280 nM against PDE1, and 6.3 μM against PDE4 [3]. Thus, in the concentration range tested, sildenafil might have inhibited more than PDE5 to achieve its maximal effect. Another possible contributing factor is that there is cross talk between cAMP and cGMP pathways. Elevated cGMP levels due to PDE5 inhibition result in the inhibition of PDE3, which in turn increases cAMP levels. Inhibition of PDE2 could increase both cAMP and cGMP concentrations and EHNA produced decent relaxation. Taken together, the data seem to suggest that when PDE inhibitors are used alone, stimulation of both cAMP and cGMP pathways is more effective than stimulating just

one pathway. It is also worth noting that the relative abundance of PDE isoenzymes does not always correlate with the significance of the functional roles they play. Intracellular compartmentalization is critical physiologically.

Acknowledgement The authors wish to thank Muh-Tsann Lai for technical assistance in obtaining rat and rabbit bladder tissues.

References

1. Anderson K-E (1999) Pathways for relaxation of detrusor smooth muscle. In: Hayward BA (ed) Pathways for relaxation of detrusor smooth muscle. Kluwer /Plenum, New York, p 241
2. Beavo, JA (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 75: 725
3. Boolell M, Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM, Osterloh IH, Gingell C (1996) Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. Int J Impot Res 8: 47
4. Eardley I (1997) The role of phosphodiesterase inhibitors in impotence. Exp Opin Invest Drugs 6: 1803
5. Fawcett L, Baxendale R, Stacey P, McGrouther C, Harrow I, Soderling S, Hetman, J, Beavo JA, Phillips SC (2000) Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. Proc Natl Acad Sci U S A 97: 3702
6. Harrison SA, Reifsnyder DH, Gallis B, Cadd GG, Beavo JA (1986) Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs. Mol Pharmacol 29: 506
7. Meacci E, Taira M, Moos MJr, Smith CJ, Movsesian MA, Degerman E, Belfrage P, Manganiello V (1992) Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase. Proc Natl Acad Sci U S A 89: 3721
8. Morita T, Wheeler MA, Weiss RM (1986) Relaxant effect of forskolin in rabbit detrusor smooth muscle: role of cyclic AMP. J Urol 135: 1293
9. Polson JB Strada SJ (1996) Cyclic nucleotide phosphodiesterases and vascular smooth muscle. Annu Rev Pharmacol 36: 403
10. Qiu Y, Kraft P, Lombardi E, Clancy J (2000) Rabbit corpus cavernosum smooth muscle shows a different phosphodiesterase profile than human corpus cavernosum. J Urol 164: 882
11. Sheppard H Wiggan GATW (1972) Structure-activity relationships for inhibitors of phosphodiesterase from erythrocytes and other tissues. Adv Cyclic Nucleotide Res 1: 103
12. Soderling SH Beavo JA (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr Opin Cell Biol 12: 174
13. Torphy JT Cieslinski LB (1989) Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. Mol Pharmcol 37: 206
14. Torphy TJ, Undem BJ, Cieslinski LB, Luttmann MA, Reeves ML, Hay DWP (1993) Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. J Pharmacol Exp Therapeut 265: 1213
15. Truss MC, Uckert S, Steif CG, Jess R, Forssmann W-G, Jonas U (1995) Porcine detrusor cyclic nucleotide phosphodiesterase isoenzymes: characterization and functional effects of various phosphodiesterase inhibitors in vitro. Urology 45: 893
16. Truss MC, Ueckert S, Stief CG, Kuczyk M, Jonas U (1996) Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in the human detrusor smooth muscle II. Effect of various PDE inhibitors on smooth muscle tone and cyclic nucleotide levels in vitro. Urol Res 24: 129
17. Truss MC, Ueckert S, Stief CG, Kuczyk M, Jonas U (1996) Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in the human detrusor smooth muscle. I. Identification and characterization. Urol Res 24: 123
18. Truss MC, Ueckert S, Stief CG, Kuczyk M, Schulz-Knappe P, Forssmann W-G, Jonas U (1996) Effects of various phosphodiesterase-inhibitors, forskolin, and sodium nitroprusside on porcine detrusor smooth muscle tonic responses to muscarinergic stimulation and cyclic nucleotide levels in vitro. Neurourol Urodyn 15: 59
19. Yuasa K, Kotera J, Fujishige K, Michibata H, Sasaki T, Omori K (2000) Isolation and characterization of two novel phosphodiesterase PDE11 A variants showing unique structure and tissue-specific expression. J Biol Chem 275: 31469