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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

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Abstract Phosphodiesterases (PDEs) regulate intracellular cyclic nucleotide metabolism and, thus, contraction and relaxation of smooth musculature. The aim of the present study was to evaluate the functional effects of isoenzyme-selective inhibitors and their effects on cyclic nucleotide levels in the human detrusor smooth muscle. In addition, the functional relevance of the cAMP versus the cGMP pathways in the regulation of the detrusor smooth muscle tone was assessed. Relaxant responses to various PDE inhibitors, forskolin and sodium nitroprusside (SNP) were investigated in vitro using a standard organ bath setup. Cyclic nucleotide levels were measured after incubation with the same substances using cAMP and cGMP radioimmunoassays (RIAs). Significant relaxant responses were only induced by non-selective PDE inhibition, the PDE I inhibitor vinpocetine and the adenylate cyclase activator forskolin. Relaxant responses to these substances were paralleled by increases in cyclic nucleotide levels. Our data suggest that the cAMP pathway and calcium/calmodulin-stimulated PDE (PDE I) may be of functional importance in the regulation of the human detrusor smooth muscle tone in vitro.

Key words Phosphodiesterase · Detrusor · cAMP · cGMP

Introduction

Relaxation of smooth muscle can be mediated by cyclic adenosine monophate (cAMP) and cyclic guanosine monophosphate (cGMP) generation. cAMP and cGMP are synthesized from adenosine triphosphate (ATP) and guanosine triphosphate (GTP) by their respective membrane-bound or soluble adenylate (AC) or guanylate cyclases (GC) [5, 6]. The breakdown of cAMP and cGMP is controlled by cyclic nucleotide phosphodiesterases (PDEs, Fig. 1). The existence of five different PDE isoenzyme families is now well established, and several drugs are known to selectively inhibit PDE isoenzymes [2, 10]. The role of cyclic nucleotides and PDEs has been investigated in various tissues and the use of isoenzyme-selective inhibitors for modulation of tissue function has been proposed, i.e. in patients with refractory congestive heart failure and asthma [1, 9]. The role of cyclic nucleotides and PDEs in the intracellular regulation of smooth muscle tone of the lower urinary tract is less clear; however, some authors postulate that predominantly cAMP, in the rabbit, pig and rat animal models, may be involved in the smooth muscle tone regulation of the detrusor while cGMP may be primarily responsible for urethral relaxation [8, 12, 13].

Current concepts for the modulation of detrusor function in various disorders of micturition are limited by a lack of efficacy and significant side effects of available drugs. Interestingly, a modulation of intracellular concentration of cyclic nucleotides by PDE inhibition was postulated as one possible mode of action in some widely used substances for the treatment of detrusor hyperreflexia with "mixed" or "direct" action, although the evidence was not convincing [3, 14]. The aim of the present study was to further delineate the role of the AC-cAMP and GC-cGMP pathways in the human detrusor smooth muscle, to evaluate the effects of PDE-isoenzyme-specific inhibitors on muscarinergic contractions and on intracellular cyclic nucleotide levels in this tissue.

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Fig. 1 Synthesis and degradation of adenosine cyclic 3′5′-monophosphate (*cAMP*) and guanosine cyclic 3′5′-monophosphate (*cGMP*). *ATP* adenosine triphosphate, *GTP* guanosine triphosphate, *AC* adenylate cyclase, *GC* guanylate cyclase, *PDEs* cyclic nucleotide phosphodiesterases

Materials and methods

Tissue preparation

Human detrusor smooth musculature was obtained from 22 patients undergoing radical surgery for pelvic malignancies. None of the patients had symptoms of urge or urge incontinence by history. Macroscopically normal, non-tumorous tissue was taken from the bladder dome and lateral walls and was immediately placed in a chilled organ protective solution (NaCl 15 mM, KCl 9 mM, $KHC_5H_6O_5$ 1 mM, $MgCl_2$ 4 mM, histidine 18 mM, tryptophan 2 mM, mannitol 30 mM, $CaCl_2$ 0.015 mM). After the urothelium and connective tissue had been removed, bladder strips ($0.6 \times 0.3 \times 0.3$ cm) were mounted in 10-ml organ bath chambers containing Ringer-Krebs solution (NaCl 119 mM, $NaHCO_3$ 15 mM, KCl 4.6 mM, $CaCl_2$ 1.5 mM, $NaHPO_4$ 1.2 mM, $MgCl_2$ 1.2 mM, glucose 11 mM, pH 7.4). The organ bath solution was continuously gassed with 95% O_2 and 5% CO_2 and the temperature was maintained at 37 °C by a thermoregulated water circuit. The strips were mounted between two L-shaped hooks, one of which was connected to a pressure transducer. The other was attached to a movable unit and a pretension of 10 mN was applied. In initial experiments this pretension revealed the best results. The strips were equilibrated for 60 min during which the organ bath solution was changed every 15 min. Then the detrusor strips were contracted with carbachol (1 μM), which ensured stable and reproducible contractions. After a stable contraction plateau had been reached, increasing doses of forskolin (adenylate cyclase activator), sodium nitroprusside (SNP, guanylate cyclase activator) and the following PDE inhibitors were cumulatively added to the organ bath chambers: papaverine (non-specific PDE inhibitor), vinpocetine (inhibitor of the calcium/calmodulin-stimulated PDE I), milrinone (inhibitor of the cGMP-inhibited PDE III), rolipram (inhibitor of the cAMP-specific PDE IV), zaprinast (inhibitor of the cGMP-specific PDE V and PDE I) and dipyridamole (inhibitor of the cGMP-specific PDE V). Drugs were made up as solutions to obtain final concentrations of 0.01–100 μM (forskolin and SNP) and 0.01–200 μM (PDE inhibitors) in the organ bath chambers. Isometric responses of the tissue were amplified and plotted or digitalized with an analog-digital converter and recorded for further evaluation and statistical analysis. Relaxation was expressed as percentage of the contraction plateau induced by carbachol (100%). All experiments were repeated 8–12 times.

There were only negligible effects of the highest concentration of the vehicles on tissue tension. Only dimethylsulphoxide (DMSO) induced 15–20% relaxation in the control experiments. These values were subtracted from the relaxant responses of the tissue strips to dipyridamole.

Assay of cyclic nucleotides

In parallel experiments, detrusor strips (approximate size $3 \times 3 \times 3$ mm) from the same preparations were dissected free of urothelium and connective tissue, incubated in Ringer-Krebs solution at 37 °C and gassed with carbogen (95% O_2, 5% CO_2). As in the organ bath studies, the detrusor strips were exposed to 1 μM carbachol for approximately 30 min. Then, forskolin (0.01, 1, 100 μM), SNP (0.01, 1, 100 μM), the PDE inhibitors mentioned above (0.1, 10 and 100 μM) or a corresponding amount of vehicle was added. Samples with PDE inhibitors were incubated for 10 min while samples with forskolin and SNP were incubated for 2 and 10 min. The reaction was stopped by freezing the tissue strips in liquid nitrogen. After homogenization with a tissue plotter, cyclic nucleotides were extracted with 70% ethanol (v/v). Samples were centrifuged at 3000 g for 10 min at 4 °C. The supernatant was lyophylised and redissolved in sodium acetate buffer (50 mM, pH 6.0). Aliquots of the samples were acetylated and assayed for cAMP and cGMP contents by specific radioimmunoassays. Each concentration was tested sixfold and assayed in duplicate for cAMP and cGMP.The protein contents of the pellets were measured according to the method of Lowry using bovine serum albumin as a standard [7].

Chemicals and drugs

Vinpocetine was from Biomol (Hamburg, Germany), milrinone from Sanofi Winthrop Germany (Munich, Germany), rolipram (ZK 67211) from Schering AG (Berlin, Germany) and zaprinast (M&B 22984) from Rhône-Poulenc Rorer (Dagenham, UK). All other chemicals were purchased from Sigma (St. Louis, Mo., USA). Ten-millimolar stock solutions of PDE inhibitors were obtained using saline (milrinone), ethanol (rolipram, vinpocetine), methanol (papaverine), 0.1 N NaOH (zaprinast) or DMSO (dipyridamole) as solvents. Stock solutions were further diluted using saline.

Analysis of data

Relaxant responses of tissue preparations during functional experiments are expressed as percentages of the contraction induced by carbachol (1 μM). Mean EC_{50} values were determined graphically from non-linear regression analyses of data and represent 50% relaxation of the carbachol-induced contraction plateau. All data are given as means ± SD. Statistical analysis was conducted by Student's *t*-test. A probability (*P*) value of less than 0.05 was accepted as significant.

Results

Effects on precontracted muscle strips

Figure 2 and Table 1 summarize the data of the functional organ bath studies. Significant, dose-dependent relaxations of carbachol-contracted detrusor strips were induced by forskolin ($EC_{50} = 5.9$ μM), papaverine ($EC_{50} = 80$ μM) and vinpocetine ($EC_{50} = 150$ μM) starting at a concentration of 10 μM. SNP, milrinone, rolipram, zaprinast and dipyridamole were significantly less potent (relaxant responses <20%). Higher PDE inhibitor concentrations than 200 μM could not be tested in order to avoid solubility problems.

Effects on cyclic nucleotide contents

Forskolin significantly increased cAMP levels dose dependently and time dependently starting at a

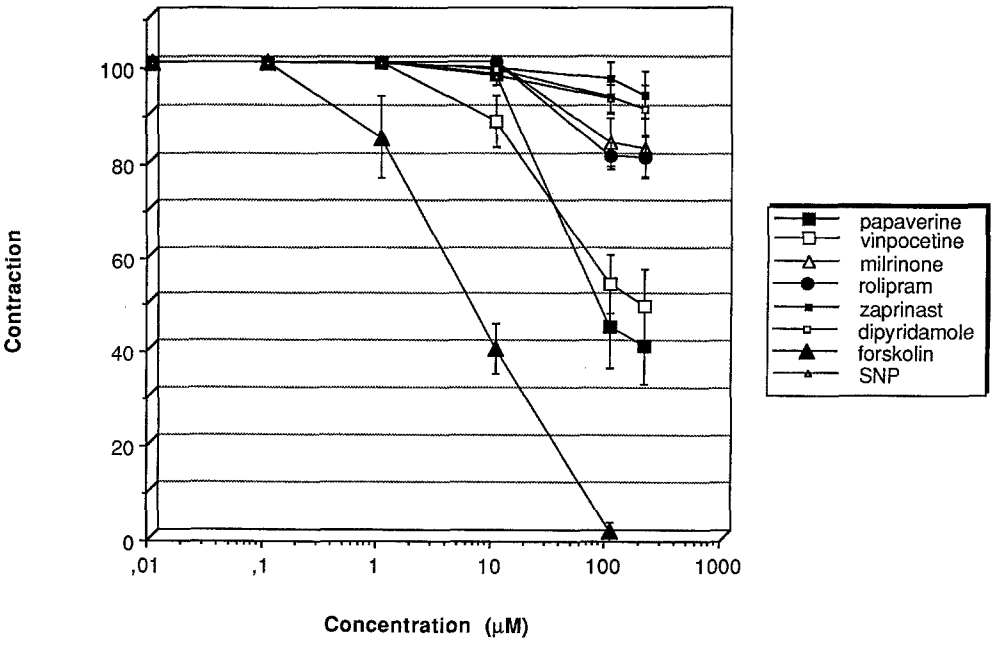


Fig. 2 Relaxation of human detrusor strips by cyclic nucleotide PDE inhibitors after contraction with carbachol (1 μM). Each data point represents 8–12 experiments. Data are given as means ± SD. * $P < 0.00001$, relaxation response to papaverine and vinpocetine when compared with milrinone, rolipram, zaprinast and dipyridamole

Table 1 Relaxation of human detrusor smooth muscle strips by cyclic nucleotide PDE inhibitors, forskolin and sodium nitroprusside (*SNP*) after contraction with carbachol (1 μM). [EC_{50} concentration of PDE inhibitor resulting in 50% relaxation of the contractile response to carbachol; E_{max} maximum relaxation at 200 μM inhibitor concentration; *ND* not determined ($EC_{50} > 200$ mM)]. Data are given as mean ± SD, $n = 8$–12

	EC_{50} (μM)	E_{max} (%)
Papaverine	80	59.9 ± 8.2
Vinpocetine	150	51.4 ± 7.9
Milrinone	ND	17.9 ± 6.2
Rolipram	ND	19.7 ± 4.0
Zaprinast	ND	6.8 ± 5.1
Dipyridamole	ND	9.9 ± 5.1
Forskolin	5.9	99.2 ± 2.0
SNP	ND	9.9 ± 2.5

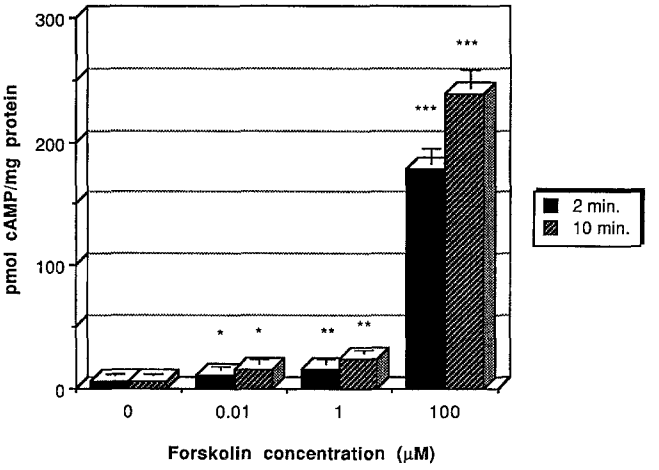


Fig. 3 Effect of the adenylate cyclase activator forskolin on cyclic adenosine monophosphate (*cAMP*) levels in human detrusor smooth muscle (* $P < 0.00001$, ** $P < 0.0000001$, *** $P < 0.000000001$ when compared with baseline values, Student's *t*-test). Two- and ten-min values were significantly different at all concentrations ($P < 0.00001$, Student's *t*-test)

concentration of 1 μM. The maximum concentration of forskolin (100 μM) increased cAMP contents 32.6- and 43.9-fold over baseline at 2 and 10 min, respectively (Fig. 3). No significant effect of forskolin on cGMP levels was noted (data not shown), and 100 μM SNP increased cGMP levels 2.8- and 2.1-fold at 2 and 10 min, respectively ($P < 0.05$ each) (Fig. 4). No significant changes were detected after incubation with 0.01 or 1 μM SNP. cAMP levels were unaffected by SNP (data not shown).

cAMP levels were dose dependently elevated by papaverine, vinpocetine and rolipram, however, at a PDE inhibitor concentration of 10 μM, statistical significance was only reached for papaverine ($P < 0.05$). At a PDE inhibitor concentration of 100 μM cAMP elevations were statistically significant for all three PDE inhibitors ($P < 0.05$). Milrinone, zaprinast and dipyridamole did not alter cAMP levels significantly (Fig. 5).

cGMP contents were dose dependently elevated by papaverine, vinpocetine and dipyridamole. Statistical significance ($P < 0.05$) was reached at 100 μM with papaverine and dipyramidole and at 10 μM for vinpocetine (Fig. 6).

Discussion

cAMP and cGMP are important intracellular second messengers formed following stimulation of adenylate

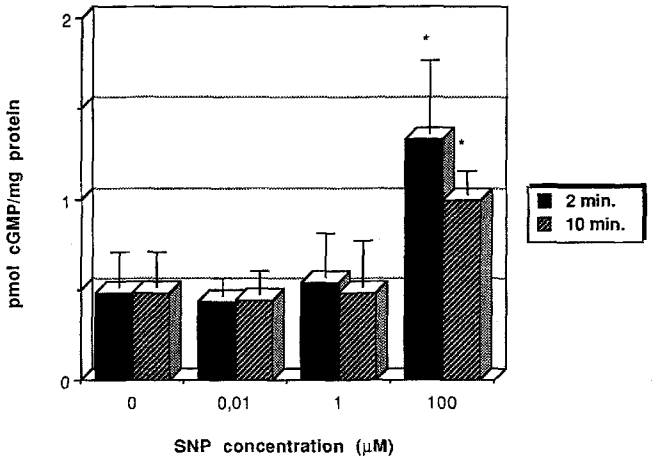


Fig. 4 Effect of the guanylate cyclase activator sodium nitroprusside (*SNP*) on cyclic guanosine monophosphate (*cGMP*) levels in human detrusor smooth muscle (* $P < 0.00001$ when compared with baseline values, Student's *t*-test). Two- and ten-min values were significantly different only at 100 μM ($P < 0.0001$, Student's *t*-test)

cyclase and guanylate cyclase, respectively [5, 6]. The effects of many hormones and neurotransmitters are mediated through specific receptors coupled to these two enzymes. The degradation of cAMP and cGMP is regulated by the activity of cyclic nucleotide PDEs [2]. cAMP and cGMP can both produce smooth muscle relaxations but their precise roles and the regulation of their levels in various tissues is not well understood. Discrepancies have been repeatedly reported between relaxation and cAMP and cGMP accumulation induced in smooth muscle by drugs which either stimulate the production or inhibit the degradation of cAMP or cGMP in the same tissue [15, 17, 18]. The aim of the present study was to further delineate the role of the AC-cAMP and GC-cGMP pathways in the human detrusor smooth muscle, to evaluate the effects of isoenzyme-specific inhibitors on muscarinergic contractions and on intracellular cyclic nucleotide levels in this tissue.

Fig. 5 Effect of various PDE inhibitors on cyclic adenosine monophosphate (*cAMP*) levels in human detrusor smooth muscle (* $P < 0.05$, Student's *t*-test)

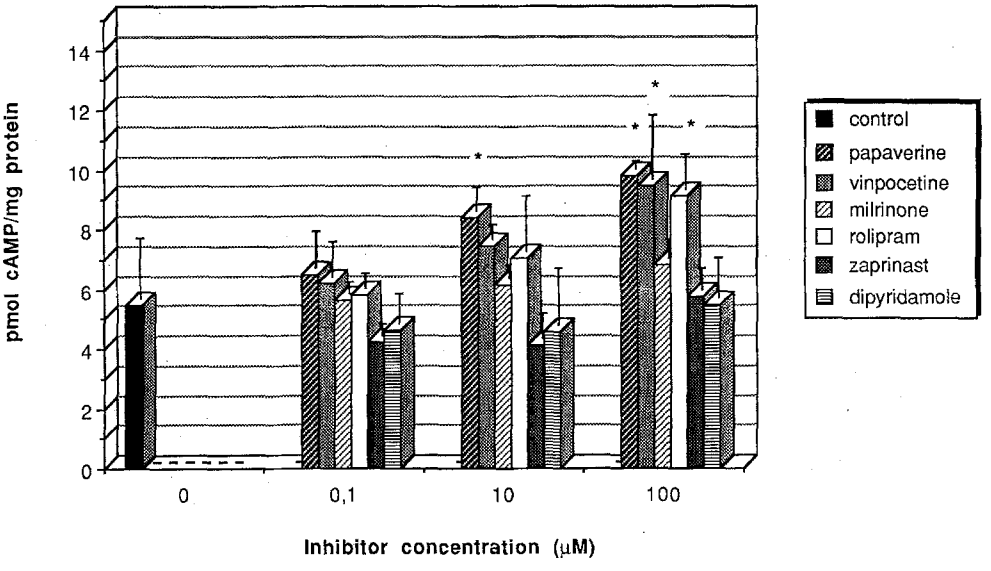
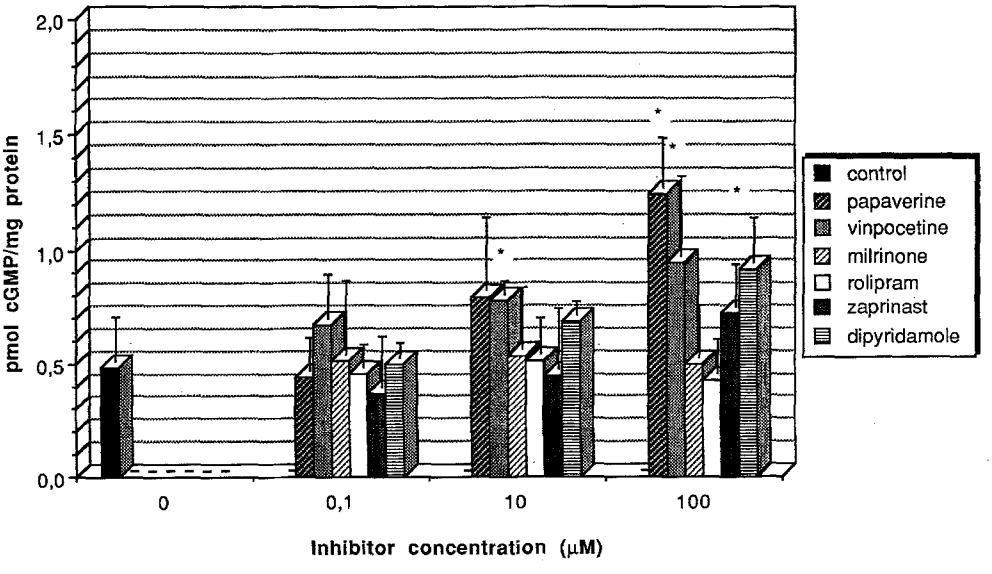


Fig. 6 Effect of various phosphodiesterase inhibitors on cyclic guanosine monophosphate (*cGMP*) levels in human detrusor smooth muscle (* $P < 0.05$, Student's *t*-test)



In functional studies the PDE I selective inhibitor vinpocetine was significantly more potent than the other selective inhibitors tested but less potent than the non-selective PDE inhibitor papaverine. The functional effects of papaverine and vinpocetine are paralleled by their ability to increase intracellular cyclic nucleotide levels. The fact that papaverine and vinpocetine inhibit both cAMP and cGMP hydrolysis – unlike in other tissues where vinpocetine primarily inhibits cGMP hydrolysis [4] – gives rise to the speculation that modulation of both pathways is necessary to influence smooth muscle tone. In contrast, rolipram and dipyridamole were able to increase cAMP and cGMP levels, respectively, but did not exert major functional effects in vitro. The apparent dissociations between cAMP and cGMP levels and functional response may also be explained by possible, additional modes of actions other than PDE inhibition or intracellular compartmentation of cyclic nucleotides. Thus, different inhibitors may elevate cyclic nucleotide levels in different intracellular pools and compartments. Therefore, cyclic nucleotides could act in such a way that very small, compartmentalized changes in cAMP or cGMP could cause major changes in intracellular calcium and, thus, in smooth muscle tone as suggested in other tissues [6, 16]. In addition, some PDE inhibitors might penetrate and be distributed within the cell differently from others.

Our functional studies suggest that the calcium/calmodulin-stimulated PDE I may be of importance in the regulation of smooth muscle tone in the human detrusor and most of the functional effect of PDE inhibition in the human detrusor may be related to PDE I. However, one has to keep in mind that vinpocetine may have different modes of action other than inhibition of PDE I, such as inhibition of the lipooxygenase pathway [11].

In general, functional organ bath studies should be interpreted with caution since cyclic nucleotide turnover rates in isolated tissue preparations are low; therefore high concentrations of PDE inhibitors are needed to elevate cyclic nucleotide levels in order to produce a significant tissue response. In in vivo systems where cyclic nucleotide turnover rates are much higher, PDE inhibitors tend to be much more effective [10].

The pronounced relaxant response to forskolin and the dramatic increase in cAMP levels after incubation with forskolin shows that the cAMP system is involved as a second messenger in the relaxation of the human detrusor smooth muscle. While measurements of cGMP after incubation with SNP, papaverine, vinpocetine and dipyridamole show that a cGMP system exists in the human detrusor smooth muscle, our functional results do not support the view that selective inhibition of cGMP hydrolysis can elicit relaxation in the human detrusor per se.

When comparing relaxant responses to forskolin and SNP and the increase in cyclic nucleotide levels after administration of these substances, cAMP seems to be the more important second messenger involved in the regulation of relaxation and contraction in the human detrusor smooth muscle. This view is in accordance with findings of others who found that, in the animal model, the cGMP pathway is predominantly involved in the regulation of the smooth muscle tone of the bladder outlet and urethra rather than the detrusor body [8, 12, 13].

In conclusion, our data suggest that relaxation of human detrusor smooth muscle is regulated by intracellular cyclic nucleotide levels. While selective inhibitors of PDE isoenzymes III, IV and V exert only a weak relaxant response on carbachol precontracted detrusor strips, the PDE I inhibitor vinpocetine was shown to be significantly more potent in vitro. Discrepancies between functional responses to PDE inhibitors and cyclic nucleotide levels may be explained by intracellular compartmentation of cyclic nucleotides. The cAMP pathway seems to be the dominant messenger system involved in the regulation of relaxation and contraction in the human detrusor smooth muscle in vitro. The functional relevance of cGMP in the regulation of relaxation and contraction is less clear.

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