Urol Res (1996) 24:123–128

ORIGINAL PAPER

M. C. Truss · S. Ückert · C. G. Stief
M. Kuczyk · U. Jonas

Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in the human detrusor smooth muscle

I. Identification and characterization

Received: 12 September 1995 / Accepted: 28 December 1995

Abstract Phosphodiesterases (PDEs) are key enzymes involved in the regulation of intracellular cyclic nucleotide metabolism. The aim of the present study was to identify and to characterize the PDE isoenzymes present in the human detrusor smooth muscle. Human detrusor PDE isoenzymes were separated by Q-Sepharose anion exchange and calmodulin-agarose affinity chromatography and characterized upon their kinetic characteristics and their sensitivity to allosteric modulators and inhibitors. All five presently known PDE isoenzyme families were identified: one high-affinity, low-K_m calcium/calmodulin-stimulated PDE I with a slight preference for cGMP over cAMP, one cGMP-stimulated PDE II, one cGMP-inhibited PDE III, one cAMP-specific PDE IV and one cGMP-specific PDE IV. All five known PDE isoenzyme families exist in human detrusor smooth musculature. The kinetic characteristics, together with functional in vitro studies, suggest that the PDE I may be of importance in the intracellular regulation of the human detrusor smooth muscle tone.

Key words Phosphodiesterase · Detrusor · cAMP · cGMP

Introduction

The fundamental role of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in the regulation of muscle tone and contractility is well established in various tissues, such as in vascular smooth muscle [4, 9], myocardium [6] and airway smooth muscle [16]. Cyclic nucleotides are synthesized from the corresponding nucleoside triphosphates by their respective membrane-bound or soluble adenylate or guanylate cyclases. cAMP and cGMP are degraded by cyclic nucleotide phosphodiesterases (PDEs) by hydrolytic cleavage of the 3′-ribose-phosphate bond. Because of their central role in the regulation of cyclic nucleotides and the considerable variation of PDE isoenzymes with respect to species and tissues, phosphodiesterases have become an attractive target for drug development [5]. Currently, five families of PDE isoenzymes can be distinguished [2]. Each family can be divided into subfamilies and single family members. The identification of PDE families has been paralleled by the synthesis of selective or partially selective inhibitors (Table 1) [5]. Theoretically, at least a partial pharmacological tissue selectivity of a drug may be achieved by isoenzyme-selective PDE inhibition. The aim of the present study was to analyse the PDE isoenzymes present in the human detrusor smooth muscle.

M. C. Truss (✉) · S. Ückert · C. G. Stief · M. Kuczyk · U. Jonas
Department of Urology, Hannover Medical School,
D-30623 Hannover, Germany

M. C. Truss · S. Ückert
Lower Saxony Institute for Peptide Research

Materials and methods

Tissue preparation

Human detrusor smooth musculature was obtained from patients undergoing radical surgery for pelvic malignancies. Macroscopically normal, non-tumorous tissue was taken from the bladder dome and lateral walls and was immediately placed in a chilled organ-protective solution and transported to the laboratory. All subsequent steps were performed at 4 °C. Detrusor smooth muscle was carefully dissected free of connective tissue and bladder urothelium. For one preparation approximately 15 g tissue was cut into small pieces and homogenized with a high-speed blender in four volumes (v/w) of buffer A (pH 6.5) containing 20 mM TRIS, 1 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol (DTT) and a mixture of the following protease inhibitors: 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 100 nM leupeptin, 0.1 mM *para*-tosyl-L-lycine chloromethyl ketone (pTLCK), 20 μg/ml soybean trypsin inhibitor and 100 μg/ml bacitracin. The

Table 1 Current classification of phosphodiesterase (PDE) isoenzyme families and isoenzyme-selective PDE inhibitors

PDE family	Characteristics	Main substrate	Inhibitor(s)
PDE I	Calcium/calmodulin stimulated	cGMP (cAMP)	Vinpocetine
PDE II	cGMP stimulated	cAMP	Not known
PDE III	cGMP inhibited	cAMP	Milrinone Enoximone Amrinone Piroximone and others
PDE IV	cAMP specific	cAMP	Rolipram RO20-1724 Etazolate Denbufylline ICI63197 and others
PDE V	cGMP specific	cGMP	Zaprinast Dipyridamole MY5445
Non-selective			Papaverine IBMX, etc.

homogenate was filtered through four layers of gauze and centrifuged at 42 000 × *g* for 45 min. The resulting supernatant was filtered through a 0.45-μm membrane and used as a source of cytosolic PDE activity. The pellet was washed twice in buffer A, resuspended, homogenized, sonicated for 5 min, centrifuged again and used as a source of microsomal PDE activity.

Anion exchange chromatography

PDE isoenzyme separation was performed on a Q-Sepharose high-performance column (26 × 39 mm, 20 ml bed volume) equilibrated with 10 bed volumes of the homogenization buffer A containing 70 mM sodium acetate. Four to five milliliters of the entire 42 000 × *g* supernatant was applied to the column. The column was then washed with at least 2 bed volumes of the buffer A to remove unbound material. PDE isoenzymes were then eluted with 200 ml (10 bed volumes) of a linear (70–1000 mM) sodium acetate gradient in buffer A. Flow rate was set at 2 ml/min, and 3-ml fractions were collected over the entire gradient and kept on ice. PDE activity was assayed in every fraction within 72 h.

Affinity chromatography

A 10-ml (100 × 11.5-mm) calmodulin-agarose affinity column was prepared and equilibrated with a buffer (pH 7.5) containing 20 mM TRIS, 0.5 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and the protease inhibitor mixture described above. For further purification of PDE I containing fractions identified after Q-Sepharose chromatography a pooled sample representing the main PDE I activity was prepared and applied to the affinity column in the presence of excess Ca^{2+} (2 mM). The unbound activity of calmodulin-insensitive isoenzymes was then washed from the column with 40 ml of the equilibration buffer. The calmodulin-bound activity was removed from the column with 40 ml of the equilibration buffer containing 2.5 mM ethyleneglycoltetraacetic acid (EGTA) in the absence of $CaCl_2$. The flow rate was set at 0.17 ml/min, and 2-ml fractions were collected and assayed for PDE activity using cAMP and cGMP as substrates in the presence and absence of 2 mM Ca^{2+}/50 units calmodulin.

PDE assay

PDE activity was measured using a modification of the two-step radioisotope assay of Thompson and Appleman [11]. Briefly, the standard assay mixture contained 5 mM $MgCl_2$, 1 mM DTT, 1 μM unlabelled cAMP or cGMP and ^{3}H-labelled cyclic nucleotide (100 000 cpm) in 50 mM TRIS/HCl buffer. The reaction was initiated by adding 50 μl of the chromatographic fractions to the assay mixture. The final assay volume was 200 μl. Reactions were conducted in the linear range where no more than 25% of the initial substrate was hydrolysed. All assays were performed in duplicate and repeated at least 4 times. Inhibitors were tested in the concentration range from 0.01 to 1000 μM. The drug concentrations producing 50% inhibition of PDE activity (IC_{50} value) were determined graphically by non-linear regression curve fitting. Final concentrations of vehicles of PDE inhibitors did not substantially affect basal PDE activities (6% or less inhibition). For determination of K_m and v_{max} values the amount of labelled cyclic nucleotide was kept constant whereas the amount of unlabelled substrate was varied between 0.1 and 200 μM (0.1, 0.5, 1.0, 10, 25, 50, 100, 200 μM). All experiments were repeated 3 times and performed in duplicate using material from two different preparations.

Results

Analysis of PDE activity showed that approximately 78% and 81% of the total cAMP- and cGMP-hydrolysing activity was present in the supernatant fraction.

Q-Sepharose anion exchange chromatography

For identification of PDE isoforms, all fractions were tested for cAMP- and cGMP-hydrolysing activity. Typical elution profiles of human detrusor PDEs are shown in Fig. 1a, b. cAMP-hydrolysing activity eluted in two peaks, one at low (peak fraction at 0.35 M sodium acetate gradient) and one at high (peak fraction

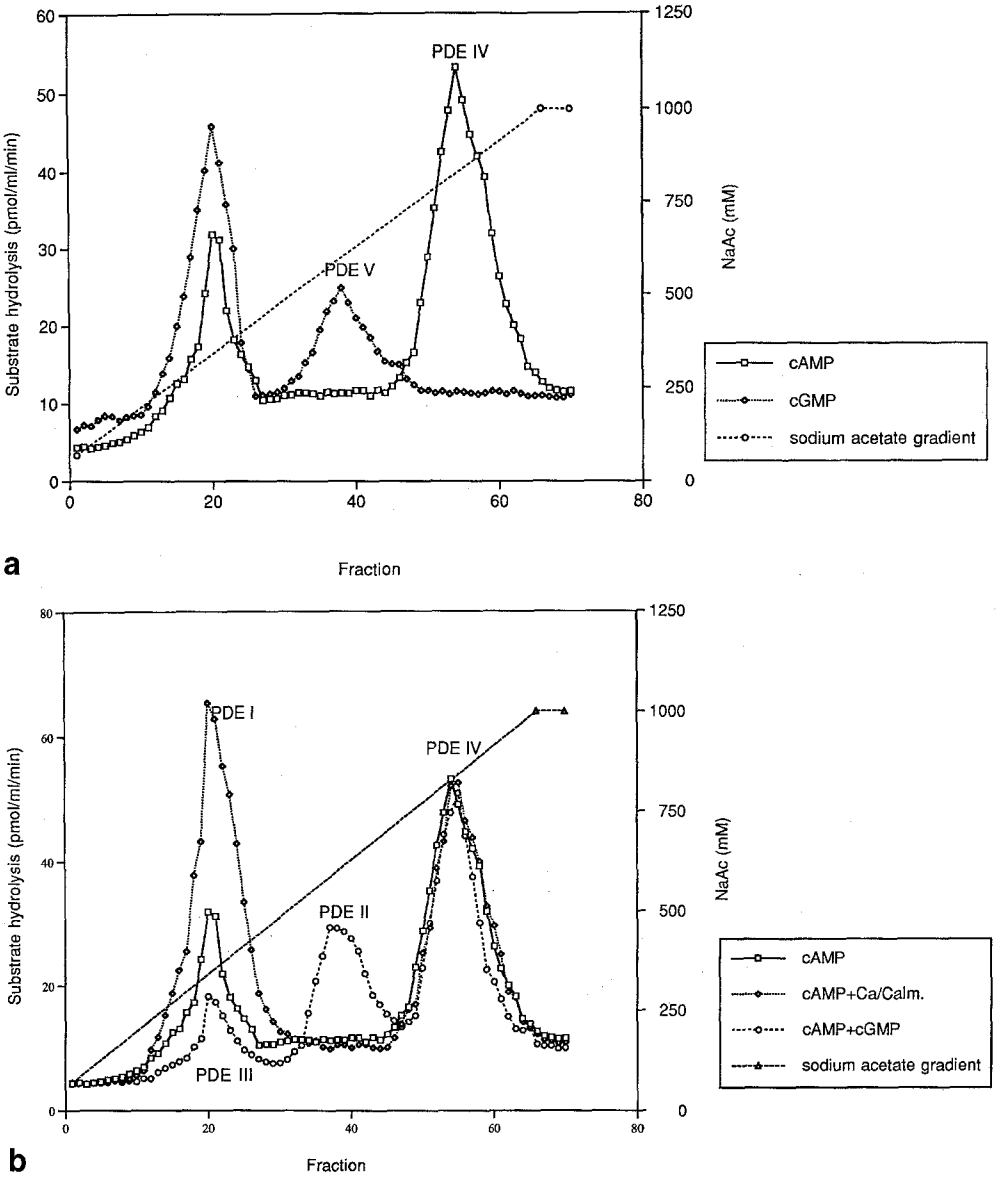

Fig. 1 **a** Typical elution profile of human detrusor homogenate from Q-Sepharose anion exchange chromatography. The tissue homogenate was applied to the column and eluted with a linear sodium acetate gradient (70–1000 mM). Peaks hydrolysing cAMP and cGMP coelute at low ionic strength (350 mM). Additional peaks elute at 600 mM (cGMP-specific PDE V) and 830 mM (cAMP-specific PDE IV). **b** Elution profile in the absence and presence of 2 mM Ca^{2+}/50 units calmodulin and 5 μM cGMP, indicating the presence of PDE I, II and III (substrate: cAMP). See text for details

at 0.83 M sodium acetate gradient) ionic strength. cGMP-hydrolysing activity coeluted at 0.35 M sodium acetate gradient (cGMP/cAMP ratio 1.44:1) and was also detected at 0.60 M sodium acetate gradient (Fig. 1a). This peak was designated as containing cGMP-specific PDE (PDE V) since no cAMP-hydrolysing activity was detected. In contrast, the cAMP peak at 0.83 M was designated as containing cAMP-specific PDE (PDE IV) since no cGMP-hydrolysing activity was detected in these fractions.

For further characterization of PDE-hydrolysing activity all fractions were assayed for cAMP hydrolysis in the presence of Ca^{2+}/calmodulin (2 mM/50 units) and cGMP (5 μM) (Fig. 1b). The rate of cAMP hydrolysis in fractions 12–31 was stimulated about two fold in the presence of 2 mM Ca^{2+} and 50 units calmodulin, whereas addition of 5 μM cGMP diminished cAMP hydrolysis to the same ratio. This indicated that the first peaks contained a mixture of PDE I (Ca^{2+}/calmodulin-stimulated PDE) and PDE III (cGMP-inhibited cAMP PDE), which eluted from the column at the same salt concentration (0.35 M sodium acetate). The PDE activity in fractions 34–45 was stimulated up to 2.6-fold by the addition of 5 μM cGMP, which was indicative for the presence of cGMP-stimulated cAMP PDE (PDE II), thus confirming coelution of PDE isoforms II and V in these fractions.

The cAMP-hydrolysing activity of the peak at 0.83 M sodium acetate was not increased by the addition of Ca^{2+}/calmodulin nor altered by 5 μM cGMP, confirming the presence of a cAMP-specific PDE (PDE IV).

Calmodulin-agarose affinity chromatography

For further purification of PDE I coeluting with PDE III from the Q-Sepharose column the peak fractions

were pooled and applied to a calmodulin-agarose affinity column in the presence of excess Ca^{2+}. Elution with a Ca^{2+}-containing buffer revealed a single peak of PDE activity which hydrolysed cAMP and was not enhanced by Ca^{2+}/calmodulin but inhibited in the presence of 5 μM cGMP (Fig. 2a). These characteristics are indicative of PDE III. After changing the eluant to a Ca^{2+}-free buffer containing 2.5 mM EGTA, another single peak of activity was eluted which had a preference for cGMP over cAMP with a ratio of 1.2:1 (Fig. 2a, b). cAMP- and cGMP-hydrolysing activity was stimulated approximately two fold in the presence of Ca^{2+}/calmodulin. These characteristics are consistent with the presence of PDE I.

Kinetic characteristics of PDE isoenzymes

The kinetic characteristics (K_m and v_{max}) of human detrusor PDE isoenzymes are presented in Table 2. In the presence of Ca^{2+}/calmodulin, PDE I hydrolysed cAMP with an apparent K_m of 5.8 μM and cGMP with a K_m of 3.1 μM. This reflects the slight substrate preference of this isoenzyme for cGMP over cAMP. Due to the low recovery of PDE III activity from the affinity column and its minor functional significance in functional studies (data not shown), no determination of kinetic parameters of PDE III was done. Activity of PDE V was measured with cGMP as substrate, whereas PDE II activity was assessed with cAMP as substrate in the presence of 5 μM cGMP. $K_m app$ values of

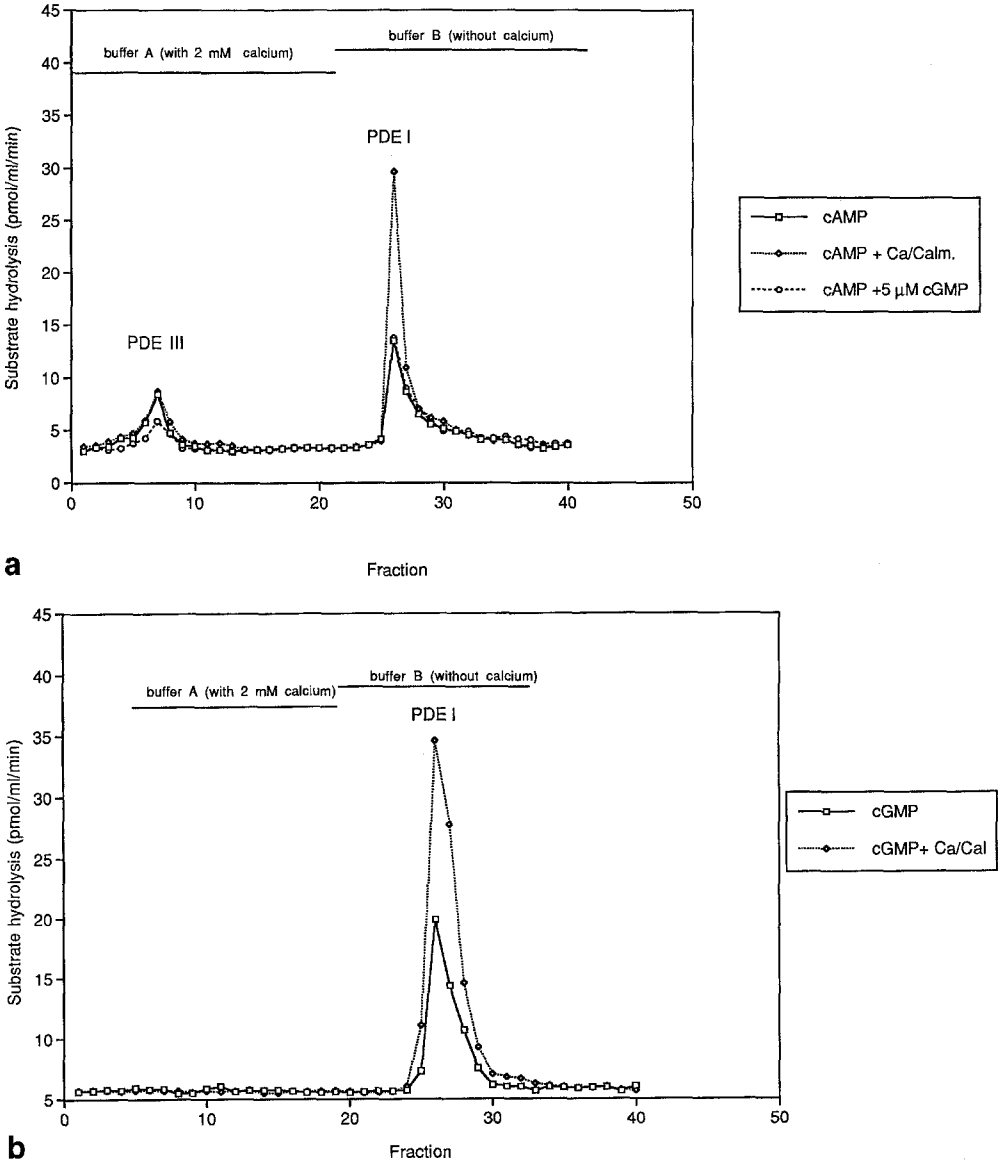

Fig. 2 **a** Elution profile from affinity chromatography (substrate cAMP). Peak fractions containing PDE I from anion exchange chromatography were pooled and applied to a calmodulin-agarose column. In the presence of excess Ca^{2+} cAMP-hydrolysing activity was eluted which was inhibited by 5 μM cGMP (PDE III). After change of buffer, cAMP-hydrolysing activity was eluted, which was stimulated by the addition of 2 mM Ca^{2+}/50 units calmodulin (PDE I). **b** The same fractions as in **a** were assayed for cGMP hydrolysis. There was no detectable activity in the first 20 fractions. After change of buffer, a peak of cGMP-hydrolysing activity was detected which was stimulated by the addition of 2 mM Ca^{2+}/50 units calmodulin (PDE I)

91.6 μM for PDE II and 166 μM for PDE V were determined. PDE V displayed positive cooperativity with respect to the hydrolysis of cGMP. PDE IV hydrolysed cAMP with a $K_m app$ of 54.5 μM.

Effect of PDE inhibitors

IC_{50} values were assessed using PDE preparations isolated as described above. The effects of several PDE isoenzyme-selective inhibitors on human detrusor PDEs are shown in Table 3. Papaverine non-specifically inhibited each isoenzyme dose dependently (the effect on PDE III was not assessed). PDE I eluted from affinity chromatography was inhibited by vinpocetine (IC_{50} 60 μM with cAMP as substrate) and vinpocetine and zaprinast (IC_{50} 200 μM with cGMP as substrate). Vinpocetine is classified as a selective PDE I inhibitor while zaprinast is regarded as a PDE V/I inhibitor. Milrinone (PDE III inhibitor), rolipram (PDE IV inhibitor) and dipyridamole (PDE V inhibitor) were significantly less potent. The cAMP-hydrolysing activity designated as PDE II was only inhibited by papaverine with an IC_{50} value of 38 μM. All other inhibitors were significantly less potent with IC_{50} values of 750 μM or above. Rolipram and papaverine inhibited PDE IV with IC_{50} values of 4.5 and 18 μM, respectively; the other inhibitors tested had IC_{50} values of 500 μM (zaprinast) or >1000 μM (vinpocetine, milrinone, dipyridamole). PDE V was inhibited by papaverine with an IC_{50} of 15 μM and by dipyridamole with an IC_{50} value of 100 μM.

Table 2 Kinetic characteristics of PDE isolated from Q-Sepharose anion exchange and calmodulin-agarose affinity chromatographies (v_{max} maximum reaction velocity, $K_m app$ apparent K_m)

PDE isoenzyme	$K_m app$ (μM)	v_{max} (mM/l/min)
I cAMP	5.8	0.078
I cGMP	3.1	0.030
II	91.6	0.83
III	ND	ND
IV	54.5	0.47
V	166	1.6

Discussion

cAMP and cGMP are important intracellular second messengers formed following stimulation of adenylate cyclase and guanylate cyclase, respectively. 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. Because the distribution of PDE isoenzymes varies in different tissues, selective inhibitors of the isoenzymes have the potential to exert at least partially specific tissue effects. The major clinical focuses for the use of selective PDE inhibitors have been as positive inotropic agents for use in heart failure and cardiac surgery, vasodilators, antithrombotic agents, antidepressants, anti-inflammatory agents and bronchodilators [5]. Recent basic research suggests that the concept of isoenzyme-selective PDE inhibition may also be applicable in the treatment of urinary stone disease and erectile dysfunction [10, 15].

Today, many drugs are available to treat bladder hyperactivity; however, their use is often limited because of a lack of efficacy or severe side effects. The most widely used substances are drugs with anticholinergic, "direct" or mixed action [1]. Interestingly, PDE inhibition has been proposed as a possible mode of action of some drugs with "direct" or mixed action [7, 8] although this has never been clearly documented. In addition, the nomenclature on PDEs was inconsistent in the early literature, which resulted in much confusion. Recently, it was shown that Ca^{2+}/calmodulin-stimulated PDE I may be of functional importance in the porcine detrusor smooth muscle in vitro [14]. We therefore conducted this study in order to determine the PDE isoenzyme profile in the human detrusor.

This study shows that all five known PDE isoenzyme families are present in the human detrusor. Ca^{2+}/calmodulin-stimulated PDE I could be identified from anion exchange chromatography and was further purified by calmodulin agarose affinity chromatography. Unlike in other tissues, PDE I did not coelute with PDE V [12] but with PDE III. Kinetic analysis revealed a K_m of 5.8 μM for cAMP and a K_m of 3.1 μM for cGMP.

Table 3 Effect of various inhibitors on human detrusor PDE isoenzymes. IC_{50} values represent molar concentrations of cyclic nucleotide PDE inhibitors resulting in 50% inhibition of PDE activity (*ND* not determined)

Inhibitor	IC_{50} (μM)					
	PDE I (cAMP)	PDE I (cGMP)	PDE II	PDE III	PDE IV	PDE V
Papaverine	40	70	38	ND	18	15
Vinpocetine	60	200	>1000	ND	>1000	600
Milrinone	>1000	>1000	>1000	ND	>1000	350
Rolipram	>1000	600	>1000	ND	4.5	>1000
Zaprinast	>1000	200	>750	ND	500	700
Dipyridamole	>1000	>1000	>1000	ND	1000	100

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. These data suggest that 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 [13]. These functional results are paralleled by the low K_m values for PDE I presented in this paper.

The assessment of the kinetic characteristics of PDE isoenzymes II and V has to be regarded as an approximation since the isoenzyme preparations after Q-Sepharose chromatography used for kinetic studies were not pure but contaminated to a certain degree by the respective coeluting isoenzyme. Nevertheless, assay conditions should have reduced the contribution of the contaminating isoenzyme.

Also, the functional relevance of the cGMP-stimulated cAMP PDE (PDE II) in the human detrusor could not be assessed based on the data presented because selective inhibitors of PDE II have not been available until now. Although unlikely, inhibitors of PDE II, once available, may prove to be efficacious in modulation of detrusor function.

Only little cGMP-inhibited cAMP-hydrolysing activity (PDE III) could be detected in the eluate from the Q-Sepharose column. In addition, the PDE III inhibitor milrinone had only little effect on carbachol-contracted detrusor strips [13]. This indicates a minor functional importance of PDE III in the regulation of the human detrusor smooth musculature in vitro. Theoretically, a minor role of the "cardiovascular" PDE III in detrusor smooth muscle may be seen as an advantage should the concept of isoenzyme-selective PDE inhibition prove to be valuable in the treatment of micturition disorders in the future.

Despite the presence of significant amounts of PDEs IV and V in the human detrusor homogenate, the PDE IV inibitor rolipram and the PDE V inhibitors zaprinast and dipyridamole were surprisingly impotent in vitro, exerting effects only at high and presumably non-specific concentrations. This may be explained by the relatively high K_m values of these isoenzymes. In addition, one should keep in mind that it is well accepted that the demonstration of the presence of a PDE isoenzyme in a specific tissue does not necessarily imply the presence of a physiologically important enzyme activity in the intact cell or tissue. For example, in airway smooth muscle PDE IV accounts for only 5% of the cAMP-hydrolysing activity in tissue homogenates but seems to be most important for the control of cAMP levels and smooth muscle tone in intact tissue [3]. One possible explanation for this phenomenon is compartmentation of PDE isoenzymes within the cell.

In summary, our data show, for the first time, that all five known phosphodiesterase isoenzyme families exist in human detrusor smooth musculature. A high-affinity, low-K_m calcium/calmodulin-stimulated PDE I was purified by calmodulin-agarose chromatography. The kinetic characteristics, together with functional in vitro studies, suggest that the PDE I may be of importance in the intracellular regulation of the human detrusor smooth muscle tone.

Acknowledgements The authors wish to thank K. E. Andersson (Department of Clinical Pharmacology, University of Lund, Sweden) for helpful comments and I. Uhrlandt for her outstanding technical assistance. We are indebted to N. van Dornick for reviewing the linguistic style of the manuscript. This work was supported by grants from Deutsche Forschungsgemeinschaft DFG Tru 343/1-2.

References

1. Andersson KE (1988) Current concepts in the treatment of disorders of micturition. Drugs 35:477
2. Beavo J (1990) Multiple phosphodiesterase isoenzymes: background, nomenclature and implications. In: Beavo J, Houslay HM (eds) Cyclic nucleotide phosphodiesterases: structure, regulation and drug action. Wiley, Chichester, p 3
3. Hall I, Walker D, Hill S, Tattersfield A (1990) Effect of isoenzyme selective phosphodiesterase inhibitors on bovine tracheal smooth muscle tone. Eur J Pharmacol 183:1096
4. Lincoln TM (1989) Cyclic CMP and mechanisms of vasodilation. Pharmac Ther 41:479
5. Nicholson C, Challiss RA, Shahid M (1991) Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. Trends Pharmacol Sci 12:19
6. Opie LH (1982) Role of cyclic nucleotides in heart metabolism. Cardiovasc Res 16:483
7. Pharmacia (1991) Dridase (Oxybutynin) product information. Pharmacia, Erlangen 49
8. Ruffmann R, Sartani A (1987) Flavoxate, a drug with smooth-muscle relaxing activity. Drugs Exp Clin Res 13:57
9. Schmidt HHW, Lohmann SM, Walter U (1993) The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim Biophys Acta 1178:153
10. Taher A, Schulz-Knappe P, Meyer M, Truss MC, Forssmann WG, Stief CG, Jonas U (1994) Characterization of cyclic nucleotide phosphodiesterase isoenzymes in the human ureter and their functional role in vitro. World J Urol 12:286
11. Thompson WJ, Appleman MM (1971) Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochemistry 10:311
12. Torphy TJ, Cieslinski LB (1990) Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isoenzymes in canine tracheal smooth muscle. Mol Pharmacol 37:206
13. Truss MC, Ückert S, Stief CG, Forssmann WG, Jonas U (1995) 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. Urol Res (submitted for publication)
14. Truss MC, Ückert S, Stief CG, Schulz-Knappe P, Hess R, Forssmann WG, Jonas U (1995) Porcine detrusor cyclic nucleotide phosphodiesterase (PDE) isoenzymes: characterization and functional effects of various PDE inhibitors. Urology 45(5):893
15. Ückert S, Stief CG, Becker AJ, Truss MC, Djamilian MH, Thon WF, Jonas U (1994) The effect of the specific phosphodiesterase (PDE III) inhibitor milrinone on human and rabbit cavernous tissue in vitro and in vivo. J Urol 151 (Suppl):495A
16. Zhou HL, Torphy TJ (1991) Relationship between cyclic guanosine monophosphate accumulation and relaxation of canine trachealis induced by nitrovasodilators. J Pharmacol Exp Ther 258:972