Invited Review

Regulation of cyclic nucleotides in the urinary tract

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Abstract

Cyclic nucleotide levels are controlled through their synthesis from nucleotide triphosphates by cyclases and their degradation to 5'-monophosphates by phosphodiesterases (PDEs). Components controlling cyclic AMP-induced relaxation in the urinary tract include receptors, inhibitory and stimulatory G-proteins, isoforms of adenylyl cyclase and PDEs. The responsiveness of PDEs to a variety of physiological challenges is related to the presence of multiple families of isoenzymes with specific localization within tissues and within cells. At least 11 families of PDEs encode more than 50 PDE proteins produced in mammalian cells. In the urinary tract, characterization of PDE isoforms has lagged behind other systems and much of the literature was published prior to identification of PDE7, 8, 9, 10, 11. Specific PDE inhibitors regulate smooth muscle function in the bladder, urethra, prostate and ureter. The pharmacological potential of these inhibitors may include treatment of urge incontinence and the low compliance bladder, and treatment of prostate cancer. G-proteins also regulate cyclic AMP production. Changes in specific G- protein isoforms with aging, most prominently Giα2, cause decreased relaxation response in the aging bladder. As we have seen here with aging and certainly in other disease processes, levels of the components of adenylyl cyclase/phosphodiesterase/protein kinase can change and thus affect the relaxation response. By exploitation of differences in PDE expression in disease, such as the overexpression of PDEs in cancer, treatment options may present themselves.

Key words: phosphodiesterase, urinary tract, cyclic AMP, cyclic GMP

Phosphodiesterases

Phosphodiesterases (PDEs) are critical components in the cyclic AMP/protein kinase A (PKA) and the cyclic GMP/phosphokinase G (PKG) signaling pathways. Previously PDEs were thought of as relatively uninteresting housekeeping enzymes. Physiologists, pharmacologists and biochemists in smooth muscle research added PDE inhibitors to enhance muscle relaxation or cyclic nucleotide production. Rather than passive "housekeeping enzymes", PDEs control

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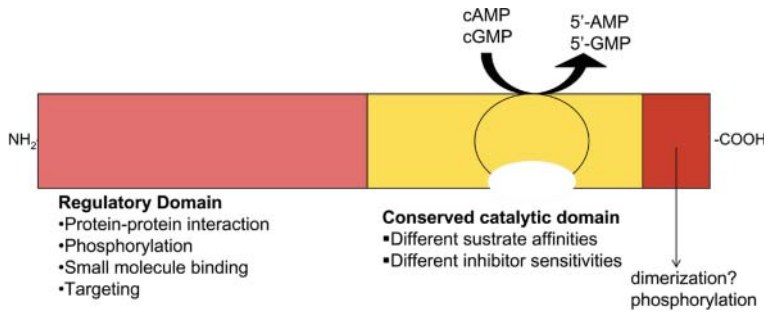

Fig. 1. Common structural organization of PDE domains.

the access of second messengers to their intra-cellular effectors and PDEs also act as signaling effectors. One line of evidence for the importance of PDE in signaling comes from recent studies in knockout mice with PDE deletions. These studies emphasize the role of PDEs in controlling the balance between positive and negative stimuli in cyclic nucleotide pathways. For example, mice deficient in PDE4D exhibit delayed growth, reduced viability and decreased female fertility. The reduced fertility is due to impaired ovulation and diminished sensitivity of the granulosa cells to gonadotropins in null females (Jin *et al.*, 1999). Additionally, PDE4D deficient mice lose cholinergic responsiveness and hyper-reactivity following exposure to antigens. This effect is specific to the airways (Hansen *et al.*, 2000).

The responsiveness of PDEs to a variety of physiological challenges is related to the presence of multiple families of isoenzymes with specific localization within tissues and within cells. At least 11 families of PDEs encompass one to four distinct genes to allow for 20–25 genes coding for PDEs. Splice variants and multiple promoters encode more than 50 PDE proteins produced in mammalian cells. Using the most widely accepted nomenclature, the 11 PDE families are indicated by an Arabic numeral, *i.e.*, PDE1, PDE11, followed by a capital letter indicating the gene within the family. A second Arabic numeral indicates the protein isoforms. For example PDE4C3 indicates a member of the PDE4 family, coding from gene C, protein isoform 3.

Family specificity of PDE enzymes is determined by domains present in PDEs, including the catalytic domain and those near the amino and carboxy terminus (Fig. 1). The conserved catalytic domain is located near the C-terminus of PDE proteins. Crystal structure of PDE4 indicates that the catalytic domain is a compact structure composed of 17α-helices, divided into 3 subdomains, with the most conserved residues involved in the formation of the catalytic pocket (Conti, 2000). The catalytic pocket contains metal ions, Zn^{++} and Mg^{++} (Francis *et al.*, 2000, Xu *et al.*, 2000). In addition to the conserved residues for catalysis in all PDEs, other areas of the catalytic domain consist of conserved areas of amino acids specific to each family. These amino acids are probably involved in defining substrate specificity and family specific sensitivity to inhibitors (Table 1). PDEs show three types of substrate specificity (Dousa, 1999). The PDE4, PDE7 and PDE8 families hydrolyze cyclic AMP specifically. PDE5, PDE6 and PDE9 are cyclic GMP specific and PDE1, PDE2, PDE3, PDE10 and PDE11 are dual substrate PDEs, that

Table 1 Family specific PDE inhibitors

PDE Family	INHIBITOR*
PDE1	vinpocetine, W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, 8-methoxy-MIX
PDE2	EHNA (*erythro*-9-(2-hydroxy-3-nonyl)adenine)
PDE3	cilostamide, milrinone, zardaverine, olprinone, cilostazol, quazinone, siguazodan
PDE4	rolipram, Ro 20-1724, piclamilast, zardaverine, cilomilast, roflumilast
PDE5	zaprinast (130 nM), sildenafil, vardenafil, tadalafil, E4021, morpholinosulfonyl-prazolopyrimidine (MSPP), dipyridamole
PDE6	zaprinast (400 nM), dipyridamole (125 nM), sildenafil
PDE7	MIX, dipyridamole (600 nM)
PDE8	Dipyridamole (9 μM), IBMX insensitive
PDE9	zaprinast (35 μM)
PDE10	dipyridamole (1 μM)
PDE11	zaprinast (12 μM), dipyridamole (400 nM), tadalafil

*(Ki) for dipyridamoleand zaprinast (Maurice *et al.*, 2003).

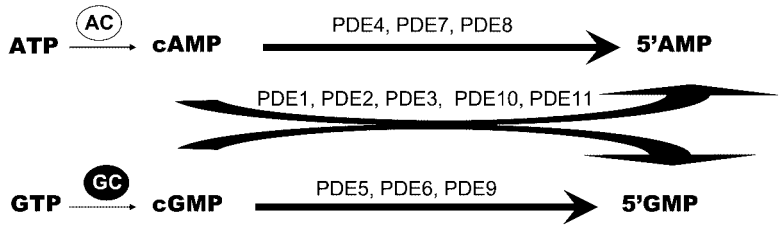

Fig. 2. Catalytic domains of PDE hydrolyze cyclic AMP, cyclic GMP or both.

is, they hydrolyze both cyclic AMP and cyclic GMP (Fig. 2).

The catalytic domain is flanked by regulatory domains near the N-terminus which have been well characterized for each of the PDE families (Table 2). These regulatory domains are family specific and include phosphorylation sites, binding sites for small molecules, membrane targeting domains and domains for protein-protein interaction (Conti and Jin, 1999). PDE1A, B, C contains a Ca^{++}-calmodulin (CaM) binding domain. PDE1s are phosphorylated by Ca^{++}-CaM dependent kinase and by PKA. Phosphorylation of PDE1s increases their affinity for CaM, resulting in a higher catalytic activity. PDE1 can become the dominant PDE during conditions when intracellular Ca^{++} is high, for example, during muscle contraction (Rybalkin *et al.*, 2003).

GAF domains in PDE2A, PDE5A, PDE6A,B,C, PDE10A, and PDE 11A bind cyclic GMP with high efficiency due to a non-catalytic high affinity binding domain, which allosterically modifies PDE activity. A novel GAF domain overlaps with the cyclic GMP binding site and binds small molecules. PDE3A, B, cyclic GMP-inhibited PDEs, contain a membrane association domain consisting of 6 transmembrane hydrophobic helices which anchors PDE3A, B to the endoplasmic reticulum. PDE3s may be particulate or soluble. PDE4A, B, C, D, cyclic AMP specific PDEs, contain two upstream conserved regions or UCR domains. UCR1 domains contain regulatory phosphorylation sites. The UCR2 domains correspond to auto-inhibitor domains that negatively regulate catalytic activity. PDE6A, B, C are cyclic GMP specific PDEs that are localized in photoreceptors and respond to light activated rhodopsin (Miki *et al.*, 1975,

Table 2 Properties of PDE families

PDE	N-terminal Regulatory Domains	Cyclic nucleotide substrates
PDE1A, B, C	Ca^{++}-CaM	Dual substrate
PDE2A	GAF (cGMP, other ligand binding)	cGMP-stimulated
PDE3A, B	Membrane association	cGMP-inhibited
PDE4A, B, C, D	UCR1-Phosphorylation UCR-2-Auto-inhibitory	cAMP-specific
PDE5A	GAF (cGMP, other ligand binding)	cGMP-specific
PDE6A, B, C	GAF (cGMP, other ligand binding)	Photoreceptor, cGMP-specific
PDE7A, B	Hydrophobic or hydrophilic	cAMP high-affinity
PDE8A, B	PAS putative small ligand binding	cAMP high-affinity
PDE9A	No non-catalytic cGMP binding domain	cGMP high-affinity
PDE10A	GAF (cGMP, other ligand binding)	Dual substrate
PDE11A	GAF (cGMP, other ligand binding)	Dual substrate

Wheeler *et al.*, 1977). The soluble PDE7A1 contains a unique hydrophilic 45 residue N-terminus sequence (Bloom and Beavo, 1996), while in contrast, the membrane bound PDE7A2 contains a unique hydrophobic 20 residue N-terminus sequence (Han *et al.*, 1997). The Period-ARNT-Sim (PAS) domains in PDE8s are putative, small ligand binding domains that are ubiquitous sensors of the extracellular environment. These domains are found in proteins from bacteria to mammals and serve as sensors for oxygen or light in *Archaea*. No ligand or other protein sensors for oxygen or light have been found in PDE8s; however this domain might serve as a sensor of the extracellular environment (Mehats *et al.*, 2002). PDE9A is a high affinity cyclic GMP PDE that lacks a non-catalytic cyclic GMP binding domain (Fisher *et al.*, 1998).

Domains are also conserved at the carboxy terminus although few studies have been done to test their role in PDE function (Fig. 1). Domains at the carboxy terminus of PDE include sites that may be involved in dimerization as are present in PDE4 and PDE1 or sites that may function as a target for phosphorylation (Lenhard *et al.*, 1996, Kovala *et al.*, 1997, Hoffmann *et al.*, 1999).

Within the cell, PDEs can hydrolyze ten times more cyclic AMP and/or cyclic GMP than is synthesized by adenylyl cyclases or guanylyl cyclases. Partial PDE inhibition results in large increases in cyclic AMP and cyclic GMP and subsequent kinase activation. PDEs can modulate the duration of signal and even cause the rapid oscillation of cyclic AMP and cyclic GMP. The tight regulation of cyclic nucleotide levels by PDEs occurs because PDEs can decrease the amount of cyclic nucleotide prior to the activation of the protein kinases (Fig. 3). This results in a complex spatial and temporal cyclic nucleotide gradient. For activation of PKA, the following scheme explains how PDEs influence function (Houslay and Milligan, 1997). Adenylyl cyclases are localized in defined regions of the plasma membrane, while PDEs can be anchored (PDE3) or free in the cytosol. The cyclic AMP generated by a given adenylyl cyclase diffuses into the cytosol, where PDEs establish gradients of cyclic AMP. Changes in cyclic AMP levels in specific intracellular compartments can be monitored by anchored protein kinase A-RII isoforms, while PKA-RI isoforms monitor cyclic AMP in the cytosol. The pattern of PKA activation depends not only on where the cyclic AMP is produced but also on how much is hydrolyzed by the PDEs, since PDEs can hydrolyze much more cyclic nucleotide than is

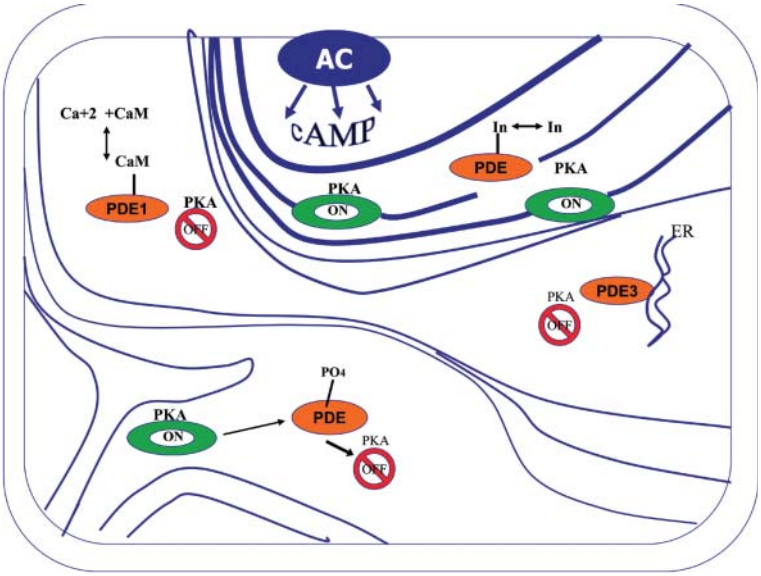

Fig. 3. The Complex Spatial-Temporal Distribution of cAMP as regulated by various PDEs and their consequent regulation of PKAs. Phosphodiesterases (PDEs) can hydrolyze cAMP produced by adenylyl cyclase (AC) and prevent activation of Protein Kinase A (PKA). PDEs are: inhibited by PDE Inhibitors (In), activated by Calmodulin (CaM), can be free in the cytosol or bound to intracellular membranes (ER). The many forms present are being activated and deactivated on a variety of time scales giving rise to complex local cAMP gradients and subsequent PKA activation.

generated. Furthermore, the localization of both PDEs and PKAs may undergo dynamic translocation, i.e. PKAs can move to the nucleus. Therefore, the amount and location of the cyclic AMP generated by adenylyl cyclases on the plasma membrane is tightly regulated by membrane bound and soluble PDEs (Fig. 3). This allows spatially directed changes in the responsiveness of PKA.

Characterization of PDEs in the urinary tract

In the urinary tract, characterization of PDE isoforms has lagged behind other systems and much of the literature was published prior to identification of PDE7, 8, 9, 10, 11 (Table 3). PDE 1, 2, 3, 4, 5 were identified in the rabbit bladder and inhibitors of PDE1 and PDE5 relax rabbit detrusor smooth muscle (Qui *et al.*, 2002). Similarly, PDE1 through PDE5 were identified in rat bladder (Qui *et al.*, 2001). In porcine detrusor, PDE1, PDE2, PDE4A and B and PDE5A and B have been identified (Truss *et al.*, 1995). In humans, one high-affinity, low-Km Ca^{++}/CaM PDE1 with a slight preference for cyclic GMP, one cyclic GMP stimulated PDE2, one cyclic GMP-inhibited PDE3, one cyclic AMP specific PDE4 and one cyclic GMP specific PDE5 have been identified by Truss *et al.* (1996A). Only PDE5 isoforms are well characterized and this was done in conjunction with studies in the corpora. In summary, in the human bladder, PDE1, 2, 3, 4, 5 (Truss *et al.*, 1996A) A1, 2 (Stacey *et al.*, 1998) and 3 (Lin *et al.*, 2000) and PDE 9A (Rentero *et*

Table 3 Characterization of PDE families in urinary tract

PDE	Bladder	Prostate	Ureter	C. Cavern osum
1	+	1A, 1B	+	1A, 1B, 1C
2	+	2A	+	2A
3	+		3A	
4	+	4A, B, C, D	+	4A, B, C, D
5	5A1, 2, 3	5A1, 2, 3	+	5A1, 2, 3
7		7A	?	7A
8		8A	?	8A
9A	+	+++		+
10		10A		10A
11A	-	+++		

al., 2003) have been identified.

Characterization of PDE isoforms in the human prostate indicates that most of the isoform families have been identified in the prostate (Uckert *et al.*, 2001, Table 3) and that PDE9A is present in very high levels compared to other tissues, except the intestines. One variant of PDE9, PDE9A12 is largely expressed in the human prostate (Rentero *et al.*, 2003). PDE11As which hydrolyze both cyclic AMP and cyclic GMP also are highly expressed in the prostate (Fawcett *et al.*, 2000). PDE 11A4 is expressed almost exclusively in human prostate, compared to 11A3 which is expressed in the testes (Yuasa *et al.*, 2001a; 2001b).

In the human ureter PDE1, PDE2, PDE4 and PDE5 have been identified (Table 3, Kuhn *et al.*, 2000), along with a cyclic AMP PDE which is insensitive to most PDE Inhibitors (Weiss *et al.*, 1981). Turning to PDE isoforms present in ureter, Dr. Robert Weiss characterized canine ureteral PDE isoforms in the 1980s (Weiss *et al.*, 1981). Eighty-five percent of cyclic AMP and cyclic GMP PDE activity was found in the supernatant of canine ureteral homogenates. In the supernatant, cyclic GMP hydrolysis was 4.5 times higher than cyclic AMP hydrolysis. DEAE cellulose chromatography of the ureteral supernatant fraction resolved two peaks of PDE activity; peak 1 which hydrolyzed 15 times as much cyclic GMP as cyclic AMP, and peak 2 which was relatively specific for cyclic AMP (Fig. 4A). Peak 1 hydrolysis of cyclic AMP (shown) and cyclic GMP was stimulated by CaM, indicating the presence of a PDE1. The two subpeaks within peak 1 were resolved using a second DEAE cellulose column with a shallower ammonium sulfate gradient (Fig. 4B).

Analysis of these fractions from Peak 1 shows both Ca^{++}-CaM insensitive and sensitive cyclic AMP hydrolysis. The larger peak is sensitive to Ca^{++}-CaM and hydrolyzes both cyclic AMP and cyclic GMP. The first peak eluted, (Peak 1A), was not sensitive to Ca^{++}-CaM and was specific for cyclic AMP.

Inhibition of these forms of soluble ureteral PDE activity was characterized using several PDE inhibitors (Table 4). Peak 1B probably corresponds to PDE1, since it is a dual substrate Ca^{++}-CaM-dependent enzyme that was inhibited by the PDE1 inhibitor, 8-methoxy methyl MIX. Both cyclic AMP and cyclic GMP hydrolysis were also inhibited by papaverine, 3-isobutyl-1-methylxanthine (MIX), and theophylline. The IC_{50} for papaverine and theophylline is lower in Peak 2 compared to Peak 1B, while inhibition by MIX and 8-methoxy-methyl MIX (PDE1) by

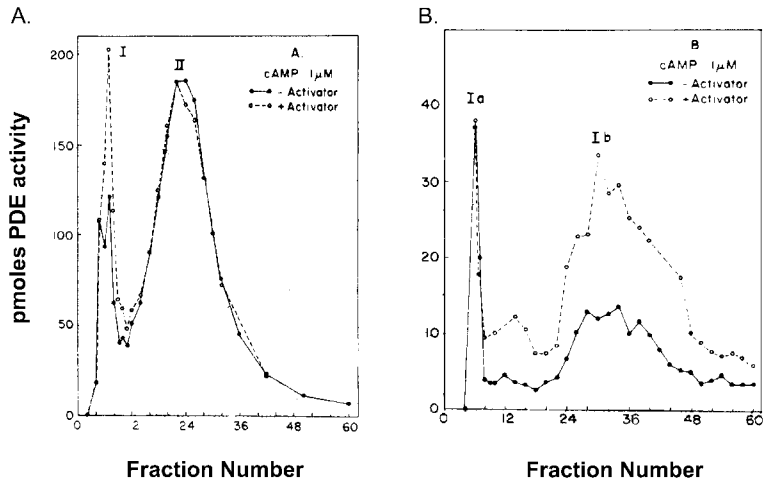

Fig. 4. DEAE-cellulose chromatography of cyclic AMP PDE activity from canine ureter. Cyclic AMP hydrolytic activity in fractions collected from an exponential gradient elution made with and homogenization buffer and A. 0.5 M $(NH_4)SO_4$, or B. 0.1 M $(NH_4)SO_4$. Fractions were assayed in the presence and absence of pig brain calmodulin (activator).

Table 4 Effect of PDE inhibitors on cyclic AMP and cyclic GMP hydrolysis of ureteral PDE isoforms (IC_{50})

Inhibitor	Peak 1A cAMP μM	Peak 1B cAMP μM	Peak 1B cGMP μM	Peak 2 cAMP μM
Papaverine	–	3	13	1.5
MIX	–	1.2	2.5	15
8-Methoxy-methyl-MIX	–	1.0	3.2	60
Theophylline	–	550	650	200

Peak 2 has a considerably higher IC_{50} than Peak IB. Peak 1A was quite unique, since hydrolysis of cyclic AMP by this peak was resistant to all common PDE inhibitors (Weiss *et al.*, 1981). The cyclic AMP PDE present in peak 1A may well be a PDE8, which is cyclic AMP dependent and highly resistant to specific and non specific PDE inhibitors with the exception of dipyridimole, a PDE2,4,5,6 inhibitor.

PDE inhibitors - pharmacological potential in the urinary tract

Bladder and urethra: In whole bladders from female guinea pigs, IBMX (a non-specific PDE inhibitor), zardavarine (PDE3, PDE4) and Ro 20-1724 (PDE4) reduced the frequency and amplitude of the phasic activity induced by the muscarinic agonist arecaidine, while EHNA (PDE2), zaprinast (see Table 1) and siquazodan (PDE3) had no effect. Ro 20-1724 also inhibited the nerve mediated rise in intravesical pressure (Gillespie 2004). This suggests that one or more isoforms of PDE4, a cyclic AMP specific PDE, may play a functional role in generating

phasic activity in the isolated guinea pig bladder. Dipyridimole but not zaprinast, caused a transient increase in frequency of the muscarinic agonist induced activity followed by inhibition in whole guinea pig bladder (Gillespie and Drake, 2004). However, the lack of specificity of the PDE inhibitors, zaprinast (PDE5, 6, 9 and 11 inhibitor) and dipyridimole (PDE5, 6, 7, 8, 9, 10 and 11), makes it difficult to determine which PDE isoform is involved in these responses in the guinea pig bladder and if this effect relies on increases in cyclic AMP or cyclic GMP. Zaprinast also potentiated reflex-evoked changes in urethral pressures in anaesthetized female rats, however both L-NAME and zaprinast increased baseline urethral pressure and urethral striated muscle (external urethral sphincter) activity (Wibberley *et al.*, 2002). Whole animal studies of bladder (Gillespie, 2004, Gillespie and Drake, 2004) and urethral (Wibberley *et al.*, 2002) function are compromised by the use of nonspecific PDE inhibitors.

The non-specific inhibitor papaverine was most effective in relaxing pre-contracted rat bladder muscle strips while isoenzyme-selective inhibitors, vinpocetine (PDE1), EHNA (PDE2), and sildenafil (PDE5) induced more relaxation than milrinone (PDE3) or rolipram (PDE4) (Qui *et al.*, 2001). Another study using isolated muscle strips from rat bladder showed that zaprinast, a PDE5, 6, 8, 9 and 11 inhibitor (Maurice *et al.*, 2003), potentiates electrically-evoked relaxations in the urethra (Dokita *et al.*, 1994).

Ureter: Papaverine reduced peristaltic motility, endoluminal pressures and longitudinal muscle contractions in guinea pig ureter (Cazzulani *et al.*, 1985). Relaxation effects on KCl-induced tension of human ureteral smooth muscle were exerted by the PDE3 inhibitor quazinone, the PDE4 inhibitor rolipram, and zaprinast (Table 1) (Steif *et al.*, 1995, Kuhn *et al.*, 2000). The PDE5 specific inhibitors, E4021 and morpholinosulfonyl-pyrazolopyrimidine (MSPP) also relaxed human ureteral smooth muscle. These inhibitors induced larger increases in cAMP levels than cyclic GMP levels (Kuhn *et al.*, 2000). Rolipram caused a relaxation response in ureteral strips in rabbits that was similar to the response in humans. Furthermore, intravenous rolipram decreased the frequency of ureteral peristalsis in rabbits while having little effect on systemic blood pressure. The non-specific PDE inhibitors papaverine and theophylline relaxed ureters for a shorter time and were accompanied by severe circulatory effects (Becker *et al.*, 1998). *In vivo* studies in which rolipram was infused into pig ureter or administered topically showed no change in the frequency or amplitude of ureteral contractions. The non-specific PDE inhibitor, papaverine, decreased contraction frequency and amplitude when administered intraveneously, but not topically (Danuser *et al.*, 2001). As was the case in studies in the rabbit (Becker *et al.*, 1998), intravenous papaverine decreased arterial blood pressure in the pig. Whether there is clinical relevance for PDE4 inhibitors in the treatment of renal colic or stones remains to be addressed.

Prostate: Adrenergic induced tension in human prostatic strip preparations was reversed by rolipram, zaprinast and sildenafil. Although these agents were not as potent as forskolin and sodium nitroprusside, these results suggest the presence and functional relevance of PDE isoenzymes 4 and 5 in human prostatic tissue (Uckert *et al.*, 2001).

PDEs as therapeutic targets in the urinary tract

Because of the intra- and inter- cellular diversity of PDEs, they would appear to be ideal targets for pharmacological manipulation, but the specificity of PDE inhibitors remains a problem. A third generation of PDE inhibitors (Kuhn *et al.*, 2000) which target specific gene products, rather than entire PDE families (second generation) or PDEs non-specifically (first generation) may provide the specificity for pharmacological intervention in the urinary tract. Other approaches, owing to the unique sequences of PDE isoform-specific exons and mRNAs may utilize antisense-mediated (Soderling and Beavo, 2000, Rybalkin *et al.*, 2002) or siRNA mediated down-regulation. The use of PDE5 inhibitors for treatment of erectile dysfunction is perhaps the best known use for PDE inhibitors. Even though many tissues, including other tissues in the urinary tract express PDE5, they do not have the same physiology as penile tissue. In penile tissue, cyclic GMP levels increase rapidly after sexual stimulation, thus increasing the therapeutic benefits PDE5 inhibitors (Corbin and Francis 1999, Lin *et al.*, 2003). Since the use of PDE5 inhibitors in the treatment of erectile dysfunction has been well characterized in other reviews, they are not the subject of this review.

The pharmacological potential of only a few PDE inhibitors has been assessed in the urinary tract. Since PDE1 may play a role in the regulation of human detrusor smooth muscle contractility (Truss *et al.*, 1996B), the PDE1 inhibitor vinpocetine has been tested for treatment of urge incontinence and the low compliance bladder. In a study by Truss *et al.* (2000), vinpocetine treatment in non-responders to standard pharmacological therapy resulted in improvement in 11/19 patients (57.9%) as assessed by clinical symptoms and/or urodynamic parameters (Truss *et al.*, 2000). Although the results of a larger randomized, double-blind, placebo-controlled, multicenter trial with vinpocetine show a tendency in favor of vinpocetine over placebo; however, significant results were documented for only one parameter (Truss *et al.*, 2001).

One potential use for PDE inhibitors is in the treatment of prostate cancer, and perhaps bladder cancer. Exisulind, which is an oxidative metabolite of the anti-inflammatory drug, sulindac, inhibits cyclic GMP PDE(s), overexpressed in precancerous and cancerous colorectal cells, and induces apoptosis in such cells with minimal effects on normal cells. Exisulind is believed to work by causing sustained increases in cyclic GMP levels and in cyclic GMP-dependent protein kinase induction which is not found with selective PDE5 inhibitors or most other PDE inhibitors. β-catenin, shown to be a substrate for PKG, is decreased by exisulind and this may cause apoptosis induction in neoplastic cells. Exisulind has been characterized as a PDE5/2 inhibitor (Thompson *et al.*, 2000). Exisulind induces apoptosis and inhibits growth of tumor cell lines including human prostate and bladder cancer cell lines (Lim *et al.*, 1999, Piazza *et al.*, 2001). Exisulind also inhibits tumor growth in rodent cancer models including models of prostate and bladder cancer (Goluboff *et al.*, 1999, Piazza *et al.*, 2001). Overexpression of PDE5 was noted in a bladder cancer cell line (HT1376) and in human squamous and transitional cell carcinomas compared with normal urothelium (Piazza *et al.*, 2001). In randomized, placebo-controlled studies of prostate cancer patients, exisulind lengthened the median PSA doubling time in men who had increasing PSA levels after radical prostatectomy (Goluboff *et al.*, 2001;

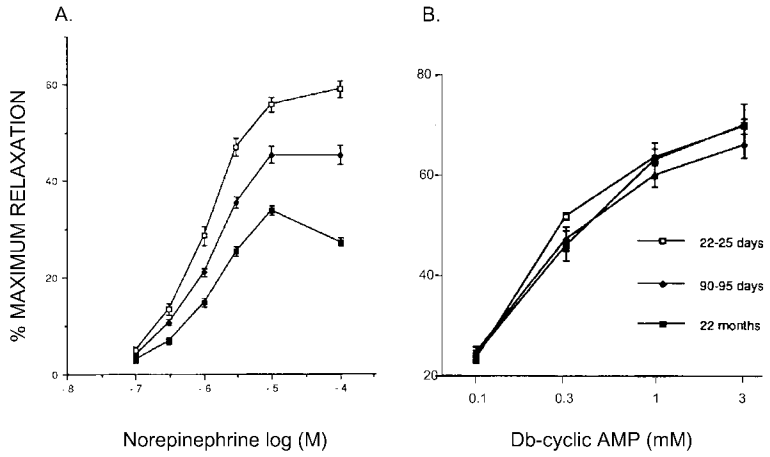

Fig. 5. Dose response curves of the relaxant effects of A. norepinephrine and B. Dibutyryl (Db) cyclic AMP on KCl contracted strips of urinary bladder dome from weanling, young adult and aged rats.

2002). Because preclinical studies have suggested synergistic interactions between docetaxel (Taxotere) and exisulind, a phase I/II clinical trial combining these agents has been performed in patients with hormone resistant prostate cancer (Ryan *et al.*, 2001).

Changes in isoproterenol responsiveness of the bladder detrusor

Our laboratory embarked on a multi year study to examine the role of aging in β-adrenergic induced relaxation of the bladder. With aging, there is a decrease in bladder capacity, bladder compliance, urinary flow rates and the ability to postpone voiding (Brocklehurst and Dilane, 1966, Chun *et al.*, 1988, Drach *et al.*, 1979). β-Adrenergic receptor activation plays an important role in the facilitation of urine storage (Edvardsen, 1968, DeGroat and Saum, 1976). In the rabbit urinary tract, the relaxation responses and increases in cyclic AMP levels produced by the β-adrenergic agonist, isoproterenol, are greater in the bladder dome than in the bladder base or the urethra (Morita *et al.*, 1992).

Dose response curves to norepinehrine and isoproterenol show at least a 40% decrease in relaxation responses when bladders muscle strips from old male rats (22 month-old) are compared to those from weanling male rats (22–25 day-old). Relaxation responses to norephinephrine (Fig. 5A) and isoproterenol decreased 23% and 16%, respectively, when bladders from young adult rats (90–95 day-old) were compared to bladders from weanling rat bladders. This age dependent decrease occurred whether bladder strips were pre-contracted with KCl or by field stimulation (Nishimoto *et al.*, 1995). The ED_{50} values for isoproterenol but not norephinephrine increased with age. Forskolin activates the catalytic unit of adenylyl cyclase by increasing the levels of cyclic AMP, as has been shown in the rabbit bladder (Morita *et al.*, 1986). Percent relaxation by forskolin decreased 20% and 30%, respectively, when 90 day and 22 month detrusor strips were compared to detrusor muscles from 22 day rats (Fig. 6).

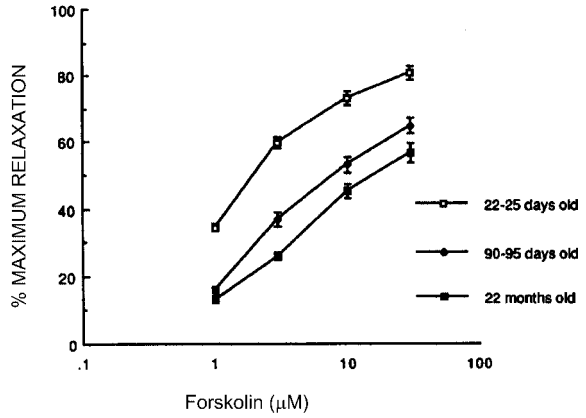

Fig. 6. Dose response curves of relaxant effects of forskolin on KCL-induced contractions of urinary bladder from weanling, young adult and aged rats.

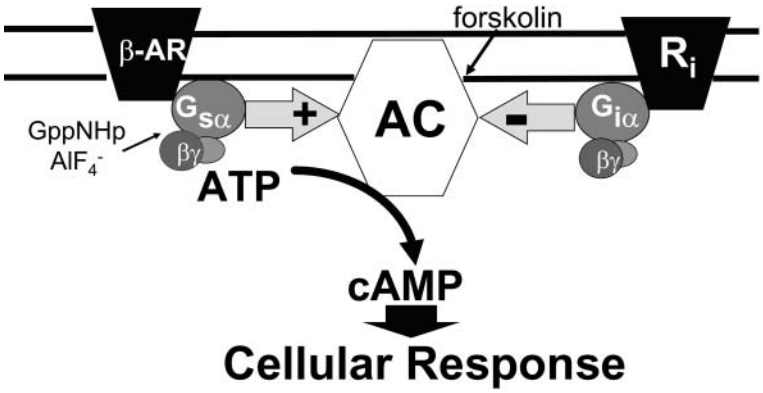

Fig. 7. Schematic diagram of the components of the Receptor-G-Protein- Adenylyl Cyclase complex.

If the decreases in β-adrenergic induced relaxation are due to events distal to cyclic AMP synthesis, we would expect that dibutyryl cyclic AMP would cause less relaxation response in old rat bladder compared to the bladder from weanling rats. However, while relaxation response to dibutyryl cyclic AMP increased with concentration, it did not change with aging (Fig. 5B). Therefore, we focused our efforts on an examination on the components responsible for production of cyclic AMP including β-adrenergic receptors, stimulatory and inhibitory guanine regulator subunits (G-proteins), and adenylyl cyclase enzyme activity.

Components of the adenylyl cyclase system

Cyclic AMP is a second messenger that delivers a signal from upstream receptor-G-protein adenylyl cyclase complexes by altering the behavior of various downstream target enzymes, such as protein kinase A (Fig. 7). Binding of an agonist to the β-adrenergic receptors results in

Table 5 Characterization of β-receptor in aging rat bladder dome

Age	B_{max} (fmol/g tissue)	Total Receptor (fmol/dome)	KD (pM)
22–25 days weanling	401 ± 18	13.5 ± 0.8	32.6 ± 2.0
90–95 days young adult	264 ± 7*	14.7 ± 0.5	36.4 ± 0.9
22 months old	160 ± 8*,#	12.5 ± 1.1	32.4 ± 2.1

*P<0.05 compared to values in weanling rat bladder dome. #P<0.05 compared to values in 90–95 day old rat bladder.

the binding of GTP to the stimulatory G-protein, Gs, which activates the catalytic component of adenylyl cyclase (Gilman, 1995). Activated β-adrenergic receptors can activate both Gs and the inhibitory G-protein, Gi (Asano *et al.*, 1984, Raymond, 1995, Lefkowitz, 1998). Because stimulation of Gi inhibits cyclic AMP production, increases in Gi levels with aging may cause decreases in cyclic AMP production. The diterpene, forskolin, increases cyclic AMP by interaction at the catalytic unit of adenylyl cyclase (Morita *et al.*, 1986).

Changes in components of adenylyl cyclase in the aging bladder detrusor

When we examined changes in β-adrenergic receptors with aging, we found a significant decrease in the B_{max}, but no change in the K_D for β-adrenergic receptors when normalized to tissue weight or to protein. However, there was no age dependent change in the total β-adrenergic receptor number/bladder dome (Table 5). These data indicate that there is no loss in the total number or affinity of β-adrenergic receptors with aging. Therefore, changes in the β-receptor were ruled out as causing the age dependent decrease in β-adrenergic induced relaxation in detrusor (Nishimoto *et al.*, 1995).

We then examined isoproterenol activated adenylyl cyclase in bladder dome homogenates from weanling, young adult and old rats. Isoproterenol produced a concentration dependent stimulation of adenylyl cyclase, which decreased with aging (Fig. 8). In fact in old rats, isoproterenol did not significantly increase adenylyl cyclase in bladder dome (Wheeler *et al.*, 1990).

We examined activation of G-protein and catalytic components of the adenylyl cyclase system in rat bladder homogenates (Wheeler *et al.*, 1990). While the ED_{50} for forskolin did not change with aging, forskolin activated adenylyl cyclase was lower in the bladders from adult and old rats when compared to those from weanling rats (Table 6). This pattern of decrease was different than that seen with isoproterenol activated adenylyl cyclase, where decreased activity was lowest in the aged rat bladder. Decreases in basal adenylyl cyclase paralleled changes in forskolin induced activity; both being a measure of catalytic activity. The non-hydrolyzable GTP analogue, 5'-guanylimido-diphosphate (GppNHp) and fluoride (10 mM NaF and 10 μM $AlCl_3$), (Sternweis and Gilman, 1982) were used as probes of the stimulatory guanine regulatory subunit (Gs). Both GppNHp and fluoride showed a distinctly different pattern of adenylyl cyclase activation in the aging rat bladder compared to that of isoproterenol or forskolin (Table 6). Adenylyl cyclase activation by GppNHp in old rats was almost double that seen in adult rats.

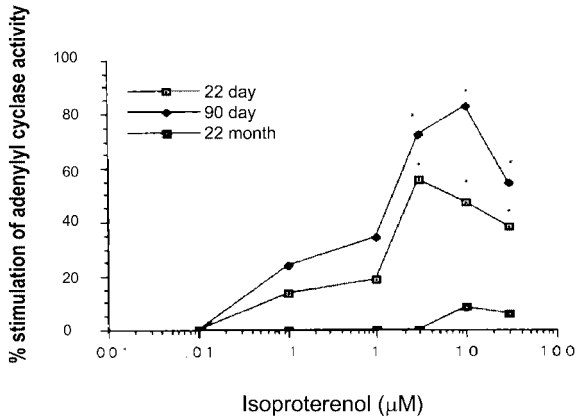

Fig. 8. Effect of isoproterenol on adenylyl cyclase activity in aging rat bladder dome. Adenylyl cyclase is assayed (10 min) in the presence of increasing concentrations of isoproterenol, 0.5 μM phentolamine and 1 μM GppNHp. Results are expressed as mean ± SEM for three experiments at each age group. *P<0.05 compared to basal levels.

Table 6 Adenylyl cyclase activity (pmol/mg tissue) in aging rat bladder dome

Age	Basal	Gpp(NH)p (1 μM)	Forskolin (30 μM)
22–25 days weanling	1.2 ± 0.1	1.3 ± 0.1	18.8 ± 1.6
90–95 day Young adult	0.7 ± 0.0	0.9 ± 0.0	10.4 ± 1.8*
22 months old	0.8 ± 0.1	1.7 ± 0.1*,#	12.2 ± 0.8*

*P<0.05 compared to values in weanling rat bladder dome. #P<0.05 compared to values in 90–95 day old rat bladder.

Additionally fluoride stimulated adenylyl cyclase to a greater extent in old compared to adult rat bladder. Because isoproterenol but not Gs induced adenylyl cyclase activity decreases with aging and because activated β-adrenergic receptors can activate both Gs and Gi, we began an examination of G-proteins in the aging rat bladder.

G-Proteins in the aging bladder

Heterotrimeric G-proteins are the key functional link between an extracellular signal that binds to a receptor and the intracellular targets for which it is intended. Receptors comprise a superfamily of more than 100 members that transduce the signals of numerous signaling molecules, including the majority of peptide hormones, monoamine neurotransmitters, prostaglandins, photoactivated rhodopsin and extracellular Ca^{++} (Alberts *et al.*, 1994). G-proteins consist of three polypeptide gene products (α, β and γ subunits) that are associated with the inner surface of the plasma membrane (Fig. 7). Each of the α, β and γ subunits can modulate multiple downstream effectors (Gudermann, 1997), but the α-subunit confers much of the specific functionality to a G-protein heterotrimer. When stimulated by an extracellular ligand via its coupled receptor, the α-subunit of the G-protein disassociates from the $\beta\gamma$ dimer,

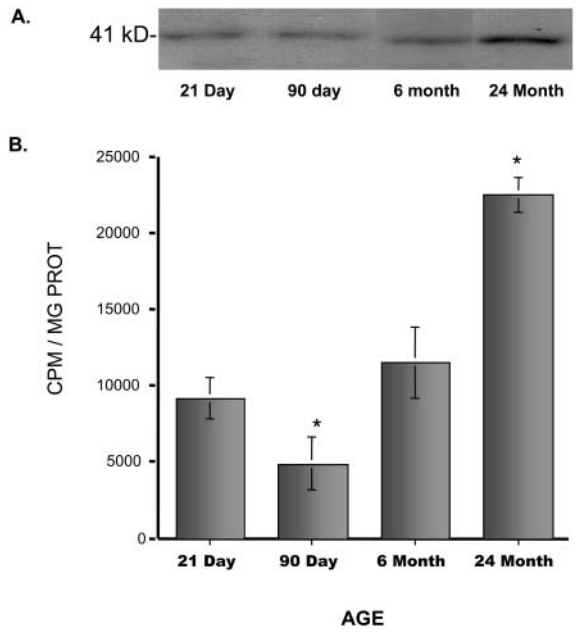

Fig. 9. A. Representative autoradiogram showing a single band that was ADP-ribosylated by [^{32}P]-NAD^{+} in the presence of pertussis toxin in rat bladder dome membranes. B. Analysis of ADP-ribosylation with pertussis toxin in aging rat bladder dome membranes (n=3/age group). *P<0.05 compared to 21 day-old results.

releases a tightly bound molecule of GDP, and binds a molecule of GTP.

The currently identified 23 mammalian α-subunits are divided into four subfamilies (Alberts *et al.*, 1994). The members of the Gαs subfamily are cholera toxin-sensitive, and their main function is to activate adenylyl cyclase, which converts ATP into cyclic AMP. A second family is the Gi/o family. Members of the Gi/o family are sensitive to ADP ribosylation by pertussis toxin. The Gαi1, 2, 3 subunits inhibit some forms of AC, as well as activate K^{+} channels in muscle cell plasma membranes. Gαo likewise activates K^{+} channels, but also inhibits neuronal Ca^{++} currents. The third subfamily, Gαq, consists of non-pertussis toxin-sensitive G-proteins that activate phospholipase C (PLC) hydrolysis of phosphoinositides. The last subfamily consists of the Gα12/13 subunits which modulate Na^{+}-H^{+} exchangers.

Focusing on the role of G-proteins with aging, we examined ADP ribosylation of the Gi/o family by pertussis toxin (Derweesh, 2000) in bladders from weanling, young adult (90 days), adult (6 month-old) and old rats. After treatment of bladder membranes from rats of four ages, with [^{32}P]-NAD^{+} and pertussis toxin, a signal was detected after gel electrophoresis (SDS-PAGE) of bladder membranes. SDS-PAGE revealed a single band at 41 K, the molecular weight of Gi in membranes treated with pertussis toxin. Furthermore, this signal increased with aging and was higher in old rat bladder compared to weanling rat bladder (Fig. 9). ADP ribosylation in young adult rat bladder was lower than weanling levels. This indicated that the activity of Gi/Go was higher in old rat bladder compared to young.

Because stimulation of Gi protein inhibits cyclic AMP production and because isoproterenol

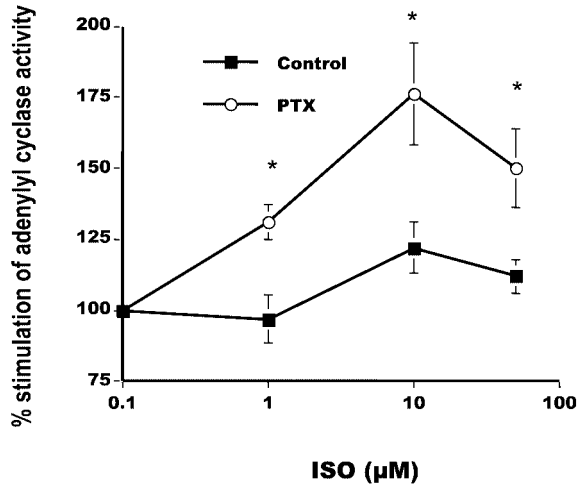

Fig. 10. Effect of isoproterenol on adenylyl cyclase activity in rat bladder dome homogenates from control and pertussis toxin-treated adult (6 month) rats. **P*<0.05 compared to basal (n=4/group).

responsive cyclase is decreased in bladders from old (22–24 months-old) rats, we measured the effect of pertussis toxin on β-adrenergic responsiveness of adult (6 months) rat bladder (Fig. 10). When bladder membranes from adult rats were treated with pertussis toxin, isoproterenol (1–100 μM) stimulated adenylyl cyclase increased compared to membranes treated with vehicle. The increase in cyclase was similar in extent to the isoproterenol-induced increase in adenylyl cyclase activity previously noted in the bladder from weanling rats (Wheeler *et al.*, 1990). This reversal indicates that pertussis toxin rescues the age dependent decrease in isoproterenol stimulated cyclase and that G-proteins are major players in this age-dependent decrease.

We then used western blot analysis to compare levels of the alpha subunit of G-proteins in weanling, young adult, adult and old rat bladder. Using an antibody that detects all Giα subunits, we found that Giα increased with aging. Relative intensity for Giα in weanling rat bladder was 100%, compared to 64% in young adult, 142% in adult and 260% in old rat bladders (Derweesh *et al.*, 2000). Further dissection of the subunits of G-proteins (Gs, Go, Gq, $Gi_{1,2,3}$) in the bladder of young adult and aged rats, indicated that levels of Gαs, Gαo and Gαi2 were higher in aged rats compared to young rats. Compared to the young adult animals, bladders from aged animals expressed 76 ± 9% more Gαs, 85 ± 12% more Gαo, and 137 ± 12% more Gαi2 (Fig. 11). In contrast, aged rats expressed 21 ± 9% less immunoreactive Gαq in their detrusor muscle than did young adult rats (Fig. 11). No age-dependent differences were found in expression of Gαi1 or Gβ subunit in bladder (Fig. 11). Gαi3 was not found to be expressed in detectable amounts in bladders from either age group. Thus the decrease in isoproterenol induced AC with aging is probably due to increases in Giα.

A theory proposed by Lefkowitz (1998) suggests that PKA phosphorylation of the β-receptor rapidly uncouples it from Gs and facilitates the coupling of the β-receptor to Gi, thus decreasing isoproterenol activated AC. The increase in Giα2 seen in the aging rat bladder may enhance AC

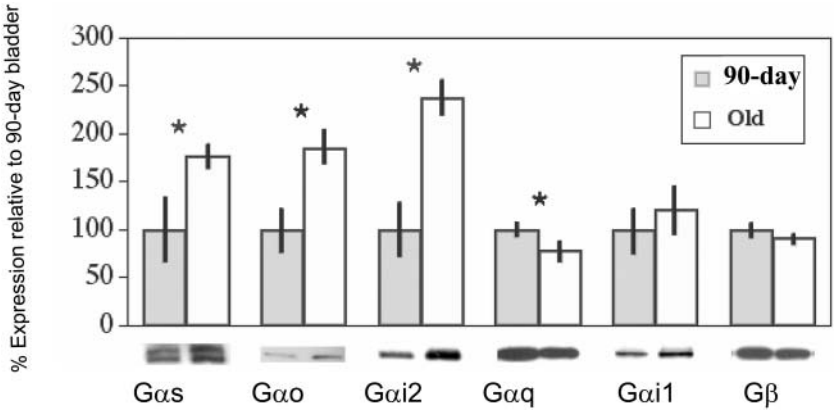

Fig. 11. Characterization of α and β subunits in bladders from 90-day-old (young adult) and 24-month-old (old) rat bladder dome. Representative western blots are shown beneath each data set. *P<0.5 (n=7–12 rats bladders/group).

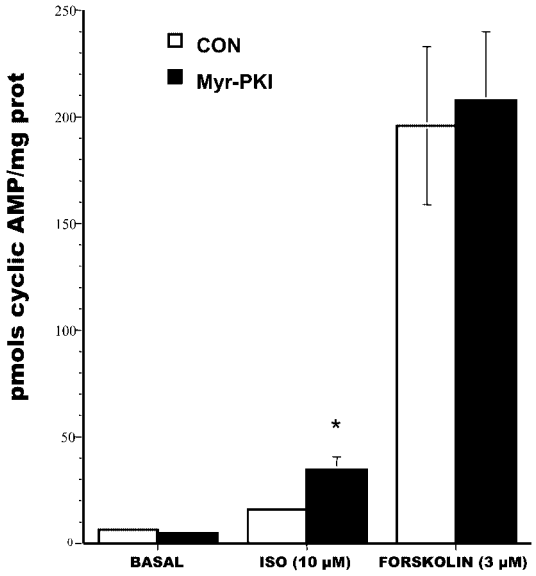

Fig. 12. Effect of PKA inhibitors on cyclic AMP accumulation in aged rat bladder. Incubation (30 min, 37°C) with PKA inhibitor Myr-PKI (5 μM) with cubes from old rat bladders (n=4) prior to incubation with isoproterenol or forskolin results in increased response to isoproterenol but not to forskolin. Results are expressed as mean ± SEM. *P<0.05 compared to control values.

inhibition by increasing the rate or the amount of phosphorylation of the β-receptor. We found that incubation with PKA inhibitors, myr-PKI, a peptide sequence and KT-5720, a nonpeptide antagonist increased isoproterenol responsive AC, but not forskolin responsive AC in homogenates from old rat bladder (Fig. 12). This indicates that PKA dependent phosphorylation of the β-receptor through Gi may be a possible mechanism for the age-

dependent decline in isoproterenol induced relaxation response.

Percent relaxation by forskolin decreased 20% and 30%, respectively, when detrusor muscle strips from young adult and old rats were compared to detrusor muscles from weanling rats (Fig. 6). Furthermore, forskolin activated adenylyl cyclase is decreased in both the bladder from the young adult and old rats compared to the young rat bladder (Table 6). Mechanisms which could be involved in the decreased relaxation response include decreased cyclic AMP synthesis by adenylyl cyclase or increased cyclic AMP degradation by PDE. Decreased synthesis seems unlikely since in the young adult rat bladder there is no decrease in G-protein activated adenylyl cyclase. Therefore the age dependent decrease in relaxation response to forskolin may be due to an increase in PDE or its phosphorylation.

The following mechanism is proposed. PDE3 or PDE4 are both present in bladder, and if the amount of PDE3, 4 or its phosphorylation increases with aging, this could be a mechanism for the decreased response to forskolin. An increase in cyclic AMP activates PKA which in turn phosphorylates PDE3A/B or PDE4D3. This phosphorylation causes an increase in PDE activity and a decrease in cyclic AMP levels, thus leading to a lesser relaxation response. The long-term accumulation of cyclic AMP produces an increase in PDE activity as a result of a *de novo* synthesis of the short-form variants of the PDE4 family, for example, PDE4D1/2 (Mehats *et al.*, 2002).

Conclusions

A number of PDE isoforms have been identified in the urinary tract, some with functional consequences. However, the lack of specificity of PDE inhibitors used in these studies and the identification of new PDE families make some of this work difficult to evaluate. PDE isoforms have not been localized within the tissues of the urinary tract and we have thought of the functional consequences of PDE inhibition only in terms of relaxation response. Localization of PDE isoforms within the urothelium may provide a different physiological response than PDE isoforms localized in bladder muscle. Furthermore, as we have seen here with aging and certainly in other disease processes, levels of the components of the adenylyl cyclase system can change and thus affect the relaxation response. By exploitation of differences in PDE expression in disease, such as the overexpression of PDEs in cancer, treatment options may present themselves. Recent studies suggest the efficacy of treatment of prostate cancer with exisulind, a PDE inhibitor (Goluboff *et al.*, 2001, 2002).

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