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Relaxation of human ureteral smooth muscle in vitro by modulation of cyclic nucleotide-dependent pathways

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Abstract Phosphodiesterases (PDE) are key enzymes regulating intracellular cyclic nucleotide turnover and, thus, smooth muscle tension. Recent reports have indicated the presence of PDE isoenzymes 1, 2, 4, and 5 in cytosolic supernatants prepared from human ureteral smooth muscle homogenates and the ability of second-generation inhibitors of PDE 3, 4, and 5 to relax KCl-induced tension of human ureteral muscle in vitro. The aim of the present study was to evaluate the functional effects of recently developed, third-generation isoenzyme-selective PDE inhibitors, the nitric oxide (NO)-donating agents sodium nitroprusside (SNP) and dihydropyridine (DHP), which is also described as an antagonist of L-type calcium channels, and the adenylyl cyclase-stimulating drug forskolin on tissue tension and cyclic nucleotide levels of human ureteral smooth muscle segments in vitro. Relaxant responses of human ureteral smooth muscle were investigated in vitro using the organ bath technique. Cyclic nucleotides cAMP and cGMP were determined by specific radioimmunoassay following time and dose-dependent incubation of the ureteral tissue with the drugs. The most pronounced relaxing effects on KCl-induced tension of ureteral smooth muscle were exerted by nitrovasodilator SNP, PDE4 inhibitor rolipram, and PDE5 inhibitors E 4021 and morpholinosulfonyl-pyrazolopyrimidine (MSPP). Relaxing potency of the drugs was paralleled by their ability to elevate intracellular levels of cGMP and cAMP, respectively. Our data suggest the possibility of using selective inhibitors of PDE isoenzymes 4 and 5 in the treatment of ureteral stones and ureteral colic.

Key words Human ureter · Phosphodiesterases · Phosphodiesterase inhibitors · Cyclic nucleotide monophosphates

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Introduction

Cyclic nucleotide monophosphates (cNMP) cyclic adenosine-3′,5′-monophosphate (cAMP) and cyclic guanosine-3′,5′-monophosphate (cGMP) are ubiquitous intracellular second messengers also involved in the relaxation of smooth muscle cells. The fundamental role of cNMP in the regulation of muscle tone is well established in various tissues such as vascular smooth muscle [10, 11], myocardium [16, 17], and airway smooth muscle [20, 21]. cNMP are synthesized from their corresponding nucleoside triphosphate by adenylyl cyclase (AC) and guanylyl cyclase (GC) and are degraded by phosphodiesterases (PDE), a heterogenous group of hydrolytic enzymes, through cleavage of the 3′-ribose phosphate bond. Because of their central role in smooth muscle tone regulation and the considerable variation of PDE isoenzymes regarding their distribution and functional importance in certain tissues, PDE have become an attractive target for drug development. Currently, nine families of PDE isoenzymes can be distinguished: Ca^{2+}/calmodulin-stimulated PDE (PDE1), cGMP-stimulated PDE (PDE2), cGMP-inhibited PDE (PDE3), cAMP-specific PDE (PDE4), cGMP-specific PDE (PDE5), and cGMP-binding, cGMP-specific PDE of mammalian rods and cones (PDE6) [1]. A type 7 PDE was isolated in a screen for human proteins that completed a deficiency in endogenous PDE in *Saccharomyces cerevisiae*. PDE7 was described to hydrolyze only cAMP with low K_m and to be insensitive to inhibition by PDE4 inhibitor rolipram [14]. PDE7 is abundant in skeletal muscle [14, 8] and has been found in human kidney, brain, and

pancreas, too. More recently, the cloning, expression, and molecular characterization of two novel human phosphodiesterases, cAMP-specific PDE 8 and cGMP-specific PDE9, was reported [5, 6]. The identification of different PDE-families has been paralleled by the synthesis of selective inhibitors. Theoretically, partial pharmacological tissue selectivity of a drug may be achieved by selective inhibition of PDE isoenzymes [16]. The role of cyclic nucleotides and PDE enzymes was investigated in various tissues and the use of isoenzyme-selective inhibitors to modulate tissue function was proposed in patients with refractory congestive heart failure [3, 4], asthma [20], and urge incontinence [22, 23]. However, the importance of cyclic nucleotides and PDE in the regulation of smooth muscle tone of the upper urinary tract is less clear. Urinary stone disease is an indication where pharmacological relaxation of ureteral smooth muscle would present an attractive therapeutic alternative. In the case of an uncomplicated renal or ureteral concretion, the intravenous administration of analgesics is the most effective way to relieve pain [9]. With respect to the potential beneficial effect of ureteral relaxation on stone passage, some spasmolytic agents such as phentolamine and orciprenaline have been shown to dilate the ureteral lumen at the position of an artificial concretion to allow increased fluid flow beyond the concretion. Many drugs have been used in ureteral colic management, but a drug that can relieve pain and facilitate stone passage with minimal side effects is not yet available. Recently, we demonstrated the presence of PDE isoenzymes 1, 2, 4, and 5 in cytosolic supernatants prepared from human ureteral smooth muscle homogenates and the ability of inhibitors of PDE 4 and 5 to relax KCl-induced tension of ureteral segments in vitro [19]. To further elucidate the relevance of cNMP-mediated pathways in ureteral tone regulation, we investigated the functional effects of recently developed selective inhibitors of PDE isoenzyme 5 E 4021, morpholinosulfonyl-pyrazolopyrimidine (MSPP), and diethylaminosulfonyl-pyrazolopyrimidine (DASPP) on KCl-induced tension and on cNMP levels of human ureteral tissue. Effects were compared with those of AC-stimulating agent forskolin, NO-releasing substance Na^+-nitroprusside (SNP), and dihydropyridine (DHP), which was originally described as being an antagonist of L-type calcium channels but recently has been found to release NO (Bayer, personal communication), and second-generation PDE inhibitors quazinone (Ro 13-6438), trequinsin (HL 725), rolipram (ZK 62711), and zaprinast (M&B 22948).

Materials and methods

Tissue preparation

Human ureters were obtained from patients who had undergone nephrectomy surgery for malignant renal tumors. Macroscopically normal tissue specimens were excised and immediately placed in a chilled organ-protective solution (Custodiol; Dr. Franz Köhler Chemie GmbH, Alsbach, Germany). Prior to the functional experiments, the ureteral smooth muscle wall was carefully freed of fat and connective tissue.

Organ-bath studies

Circular ureteral segments were mounted in 10-ml chambers of a vertical organ-bath system (Isolated Organ Apparatus IOA 5306; Föhr Medical Instruments, Seeheim, Germany), containing a modified Ringer-Krebs solution (pH 7.4) of the following composition: 120 mM NaCl, 25.6 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 22 mM glucose, 0.1 mM $2Na^+$ (Ca^{2+}) EDTA. The solution was continuously gassed with 95% O_2 and 5% CO_2, and the temperature maintained at 37 °C. The tissue segments were mounted between two hooks, one of which was connected to an isometric force transducer (Radnoti Glass Technology, Monrovia, Calif., USA). A pretension of 10 mN (1 g) was applied and the tissue was allowed to equilibrate for 60 min without further mechanical manipulation. The musculature was then stimulated with KCl (80 mM), which ensured stable and reproducible contractions. After stable contraction plateaus had been reached, increasing doses of forskolin, SNP, DHP, and PDE inhibitors were added to the bath chambers in a cumulative manner (0.01–10/100 μM). Isometric responses of the tissue were amplified and recorded with a MacLab data recording and analysis system (Analog Digital Instruments, Castle Hill, NSW, Australia).

Assays for cyclic nucleotides

To determine dose- and time-dependent effects of the drugs on cyclic nucleotide levels, circular segments were incubated in 2-ml reaction vials containing Krebs-Ringer solution continuously gassed with carbogen. Tissue preparations were incubated with forskolin and SNP (0.01, 1, and 100 μM) for 2, 5, and 10 min. PDE inhibitors (1, 10, and 100 μM) were allowed to incubate the tissue for 10 min. Incubation of the muscle segments with forskolin and SNP was performed in the presence of 1 mM isobutylmethylxanthine (IBMX) to prevent degradation of cyclic nucleotides by PDE activity. Following the incubation period, the tissue was rapidly frozen in liquid nitrogen. The tissue was homogenized in a frozen state and camp were extracted using 70% ethanol. After centrifugation at 3000*g* for 10 min at 4 °C, the ethanolic phase was removed and lyophilized, and the remaining dry particulate fraction was resuspended in 50 mM sodium acetate buffer. Aliquots of the samples were acetylated and assayed for cAMP and cGMP contents by specific radioimmunoassays (RIA). The protein content of the particulate fractions was measured according to the method of Lowry [12] using bovine serum albumin as a standard.

Data analysis

Relaxant responses of ureteral segments during organ-bath experiments are expressed as percentage of the maximum contraction induced by 80 mM KCl. Mean EC_{50} values were determined graphically from the linear plot of the percentage of response versus drug concentration by nonlinear regression and represent 50% relaxation of the KCl-induced contraction plateau. The magnitude of drug effect at maximum concentration (R_{max}) is given as percentage relaxation of maximum tension. All data are given as the mean ± SD. Statistical analysis was conducted by Gosset *t*-test. A probability (*P*) value of less than 0.05 was accepted as significant. All experiments were repeated eight to ten times on tissue segments originating from at least two different tissue preparations. In RIA experiments, each concentration was tested threefold and assayed in duplicate for cAMP and cGMP. Stimulating effects of IBMX on cNMP levels were subtracted when processing the data. Statistical analysis was also conducted using the Gosset *t*-test. There were only negligible effects of the highest concentration of the solvent vehicles on tissue tension and cNMP content.

Drugs

Forskolin, SNP, and IBMX were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Rolipram was kindly provided by Schering AG, Berlin, Germany, zaprinast by Rhone-Poulenc Rorer, Dagenham, UK, and quazinone was obtained from Biomol, Hamburg, Germany. ^{125}[I]cAMP and ^{125}[I]cGMP were obtained from Amersham Pharmacia Biotech Europe, Freiburg, Germany. Antibodies raised in rabbits against cAMP and cGMP were generously provided by the Lower Saxony Institute of Peptide Research, Hannover, Germany. All other drugs were supplied by the Pharmaceutical Division of Bayer AG, Wuppertal, Germany. Drugs were made up as stock solutions (0.5–10 mM, depending on the solubility properties of the individual drugs) using saline, methanol, ethanol, or dimethylsulfoxide (DMSO) and were further diluted with saline or Krebs solution.

Results

Effects of drugs on KCl-induced tension

None of the ureteral smooth muscle segments showed any spontaneous activity. All substances tested induced dose-dependent relaxation of different potency and efficacy. Rolipram, an inhibitor of PDE4, as well as semi-selective and selective inhibitors of PDE5 turned out to be the most potent relaxing compounds: KCl-induced tension was significantly reversed by PDE4 inhibitor rolipram ($EC_{50} = 0.1$ μM, $R_{max} = 100\%$), dual PDE5/1 inhibitor zaprinast ($EC_{50} = 40$ μM, $R_{max} = 76 \pm 7.0\%$), and selective PDE5 inhibitors E 4021 ($EC_{50} = 50$ μM, $R_{max} = 59 \pm 12\%$) and MSPP ($EC_{50} = 20$ μM, $R_{max} = 67 \pm 10.6\%$). PDE3 inhibitors quazinone and HL 725 induced relaxant effects with EC_{50}values of 25 μM and 100 μM, respectively. Nitrovasodilator SNP reversed KCl-induced tension of the ureteral segments with an EC_{50} of 4 μM; R_{max} was determined as $62 \pm 5\%$. In contrast, effects of forskolin and NO-donating substance DHP on tissue tension were significantly less potent. R_{max} values were determined as $39 \pm 2\%$ (forskolin) and $22.5 \pm 11\%$ (DHP). Figs 1–3 and Table 1 summarize the data of the functional organ-bath studies.

Effects of drugs on cyclic nucleotide levels

Forskolin significantly increased cAMP levels dose-dependently and time-dependently starting at a concentration of 0.01 μM. Maximum concentration of forskolin (100 μM) increased cAMP level 22-fold, 42-fold, and 51-fold over control (control 31.9 ± 2.7 pmol/mg protein) after 2, 5, and 10 min of incubation, respectively (Fig. 4). No time- and dose-dependent effects of forskolin on cGMP level were noted (data not shown). Incubation with SNP resulted in an increase in cGMP level (control 1.7 ± 1.16 pmol/mg protein) 13-fold after 2 min of incubation with 0.01 μM SNP to 90-fold after 10 min incubation with 100 μM SNP ($P < 0.01$ for each; Fig. 5). No effects of SNP on cAMP levels with respect to dose and time were noted (data not

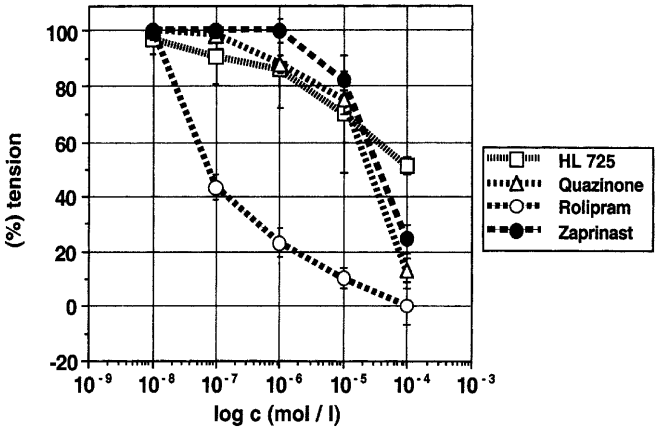

Fig. 1 Relaxation of human ureteral segments in vitro induced by cumulative addition of phosphodiesterase 3 (PDE3) inhibitor HL 725 and second-generation PDE inhibitors rolipram, quazinone, and zaprinast. Each point is expressed as percentage of maximum KCl-induced tension and represents mean ± standard deviation (SD) of the mean of $n = 8$–10 determinations

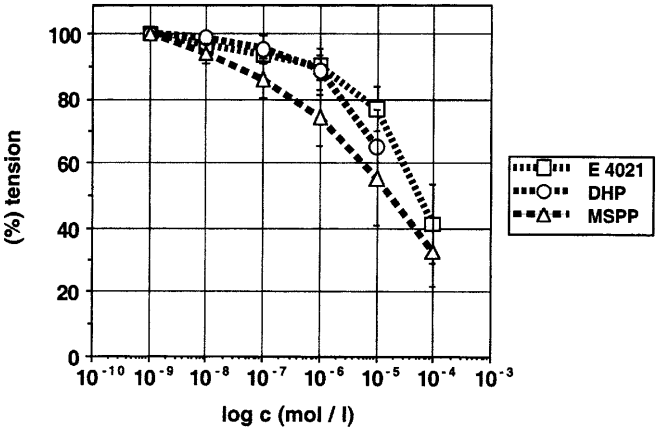

Fig. 2 Relaxing effects of PDE5 inhibitors E 4021 and morpholinosulfonyl-pyrazolopyrimidine (*MSPP*), and NO-donor dihydropyridine (*DHP*) on KCl-induced tension of circular human ureteral segments. Each point is expressed as a percentage of maximum KCl-induced tension and represents mean ± SD of $n = 8$–10 determinations

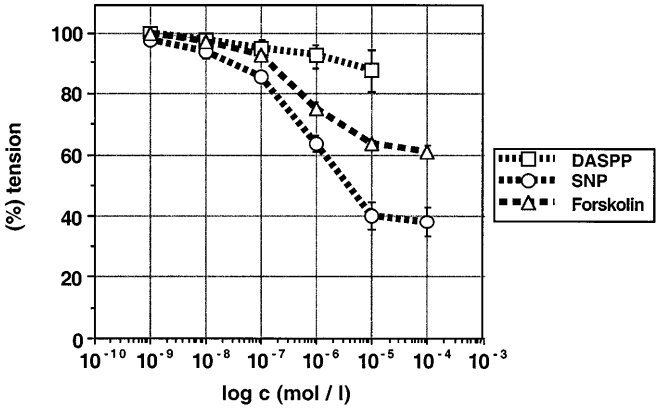

Fig. 3 Relaxing effects of PDE5 inhibitor diethylaminosulfonyl-pyrazolopyrimidine (*DASPP*), NO-donor sodium nitroprusside (*SNP*), and adenylyl cyclase stimulating agent forskolin on KCl-induced tension of circular human ureteral segments

Table 1 Effects of drugs on KCl-induced tension of human ureteral segments by means of EC_{50} (EC_{50} > > 100 μM) and R_{max} values (EC_{50} drug concentration inducing 50% reversion of KCl-induced tension, R_{max} relaxation at final drug concentration, *ND* not determined, *SNP* sodium nitroprusside)

Substance	R_{max} ± SD (%)	EC_{50} (μM)
Quazinone (Ro 13–6438)	87 ± 4.0	25.0
HL 725	49 ± 3.0	100.0
Rolipram (ZK 62711)	100	0.1
Zaprinast (M&B 22948)	76 ± 7.0	40.0
E 4021 (PDE5 inhibitor)	59 ± 12.5	50.0
DHP (dihydropyridine)	35 ± 11.8 (10 μM)	ND
Morpholinosulfonyl-pyrazolopyrimidine (MSPP, PDE5 inhibitor)	67.3 ± 10.6	20.0
Diethylaminosulfonyl-pyrazolopyrimidine (DASPP, PDE5 inhibitor)	12.6 ± 7.0 (10 μM)	ND
Forskolin	39.2 ± 2.3	ND
SNP	62 ± 5.0	4.0

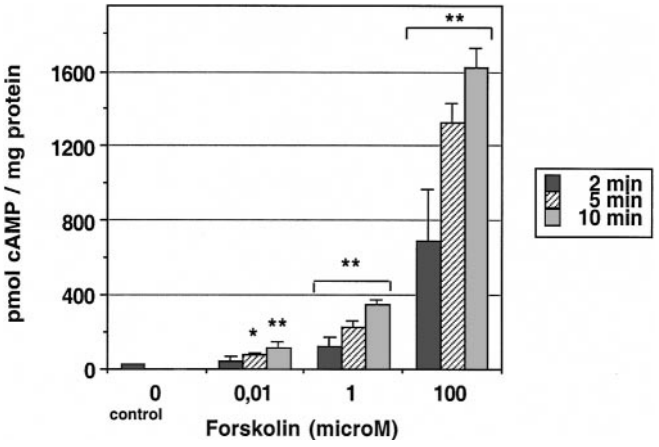

Fig. 4 Time course of cAMP accumulation in the presence of forskolin in human ureteral segments. Each *bar* represents the mean ± SD of three individual determinations. *Values significantly different from control (*$P < 0.05$, **$P < 0.01$)

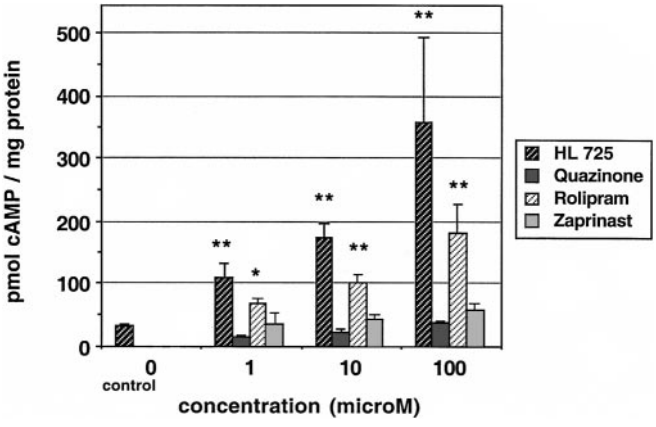

Fig. 6 Levels of cAMP in isolated human ureteral segments incubated with PDE inhibitors HL 725, rolipram, quazinone, and zaprinast. Each *bar* represents the mean ± SD of three individual determinations. *Values significantly different from control (*$P < 0.05$, **$P < 0.01$)

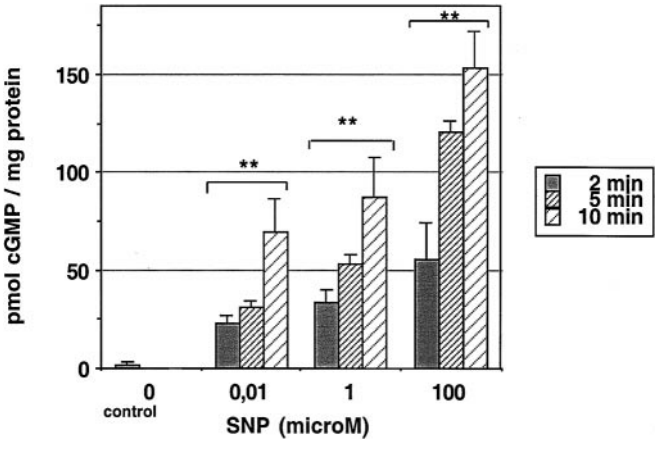

Fig. 5 Time course of cGMP accumulation in the presence of SNP in human ureteral segments. Each *bar* represents the mean ± SD of three individual determinations. *Values significantly different from control (*$P < 0.05$, **$P < 0.01$)

shown). Ten minutes of incubation with 10 μM and 100 μM rolipram caused increases in cAMP threefold and sixfold, respectively (Fig. 6). Stimulation by HL 725 was 3-fold, 5-fold, and 11-fold at 1 μM, 10 μM, and 100 μM (Fig. 6). E 4021 increased cAMP levels dose-dependently 12-fold to 62-fold; stimulation by MSPP was 7-fold to 31-fold. All values were significant ($P < 0.05$). cGMP levels were elevated twofold by 100 μM rolipram, zaprinast, and E 4021 (Figs. 7, 8).

Discussion

cAMP and cGMP are important intracellular second messengers formed following stimulation of AC and GC, respectively. The action of many hormones and neurotransmitters involved in the regulation of smooth muscle tone is mediated through specific receptors coupled to these enzymes. The degradation of cNMP is regulated by the activity of cyclic nucleotide PDE. The major clinical focuses for selective PDE inhibitors were determined as positive inotropic agents, antidepressants, anti-inflammatory agents, and bronchodilators. Recent basic and clinical research has confirmed that the concept of PDE inhibition is also applicable in the treatment of male erectile dysfunction [2, 7, 13]. The aim of the present study was to further delineate the functional importance of cAMP- and cGMP-

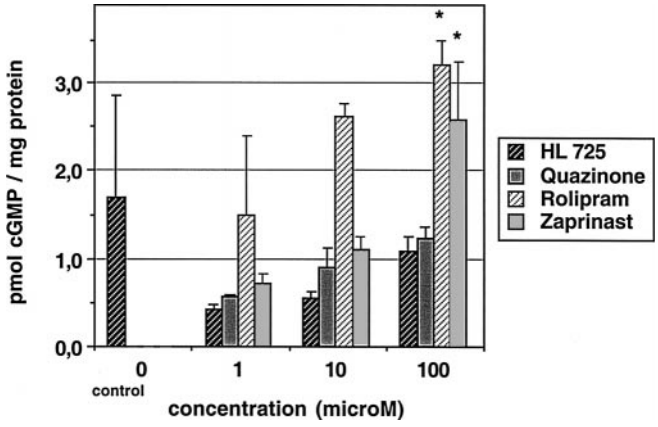

Fig. 7 Levels of cGMP in isolated human ureteral segments incubated with PDE inhibitors HL 725, rolipram, quazinone, and zaprinast. Each *bar* represents the mean ± SD of three individual determinations. *Values significantly different from control (*$P < 0.05$, **$P < 0.01$)

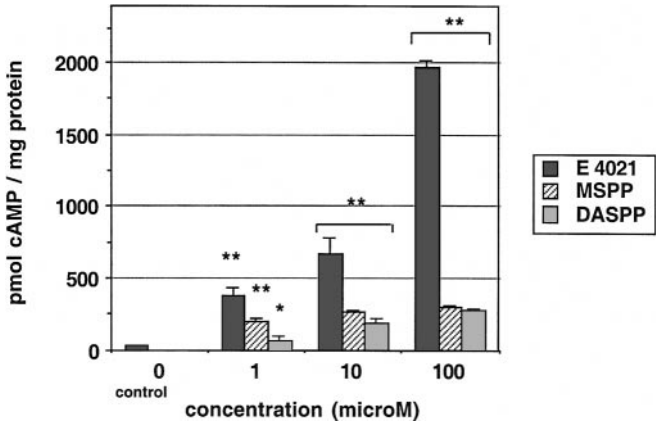

Fig. 8 Levels of cAMP in isolated human ureteral segments after 10 min of incubation with PDE5 inhibitors E 4021, MSPP, and DASPP. Each *bar* represents the mean ± SD of three individual determinations. *Values significantly different from control (*$P < 0.05$, **$P < 0.01$)

mediated pathways in human ureteral smooth muscle. Therefore, we examined the effects of various third-generation isoenzyme-selective PDE inhibitors on ureteral tension and cyclic nucleotide levels in vitro.

As previously shown, the PDE4 inhibitor rolipram, PDE3 inhibitor quazinone, and dual PDE5/1 inhibitor zaprinast strongly reversed KCl-induced tension of human ureteral segments. We were able to demonstrate that these relaxing properties are paralleled by the ability of the drugs to elevate cAMP and cGMP levels. Our organ-bath studies revealed the relaxing potency of nitrovasodilator SNP, PDE3 inhibitor HL 725, and PDE5 inhibitors E 4021 and MSPP. While inhibitors of PDE3, which act on cAMP-hydrolyzing PDE isoenzymes, significantly increased cAMP levels, E 4021 and MSPP, selective inhibitors of cGMP-specific PDE5, did not exert major effects on cGMP levels – unexpected from their mode of action – but considerably elevated

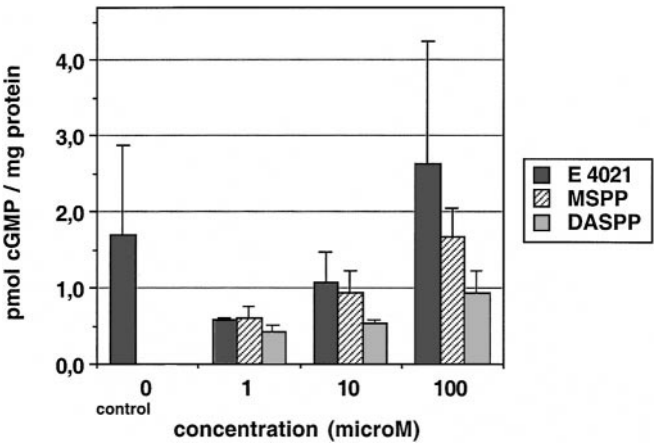

Fig. 9 Levels of cGMP in isolated human ureteral segments after 10 min of incubation with PDE5 inhibitors E 4021, MSPP, and DASPP. Each *bar* represents the mean ± SD of three individual determinations. *Values significantly different from control (*$P < 0.05$, **$P < 0.01$)

cAMP. This fact gives rise to the speculation that, in ureteral smooth muscle, cAMP might be the main mediator of muscle relaxation. In contrast, the low relaxing potency of forskolin, despite its remarkable effects on intracellular cAMP and the pronounced relaxant response to SNP in combination with the dramatic increase in cGMP levels after incubation with SNP, demonstrates that cGMP is also involved in the relaxation of human ureteral smooth muscle. This is in accordance with previous findings that ureteral relaxation may involve the NO/cGMP pathway [18]. Therefore, in the human ureter, cGMP and cAMP either appear to have similar physiological effects and work synergistically or a slight increase in cGMP may exert an inhibitory effect on PDE3, leading to an elevation of cellular cAMP and finally ureteral relaxation. A comparable mechanism has already been assumed by Maurice and Haslam from experiments on rat aortic smooth muscle [15]. Although PDE3 was not revealed in the cytosolic fraction of ureteral smooth muscle [19], it might be localized in cellular compartments of the microsomal fraction. This may explain the in vitro effects of PDE3 inhibitor HL 725 on ureteral smooth muscle. The apparent discrepancy between cAMP and cGMP levels and functional responses may also be explained by possible intracellular compartmentation of cyclic nucleotides and important regulatory PDE isoenzymes. Thus, different inhibitors may elevate cyclic nucleotide levels in different intracellular compartments. Cyclic nucleotides could act in such a way that very small, compartmentalized changes, e.g., in cGMP, could cause calcium desensitization by stimulation of light chain myosin phosphatase activity or major changes in intracellular calcium by activation of cGMP-dependent protein kinase, thus resulting in changes of smooth muscle tone. Penetration and distribution of some PDE inhibitors may vary within the cell. The present data demonstrate that pharmacological modulation of in-

tracellular cNMP-dependent signal transduction pathways resulted in changes of ureteral tension in vitro, which is accompanied by an increase in cNMP levels. The effects of PDE5 inhibitors E 4021 and MSPP on cAMP levels indicate that cGMP and cAMP-dependent signal transduction pathways are not generally parallel or independent in smooth muscle tissue, and manipulation of cGMP-downstream might interfere with regulatory cAMP cascades. It is important to emphasize that cyclic nucleotide turnover in isolated tissue preparations is low, since only basal cyclase activity is maintained, thus high concentrations of PDE inhibitors are needed to elevate cyclic nucleotides in order to produce a significant tissue response. In contrast to this situation, cyclic nucleotide turnover rates are much higher in in vivo systems where PDE inhibitors tend to be much more effective [16]. Therefore, future studies on ureteral smooth muscle relaxation and determination of cyclic nucleotide levels under better physiological conditions, i.e., by addition of subsaturating concentrations of SNP or forskolin and various concentrations of PDE inhibitors, would gain importance.

Generally, there are three characteristics of PDE4 and PDE5 inhibitors that might be beneficial in the treatment of ureteral colic: (1) relaxation of ureteral muscle at the site of the concretion might relieve colic pain and facilitate stone passage; (2) inhibitors of PDE isoenzymes 4 and 5 are thought to induce minimal systemic side-effects at the dosage needed; and (3) improved isoenzyme selectivity of third-generation PDE inhibitors will help to focus drug effects on the target tissue. Clinically, the use of inhibitors PDE4 and 5 seems promising due to the anti-inflammatory effect as well as the favorable effect to side-effects ratio of the drugs.

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