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ORIGINAL PAPER

In vitro effects of PDE5 inhibitors sildenafil, vardenafil and tadalafil on isolated human ureteral smooth muscle: a basic research approach

Christian Gratzke · Stefan Ückert · Giorgi Kedia · Oliver Reich · Boris Schlenker · Michael Seitz · Armin J. Becker · Christian G. Stief

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Abstract Cyclic nucleotide phosphodiesterase (PDE) isoenzymes are key proteins regulating intracellular cyclic nucleotide turnover and thus smooth muscle tension. Several in vitro studies have indicated that the cyclic GMP and cyclic AMP-mediated signaling may play a role in the control of human ureteral muscle. The aim of the present study was to evaluate the functional effects of PDE5 inhibitors sildenafil (Sil), vardenafil (Var) and tadalafil (Tad), as well as nitric oxide (NO)-donating agent sodium nitroprusside (SNP) and non-selective muscarinic antagonist butylscopolamine (BSC) on the tension induced by KCl and the turnover of cyclic nucleotides in isolated human ureteral smooth muscle. In vitro relaxant responses of human ureteral smooth muscle to the PDE5 inhibitors mentioned above were investigated using the organ bath technique. Cyclic nucleotides cAMP and cGMP were determined by means of specific radioimmunoassay following incubation of the tissue with Sil, Var, Tad and SNP. The tension induced by KCl of the ureteral tissue was dose dependently reversed by the drugs with the following rank order of efficacy: SNP > Var ≥ Sil > Tad > BSC. R_{max} values ranged from $25 \pm 9\%$ (SNP) to $5 \pm 3\%$ (BSC). Relaxant responses were paralleled by threefold to fourfold increase in tissue levels of cGMP. Our results indicate that PDE5 inhibitors can reverse the tension of isolated human ureteral smooth muscle via cGMP-mediated pathways. Nevertheless, further studies are indicated in order to evaluate as to whether there might be a use for PDE5 inhibitors in the treatment of ureteral stone disease.

Keywords Urinary stone disease · Human ureter · Phosphodiesterase (PDE) isoenzymes · PDE5 inhibitors · Cyclic GMP

C. Gratzke (✉) · O. Reich · B. Schlenker · M. Seitz · A. J. Becker · C. G. Stief
Department of Urology, Ludwig-Maximilians-University, University Hospital Großhadern, Marchioninistr. 15, 81377 Munich, Germany
e-mail: christian.gratzke@med.uni-muenchen.de

S. Ückert · G. Kedia
Department of Urology, Hannover Medical School, 30625 Hannover, Germany

Introduction

Urolithiasis is a disease which is constantly increasing in many of the westernized countries [1]. Since several factors contribute to the inhibition of spontaneous passage of ureteral calculi, symptomatic ureterolithiasis represents one of the most common emergency conditions in urological practice. Factors to determine the severity of the condition are stone size, configuration and location, smooth muscle spasm, as well as the local anatomy of the ureter [2, 3].

Several drugs have been evaluated in clinical settings over the last 10 years in order to help ease spontaneous passage of distal ureteral stones and relieve ureteral colic pain. Among these drugs are Ca^{2+}-channel blocking agents, such as nifedipine, inhibitors of prostaglandin synthesis, the nitric oxide (NO) donor glyceryl trinitrate, non-specific PDE inhibitor papaverine, as well as the $alpha_{1A/1D}$-adrenoceptor antagonist tamsulosin [4–8].

Cyclic adenosine-3′,5′-monophosphate (cAMP) and cyclic guanosine-3′,5′-monophosphate (cGMP) are important intracellular second messengers mediating cellular responses. An increase in cAMP and/or cGMP triggers a signal transduction cascade encompassing the activation of cyclic nucleotide-dependent protein kinases, subsequent phosphorylation of the actin–myosin system, as well as membrane-located Ca^{2+}-channels. This cascade leads to a reduction in cytosolic Ca^{2+} and, finally, smooth muscle relaxation [9, 10]. Cyclic nucleotides are degraded by phosphodiesterases (PDEs), a heterogenous group of hydrolytic enzymes. Because of their central role in the control of smooth muscle tone, PDEs have become an attractive target for drug development. Clinical trials have suggested that, in urological routine, the use of PDE inhibitors might not only be applicable to the treatment of male erectile dysfunction (ED) but also to lower urinary tract symptomatology (LUTS) secondary to benign prostatic hyperplasia (BPH), urinary incontinence, and symptoms of female sexual dysfunction [11–15].

Taher et al. [16] reported the presence of PDE isoenzymes 1, 2, 4 and 5 in cytosolic supernatants prepared from human ureteral tissue and—using the organ bath technique—demonstrated the potency of the PDE3 inhibitor quazinone, PDE4 inhibitor rolipram and dual PDE5/PDE1 inhibitor zaprinast to reverse the tension induced by KCl of circular ureteral segments in vitro. Kuhn et al. [17] later confirmed the relaxing properties of inhibitors of PDE4 (rolipram) and PDE5 (E 4021, MSPP) on isolated human ureteral smooth musculature and demonstrated that these effects were paralleled by an elevation in intracellular levels of cAMP or cGMP. Based on the results from experiments on the effects of the NO donor drugs sodium nitroprusside (SNP) and molsidomine (SIN-1) and the PDE5-inhibitor zaprinast on the tension induced by KCl of proximal segments of the human ureter, Saighi et al. [18] concluded that cGMP is an important second messenger in the signalling pathway leading to the relaxation of the ureteral smooth muscle. In a rabbit model, Becker et al. [19] examined the in vivo potential of rolipram in comparison to papaverine, theophylline and butylscopolamine (BSC) to induce ureteral relaxation. They found that only rolipram induced a pronounced relaxation of the rabbit ureter with no considerable effects on the systemic circulation, whereas the application of BSC, papaverine or theophylline exerted no or only short-lasting relaxation of the ureter but significantly affected systemic blood pressure.

Up until now, the effects of selective PDE5 inhibitors, today representing first-line drugs in the oral pharmacotherapy of ED, on human ureteral smooth muscle have not been examined. Therefore, we investigated the functional effects of sildenafil (Sil), vardenafil (Var) and tadalafil (Tad), as well as the NO-releasing compound SNP and BSC on the tension induced by KCl and on cyclic nucleotide levels in isolated human ureteral tissue.

Materials and methods

Tissue source

Human ureteral tissue was obtained from 16 male patients (mean age 62 years) who had undergone removal of a kidney due to renal cancer. Macroscopically normal specimens of the proximal ureter were excised and immediately placed in a chilled organ protective solution (CUSTODIOL®, Dr. Franz Köhler Chemie GmbH, Alsbach, Germany). Maximum time between harvesting the sample and the experimental studies was 6 h.

Organ bath studies

Ureteral smooth muscle was carefully dissected free of fat and connective tissue. Circular segments were mounted in 10 ml chambers of a IOA 5306 vertical organ bath system under standard conditions as has been described earlier [16, 17] (Föhr Medical Instruments GmbH, Seeheim, Germany). Bath chambers contained modified Ringer-Krebs solution, pH 7.4, composed of 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 and 0.1 mM $2Na^+$ (Ca^{2+}) ethylenediaminetetraacetic acid. The solution was continuously gassed with 95% O_2 to 5% CO_2 and the temperature was maintained at 37°C. The strips were mounted between two hooks, of which one was connected to a force transducer (Ragnotti-Grass, Quincy, MA). A pretension of 10 mN (1 g) was applied and the tissue allowed to equilibrate for 60 min without further mechanical manipulation. The musculature was then exposed to KCl (80 mM). After stable contractions had been reached, increasing doses of the PDE5 inhibitors Sil, Var, Tad and BSC were added to the bath chambers in a cumulative manner (0.0001–10 μM). Some strips were challenged by a single dose (10 GM) of the NO donor SNP. Isometric responses of the tissue were amplified and recorded with a MacLab™ data recording and analysis system (AD Instruments, Castle Hill, Australia).

Assays for cyclic nucleotides

Effects of the drugs on cyclic nucleotide levels in isolated specimens of human ureteral smooth muscle were investigated as described by Kuhn et al. [17]. Following exposure of the tissue to the final concentration (10 μM) of Sil, Var, Tad and SNP during the organ bath experiments, specimens were immediately frozen in liquid nitrogen and then processed for the measurement of cyclic nucleotides. In order to assay the tissue content of cAMP and cGMP, specific radioimmunoassays (RIA) were used according to the manufacturer's manual (IBL GmbH, Hamburg, Germany).

Data analysis

Relaxant responses of the ureteral tissue are expressed as percentage of the maximum contraction induced by 80 mM KCl. The magnitude of drug effect at maximum concentration (R_{max}) is given in percent relaxation of maximum tension. All data are given as the mean ± SD. Statistical analysis was conducted by Gosset *t* test. A probability (*P*) value less than 0.05 was accepted as significant. All experiments were repeated 8–12 times using tissue segments originating from at least four different individuals. In the RIA experiments, all samples were assayed in duplicate for cAMP or cGMP. Statistical analysis was also conducted using the Gosset *t* test.

Drugs

SNP and BSC were purchased from Sigma Chemical Co., St Louis, USA. The PDE5 inhibitors Sil and Var were kindly provided by Bayer Vital GmbH, pharmaceutical business unit (Leverkusen, Germany), Tad was a generous gift from Tanabe Pharmaceutical Co. (Osaka, Japan). Drugs were made up as stock solutions (10 mM) using saline (SNP) or dimethylsulfoxide (DMSO) and further diluted with KREBS-solution. All other laboratory chemicals were either purchased from Merck KGaA (Darmstadt, Germany), Mallinckrodt-Baker BV (Deventer, The Netherlands) or ACR OS Organics NV (Geel, Belgium).

Results

Organ bath studies

None of the circular ureteral segments showed any spontaneous contractile activity after they had been mounted to the organ chambers. Although all compounds tested exerted relaxation of the KCl-induced tension, none of the drugs induced a 50% reversion of the initial contractile force induced by KCl (EC_{50}). The most pronounced relaxing effect was registered in response to the NO donor SNP, R_{max} was determined as 25 ± 9% (data not shown). The PDE5 inhibitors dose-dependently reversed the tonic contraction of ureteral smooth muscle with the following rank order of efficacy: Var ($R_{max} = 23 \pm 8\%$) ≥ Sil ($R_{max} = 20 \pm 4\%$) > Tad ($R_{max} = 6 \pm 4\%$) > BSC ($R_{max} = 5 \pm 3\%$). No significant differences were noted with regard to the relaxation induced by Var and Sil, whereas Tad and BSC were significantly less efficacious. The results from the organ bath studies are summarized in Fig. 1.

Assays for cyclic nucleotides

The nitric oxide donor drug SNP (10 μM) significantly increased tissue levels of cGMP 3.1-fold over the control readings (1.7 ± 1.16 pmol/mg protein). Cyclic GMP was also elevated 3.3-fold in response to exposure of ureteral smooth muscle to 10 μM of Var. Interestingly, incubation of the tissue with Sil or Tad did not result in a marked enhancement of cGMP. Not unexpectedly, no significant stimulation of cAMP accumulation was noted following exposure of the musculature to the test compounds. SNP and the PDE5 inhibitor Tad only incuded a slight 1.7-fold and 1.6-fold rise, respectively, in tissue cAMP (control 31.9 ± 2.7 pmol/mg protein), whereas no elevation was registered in response to Sil and Var. The results from the measurements of cAMP and cGMP are displayed in Fig. 2a, b.

Discussion

Although it has been shown earlier that PDE inhibitors can reverse the contraction of isolated human ureteral

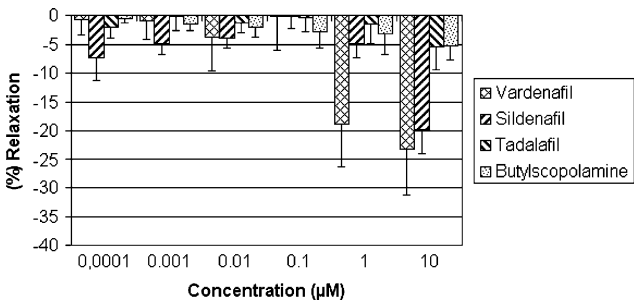

Fig. 1 Relaxation of circular human ureteral segments in vitro induced by the cumulative addition of the PDE5 inhibitors sildenafil, vardenafil and tadalafil as well as the non-selective muscarinic antagonist butylscopolamine. Each point is expressed as percentage of the maximum KCl-induced tension and represents mean ± SD of the mean of *n* = 8–12 determinations

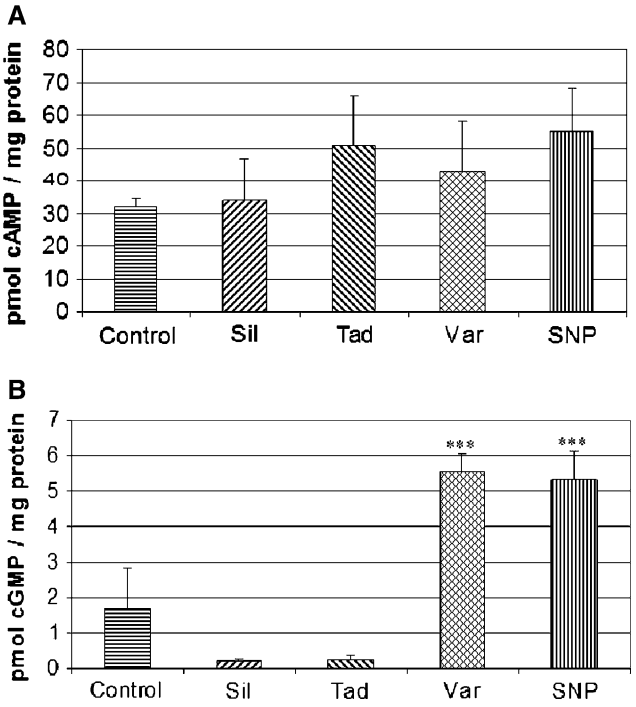

Fig. 2 Effects of a single concentration (10 μM) of the NO donor drug SNP and PDE5 inhibitors sildenafil, vardenafil and tadalafil on the accumulation of cAMP (**a**) and cGMP (**b**) in isolated human ureteral smooth muscle tissue. Each *bar* represents the mean ± SD of three individual determinations. *Asterisk* indicates values are significantly different from control readings (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$)

smooth muscle [16–18], up until now, the properties of the PDE5 inhibitors Sil, Var and Tad to antagonize the tension of the smooth muscle of the ureter have not been investigated. Thus, it was the aim of the present study to evaluate the effects of said compounds on tissue tension and the accumulation of cyclic nucleotides in isolated ureteral tissue.

We were able to demonstrate that the PDE5 inhibitors induced a dose-dependent reversion of tissue tension, which was, in part, accompanied by an increase in tissue cGMP but not cAMP. In the present study, SNP, Var and Sil turned out to be more effective than Tad with regard to the reversion of tension. Nevertheless, although the relaxation induced by SNP and Var was accompanied by a marked increase in tissue cGMP, none of the compounds induced a 50% inhibition of the initial contraction. This apparent discrepancy between cGMP levels and relaxant responses might be due to a possible intracellular compartmentation of cyclic nucleotides and PDE isoenzymes. Thus, different inhibitors may elevate cGMP in different intracellular compartments. As a consequence, a pronounced local elevation in cyclic nucleotides may not result in major changes in intracellular Ca^{2+} and, subsequently, smooth muscle tone.

Moreover, it should be taken into consideration that cyclic nucleotide turnover in isolated tissue preparations is low, since only a basal activity of adenylyl cyclase and guanylyl cyclase is maintained. Thus, high concentrations of PDE inhibitors are needed to elevate cyclic nucleotides in order to produce a significant tissue response. In contrast, cyclic nucleotide turnover rates are much higher in in vivo systems where PDE inhibitors tend to be much more effective [20]. In view of this, the poor relaxation induced by the PDE5 inhibitors and the finding that Sil and Tad per se did not enhance the intracellular accumulation of cGMP in the ureteral tissue is not necessarily in contrast to the mechanism of action of a selective PDE5 inhibitor. It has been demonstrated that the synergistic mechanism of cGMP-production and the inhibition of its degradation by cGMP-PDEs can be amplified in the presence of an exogenous NO donor known to trigger the cGMP-cascade [21, 22]. Not unexpectedly, we observed no increase in tissue cAMP following the incubation with Sil, Var or Tad. This is in favour of the hypothesis that the action of PDE5 inhibitors on human ureteral smooth muscle is exclusively mediated via the NO/cGMP pathway and that this signalling is, to a certain degree, involved in the control of ureteral relaxation [17, 23].

The discrepancy between cGMP levels and relaxant responses might also be explained by observations indicating that PDE5-inhibitors facilitate muscle relaxation via an additional mechanism independent of the classical NO-cGMP-pathway. Lau et al. [24] reported concentration-dependent relaxation of rabbit corpus cavernosum smooth muscle precontracted with NA for Sil, Var and Tad. The rank order of potency was, like in our study, Var > Sil > Tad. Pre-incubation of cavernosal muscle strips with an inhibitor of NO synthase or a selective inhibitor of soluble guanylate cyclase culminated in only a 20–30% reduction in muscle relaxant action of the three PDE inhibitors, which suggested that another mechanism of relaxation independent of NO-cGMP pathway was involved. On K^+ depolarized tissues, Sil was as potent as Var whereas Tad was the least effective in relaxing K^+-induced tone. In addition, Sil and Var were more efficacious than Tad in reversing tonic contractions by a Ca^{2+} channel activator (BAY K-8644). The authors concluded that Var and Sil possess direct muscle relaxant potential possibly via inhibiting Ca^{2+} influx through both receptor-operated and voltage-dependent Ca^{2+} channels whereas Tad appears capable of inhibiting receptor-operated transmembrane Ca^{2+} entry only. Sil might have yet another relaxant action than through PDE inhibition. cGMP is, in addition to degradation by PDEs exported from

cells by multidrug resistance protein 5 which is an adenosine triphosphate dependent export pump for cGMP. Nies et al. [25] showed co-expression of multidrug resistance protein 5 and PDE5 in smooth muscle cells of the genitourinary system; Sil potently inhibited multidrug resistance protein 5 independent of NO. Therefore, binding of Sil to multidrug resistance protein 5 may constitute another pathway contributing to smooth muscle relaxation.

Butylscopolamine is a non-selective antimuscarinic quaternary ammonium derivative of scopolamine, which effectively suppresses motility in the gastrointestinal tract [26]. Antimuscarinic agents may help to provide analgesia in ureteral colic by inducing smooth muscle relaxation, which reduces ureteral spasm and therefore are still recommended in the treatment of renal colic today, usually as adjuvant therapy to NSAIDS and/or opioids [27]. In our study, BSC did not exert a significant relaxation on human ureteral smooth muscle ($R_{max} = 5 \pm 3\%$). These results confirm findings by Wanajo et al. [28] who tested the potency of BSC on the tonic contraction induced by KCl (80 mM) in isolated canine ureteral preparations. They found that BSC had no effect at all on the KCl-induced contraction. In the study by Becker et al. [19] described earlier, the application of BSC exerted no or only short-lasting relaxation of the ureter in vivo in a rabbit model. In a recent randomized controlled trial including 192 patients, Holdgate et al. [29] also found no evidence that hyoscine butylbromide (Buscopan™) reduces opioid requirements or the need for ongoing opioid analgesia in acute renal colic. Thus, the role of antimuscarinics in general and BSC in particular in the treatment of ureteral colic seems to be limited.

In conclusion, our study presented further evidence that human ureteral smooth muscle tension can be influenced by compounds interacting with the NO/cGMP-signaling pathway, such as PDE5 inhibitors. Nevertheless, it remains to be elucidated as to whether the in vitro effects of PDE5 inhibitors we observed can be put into a clinical use. Thus, future studies on ureteral smooth muscle relaxation and cyclic nucleotide turnover under more physiological settings will gain importance.

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