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ORIGINAL PAPER

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Involvement of cyclic nucleotides in renal artery smooth muscle relaxation

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Abstract The elevation of vascular smooth muscle tone in the renal arteries during kidney transplantation and nephron-sparing surgery plays a major role in postsurgical organ dysfunction. Therefore, a better understanding of the intracellular mechanisms of contraction and relaxation is of fundamental interest to improve urological treatment. The present study was designed to investigate the complex intracellular system of cyclic nucleotides involved in the regulation of smooth muscle relaxation by using swine renal artery rings in the Schuler organ bath. Phenylephrine (PE) induced dose-dependent and fully reversible isometric contractions with a threshold concentration of 10 nM and an EC_{50} of 804 nM. The receptor was identified as α_{1A}-subtype by the selective antagonist WB4101. Increasing the intracellular concentration of cyclic 3':5'-adenosine monophosphate (cAMP) by dibutyryl-cAMP (5 mM) and forskolin (5 μM) resulted in a decreased contractiltity of 48.0% and 76.3%, respectively. Elevation of the cytosolic content of cyclic 3':5'-guanosine monophosphate (cGMP) using dibutyryl-cGMP (1 mM), sodium nitroprusside (100 μM) and SIN-1 (100 μM) decreased the average PE-induced contraction by 16.4%, 41.9% and 62.4%, respectively. The unselective phosphodiesterase inhibitors theophylline (1 mM), papaverine (100 μM) and IBMX (5 mM) reduced the PE-induced contraction by 37.3%, 93.1% and 95.5%, respectively. Furthermore, selective inhibition of phosphodiesterases by milrinone (PDE_3-selective) resulted in a decreased contractility by 1.3% (50 μM), 29.5% (100 μM) and 93.5% (5 mM), and using rolipram (PDE_4 selective), the PE-induced contraction was inibited by 57.9% (50 μM) and 81.9% (100 μM). The results suggest the involvement of cAMP and cGMP in the relaxation of renal artery smooth muscle cells. Moreover, phosphodiesterases, especially PDE_3 and PDE_4, seem to play a critical role in the regulation of renal artery smooth muscle tone.

Keywords Smooth muscle relaxation · Renal artery myocytes · cAMP and cGMP · Cyclic nucleotides · Phosphodiesterases

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Introduction

A precise regulation of renal artery smooth muscle tone is of great interest in the field of urological surgery [16; Karsten and Eckert, manuscript submitted]. In order to improve the postsurgical organ function, intracellular mechanisms involved in arterial smooth muscle relaxation are the subject of current research.

The intracellular secondary messengers, the cyclic nucleotides cyclic 3':5'-adenosine monophosphate (cAMP) and cyclic 3':5'-guanosine monophosphate (cGMP), are regulated by a complex system of different enzymes including adenylyl cyclases (ACs), guanylyl cyclases (GCs) and phosphodiesterases (PDEs) and play a fundamental role in the regulation of multiple cellular functions [19]. It is well established in e.g. myocardial inotropy [38, 39], airway [42, 50, 51], gastrointestinal [33], ureteral [28, 48, 49] and vascular smooth muscle relaxation [14, 26, 30, 42]. cAMP and cGMP are synthesized from the respective nucleoside triphosphates, ATP and GTP, by the membrane-integrated ACs and GCs (also in soluble forms), respectively. Up to nine distinct forms of ACs can be distinguished [6, 23], whereas four subtypes of membrane-bound GCs (GC-A, -B, -C, -P) and one soluble GC (GC-S-α_1, -β_1, -β_2) have been described [6]. A family of isoenzymes, the phosphodiesterases (PDEs), hydrolyze the nucleotides into inactive monophosphates (AMP and GMP) [10, 19]. Multiple isoforms of PDEs can be distinguished according to their biochemical and pharmacological properties. The cAMP and cGMP

metabolism provides a sensitive mechanism for the precise regulation of cellular functions and is therefore of interest for renal preservation [19]. PDEs are a heterogenous group of hydrolytic enzymes with their major function being to regulate the intracellular cyclic nucleotide turnover by modulating the amplitude, duration, and termination of the cyclic nucleotide secondary messenger signals [4]. Eleven different PDE families have already been described, representing only the initial level of complexity [9, 19, 46]. They differ in their primary structures, affinities for cAMP and cGMP, responses to specific effectors, sensitivities to specific inhibitors, mechanisms of regulation and tissue distribution [11, 12, 19, 34]. PDEs exhibit very different biochemical and pharmacological properties [34]. PDEs play a role in e.g. olfaction, atrial natriuretic peptide regulation of aldosterone, catecholamine secretion, cardiac calcium channel control, platelet aggregation, insulin action, pulmonary vascular resistance, visual transduction and regulation of smooth muscle contractility [4, 19, 32, 45]. They are regulated by intracellular cAMP and cGMP concentrations, binding of Ca^{2+}/calmodulin, phosphorylation events, interaction with regulatory proteins, subcellular localization, and alterations in protein levels [19]. As in individual cells, differential expression of PDE isozymes has been observed, and cell type-specific properties are strongly suggested [4, 46], hence participating in the integration of multiple inputs into the complex modulation and termination of cyclic nucleotide signals and responses [12].

In summary, due to the remarkable complexity in other components of the cyclic nucleotide signaling systems (i.e. receptor families, ion channels, ACs, GCs, G-proteins, etc.), the presence of multiple PDE isoenzymes and their differential expression and regulation contribute to the control of cyclic nucleotide concentrations and their biological effects [12, 19, 34].

The purpose of the present study was to investigate the involvement of the cyclic nucleotide secondary messengers cAMP and cGMP and PDEs in the mechanisms of renal vascular smooth muscle relaxation.

Materials and methods

Preparation of renal arteries

Swine kidneys were freshly obtained from the slaughterhouse and transported in cold, calcium-free tyrode solution (140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4 with NaOH) to the laboratory. There the organ was freed from fat, decapsulated, and interlobar arteries prepared and removed [16; Karsten and Eckert, manuscript submitted]. In order to detect the arterial contractions responsible for the disturbance of renal perfusion, arterial rings were dissected rather than using longitudinal strips, i.e. the circumferential contraction was measured rather than the longitudinal.

Organ bath experiments

The contractile properties of swine renal artery smooth muscle rings were studied in the Schuler organ bath FMI IOA-5301 (Föhr Medical Instruments, Seeheim, Germany; Fig. 1). The pre-tension was determined by establishing the corresponding length-tension curve of the renal artery ring (not illustrated) [15, 16]. In the present experiments, a pre-tension of 2 g was established, which is in line with the observation in canine aorta and swine renal artery [16, 22, 31]. Contractions were evoked by application of the α_1-adrenoceptor selective agonist phenylephrine (PE). In all experiments isometric contractions of the tissue were registered on a FMI GM-2 force displacement transducer (Föhr Medical Instruments, Seeheim, Germany) and stored digitally on a Pentium III computer.

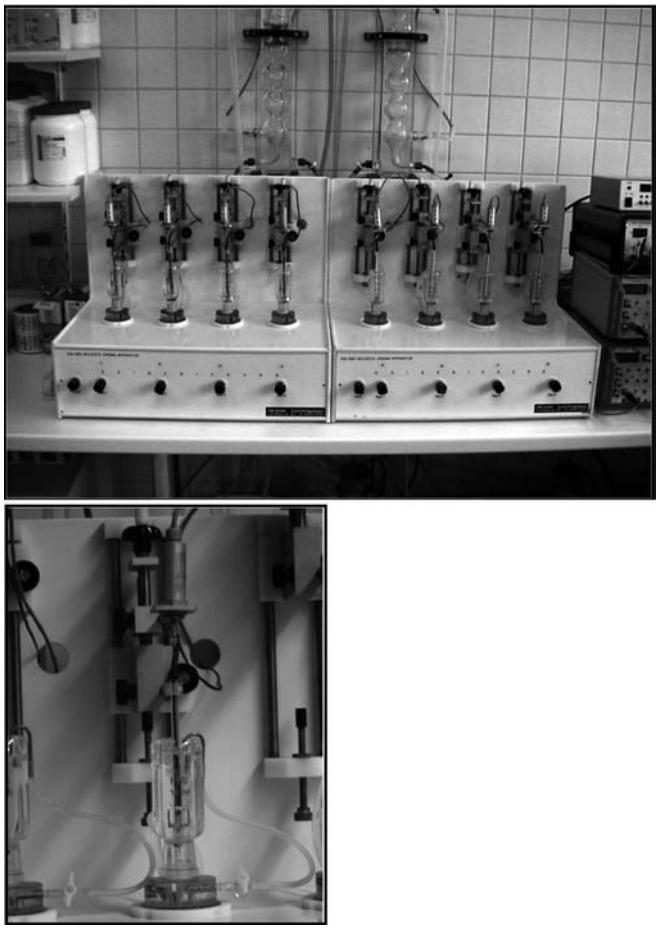

Fig. 1 Photograph of the Schuler organ bath FMI IOA-5301. The system consists of eight single organ bath units that enable simultaneous measurements. A single organ bath chamber is depicted to present the set-up: The arterial ring is placed inside the perfused chamber inbetween two stainless high-grade steel hooks, linked to the transducer. The output of the transducer is connected to the amplifier. The pre-tension of the renal artery ring is adjusted manually to 2 g at the pre-adjustment screw. The circumferential contractions of the rings are registered and stored digitally on a PC

Investigation of α-blockers. Three control contractions were evoked (10 μM PE), and the mean contractility of each arterial ring was quoted as 100%. Then the arterial rings were incubated for 20 min with the respective α-blocker non-cumulatively, i.e. a washout period of 20 min was applied before increasing the drug concentration. Dose-response curves of the α-blockers were obtained by inducing another PE contraction (10 μM) after incubation and comparing the remaining contractility with the mean contractility (100%).

Investigation of other substances. Three control contractions were evoked (10 μM PE), and the mean contractility of each arterial ring was quoted as 100%. Then the arterial rings were incubated with the respective substance in the respective concentration for 20 min. Another PE contraction (10 μM) was induced, and the remaining contractility of each arterial ring after incubation was compared to the mean contractility (100%).

Results are expressed as mean values ± standard error of mean (SEM), whereby the number of experiments and the SEM are shown in the figures.

Drugs and solutions

Krebs-Henseleit solution was composed as follows: 118 mM NaCl, 24.9 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.6 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, 1 mM sodium pyruvate, pH 7.4 with NaOH, temperature 37.0°C. The solution was aerated with 5% CO_2 and 95% O_2.

The following drugs were used: PE, HEPES, sodium pyruvate, prazosin, urapidil, WB4101, forskolin, N^6,2'-*o*-dibutyryl-cAMP (dbcAMP), N^2,2'-*o*-dibutyryl-cGMP (dbcGMP), sodium nitroprusside, 3-morpholinosydnonimine (SIN-1), theophylline, papaverine, 3-isobutyl-1-methylxanthine (IBMX), milrinone, rolipram (all from Sigma-Aldrich, Deisenhofen, Germany), phenoxybenzamine (from Calbiochem-Novabiochem, Bad Soden, Germany). All other chemicals were from Merck, Darmstadt, Germany.

Results

PE-induced contraction of renal artery smooth muscle rings

PE was used as selective α_1-adrenoceptor agonist. Application of PE resulted in dose-dependent, fully reversible and reproducible contractions of arterial rings without desensitization. Figure 2 depicts a representative time course of isometric contraction in response to cumulatively increasing concentrations of PE. The threshold concentration was 10 nM with maximal contractions in the micromolar range. The peak contraction was reached within 4.1 min ($n = 4$). By establishing the dose-response curve for the PE-induced contractions (10^{-9} M to 10^{-4} M) (not illustrated), an EC_{50} concentration of 804 nM was calculated [16; Karsten and Eckert, manuscript submitted].

Characterization of renal artery α_1-adrenoceptor

The adrenoceptor blockers phenoxybenzamine, prazosin, urapidil, and WB4101 have been used to identify the renal artery α_1-adrenoceptor subtype [8, 16, 18, 53; Karsten and Eckert, manuscript submutted]. The dose-response curves were obtained non-cumulatively, i.e. the next higher concentration was applied after several washouts. The antagonists used fully suppressed the contractions evoked by 10 μM PE at concentrations higher than 10^{-5} M. By establishing the dose-response curves for phenoxybenzamine, prazosin, urapidil and WB4101 (not illustrated here), EC_{50} concentrations of 2.46×10^{-8}, 1.19×10^{-8}, 2.43×10^{-7} and 6.11×10^{-9}, respectively, were calculated [16; Karsten and Eckert, manuscript submitted]. The renal artery α_1-adrenoceptor was identified as α_{1A}-subtype since WB4101, an α_{1A}-selective antagonist [36, 40, 53], showed the highest affinity.

Influence of increasing intracellular cAMP content on PE-induced renal artery smooth muscle contraction

Renal artery rings were incubated with the following substances in order to elevate the intracellular concentration of the secondary messenger cAMP. dbcAMP, a cAMP analogue able to permeate the cellular membrane and to mediate cAMP effects inside cells [41], was used at a concentration of 5 mM and depressed cellular contractility by 48.0% ($n = 8$, SEM = 5.8%). Furthermore, forskolin (known to directly stimulate adenylyl cyclase and hence generate cAMP [13, 23, 24]) was applied in a concentration of 5 μM and inhibited the PE-induced contraction by 76.3% ($n = 8$, SEM = 5.5%) (Fig. 3).

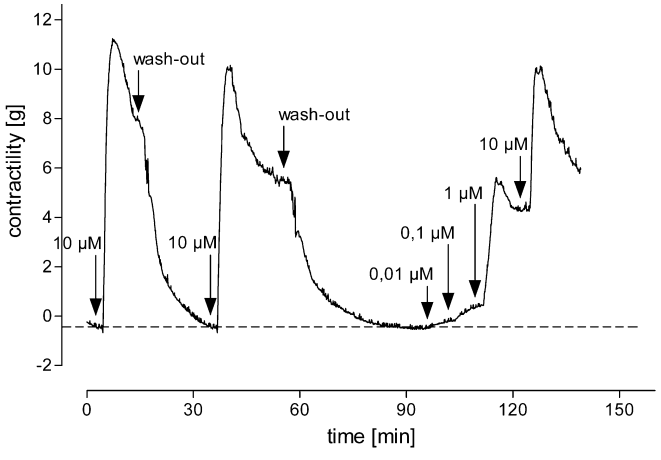

Fig. 2 Time course of an original trace of renal artery smooth muscle contraction in response to phenylephrine (PE). The *arrows* indicate the time points of PE administration, and the respective concentration is given

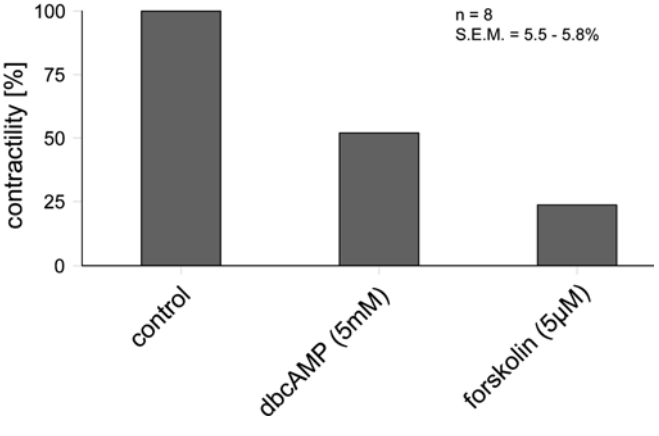

Fig. 3 Comparison of mean renal artery smooth muscle contractility decreased by maximal concentrations of N^6,2'-*o*-dibutyryl-cAMP (dbcAMP) and forskolin. Lower concentrations of the agents produced smaller inhibitory effects. Concentrations, SEM and number of experiments are given

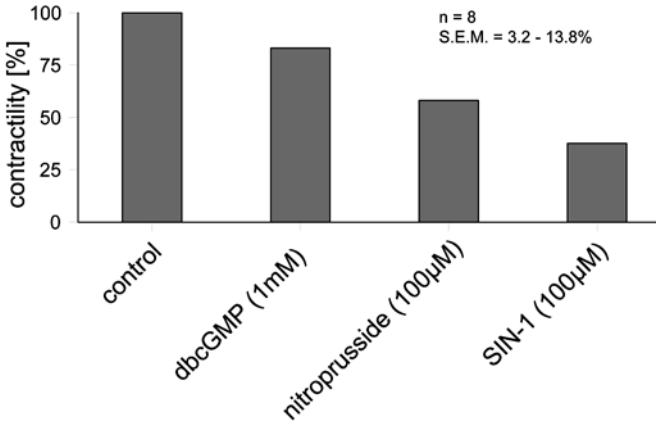

Fig. 4 Comparison of mean renal artery smooth muscle contractility decreased by maximal concentrations of dbcGMP, nitroprusside and 3-morpholinosydnonimine (SIN-1). Lower concentrations of the agents produced smaller inhibitory effects. Concentrations, SEM and number of experiments are given

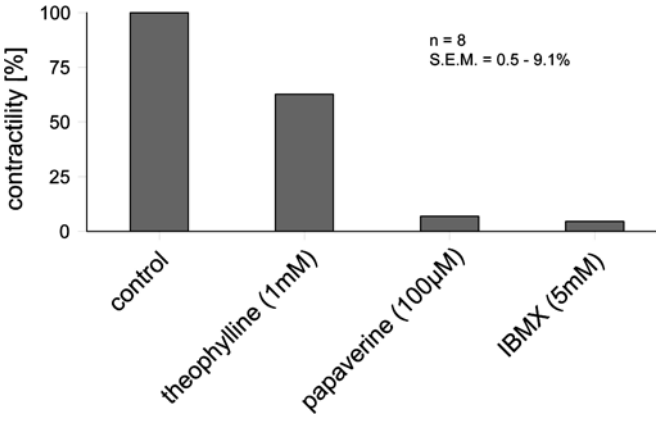

Fig. 5 Comparison of mean renal artery smooth muscle contractility decreased by maximal concentrations of unselective phosphodiesterase (PDE)-inhibitors theophylline, papaverine and 3-isobutyl-1-methylxanthine (IBMX). Lower concentrations of the agents produced smaller inhibitory effects. Concentrations, SEM and number of experiments are given

Influence of increasing intracellular cGMP content on PE-induced renal artery smooth muscle contraction

The membrane permeable cGMP analogue dbcGMP [52] at a concentration of 1 mM inhibited the PE-induced contraction by 16.4% ($n = 8$, SEM = 13.8%). Sodium nitroprusside and SIN-1 are both known to generate NO extracellularly, which permeates the cellular membrane and directly stimulates the cytoplasmic form of guanylyl cylcase (GC-S) [3, 23, 47]. Sodium nitroprusside (100 µM) depressed the contractility by 41.9% ($n = 8$, SEM = 3.2%) and SIN-1 (100 µM) by 62.4% ($n = 8$, SEM = 9.1%) (Fig. 4).

Blockade of PDEs decreases PE-induced renal artery smooth muscle contraction

To investigate the intracellular mechanisms further, various PDE-inhibitors were tested. In different experiments, the vascular rings were incubated with the unselective PDE-blockers theophylline, papaverine and IBMX [4, 5, 7, 19]. Theophylline at a concentration of 1 mM depressed the contractility by 47.3% ($n = 8$, SEM = 9.1%), papaverine (100 µM) inhibited the contraction by 93.1% ($n = 8$, SEM = 0.5%), and finally IBMX (5 mM) reduced the PE-induced contractility of the renal artery rings by 95.5% ($n = 8$, SEM = 0.5%) (Fig. 5). The PDE subtype III-selective inhibitor milrinone [1, 6, 19, 34] exerted depression of smooth muscle contractility in a dose-dependent manner. At a concentration of 50 µM, 1.3% inhibition ($n = 8$, SEM = 13.8%) was observed. When applied in a concentration of 100 µM, contractility was inhibited by 28.5% ($n = 8$, SEM = 14.8%), and with 5 mM, milrinone inhibited the contraction by 93.3% ($n = 8$, SEM = 6.5%) (Fig. 6). The PDE subtype IV-selective inhibitor rolipram [19, 34, 43] exerted stronger effects. Primarily, no effect on smooth muscle contractility was observed at a concentration of 10 µM. Application of 50 µM clearly reduced the PE-induced contraction by 57.9% ($n = 8$, SEM = 6.8%), and at a concentration of 100 µM, smooth muscle contractility was inhibited by 81.9% ($n = 8$, SEM = 4.8%) (Fig. 7).

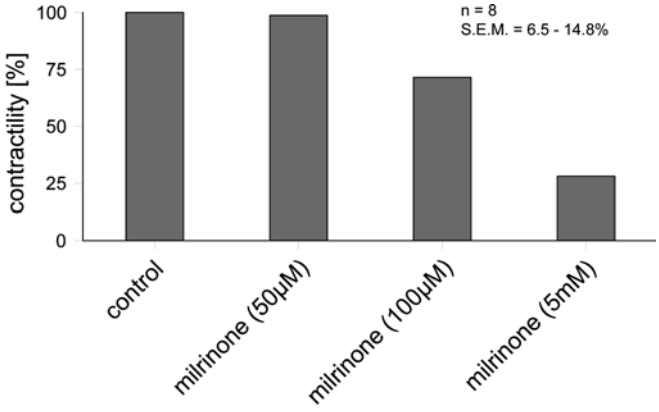

Fig. 6 Comparison of mean renal artery smooth muscle contractility decreased by the selective PDE III-inhibitor milrinone. Concentrations, SEM and number of experiments are given

Discussion

The present study was designed to investigate the involvement of cAMP, cGMP and PDEs in renal artery smooth muscle relaxation. The precise regulation of the intracellular amount of the secondary messengers cAMP and cGMP plays an important role in many physiological processes in different cellular functions. Pharmacological intervention at the level of the cyclic nucleotides is already established or has been proposed in a number of different maladies, e.g. bronchial

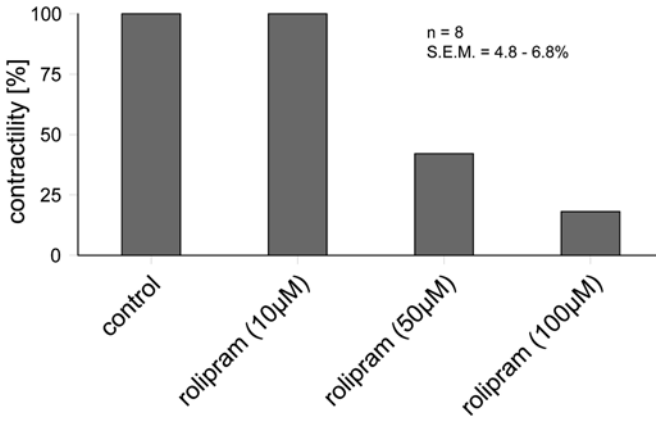

Fig. 7 Comparison of mean renal artery smooth muscle contractility decreased by the selective PDE IV-inhibitor rolipram. Concentrations, SEM and number of experiments are given

asthma, refractory congestive heart failure, hypertension, urge incontinence, erectile dysfunction. However, the treatment of postsurgical renal vasospasm at the level of cyclic nucleotide regulation by selective drugs could be a promising alternative and supplement to present therapeutic strategies. Therefore, the cellular mechanisms in renal myocytes have to be investigated [2, 16, 25, 27, 44; Karsten and Eckert, manuscript submitted].

The agonist PE induced dose-dependent, reversible and reproducible contractions in renal artery myocytes which were mediated by the α_1-receptor, identified as α_{1A}-subtype based on the highest affinity of the selective antagonist WB4101. The determination of the receptor subtype and the observed EC_{50} concentration of PE are comparable with those in other studies [8, 16, 37; Karsten and Eckert, manuscript submitted] and in accordance with radioligand binding studies performed in rat renal artery where an α_{1A}-subtype was predominant [18]. The addition of selective α_1-receptor-blockers to established conservation solutions in renal transplantation could be an interesting possibility to prevent the graft from undergoing surgically induced vasospasm.

Pretreatment of the artery rings with forskolin, a direct stimulator of adenylyl cyclase, and dbcAMP, a membrane-permeable analogue of cAMP, lead to a decreased PE-induced contractility. These results confirm the known effect of elevated intracellular cAMP that leads to smooth muscle relaxation [17, 21, 32]. Although there are no substances available yet that could be applied in a therapeutic way, increase of cAMP by selective drugs could be an interesting possibility in the prevention of renal vasospasm.

Application of dbcGMP, the membrane-permeable analogue of cGMP, resulted in a decreased contractility, indicating the vasodilating effect of cGMP [17, 21, 32] in renal arteries. Furthermore, the use of the NO-donors sodium nitroprusside and SIN-1 reduced the PE-induced contractility remarkably. As NO is known to stimulate cGMP formation by activation of soluble GC [6] and exerted vasodilatatory properties in the kidney in other studies [29], the NO/cGMP pathway also seems to be involved in renal vasodilation in the present study, which is in accordance with previous findings in ureteral relaxation [28, 48, 49]. However, NO-induced cGMP accumulation in the mouse bladder was found not to be related to smooth muscle relaxation [20]. Since NO-donors are already well established in the treatment of other diseases (e.g. hypertension), the use of drugs interfering with the NO/cGMP pathway may open a new avenue in the prevention of renal vasospasm after transplantation.

The use of unselective PDE-inhibitors resulted in a clear reduction of PE-induced contractility of renal artery rings. Hence, a general participation of PDEs in renal vascular smooth muscle relaxation is proposed, although the influence of distinct PDE-subfamilies cannot be distinguished with unselective substances. Since family-specific PDE-inhibitors, especially for PDEs III and IV, have facilitated the understanding of functions of individual PDEs in regulating specific cyclic nucleotide-mediated processes such as the relaxation of vascular smooth muscle cells [12], milrinone (PDE III-selective) and rolipram (PDE IV-selective) were used to evaluate the role of PDE III and IV in the mechanism of renal artery relaxation. Both substances showed a dose-dependent reduction of vascular contractility. While 50 µM and 100 µM milrinone only induced minor reductions of contractility, inhibitition was remarkable at a concentration of 5 mM. Rolipram already showed a remarkable inhibition of PE-induced contraction at a concentration of 50 µM, and interestingly had a great effect at a concentration of 100 µM. Compared with milrinone and the unselective inhibitors, rolipram seems to have a remarkably great potency in inhibiting adrenergically induced contractions in these cells. Hence, the participation of PDEs subtype III and, to a greater extent, subtype IV is proposed in the mechanism of renal vascular smooth muscle relaxation, which is in accordance with other studies [6, 19] and with the findings in rat aortic smooth muscle cells [35].

Due to their central role in smooth muscle tone regulation and the considerable variation of PDE isozymes in terms of their distribution and functional importance in certain tissues, PDEs have become an attractive target for drug development. Length and magnitude of the signal, the interactions of cAMP and cGMP with each other and with other signalling pathways, and the types of feedback regulation are all affected by the various PDEs that are expressed [6]. As distinct PDEs regulate specific cellular functions [46] and are involved in smooth muscle tone regulation, a specific modulation of individual PDE isoenzymes and of cAMP/cGMP metabolism with therapeutic agents could open a new avenue in pharmacological strategies of kidney preservation.

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