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ORIGINAL ARTICLE

Mechanisms involved in the adenosine-induced vasorelaxation to the pig prostatic small arteries

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Abstract Benign prostatic hypertrophy has been related with glandular ischemia processes and adenosine is a potent vasodilator agent. This study investigates the mechanisms underlying the adenosine-induced vasorelaxation in pig prostatic small arteries. Adenosine receptors expression was determined by Western blot and immunohistochemistry, and rings were mounted in myographs for isometric force recording. A_{2A} and A_3 receptor expression was observed in the arterial wall and A_{2A}-immunoreactivity was identified in the adventitia–media junction and endothelium. A_1 and A_{2B} receptor expression was not obtained. On noradrenaline-precontracted rings, P1 receptor agonists produced concentration-dependent relaxations with the following order of potency: 5′-*N*-ethylcarboxamidoadenosine (NECA)= CGS21680>2-Cl-IB-MECA=2-Cl-cyclopentyladenosine= adenosine. Adenosine reuptake inhibition potentiated both NECA and adenosine relaxations. Endothelium removal and ZM241385, an A_{2A} antagonist, reduced NECA relaxations that were not modified by A_1, A_{2B}, and A_3 receptor antagonists. Neuronal voltage-gated Ca^{2+} channels and nitric oxide (NO) synthase blockade, and adenylyl cyclase activation enhanced these responses, which were reduced by protein kinase A inhibition and by blockade of the intermediate (IK_{Ca})- and small (SK_{Ca})-conductance Ca^{2+}-activated K^+ channels. Inhibition of cyclooxygenase (COX), large-conductance Ca^{2+}-activated-, ATP-dependent-, and voltage-gated-K^+ channel failed to modify these responses. These results suggest that adenosine induces endothelium-dependent relaxations in the pig prostatic arteries via A_{2A} purinoceptors. The adenosine vasorelaxation, which is prejunctionally modulated, is produced via NO- and COX-independent mechanisms that involve activation of IK_{Ca} and SK_{Ca} channels and stimulation of adenylyl cyclase. Endothelium-derived NO playing a regulatory role under conditions in which EDHF is non-functional is also suggested. Adenosine-induced vasodilatation could be useful to prevent prostatic ischemia.

Keywords Pig prostatic small arteries · Endothelial A_{2A} purinoceptors · A_{2A} expression · NECA · Vasorelaxation · Adenylyl cyclase · IK_{Ca} channels · SK_{Ca} channels

Abbreviations

CGS21680	[4-2[[6-amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride
2-Cl-cyclopentyladenosine	2-chloro-N^6-cyclopentyladenosine
2-Cl-IB-MECA	[1-2-chloro-6-[[(3-iodophenyl) methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-*b*-D-ribofuranuronamide
ω-CgTX	ω-Conotoxin GVIA

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COX	Cyclooxygenases
DPCPX	8-Cyclopentyl-1, 3-dipropylxanthine
K_{ATP}	ATP-dependent K^+ channels
BK_{Ca}	Large-conductance Ca^{2+}-activated K^+ channels
IK_{Ca}	Intermediate-conductance Ca^{2+}-activated K^+ channels
SK_{Ca}	Small-conductance Ca^{2+}-activated K^+ channels
Kv	Voltage-gated K^+ channels
KT5720	(9*R*,10*S*,12*S*)-2,3,9,10,11, 12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo [3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, hexyl ester
L-NOARG	N^G-nitro-L-arginine
MRS1220	*N*-[9-chloro-2-(2-furanyl) [1,2,4]-triazolo[1,5-*c*] quinazolin-5-yl]benzene acetamide
NA	Noradrenaline
NECA	5′-*N*-ethylcarboxamidoadenosine
NO	Nitric oxide
PKA	Protein kinase A
PSB1115	4-(2,3,6,7-tetrahydro-2,6-dioxo-1-propyl-1*H*-purin-8-yl)-benzenesulfonic acid potassium salt
TRAM34	1-[(2-Chlorophenyl) diphenylmethyl]-1*H*-pyrazole
ZM241385	4-(-[7-amino-2-(2-furyl)[1,2,4] triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl) phenol

Introduction

Adenosine is an autacoid produced by the action of ecto-5′-nucleotidase on extracellular ATP that mediates different biological actions via membrane receptors denoted P1 purinoceptors [1]. Four subtypes of P1 receptors (A_1, A_{2A}, A_{2B}, and A_3) have been cloned and characterized based on their molecular structure, pharmacology, and mechanisms of G protein-mediated signaling mechanisms [2–4]. Adenosine A_1 and A_3 receptors are coupled to Gi/Go/Gq proteins and inhibit the activity of adenylyl cyclase, while adenosine A_{2A} and A_{2B} receptors are coupled to Gs ones and activate adenylyl cyclase, leading to cAMP accumulation and subsequent activation of protein kinase A (PKA) [3, 4]. Although all four adenosine receptors subtypes are found in vascular smooth muscle cells, only A_{2A} and A_{2B} have been shown to be present on endothelial cells [5–7]. A_3 purinoceptors have also been localized on endothelial cells in mouse aorta producing smooth muscle contraction, via cyclooxygenase-1 [8].

Purinergic signaling is involved both in short-term control of vascular tone and in longer-term control of cell proliferation, migration and death involved in vascular remodeling. There is a dual control of the vascular tone by ATP released from perivascular nerves and by ATP released from endothelial cells in response to changes in blood flow (shear stress) and hypoxia [9]. Both ATP and adenosine regulate smooth muscle and endothelial cell proliferation, these regulatory mechanisms being important under conditions associated with vascular endothelial dysfunction such as hypertension and atherosclerosis. These pathologies are risk factors for benign prostatic hypertrophy, thus opening the possibility for a pathophysiological role for purines under this disturbance [9–12]. In addition, adenosine receptor agonists have been proposed as anticancer agents, since the adenosine purinoceptor agonist 2-chloroadenosine modulates PAR-1 and IL-23 gene expression, thus suggesting a modulation of cancer metastasis and immune system activity [13].

Knowing the physiological mechanisms controlling prostatic vasculature is essential in order to a basic knowledge as well as to understand the prostate pathophysiology. Our group has recently characterized the mechanisms involved in the vasoconstriction of pig prostatic small arteries, and both endothelin 1, acting through constrictor muscle ET_A and relaxant endothelial ET_B receptors [14], and neurally released noradrenaline (NA), that activates postjunctional α_{1L}-adrenoceptors and prejunctional inhibitory autoreceptors [15], play a main role. The mechanisms involved in the relaxation of prostatic small arteries are relatively unknown [16], and there is no information about the role played by adenosine in the reactivity of the prostatic small arteries responsible for deep gland vascularization. Therefore, the aim of the present study was to investigate the mechanisms involved in the vasoactive effects induced by adenosine in the pig prostatic small arteries.

Material and methods

Collection and tissue dissection

Prostates were obtained from adult pigs at the local slaughterhouse immediately after the animals were killed, and placed in cold (4°C) physiological saline solution (PSS). The gland was opened, the adjacent connective and fatty tissues were removed and second-order branches of the prostatic artery, with an internal lumen diameter of 220–350 μm, which are distributed in the deep of the glandular parenchyma, were carefully dissected.

Western blotting

Prostatic arterial rings were homogenized in 50 mM Tris–HCl, pH 7.5, containing 125 mM NaCl, 10 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA; all from Sigma, USA), 1% Nonidet P 40 (Fluka Chemie, Buchs, Switzerland) and 1% Halt Protease Inhibitor (Thermo Scientific, Rockford, IL, USA). After centrifugation at 12,000×*g* for 5 min, the supernatant was collected and submitted to protein determination (BCA Protein Assay Kit, Rockford, IL, USA). Twenty micrograms protein samples were then separated by SDS-PAGE on a discontinuous (7.5% and 12%) acrylamide gel and transferred to a polyvinylidene fluoride membrane (Amersham, GE Healthcare, Buckinghamshire, UK). Blots were blocked with 1% casein in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Tween 20 (PBST, Bio-Rad, Hercules, CA, USA), for 1 h. The primary antibodies used were as follows: A_1 receptor, polyclonal rabbit antibody raised against rat A1 receptor (Lifespan Biosciences, Seattle, WA, USA). A_{2A} receptor, monoclonal mouse anti-human A_{2A} receptor antibody (Novus Biologicals, Cambridge, UK). A_{2B} receptor, polyclonal goat anti-human A_{2B} receptor antibody (Abnova, Walnut, CA, USA). A_3 receptor, polyclonal rabbit antibody raised against human A_3 receptor (MBL, Woburn, MA, USA). Blots were incubated with the corresponding receptor antibody, at a 1/100, 1/500, 1/100, or 1/200 dilution for the anti-A_1, anti-A_{2A}, anti-A_{2B}, or anti-A_3 receptor, respectively. Primary antibodies were incubated in PBST, at 4°C, overnight. Blots were then washed for 10 min with PBST, and incubated with a peroxydase-labeled anti-mouse (1/5,000 dilution, Amersham, GE Healthcare, Buckinghamshire, UK) or anti-goat (1/1,500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody, at room temperature, for 1 h. Enhanced chemiluminescence (ECL) was performed with an ECL Western blot detection kit (Amersham, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Blots were exposed to Hyperfilm ECL (Amersham, GE Healthcare, Buckinghamshire, UK), for 1 min. For determination of the molecular mass, a SDS-PAGE standard set (Amersham, GE Healthcare, Buckinghamshire, UK) was used.

Immunohistochemistry

Prostatic artery segments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 to 48 h at 4°C, and subsequently placed in 30% sucrose in 0.1 M phosphate buffer for cryoprotection. The tissue was frozen in CO_2 and stored at −80°C until sectioning. Transversal sections of 10 μm were obtained by means of a cryostat. The sections were processed for immunohistochemistry following the avidin–biotin–peroxidase complex (ABC) method. Samples were preincubated in 10% normal goat serum (NGS) in PB containing 0.3% Triton-X-100 for 2–3 h, an then incubated in the presence of A_{2A} receptor antibody (5 μg/ml concentration) in 2% NGS in PB containing 0.3% Triton-X-100, 4°C for 48 h. The sections were reacted with a biotinylated goat anti-mouse secondary serum (Chemicon International Inc, 1:400 dilution), for 2 h at room temperature. The specimens were incubated with ABC (Vector, 1:100 dilution), for 90 min at room temperature. The immunocomplex was visualized with 0.05% 3,3′diaminobenzidine and 0.001% H_2O_2 in PB. No immunoreactivity could be detected in sections incubated in the absence of the primary antisera.

Microvascular myograph

Arterial rings (2 mm long) were mounted on two 40-μm wires in a double microvascular myograph, by fixing one of the wires to a force transducer for isometric tension recordings and the second wire to a length displacement device. The small arteries were allowed to equilibrate in PSS at 37°C, pH 7.4, for 30 min. The relation between resting wall tension and internal circumference L_{100} corresponding to a transmural pressure of 100 mmHg for a relaxed vessel in situ was calculated. The arteries were set to an internal circumference L_1, given by $L_1=0.9\times L_{100}$. Preliminary experiments showed that the force development was close to maximal at this internal circumference in the present study.

The contractile ability of the strips was determined by exposing them to K^+-rich (124 mM) PSS (KPSS). Intact endothelium was evaluated by inducing a stable contraction with NA (1 μM), and then adding 10 μM acetylcholine. Relaxations larger than 80% were taken as evidence for the presence of endothelium. Relaxations to purinergic receptor agonists were carried out on 1 μM NA-induced tone. Responses to purinoceptor agonists were reproducible in at least two consecutive concentration–response curves (CRC). Thus, a first control curve was performed and after washing the preparation in PSS every 20 min during a total period of 80 min, the vessel was incubated with the specific treatment for 30 min, and then a new CRC was constructed. The NA-induced control level in arteries incubated with specific blockers was matched to that exhibited in control preparations. Neither of the used pharmacological treatments or mechanical removal of endothelium modify the initial basal tone. Control curves were run in parallel.

Calculations and statistics

For each relaxation CRC to adenosine receptor agonists, the drug concentration required to give 50% (EC_{50}) relaxation of the NA-induced contraction was estimated by computerized non-linear regression analysis (GraphPad Prism, San Diego, CA, USA). The sensitivity of the drugs is expressed in terms

of pD_2, where pD_2 is defined as the negative logarithms of EC_{50} ($pD_2 = -\log EC_{50}$ [M]). Results are expressed as mean± s.e.m. of *n* (number of arteries). Statistical significance of the differences was studied by Student's *t* test for paired observations and by analysis of variance (ANOVA) and an a posteriori Bonferroni method for multiple comparisons. Differences were considered significant with a probability level of $P<0.05$. *P* values are shown in the table and figure legends.

Drugs and solutions

The following drugs were used: adenosine, 4-aminopyridine (4-AP), apamin, atropine, N^G-nitro-L-arginine (L-NOARG) and NA all from Sigma (USA). [1-2-chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-*b*-D-ribofuranuronamide (2-Cl-IB-MECA), 2-chloro-N^6-cyclopentyladenosine (2-Cl-cyclopentyladenosine), ω-conotoxin GVIA (ω-CgTX), dipyridamole, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), [4-2[[6-amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl] benzene propanoic acid hydrochloride (CGS21680), forskolin, glibenclamide, iberiotoxin (IbTX), (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1, 6]benzodiazocine-10-carboxylic acid (KT5720), *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide (MRS1220), 5′-*N*-ethylcarboxamidoadenosine (NECA), 4-(2,3,6,7-tetrahydro-2,6-dioxo-1-propyl-1*H*-purin-8-yl)-benzenesulfonic acid potassium salt (PSB1115), 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM 34) and 4-(-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) all from Tocris (UK). CGS 21680, 2-Cl-IB-MECA, 2-Cl-cyclopentyladenosine, dipyridamole DPCPX, forskolin, glibenclamide, KT5720, MRS1220, NECA, PSB1115, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM34), and ZM241385 were dissolved in 1% dimethyl sulfoxide. The other drugs were dissolved in distilled water. The solvents, at the final concentration used in the bath, had no effect on the reactivity of the vessels. The composition of PSS was (in millimolar): NaCl 119, KCl 4.6, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, glucose 11, $CaCl_2$ 1.5, KH_2PO_4 1.2, EDTA 0.027. The solution was maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2 to maintain pH at 7.4. KPSS was PSS with KCl exchanged for NaCl on an equimolar basis. Stock solutions were prepared daily in distilled water.

Results

In normalized prostatic arterial rings, the amplitude of the 124 mM KPSS response amounted to 16.9±4.9 mN (*n*=106 preparations). NA (1 μM) induced a sustained contraction above basal tension of 14.3±2.8 mN (*n*=106).

Relaxations induced by adenosine receptor agonists

Adenosine and P1 receptor agonists induced relaxations in a concentration-dependent manner, with the following order of potency: NECA=CGS 21680>2-Cl-IB-MECA= 2-Cl-cyclopentyladenosine=adenosine (Fig. 1a and b and Table 1).

Effects of A_1, A_{2A}, A_{2B}, and A_3 receptor antagonists on the relaxations to NECA

ZM241385 (0.1 μM; Fig. 2b and Table 2), an A_{2A} receptor selective antagonist, reduced relaxations to NECA, whereas DPCPX (0.1 μM; Fig. 2a), PSB1115 (0.1 μM; Fig. 2c) and MRS1220 (10 μM) (Fig. 2d), antagonists of the A_1, A_{2B}, and A_3 receptors, respectively, failed to modify these responses (Table 2).

Effect of adenosine reuptake inhibition on the relaxations to adenosine or NECA

Dipyridamole (10 μM), an inhibitor of the adenosine reuptake, potently enhanced the relaxations to adenosine (pD_2 and E_{max} values being 5.2±0.1 and 5.7±0.1* and 92.7±4.3% and 100±0%, in control and in the presence of dipyridamole, respectively, $^*P<0.05$, paired *t* test versus control, $n=6$, Fig. 3a) or to NECA (pD_2 and E_{max} values being 5.1±0.1 and 6.3±0.1* and 94.3±2.8% and 100± 0%, in control and in the presence of dipyridamole, respectively, $^*P<0.05$, paired *t* test versus control, $n=6$, Fig. 3b).

Western blot of the A_1, A_{2A}, A_{2B} and A_3 receptors and immunoreactivity of the A_{2A} receptor

In western blots of prostatic small arteries, antibodies directed against the A_{2A} or the A_3 receptors recognized several bands. For the A_{2A} receptor blot, one of the bands was located at approximately 43 kDa (Fig. 4a), while the A_3 receptor blot showed a 39-kDa band (Fig. 4a); both bands corresponding to the expected molecular weights for its respective receptor. Western blots using antibodies directed against the A_1 or the A_{2B} receptor showed several bands, neither of them matching the expected molecular weight (38 and 36 kDa for the A_1 or the A_{2B} receptor, respectively). Immunohistochemical studies revealed the presence of small "patches" of A_{2A}-immunoreactives ($MsIgG_{2A}$-IR) at the boundary between muscular layer and adventitia (Fig. 4b) and in the endothelium (Fig. 4c).

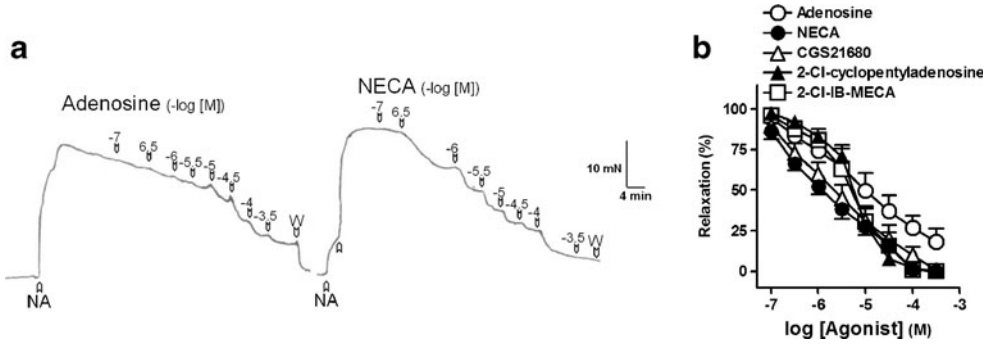

Fig. 1 **a** Isometric force recordings showing the relaxations evoked by adenosine (0.1–300 μM) and 5′-*N*-ethylcarboxamidoadenosine (*NECA*, 0.1–300 μM), on 1 μM noradrenaline (*NA*)-precontracted pig prostatic small arterial rings. *Vertical bar* shows tension in millinewtons and *horizontal bar* time in minutes. **b** Log concentration–response relaxation curves to adenosine (*open circle*), NECA (*closed circle*), [4-2[[6-amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride 2-chloro-N^6-cyclopentyladenosine (CGS 21680, *open triangle*), 2-chloro-N^6-cyclopentyladenosine (2-Cl-cyclopentyladenosine, *closed triangle*) and [1-2-chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-*b*-D-ribofuranuronamide (2-Cl-IB-MECA, *open square*). Results are expressed as a percentage of the NA-induced contraction and represent mean±s.e.m. of six to eight arteries

Effect of endothelium mechanical removal and of NO synthase and cyclooxygenases inhibitors on the relaxations to NECA

Mechanical removal of the endothelium potently reduced the relaxations to NECA (Fig. 5a and Table 3). L-NOARG (100 μM; Fig. 5b), blocker of nitric oxide (NO) synthase, potentiated the relaxations to NECA whereas, indomethacin (3 μM; Fig. 5c), a non-selective cyclooxygenases (COX) inhibitor, failed to modify these responses (Table 3).

Role of the PKA pathway and of the K^+ channels on the relaxations to NECA

A threshold concentration (30 nM) of the adenylyl cyclase activator forskolin evoked a leftward displacement of the relaxation CRC to NECA (Fig. 6a and Table 4). The PKA inhibitor KT 5720 (3 μM) induced a rightwards displacement of the relaxation CRC to NECA (Fig. 6b and Table 4).

Raising extracellular K^+ to 60 mM induced a sustained tone of 11.1±1.2 mN (n=6). NECA produced a concentration-dependent relaxation on 60 mM KPSS-precontracted arteries was lower than that obtained on NA-contracted rings (Fig. 7a, Table 5). In KPSS-precontracted rings, a situation in which the endothelial-dependent hyperpolarizing factor (EDHF) is inoperative, L-NOARG (100 μM) caused a profound reduction of the NECA relaxations (Fig. 7a, Table 5).

TRAM34 (20 nM; Fig. 7c) and apamin (0.5 μM; Fig. 7d), blockers of intermediate (IK_{Ca})- and small (SK_{Ca})-conductance Ca^{2+}-activated K^+ channels, respectively, reduced the relaxations to NECA (Table 5). However, IbTX (100 nM; Fig. 7b), glibenclamide (1 μM) and 4-AP (1 mM), inhibitors of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), ATP-dependent K^+ (K_{ATP}) and voltage-gated K^+ channels (Kv) channels, respectively, failed to modify the relaxations to NECA (Table 5).

Table 1 Relaxation induced by adenosine receptor agonists in the pig prostatic small arteries

	NECA			
	n	It (mN)	pD_2	E_{max} (%)
Adenosine	8	5.9±2.2	5.2±0.1	81.7±8.4
NECA	8	8.1±1.0	5.9±0.1*	100±0*
CGS21680	6	5.0±1.0	5.8±0.1*	99.4±0.5*
2-Cl-cyclopentyladenosine	6	6.7±1.6	5.2±0.1	100±0*
2-Cl-IB-MECA	6	6.9±1.3	5.3±0.1	99.8±0.1*

Results are expressed as mean±s.e.m. of *n* arteries. pD_2=−log EC_{50}, where EC_{50} is the concentration of agonist producing 50% of the E_{max}
It initial tension; E_{max} is the maximal relaxation, expressed as a percentage of the noradrenaline-induced contraction
*P<0.05 versus adenosine (ANOVA following Bonferroni t test)

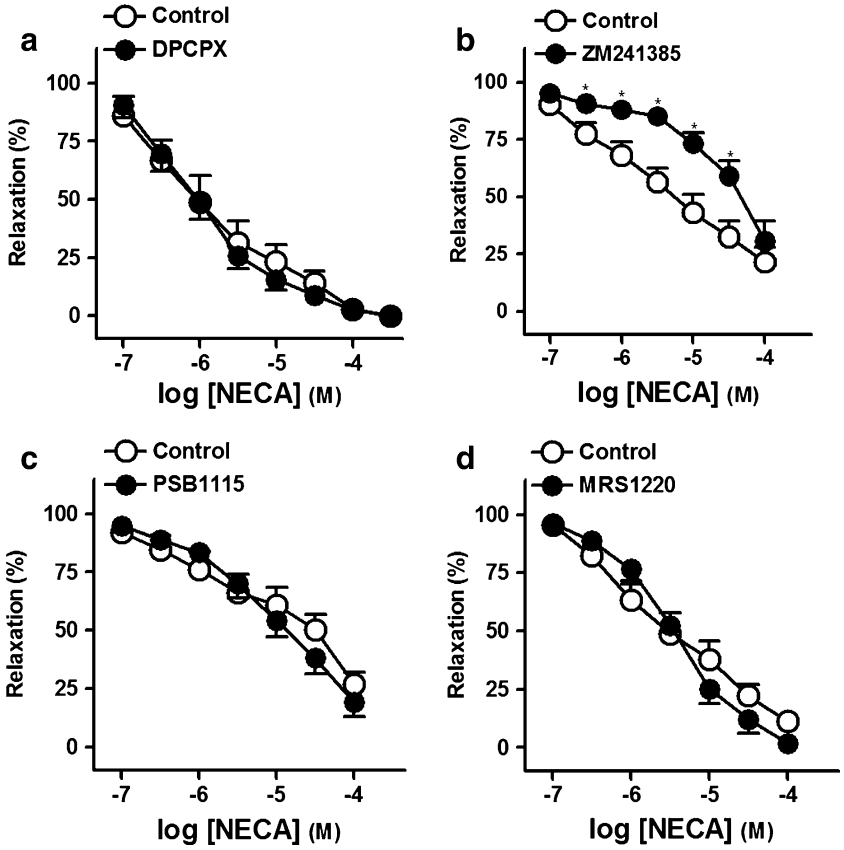

Fig. 2 Log concentration–response relaxation curves to 5′-*N*-ethylcarboxamidoadenosine (*NECA*), in the absence (*open circle*) and presence (*closed circle*) of 8-cyclopentyl-1,3-dipropylxanthine (*DPCPX*, 0.1 μM) (**a**), 4-(-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol (*ZM241385*, 0.1 μM) (**b**), 4-(2,3,6,7-tetrahydro-2,6-dioxo-1-propyl-1*H*-purin-8-yl)-benzenesulfonic acid potassium salt (*PSB1115*, 0.1 μM) (**c**) or *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide (*MRS1220*, 10 μM) (**d**), on 1 μM noradrenaline (*NA*)-precontracted pig prostatic small arteries. Results are expressed as a percentage of the NA-induced contraction and represent mean±s.e.m. of six to eight arteries. **P*<0.05 versus control value (paired *t* test)

Effect of the inhibitor of neuronal voltage-gated Ca^{2+} channels on the relaxations to NECA

ω-CgTX (1 μM; Fig. 8), a blocker of the neuronal voltage-gated Ca^{2+} channels, potentiated the relaxations to NECA (pD_2 and E_{max} values being 5.4±0.1 and 5.9±0.1* and 97.8±1.0% and 97.9±0.6%, in control and in the presence of ω-CgTX, respectively, **P*<0.05, paired *t* test versus control, *n*=6).

Table 2 Effects of blockers of adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors on relaxations evoked by 5′-*N*-ethylcarboxamidoadenosine (NECA, 0.1–300 μM) in the pig prostatic small arteries

	NECA			
	n	It (mN)	pD_2	E_{max} (%)
Control	8	5.6±1.1	6.0±0.1	100±0
DPCPX (0.1 μM)	8	4.9±1.1	6.0±0.1	100±0
Control	8	6.3±1.0	5.2±0.1	78.4±6.5
ZM241385 (0.1 μM)	8	7.0±1.2	4.3±0.1**	69.1±8.8
Control	6	6.2±1.3	4.6±0.1	72.8±5.0
PSB1115 (0.1 μM)	6	6.5±1.7	4.8±0.1	80.8±5.9
Control	6	2.7±1.2	5.5±0.2	90.8±3.1
MRS1220 (10 μM)	6	2.3±1.0	5.4±0.2	98.1±1.4

Results are expressed as mean±s.e.m. of *n* arteries. pD_2=−log EC_{50}, where EC_{50} is the concentration of agonist producing 50% of the E_{max}

It initial tension; E_{max} is the maximal relaxation, expressed as a percentage of the noradrenaline-induced contraction

***P*<0.01 versus control (paired *t* test)

Discussion

The present study was designed to investigate the involved mechanisms in the vasoactive effect of adenosine in the pig prostatic small arteries. Our results suggest that adenosine produces vasodilatation of the pig prostatic arteries through endothelial A_{2A} purinoceptors. Such relaxation is produced via NO- and COX-independent mechanisms that involve activation of IK_{Ca} and SK_{Ca} channels and stimulation of adenylyl cyclase. A prejunctional modulation of the adenosine-induced relaxation is also suggested.

Adenosine or its analogs are mostly directed toward adenosine-mediated effects on the cardiovascular system, such as the treatment of supraventricular arrhythmia, congestive heart failure, controlling blood pressure, attenuating reperfusion injury following regional myocardial infarction, reducing infarct size and incidence of arrhythmias, and improving post-ischemic cardiac function [17, 18]. Due to adenosine receptors differential coupling to either Gs (A_{2A} and A_{2B} receptors) or Gi (A_1 and A_3

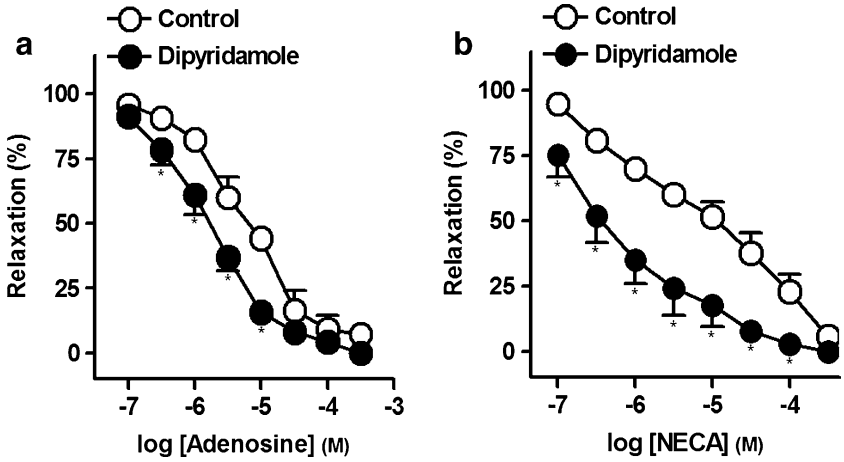

Fig. 3 Log concentration–response relaxation curves to adenosine (**a**) or 5′-*N*-ethylcarboxamido adenosine (*NECA*) (**b**), in the absence (*open circle*) and presence (*closed circle*) of dipyridamole (10 μM), on 1 μM noradrenaline (NA)-precontracted pig prostatic small arteries. Results are expressed as a percentage of the NA-induced contraction and represent mean±s.e.m. of six arteries. **P*<0.05 versus control value (paired *t* test)

receptors) proteins, along with the variable tissue distribution of adenosine receptor subtypes, adenosine elicits both relaxation (A_{2A}- and A_{2B}-mediated) and constriction (A_1- and A_3-mediated) in the peripheral and coronary vasculature. Vasodilatation is mainly due to the A_{2A} receptor [19]. A_1 and A_3 receptors inhibit coronary vasodilatation induced by A_{2A} and/or A_{2B} receptor activation [20].

Adenosine A_1 receptors cause porcine coronary arteries vasorelaxation, via activation of K^+ channels, as well as vasoconstriction [21, 22]. A negative modulatory role has been suggested for A_1 in A_{2A}- and A_{2B}-mediated coronary flow in isolated mouse hearts [20]. In fact, A_1 receptors protect against the injury caused by myocardial ischemia and reperfusion, by inhibiting adenylyl cyclase and by activating K_{ATP} channels [23]. The A_1 receptor-mediated contractile effect has been reported in mouse aorta and coronary arteries [24, 25]. A role for the phospholipase C (PLC)–protein kinase C (PKC) system has been suggested in adenosine A_1 receptor-mediated contraction of coronary vascular smooth muscle has also been demonstrated [25]. Other studies have shown that adenosine A_1 receptor enhances PKC expression in porcine coronary arteries [26]. These findings suggest that the PLC–PKC pathway has a major role in adenosine A_1 receptor-mediated vascular contraction of the coronary arteries and the aorta. In pig prostatic arteries, the low relaxant potency shown by the A_1 purinoceptor selective agonist 2-Cl-cyclopentyladenosine, and the lack of inhibitory effect shown by DPCPX, an adenosine A_1 receptor selective antagonist, on NECA relaxations, seem to rule out the mediation of an A_1 receptor in the purinergic relaxation mediation.

Adenosine A_3 receptors have previously been reported to induce a dual (contraction/relaxation) effect on vascular smooth muscle. Thus, A_3 receptors mediate in vivo

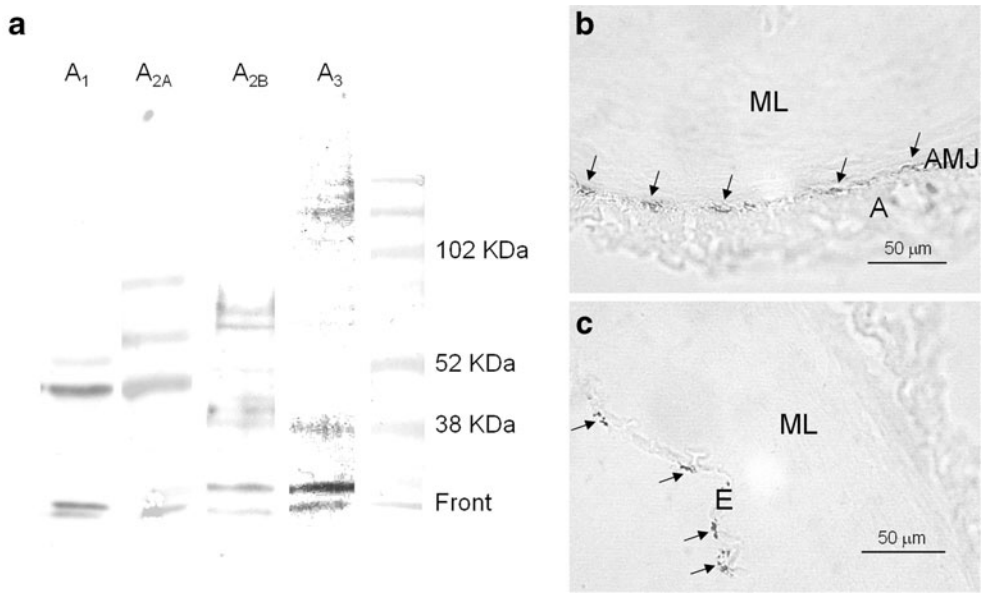

Fig. 4 **a** Western blot of pig prostatic small arteries membranes incubated with A_1, A_{2A}, A_{2B}, and A_3 receptor antibodies. The A_{2A} and the A_3 receptor antibodies show major bands of 43 and 39 kDa, respectively, compatible with the presence of these purinoceptor subtypes. **b**, **c** Immunohistochemical staining demonstrating the existence of A_{2A}-immunoreactivity (A_{2A}-IR) in the arterial wall. Small "patches" of A_{2A}-IR (*arrows*) on the border between the muscularis and adventitia layer (**b**) and in the endothelium (**c**) (*A* adventitia, *AMJ* adventitia–media junction, *ML* muscular layer, *E* endothelium)

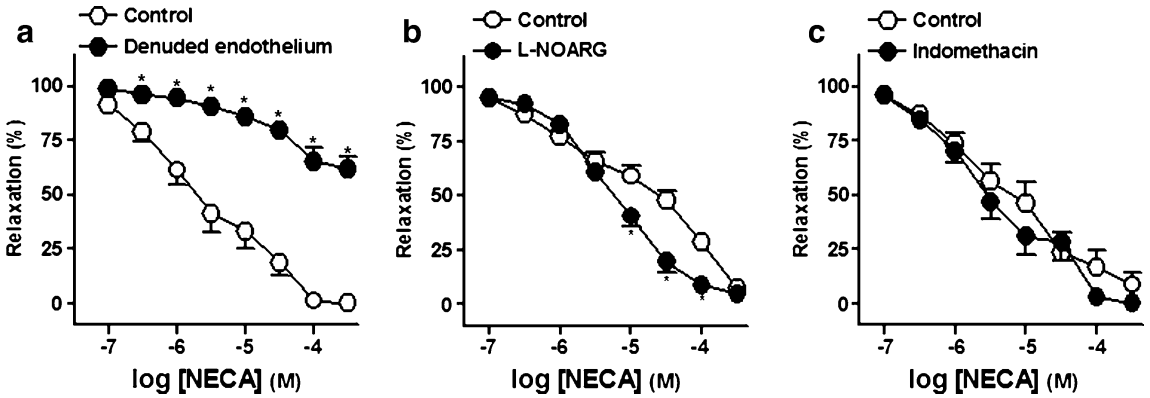

Fig. 5 Log concentration–response relaxation curves to 5′-*N*-ethylcarboxamidoadenosine (*NECA*), in the intact (control, *open circle*) and endothelium-denuded (*closed circle*) rings (**a**) and in the absence (*open circle*) and presence (*closed circle*) of N^G-nitro-L-arginine (*L-NOARG*, 100 μM) (**b**) or indomethacin (3 μM) (**c**), on 1 μM noradrenaline (*NA*)-precontracted pig prostatic small arteries. Results are expressed as a percentage of the NA-induced contraction and represent mean±s.e.m. of six to eight arteries. *$P<0.05$ versus control value (paired *t* test)

vasoconstriction by stimulation of mast cells and subsequent release of histamine and thromboxane [27] while a coronary vasodilator effect has also been demonstrated in rat [28]. In our study, the low relaxant potency shown by the A_3 receptor agonist 2-Cl-IB-MECA and the lack of inhibitory effect of the A_3 receptor selective antagonist MRS 1220 on NECA-induced relaxations seems to rule out the mediation of these receptors in adenosine responses. The fact that A_3 receptors were expressed in pig prostatic arteries agrees with the enigmatic role previously described for these receptors, whose function is not fully known [29]. However, A_3 selective ligands might be useful for the treatment of pathologies such as glaucoma, asthma, arthritis or cancer, in which inflammation is present [29].

Adenosine A_2 receptor subtypes have been involved in the vasorelaxation of the coronary and mesenteric vascular bed of several species, including humans. Thus, adenosine A_{2A} receptors have been described in coronary vessels from rat mesenteric arteries [30], while adenosine A_{2B} receptors mediate vasorelaxation of human coronary arteries [31]. It has recently been suggested that there is a compensatory upregulation of the adenosine A_{2B} receptor in an adenosine A_{2A} receptor knock-out mouse model [32]. In pig prostatic arteries, CGS21680, an A_{2A} receptor selective agonist, produced a significant vascular smooth muscle relaxation. This fact, together with the agonist potency order in producing relaxation (NECA=CGS21680>2-Cl-IB-MECA 2-Cl-cyclopentyladenosine=adenosine), suggests an A_{2A} receptor subtype mediation [33]. Agonist-based purinoceptors characterization requires, however, some caution, since the rank order of agonist potency may substantially be influenced by the different susceptibilities of purine analogs to degradation by ecto-nucleoside triphosphate diphosphohydrolase [34]. The involvement of the A_{2A} subtype in the NECA-induced vasorelaxation is confirmed by the inhibition produced by the adenosine A_{2A} receptor selective

Table 3 Effects of endothelium mechanical removal and of blockers of nitric oxide synthase and cyclooxygenases on relaxations evoked by 5′-*N*-ethylcarboxamido adenosine (NECA, 0.1–300 μM) in the pig prostatic small arteries

	NECA			
	n	It (mN)	pD_2	E_{max} *(%)*
Endothelium-intact (Control)	7	7.4±1.4	5.6±0.1	100±0
Endothelium-denuded	7	7.2±2.0	–	37.9±5.7***
Control	8	4.8±1.2	4.8±0.1	92.8±2.5
L-NOARG (100 μM)	8	5.7±1.6	5.2±0.1*	95.3±2.4
Control	6	5.1±0.9	5.2±0.1	91.1±5.5
Indomethacin (3 μM)	6	4.8±1.4	5.4±0.1	99.8±0.2

Results are expressed as mean±s.e.m. of *n* arteries. $pD_2=-\log EC_{50}$, where EC_{50} is the concentration of agonist producing 50% of the E_{max}
It initial tension; E_{max} is the maximal relaxation, expressed as a percentage of the noradrenaline-induced contraction
*$P<0.05$ and ***$P<0.001$ versus control (paired *t* test)

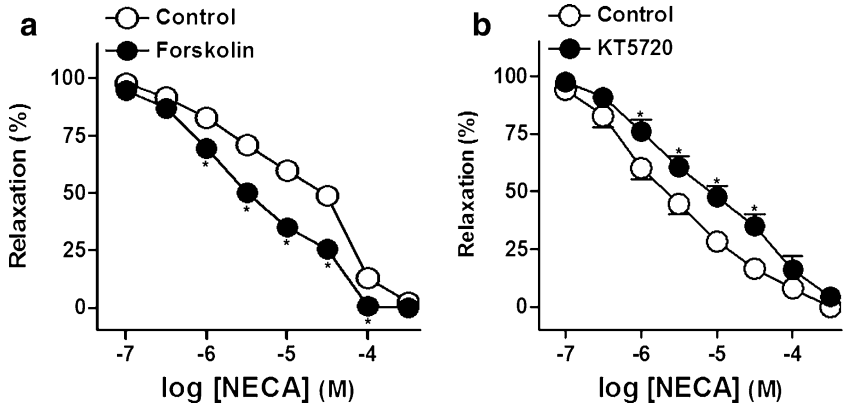

Fig. 6 Log concentration–response relaxation curves to 5′-*N*-ethylcarboxamidoadenosine (*NECA*), in the absence (*open circle*) and presence (*closed circle*) of forskolin (30 nM) (**a**) or KT 5720 (3 μM) (**b**), on 1 μM noradrenaline (*NA*)-precontracted pig prostatic small arteries. Results are expressed as a percentage of the NA-induced contraction and represent mean±s.e.m. of six to seven arteries. *$P<0.05$ versus control value (paired *t* test)

antagonist, ZM241385. A_{2A} receptors participation in pig prostatic arteries was also confirmed by adenosine A_{2A} receptor expression studies. Western blot assays showed a band at about 43 kDa, compatible with that expected for the A_{2A} receptor, and immunostaining of prostatic arteries samples with an A_{2A} antibody revealed a labeling of small A_{2A}-IR "patches" in the adventitia–media junction and in the intima layer. The fact that NECA acts not only on postsynaptic endothelial receptors but also on presynaptic ones located at nerve endings in the arterial preparations can support the variability observed in NECA-induced relaxations [15]. The fact that the non-selective adenosine analog NECA produced relaxation with a potency similar to that of the A_{2A}-selective CGS 21680 suggests the mediation of another adenosine receptor subtype (possibly the A_{2B} receptor) contributing to this relaxation. However, the lack of effect produced by the A_{2B} receptor selective antagonist PSB1115, as well as the absence, in Western blot studies, of a band compatible with the A_{2B} subtype, seems to exclude the expression of these receptors in the pig prostatic small arteries. On the other hand, dipyridimole, an inhibitor of the adenosine reuptake, potentiated the NECA- or adenosine-induced relaxation. This result could be explained on the basis that increased adenosine levels would preferentially activate receptors with a high affinity for adenosine, such as the A_1 subtype [3]. Western blot assays, however, did not show A_1 receptor expression, and an A_1 receptor selective antagonist had no effect on NECA relaxations, which seems to discard an A_1 receptor participation in the purinergic relaxations. The A_3 receptor, while being expressed in our preparation, neither seems to be involved in adenosine or NECA relaxations, as they were not modified by the A_3 receptor selective antagonist MRS1220. Purinergic relaxations in our preparation, therefore, seem to be due to the A_{2A} subtype present in the endothelium of the prostatic small arteries. These results agree with those obtained in rabbit corpora cavernosa, where dipyridamole enhances the A_{2A} mediated adenosine-induced relaxation [35]. Since adenosine plays an essential role in the vasorelaxation of the prostatic vascular bed, compounds that enhance adenosine levels, such as dipyridamole, may be useful to prevent prostatic glandular ischemia.

Table 4 Effects of adenylyl cyclase activation and of protein kinase A inhibition on relaxations evoked by 5′-*N*-ethylcarboxamidoadenosine (NECA, 0.1–300 μM) in the pig prostatic small arteries

	NECA			
	n	It (mN)	pD_2	E_{max} (%)
Control	7	7.3±0.8	4.8±0.1	97.7±1.7
Forskolin (30 nM)	7	6.0±1.0	5.4±0.1**	100±0
Control	6	8.0±3.0	5.6±0.1	100±0
KT5720 (3 μM)	6	6.0±1.6	5.1±0.1*	95.5±1.9

Results are expressed as mean±s.e.m. of *n* arteries. pD_2=−log EC_{50}, where EC_{50} is the concentration of agonist producing 50% of the E_{max}

It initial tension; E_{max} is the maximal relaxation, expressed as a percentage of the noradrenaline-induced contraction

*$P<0.05$ and **$P<0.01$ versus control (paired *t* test)

Both adenosine A_2 receptor subtypes can mediate hyperpolarization of smooth muscle and NO release from vascular endothelium [6]. Cell culture studies have demonstrated an adenosine A_{2A}- and A_{2B}-receptor-mediated NO release in porcine and human coronary endothelial cells [5, 6]. However, very few functional studies have demonstrated that NO release is responsible for adenosine A_{2A}- or A_{2B}-mediated coronary vasodilatation. It has been suggested that endogenously released adenosine and prostanoids induce NO- and/or K_{ATP} channel-dependent vasorelaxation and thereby modulate basal coronary tone [36]. Whereas adenosine A_{2A} receptor plays a significant role in background NO release, thus affecting basal coronary tone [32], the role played by adenosine A_{2B} receptors in NO release remains to be determined. In addition, both adenosine A_{2A} and A_{2B} receptors have also been implicated in endothelium-independent relaxation of coronary artery smooth muscle [37]. In our study, mechanical removal of the endothelium

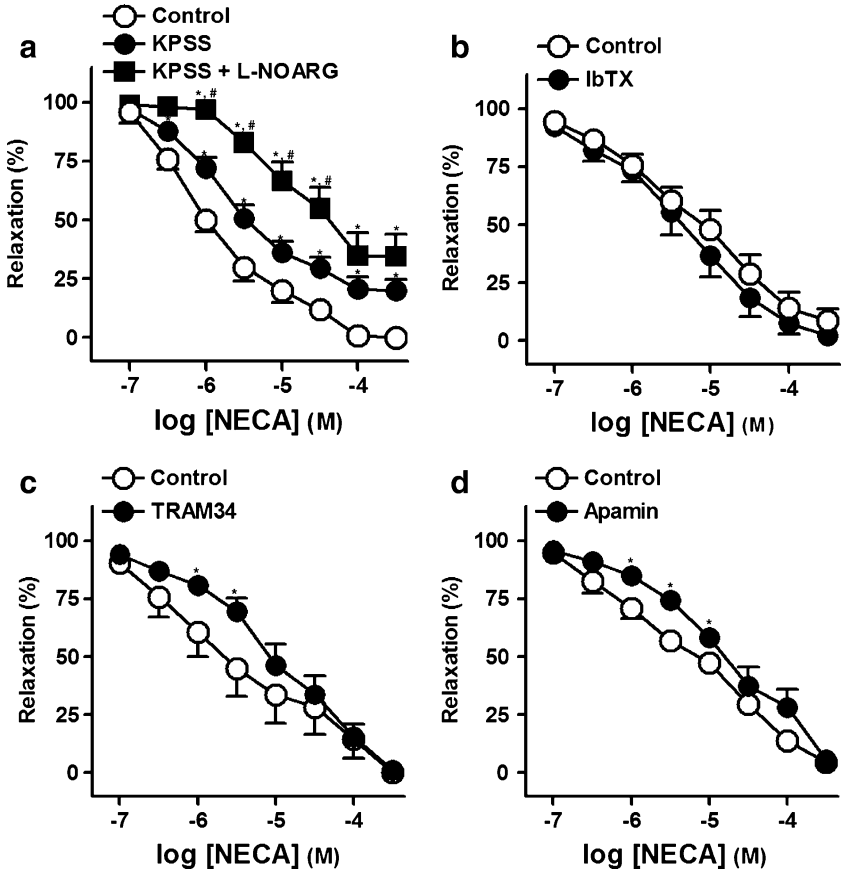

Fig. 7 **a** Log concentration–response relaxation curves to 5′-*N*-ethylcarboxamidoadenosine (*NECA*), on 1 μM noradrenaline (*NA*)- (*control*, *open circle*) or 60 mM K^+ rich physiological saline solution (*KPSS*)-precontracted pig prostatic small arteries, in the absence (*closed circle*) and presence (*closed square*) of N^G-nitro-L-arginine (L-NOARG, 100 μM). Concentration–response relaxation curves to NECA in the absence (*open circle*) and presence (*closed circle*) of iberiotoxin (*IbTX*, 100 nM) (**b**), 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM 34, 20 nM) (**c**), or apamin (0.5 μM) (**d**), on 1 μM NA-precontracted rings. Results are expressed as a percentage of the NA-induced contraction and represent mean ±s.e.m. of six to seven arteries. $^{*,\#}P<0.05$ versus control and KPSS, respectively (paired *t* test for paired observations and analysis of variance and an a posteriori Bonferroni method for multiple comparisons)

potently reduced the relaxations to NECA, thus indicating that adenosine relaxes pig prostatic arteries through adenosine receptors located on endothelial cells. On the basis of the reduction produced by endothelium removal, several mediators such as NO, prostanoids and/or EDHF could be expected to be the mediators released by the vascular endothelium. However, NO synthase blockade enhanced the relaxations to NECA, thus ruling out the involvement of NO released from vascular endothelium in the responses to adenosine. The potentiation produced ω-CgTX, a blocker of the neuronal voltage-gated Ca^{2+} channels, on the NECA relaxations suggests the involvement of neuronal mechanisms in the adenosine-induced vasodilatation. The fact that the potentiation produced by L-NOARG was observed at high concentrations could be ascribed to the mediation of prejunctional mechanisms, such as that found in lamb small coronary arteries, where the blockade of NO synthase enhanced the relaxations to ATP, suggesting a nerve modulation of purinergic relaxations [38].

In addition to NO, the COX pathway has also been involved in the vasorelaxations to adenosine [39]. In pig prostatic arteries, indomethacin, a non-selective COX inhibitor, failed to modify the relaxations to NECA, thus excluding a possible role of prostanoids in these responses. However, our results suggest the mediation of EDHF, on the basis of the L-NOARG-resistant NECA relaxations obtained in 60 mM KPSS-precontracted rings. The fact that endothelium-derived NO plays a regulatory role under conditions in which EDHF is non-functional suggests that endothelium-derived relaxing (EDRF) and EDHF factors can act via independent signaling pathways. Thus, acetylcholine produces vasorelaxation in guinea-pig basilar artery [40] and vasodilation in the rat kidney in vivo [41] by causing release of both EDRF and EDHF but through different mechanisms.

Adenosine A_{2A} receptor-induced vasodilatation is positively coupled to adenylyl cyclase, leading to a cytoplasmic cAMP elevation [42]. cAMP-dependent relaxations in vascular smooth muscle are generally mediated by PKA activation. However, other second-messenger systems, such as those of phosphatidylinositol 3-kinase, tyrosine kinase and PLC, may also be activated by adenosine receptors [20], even their roles in mediating adenosine vasoactive effects have not clearly been defined. In addition, crosstalk

Table 5 Effects of extracellular K^+ raising and of blockers of NO synthase, large-, intermediate-, small-conductance Ca^{2+}-activated-, ATP-dependent-, and voltage-gated-K^+ channels on relaxations evoked by 5′-*N*-ethylcarboxamidoadenosine (NECA, 0.1–300 μM) in the pig prostatic small arteries

	NECA			
	N	It (mN)	pD_2	E_{max} (%)
Control (on NA-induced contraction)	6	5.4±1.7	5.9±0.1	100±0
Control (on KPSS-induced contraction)	6	4.7±1.7	5.2±0.1*	79.9±4.8*
L-NOARG (100 μM) (on KPSS-induced contraction)	6	4.8±1.5	4.3±0.1*,**	65.4±9.2*
On NA-induced contraction				
Control	6	10.2±2.1	5.3±0.1	97.7±1.7
IbTX (100 nM)	6	10.3±3.1	5.2±0.1	91.2±5.1
Control	7	7.3±1.5	5.6±0.1	99.4±0.3
TRAM34 (20 nM)	7	5.9±1.3	5.0±0.1*	98.7±0.8
Control	6	6.3±1.3	5.2±0.1	95.5±2.3
Apamin (0.5 μM)	6	5.7±1.9	4.7±0.1*	94.3±2.7
Control	6	5.7±0.7	5.7±0.2	99.7±0.3
Glibenclamide (1 μM)	6	4.9±1.1	5.7±0.2	100±0
Control	6	7.2±1.6	5.6±0.2	98.6±0.8
4-AP (1 mM)	6	6.0±1.2	5.7±0.1	95.2±1.1

Results are expressed as mean±s.e.m. of *n* arteries. pD_2=−log EC_{50}, where EC_{50} is the concentration of agonist producing 50% of the E_{max}
It initial tension, E_{max} is the maximal relaxation, expressed as a percentage of the 1 μM noradrenaline (NA)- or 60 mM KPSS-induced contraction
*,**$P<0.05$ versus control and potassium-enriched (60 mM) saline solution (KPSS), respectively (paired *t* test for paired observations and analysis of variance and an a posteriori Bonferroni method for multiple comparisons)

between the cAMP/PKA pathway and the PLC/PKC pathway has also been reported [43]. In the current study, the potentiation by the adenylyl cyclase activator forskolin of the NECA relaxations and their reduction by the PKA inhibitor KT 5720 suggest that NECA produces relaxation of pig prostatic arteries in part via activation of adenosine receptors linked to the PKA pathway.

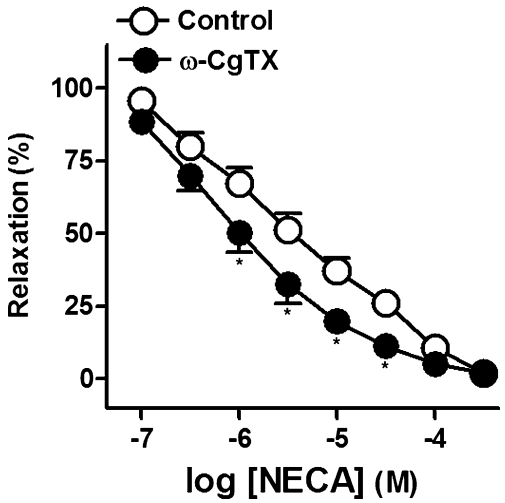

Fig. 8 Log concentration–response relaxation curves to 5′-*N*-ethylcarboxamidoadenosine (*NECA*), in the absence (*open circle*) and presence (*closed circle*) of ω-conotoxin GVIA (*CgTX*, 1 μM), on 1 μM noradrenaline (*NA*)-precontracted pig prostatic small arteries. Results are expressed as a percentage of the NA-induced contraction and represent mean±s.e.m. of six arteries. *$P<0.05$ versus control value (paired *t* test)

K^+ channels have been reported to be involved in the vasorelaxations to adenosine. Thus, BK_{Ca} channel modulation is involved in cAMP-mediated relaxation in adenosine-induced canine coronary vasodilatation [44]. In vascular smooth muscle, an IK_{Ca} channel upregulation seems to compensate a deficient activity of SK_{Ca} channels in the arteries of spontaneously hypertensive rats [45]. IK_{Ca} and SK_{Ca} channels are important for the regulation of a variety of neuronal and non-neuronal functions. Their presence in human prostate suggests that these channels are likely to have different biological functions and could be specifically targeted for human diseases, such as prostatism [46]. In the current study, the lower NECA relaxation obtained as a consequence of the elevation of extracellular K^+, the reduction produced by TRAM 34 and by apamin, inhibitors of the IK_{Ca} and SK_{Ca} channels, respectively, and the lack of effect shown by IbTX, a blocker of BK_{Ca} channels, suggest the activation of IK_{Ca} and SK_{Ca} channels in the NECA vasorelaxations. Both K_{ATP} and K_{Ca} are involved in adenosine A_{2A} and A_{2B} receptors-mediated hyperpolarization, which also leads to NO release in coronary endothelial cells [5]. In addition to these channels, Kv channels are involved in adenosine-mediated relaxation of coronary

arterioles from pig males [47]. Kv channels of the of the Kv1.3 family have also been reported in rat prostate epithelial cells, and a significant increase in Kv channel expression, particularly of the Kv1 and Kv2 subfamily members, has been shown to be involved in the proliferation of prostate carcinoma [48, 49]. In pig prostatic arteries, glibenclamide and 4-AP, blockers of the K_{ATP} and Kv channels, respectively, failed to modify the NECA relaxations thus ruling out the involvement of these channels.

Conclusion

Our results suggest that adenosine induces vasorelaxation of the pig prostatic small arteries through endothelial A_{2A} purinoceptors. Such relaxation, which is prejunctionally modulated, is produced via NO- and COX-independent mechanisms that involve activation of IK_{Ca} and SK_{Ca} channels and stimulation of adenylyl cyclase. In these arteries, the possibility that EDRF and EDHF act via independent mechanisms is also suggested. Adenosine-induced vasodilatation could be useful to prevent prostatic ischemia.

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Conflict of interest statement None.

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