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A_{2B} adenosine receptors mediate relaxation of the pig intravesical ureter: adenosine modulation of non adrenergic non cholinergic excitatory neurotransmission

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1 The present study was designed to characterize the adenosine receptors involved in the relaxation of the pig intravesical ureter, and to investigate the action of adenosine on the non adrenergic non cholinergic (NANC) excitatory ureteral neurotransmission.

2 In U46619 (10^{-7} M)-contracted strips treated with the adenosine uptake inhibitor, nitrobenzylthioinosine (NBTI, 10^{-6} M), adenosine and related analogues induced relaxations with the following potency order: 5′-N-ethylcarboxamidoadenosine (NECA) = 5′-(N-cyclopropyl)-carboxamidoadenosine (CPCA) = 2-chloroadenosine (2-CA) > adenosine > cyclopentyladenosine (CPA) = N^6-(3-iodobenzyl)-adenosine-5′-N-methylcarboxamide (IB-MECA) = 2-[p-(carboxyethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680).

3 Epithelium removal or incubation with indomethacin (3×10^{-6} M) and L-N^G-nitroarginine (L-NOARG, 3×10^{-5} M), inhibitors of prostanoids and nitric oxide (NO) synthase, respectively, failed to modify the relaxations to adenosine.

4 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 10^{-8} M) and 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385, 3×10^{-8} M and 10^{-7} M), A_1 and A_{2A} receptor selective antagonists, respectively, did not modify the relaxations to adenosine or NECA. 8-phenyltheophylline (8-PT, 10^{-5} M) and DPCPX (10^{-6} M), which block A_1/A_2-receptors, reduced such relaxations.

5 In strips treated with guanethidine (10^{-5} M), atropine (10^{-7} M), L-NOARG (3×10^{-5} M) and indomethacin (3×10^{-6} M), both electrical field stimulation (EFS, 5 Hz) and exogenous ATP (10^{-4} M) induced contractions of preparations. 8-PT (10^{-5} M) increased both contractions. DPCPX (10^{-8} M), NECA (10^{-4} M), CPCA, (10^{-4} M) and 2-CA (10^{-4} M) did not alter the contractions to EFS.

6 The present results suggest that adenosine relaxes the pig intravesical ureter, independently of prostanoids or NO, through activation of A_{2B}-receptors located in the smooth muscle. This relaxation may modulate the ureteral NANC excitatory neurotransmission through a postsynaptic mechanism.

Keywords: Pig intravesical ureter; adenosine receptors; electrical field stimulation; 8-PT; DPCPX; ZM 241385; NANC neurotransmission

Abbreviations: 2-CA, 2-chloroadenosine; 8-PT, 8-phenyltheophylline; ACh, acetylcholine; CGS21680, 2-[p-(carboxyethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine; CPA, cyclopentyladenosine; CPCA, 5′-(N-cyclopropyl)-carboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; IB-MECA, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; NBTI, nitrobenzylthioinosine; NECA, 5′-N-ethylcarboxamidoadenosine; ZM 241385, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

Introduction

ATP and related nucleotides and nucleosides have been described to be involved in non adrenergic non cholinergic (NANC) neurotransmission (Burnstock, 1972; Burnstock *et al*., 1978). ATP is converted to adenosine by the action of ecto-ATPases and nucleotidases, which are present in the surroundings of the nerve terminal (McDonald & White, 1984; Cusack & Hourani, 1984; Cusack *et al*., 1988). Once formed, adenosine acts on presynaptic receptors to depress further ATP release (Khakh & Kennedy, 1998).

Physiological and pharmacological effects of purines are mediated *via* two main classes of receptors, designated as P_1 and P_2, which primarily respond to adenosine and ATP, respectively (Burnstock, 1978). Smooth muscle P_1-purinoceptors generally cause relaxation (Burnstock, 1978) although in a number of smooth muscle preparations adenosine cause contraction *via* these receptors (Bailey *et al*., 1992). P_2-purinoceptors have recently been termed P2 receptors since they are receptors for a purine or pyrimidine nucleotide or dinucleotide. These receptors have been classified into the ionotropic P2X and the metabotropic P2Y. P2X operate *via* intrinsic ion channels mediating fast and transient responses while P2Y are G protein-coupled receptors which mediate slower responses (Abbracchio & Burnstock, 1994; Fredholm *et al*., 1994; 1997).

P_1-receptors have been divided in four subtypes, designated as A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al*., 1994; Olah & Stiles, 1995). All four are G protein-coupled receptors; the A_1 and A_3

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subtypes are coupled to inhibitory G proteins, while activation of the A_{2A}- and A_{2B}-receptors leads to adenylate cyclase stimulation (Collis & Hourani, 1993; Alexander *et al*., 1994; Fredholm *et al*., 1994). A_1- and A_2-receptor subtypes have been defined according to the relative potency on them of adenosine analogues, as well as considering the potency shown by A_1-selective antagonists such as 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX). Thus, on A_1-receptors, N^6 substituted analogues such as N^6-cyclopentyladenosine (CPA) are more potent than 5′-substituted analogues such as 5′-N-ethylcarboxamidoadenosine (NECA). Xanthines such as 8-phenyltheophylline (8-PT) and 8-sulphophenyltheophylline (8-SPT) are antagonists at both A_1- and A_2-receptors, whereas DPCPX has nanomolar affinity at A_1-receptors and micromolar affinity at A_2 receptors, and thus effectively discriminates between the two types of receptors (Lohse *et al*., 1987; Bruns, 1990; Collis *et al*., 1989).

A_2-receptors have been divided further into A_{2A} and A_{2B} subtypes, based on the potency shown by some two-substituted adenosine analogues, such as 2-[p-(carboxyethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680). Thus, CGS21680 is a potent agonist at A_{2A} receptors but is virtually inactive at A_{2B} sites (Jacobson, 1990). Recently, a non-xanthine antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol, (ZM 241385) had shown high selectivity for A_{2A} receptors (Poucher *et al*., 1995).

A_3-receptors, which are considered to be xanthine-insensitive, have been cloned from rat striatum. These receptors are coupled to an inhibitory G protein (Meyerhof *et al*., 1991; Zhou *et al*., 1992). N^6-(3-iodobenzyl)-adenosine-5′-N-methylcarboxamide (IB-MECA) has been reported as a selective A_3-receptor agonist (Jacobson *et al*., 1993; 1995; Gallo-Rodríguez *et al*., 1994). A_3-receptors are resistant to blockade with methylxanthines, although it has been reported that compounds such as 1,3-dipropyl-8-(4-acrylate)phenylxanthine (BWA1433) block them (Jacobson *et al*., 1995).

The autonomic nervous system plays an essential role in the maintenance of ureteral peristalsis. Thus, a rich catecholaminergic innervation forming dense neuromuscular, perivascular and subepithelial plexuses has been found in the intravesical ureter (Schulman, 1985; Prieto *et al*., 1993; 1994). Both noradrenaline (Hernández *et al*., 1992) and acetylcholine (Hernández *et al*., 1993; 1995a; Prieto *et al*., 1994) stimulate the two contractile components of intravesical ureteral smooth muscle (phasic activity and tone). In addition to the adrenergic and cholinergic systems, several neuropeptides, such as neuropeptide Y (NPY), vasoactive intestinal peptide and somatostatin have been identified in ureteral nerves, coexisting with different neurotransmitters (Edyvane *et al*., 1994; Smet *et al*., 1994; Prieto *et al*., 1997). These peptides act by modulating the neurotransmitter-evoked activity. Thus, NPY has been reported to enhance the noradrenaline-induced contraction in the equine intravesical ureter through Y_2-receptors (Prieto *et al*., 1997). Together with the above mentioned peptides, nitric oxide (NO) (Hernández *et al*., 1995b) and calcitonin gene related peptide (CGRP) (Maggi & Giuliani, 1991) have been involved in the ureteral non adrenergic non cholinergic (NANC) inhibitory neurotransmission, in processes operated through guanylate cyclase (Hernández *et al*., 1997) and adenylate cyclase (Maggi *et al*., 1994; 1995), respectively, involving in both cases the activation of glibenclamide-sensitive K^+ channels.

Adenosine has been reported to modulate the NANC mouse urinary bladder neurotransmission (Acevedo *et al*., 1992). The possible role exerted by adenosine in the intravesical ureter has not been described. Therefore, the present study was designed to characterize the functionally active adenosine receptor subtypes involved in the relaxation of porcine ureter, and to investigate the possible modulatory action of adenosine in the NANC motor transmission of the pig intravesical ureter.

Methods

Adult pigs of either sex with no lesions in their urinary tract were selected from the local slaughterhouse. Urinary bladders with attached ureters were removed immediately after the animals were killed, and kept in chilled physiological saline solution (PSS) at 4°C. The adjacent connective and fatty tissues were removed with care and longitudinal preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter were isolated from the bladder by dissection, as previously described (Hernández *et al*., 1992). The ureteral strips were suspended horizontally and placed parallel between two platinum electrodes, with one end connected to an isometric transducer (Grass FT 03C) and the other one to a micrometer screw which regulates the tension applied to the preparations, in 5 ml organ baths. The signal was continuously recorded on a polygraph (Graphtec Multicorder MC 6621, Hugo Sachs Elektronik, Germany). Passive tension of 2 g was applied to the ureteral preparations and they were allowed to equilibrate for 60 min.

Experimental procedure

The contractile ability of the preparations was determined by exposing the ureteral strips to 124 mM potassium-rich physiological saline solution (KPSS). Relaxations to adenosine and structurally related analogues were performed on strips incubated for 30 min with the adenosine uptake inhibitor nitrobenzylthioinosine (NBTI, 10^{-6} M) and further contracted with the thromboxane analogue U46619 (10^{-7} M). Afterwards, the tissues were washed every 20 min during 1.5 h. To study the effect of antagonists on adenosine or NECA relaxations, the preparations were incubated with NBTI alone (control curve) or NBTI plus the antagonist (30 min, except for 8-PT, which was incubated for 45 min). At the end of each experiment with adenosine and its structural analogues, papaverine (10^{-4} M) was added to the organ bath with the aim of obtaining the maximal relaxation of preparations.

In electrical field stimulation (EFS) experiments, adrenergic neurotransmission, muscarinic receptors, NO synthase activity and prostaglandins were blocked by incubation with guanethidine (10^{-5} M), atropine (10^{-7} M), L-N^G-nitroarginine (L-NOARG, 3×10^{-5} M) and indomethacin (3×10^{-6} M), respectively, during a period of 1 h, washing every 20 min, and these drugs were present throughout the experiment. EFS was performed with rectangular pulses (1 ms duration, 5 Hz, 20 s trains), at 3 min intervals, from a Cibertec CS20 stimulator (Barcelona, Spain) with constant current output adjusted to 75 mA. The strips were repeatedly washed and allowed to equilibrate for at least 1 h before they were incubated with blocking agents.

Drugs and solutions

The following drugs were used: acetylcholine (ACh), adenosine, adenosine 5′-triphosphate (ATP), atropine sulphate, guanethidine, 5-hydroxytryptamine (5-HT), indomethacin, L-NOARG, papaverine, 9, 11-dideoxy-11α, 9α-epoxymethano-

prostaglandin $F_{2\alpha}$ (U46619) and tetrodotoxin (Sigma, U.S.A.). N^6-cyclopentyladenosine (CPA), 5′-(N-cyclopropyl)-carboxamidoadenosine (CPCA), 2-chloroadenosine (2-CA), 2-[p-(carboxyethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680), 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide (IB-MECA), nitrobenzylthioinosine (NBTI), 5′-N-ethylcarboxamidoadenosine (NECA), 8-phenyl-theophylline (8-PT) (RBI, U.S.A), 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) (Tocris Cookson, U.S.A.). ACh, adenosine, ATP, guanethidine, 5-HT, L-NOARG and tetrodotoxin were dissolved in double distilled water. CGS21680, DPCPX, NBTI, NECA, IB-MECA and ZM 241385 were dissolved in dimethyl sulphoxide (DMSO). CPCA was dissolved in 0.1N HCl. 8-PT was dissolved in 25% ethanol plus 30 mM NaOH. U46619, indomethacin, 2-CA, CPA and papaverine were dissolved in 96% ethanol. These solvents, in the amount applied, had no effect on pig intravesical ureteral activity. The composition of PSS was (mM): 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 (ethylenediamine tetraacetic acid) 0.027. The solution was continuously gassed at 37°C 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 double-distilled water.

Calculations and statistics

For each concentration-response relaxation curve to adenosine and related analogues, the drug concentration required to give 40% relaxation (EC_{40}) of U46619 induced contraction was estimated by regression of the linear portion of the curve, by computerized linear regression analysis (Inplot, GraphPad, U.S.A.). The sensitivity of the drugs is expressed in terms of pEC_{40}, where pEC_{40} is defined as the negative logarithm of EC_{40} ($pEC_{40} = -\log EC_{40}$ (M). Each parameter was determined from ureters of at least 4–6 different animals. Statistical significance of differences was calculated by Student's *t*-test, for paired observations for individual concentrations or frequencies and variance analysis (ANOVA) for multiple comparisons, followed by an *a posteriori* Bonferroni test (Wallestein *et al*., 1980). Differences were considered significant with a probability level of $P<0.05$.

Results

The pig intravesical ureteral preparations were equilibrated to a passive tension of 1.8 ± 0.3 g ($n=87$). In NBTI (10^{-6} M)-pretreated tissues, the thromboxane analogue, U46619 (10^{-7} M) induced sustained contractions of 1.9 ± 0.3 g ($n=75$). Adenosine, NECA, 2-CA, CPCA, CGS 21680, CPA and IB-MECA all concentration-dependently relaxed the U46619-contracted intravesical ureteral strips, the order of potency being: NECA = CPCA = 2-CA > adenosine > CPA = IB-MECA = CGS21680 (Figure 1, Table 1). This order of potency suggests that the adenosine receptors that relax the pig intravesical ureteral smooth muscle belong to the A_{2B} subtype.

The relaxations to adenosine (10^{-7}–10^{-3} M) and NECA (10^{-7}–10^{-4} M) were reproducible. Thus, pEC_{40} values and the relaxations to 10^{-3} M adenosine were 4.1 ± 0.2 and $73.2\pm7.0\%$, and 4.0 ± 0.2 and $70.2\pm6.8\%$ ($P>0.05$, paired *t*-test, $n=9$), in a first and second concentration-response curve performed in the same preparation, respectively. pEC_{40} values and the relaxations to 10^{-4} M NECA were 5.2 ± 0.2 and $92.1\pm5.2\%$ and 5.1 ± 0.3 and $88.3\pm4.8\%$ ($P>0.05$, paired *t*-test, $n=7$), in two consecutive curves.

Removal of ureteral epithelium did not modify the adenosine concentration-relaxation curve, the pEC_{40} values being 4.0 ± 0.2 and 4.1 ± 0.2, ($P>0.05$, paired *t*-test, $n=6$) in the absence and in the presence of epithelium, respectively. Epithelium removal had also no effect on the relaxations to 10^{-3} M adenosine. These relaxations were $70.1\pm4.7\%$ and $63.2\pm5.2\%$ ($P>0.05$, paired *t*-test, $n=6$), in the absence and in the presence of epithelium, respectively (Figure 2a).

Indomethacin (3×10^{-6} M) and L-NOARG (3×10^{-5} M), inhibitors of prostanoid production and NO synthase activity, respectively, did not modify the relaxations to adenosine. The pEC_{40} values and the relaxations to 10^{-3} M adenosine were 4.1 ± 0.1 and $75.7\pm4.8\%$ and 4.1 ± 0.2 and $76.4\pm4.3\%$

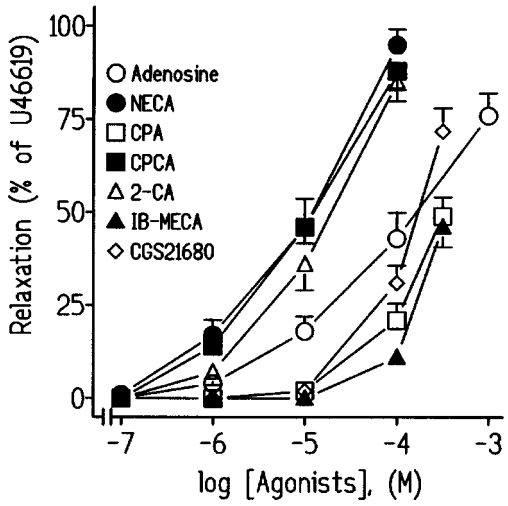

Figure 1 Log concentration-response relaxation curves to adenosine, 5′-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), 5′-(N-cyclopropyl)-carboxamidoadenosine (CPCA), 2-chloroadenosine (2-CA), N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide (IB-MECA) and 2-[p-(carboxyethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680), in pig intravesical ureteral strips contracted to 10^{-7} M U46619. Relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e. of the mean of 5–17 preparations.

Table 1 Relaxations induced by adenosine and related structural analogues in pig intravesical ureteral strips contracted with 10^{-7} M U46619

Agonist	n	*pEC_{40}*	*R(%)*
Adenosine	17	4.5 ± 0.1	75.9 ± 6.1
NECA	11	5.4 ± 0.2*	95.1 ± 4.2
CPCA	7	5.4 ± 0.1*	88.1 ± 5.4
2-CA	6	5.2 ± 0.3*	85.0 ± 5.1
CPA	6	$4.2\pm0.1^{*,\dagger,\#,+}$	48.9 ± 6.1
CGS21680	7	$4.1\pm0.1^{*,\dagger,\#,+}$	71.8 ± 6.3
IB-MECA	5	$4.0\pm0.1^{*,\dagger,\#,+}$	46.1 ± 5.2

Results are expressed as mean ± s.e.mean of *n* experiments. Differences were calculated by one-way analysis of variance (ANOVA) followed by an *a posteriori* Bonferroni *t*-test in case of significance; $^*P<0.05$ compared to adenosine, $\dagger P<005$ compared to NECA, $\#P<0.05$ compared to CPCA, $^+P<0.05$ compared to 2-CA. $pEC_{40} = -\log EC_{40}$, where EC_{40} is the concentration of agonist producing 40% relaxation of U46619 induced contraction. *R*: relaxation obtained at the highest concentration applied of each drug: Adenosine, 10^{-3} M; NECA 10^{-4} M; CPCA 10^{-4} M; 2-CA, 10^{-4} M; CPA 3×10^{-4} M; CGS21680, 3×10^{-4} M and IB-MECA, 3×10^{-4} M

($P>0.05$, paired t-test, $n=6$), in control conditions and in the presence of indomethacin, respectively (Figure 2b). pEC_{40} values and the relaxations to 10^{-3} M adenosine in the experiments with L-NOARG (3×10^{-5} M) were 4.1 ± 0.2 and $73.4\pm5.5\%$ and 4.0 ± 0.2 and $68.8\pm4.7\%$ ($P>0.05$, paired t-test, $n=7$), in the absence and in the presence of L-NOARG, respectively (Figure 2c).

Effects of 8-PT, DPCPX and ZM 241385 on relaxations to adenosine and NECA

We examined whether 8-PT (10^{-5} M), an inhibitor of both A_1- and A_2-adenosine receptors, and DPCPX (10^{-8} M and 10^{-6} M), inhibited the relaxations to adenosine or NECA in U46619-precontracted strips obtained from pig intravesical ureter. Pretreatment of strips with 8-PT (10^{-5} M), did not modify the ureteral basal tone (1.6 ± 0.4 g, in control conditions and in the presence of 8-PT), but reduced the relaxations to adenosine (Figure 4a, Table 2) and NECA (Figure 3 and 4b, Table 1). Incubation with 10^{-8} M DPCPX, a concentration selective for A_1-receptors, did not modify the relaxations to adenosine or to NECA. However, the use of 10^{-6} M DPCPX, a concentration which blocks the A_2-receptors, significantly reduced the relaxations to adenosine or NECA on ureteral strips (Figure 5a and b, Table 2). ZM 241385 (3×10^{-8} M and 10^{-7} M), a non-xanthine antagonist which shows high selectivity for A_{2A} did not modify the relaxations to adenosine or to NECA (Figure 5c and d, Table 2).

Effects of 8-PT and DPCPX on contractions evoked by EFS and exogenous ATP

Pig intravesical ureteral strips were treated with guanethidine (10^{-5} M), atropine (10^{-7} M), L-NOARG (3×10^{-5} M) and indomethacin (3×10^{-6} M), with the aim to block adrenergic neurotransmission, muscarinic receptors, NO synthase activity and prostanoids, respectively. In such conditions, both EFS (5 Hz) and exogenous ATP (10^{-4} M) evoked reproducible contractions of 1.3 ± 0.2 g and 1.2 ± 0.2 g ($P>0.05$, paired t-test, $n=7$) and 0.4 ± 0.1 g and 0.4 ± 0.2 g ($P>0.05$, paired t-test, $n=5$), in a first and second addition, respectively, on the basal tone of preparations. Treatment of ureteral strips with 8-PT (10^{-5} M) significantly potentiated the contractions induced by both EFS (1.1 ± 0.3 g and 1.7 ± 0.4 g, in control conditions and in the presence of 10^{-5} M 8-PT, respectively, $n=9$, $P<0.05$, paired t-test) (Figure 6a and b) or exogenous ATP (0.5 ± 0.2 g and 1.1 ± 0.2 g in the absence and in the presence of 10^{-5} M 8-PT, respectively, $n=11$, $P<0.05$, paired t-test) (Figure 6a and c). The incubation with a selective A_1-receptor antagonist, DPCPX (10^{-8} M), did not modify the contractions induced by EFS (0.9 ± 0.2 g and 1.0 ± 0.3 g, $P>0.05$, paired t-test, $n=7$, in control conditions and in the presence of 10^{-8} M DPCPX, respectively) or exogenous ATP (0.6 ± 0.2 g and 0.5 ± 0.2 g, $P>0.05$ paired t-test, $n=8$, in the absence and in the presence of 10^{-8} M DPCPX, respectively). The contractions elicited by EFS (5 Hz) were abolished by including

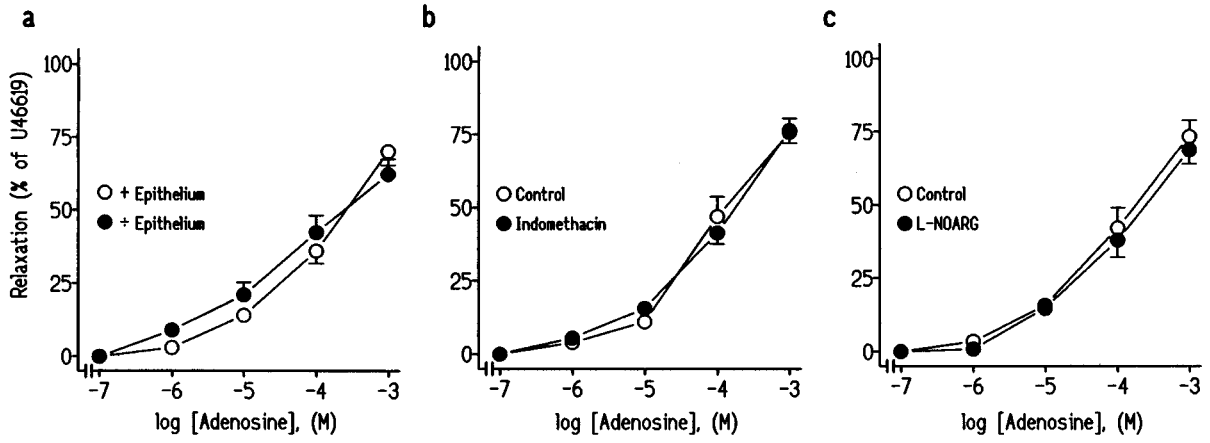

Figure 2 (a) Log concentration-response relaxation curves to adenosine in pig intravesical ureteral strips contracted to 10^{-7}M U46619 in the presence and in the absence of ureteral epithelium. (b and c) Log concentration-response relaxation curves to adenosine in control conditions or in the presence of (b) 3×10^{-6} M indomethacin or (c) 3×10^{-5} M L-N^G-nitroarginine (L-NOARG). The relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e.mean of 6–7 preparations.

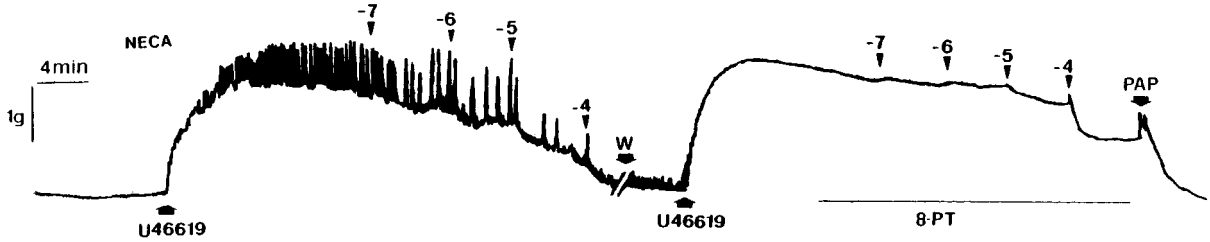

Figure 3 Isometric force recordings showing the effect of 8-phenyltheophylline (8-PT, 10^{-5} M) on the relaxant response to 5′-N-ethylcarboxamidoadenosine (NECA, 10^{-7}–10^{-4} M), in U46619 (10^{-7} M)-contracted pig intravesical ureteral strips. At the end of the experiment, papaverine (PAP, 10^{-4} M) was added to obtain the maximal relaxation of the ureteral strips. Vertical bar shows tension in g and horizontal bar time in min. Numbers indicate molar concentration in the bath. W: wash out.

10^{-6} M tetrodotoxin in the bath, thus providing evidence for the neurogenic origin of these contractions.

Effects of NECA, CPCA and 2-CA on contractions evoked by EFS

In the conditions above mentioned, the incubation during 5 min of the preparations with a maximal (10^{-4} M) concentration of NECA, CPCA and 2-CA did not modify the contractions to EFS (5 Hz). Thus, EFS evoked contractions of 1.0 ± 0.3 g and 1.1 ± 0.3 g ($P>0.05$, paired t-test, $n=7$); 0.9 ± 0.2 g and 0.8 ± 0.2 g ($P>0.05$, paired t-test, $n=5$); 1.1 ± 0.3 g and 1.2 ± 0.4 g, ($P>0.05$, paired t-test, $n=5$) in control conditions and in the presence of NECA, CPCA and 2-CA, respectively.

Effects of NECA on contractions elicited by exogenous ATP

Pretreatment of ureteral strips for 5 min with NECA (10^{-4} M), reduced the contractions to exogenous ATP (10^{-4} M). Thus, ATP (10^{-4} M) induced contractions of 0.5 ± 0.2 g and 0.2 ± 0.2 g ($P<0.05$, paired t-test, $n=5$) in the absence or presence of NECA (10^{-4} M), respectively.

Effects of 8-PT on contractions induced by exogenous ACh and 5-HT

Incubation of preparations with 8-PT (10^{-5} M) increased the contractions to ACh (10^{-5} M) or 5-HT (10^{-5} M) in the pig intravesical ureter. ACh (10^{-5} M) induced contractions of 0.9 ± 0.2 g and 1.3 ± 0.3 g ($P<0.05$, paired t-test, $n=7$) in the absence or presence of 8-PT (10^{-5} M), respectively. 5-HT (10^{-5} M) evoked contractions of 1.3 ± 0.2 g and 1.8 ± 0.2 g ($P<0.05$, paired t-test, $n=5$) in control conditions and in the presence of 8-PT (10^{-5} M), respectively.

Discussion

In the present study adenosine and six of its related structural analogues, NECA, 2-CA, CPCA, CGS21680, CPA and IB-MECA concentration-dependently and epithelium-independently relaxed the pig intravesical ureteral strips precontracted

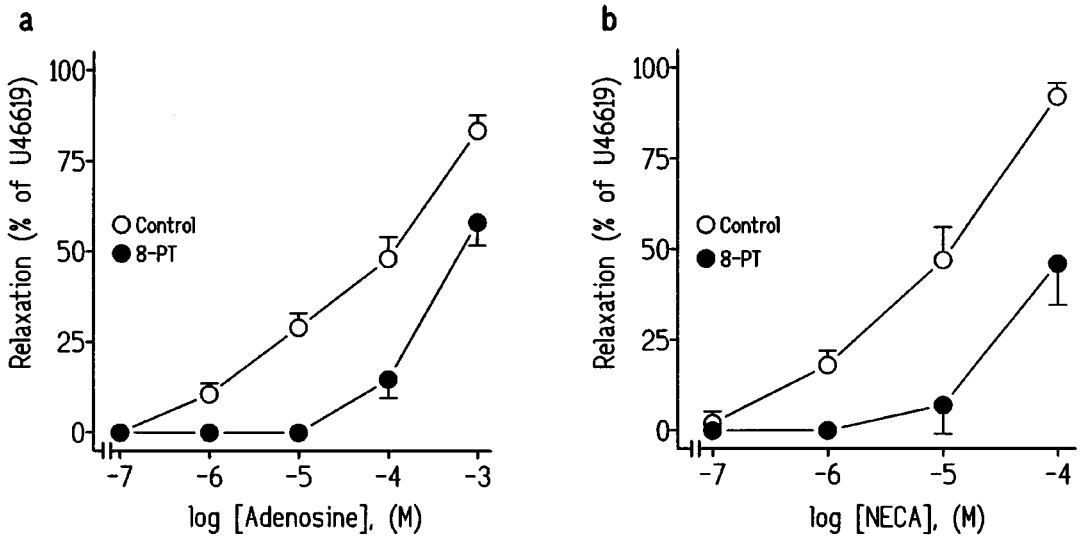

Figure 4 Log concentration-response relaxation curves to (a) adenosine and (b) 5′-N-ethylcarboxamidoadenosine (NECA) in pig intravesical ureteral strips contracted to 10^{-7} M U46619, in control conditions and in the presence of the A_1 and A_2 adenosine receptors inhibitor, 8-phenyltheophylline (8-PT, 10^{-5} M). The relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e.mean of 6–7 preparations.

Table 2 Effect of 8-PT (10^{-5} M), DPCPX (10^{-8} M), DPCPX (10^{-6} M), ZM 241385 (3×10^{-8} M) and ZM 241385 (10^{-7} M) on relaxations to adenosine and NECA of pig intravesical ureter contracted to U46619 (10^{-7} M)

	Adenosine			*NECA*		
	n	pEC_{40}	*R (%)*	n	pEC_{40}	*R (%)*
Control	7	4.4 ± 0.2	83.3 ± 4.3	6	5.3 ± 0.2	92.1 ± 0.2
8-PT	7	–	58.1 ± 6.2*	6	–	45.9 ± 11.3*
Control	9	4.3 ± 0.1	77.2 ± 6.1	7	5.2 ± 0.2	94.7 ± 4.6
DPCPX (10^{-8} M)	9	4.3 ± 0.2	78.8 ± 5.3	7	5.2 ± 0.1	86.1 ± 5.2
DPCPX (10^{-6} M)	9	–	52.2 ± 4.1#	7	–	57.9 ± 6.7#
Control	6	4.2 ± 0.2	68.1 ± 4.7	7	5.2 ± 0.2	86.4 ± 4.8
ZM 241385 (3×10^{-8} M)	6	4.2 ± 0.2	70.1 ± 5.1	7	5.1 ± 0.3	85.1 ± 5.0
ZM 241385 (10^{-7} M)	6	4.0 ± 0.3	66.2 ± 6.3	7	4.9 ± 0.3	78.2 ± 5.5

Results are expressed as mean$\pm$s.e.mean of n experiments. *Parameter significantly ($P<0.05$) different compared to control value by paired t-test. #Parameter significantly ($P<0.05$) different compared to control value by analysis of variance (ANOVA), followed by an *a posteriori* Bonferroni test. $pEC_{40}=-\log EC_{40}$, where EC_{40} is the concentration of agonist producing 40% relaxation of U46619 induced contraction. *R*: relaxation obtained at the highest concentration used: adenosine, 10^{-3} M and NECA, 10^{-4} M.

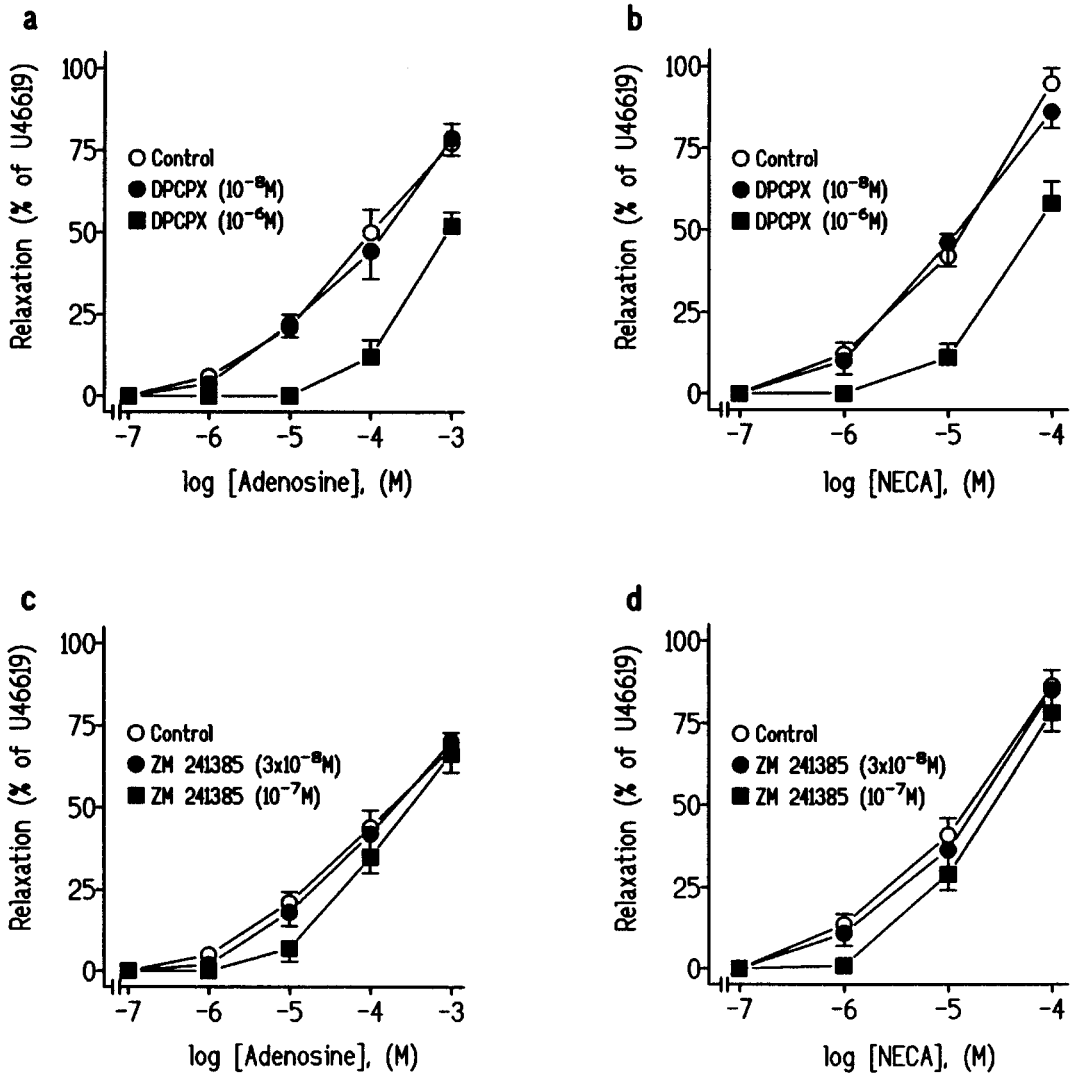

Figure 5 Log concentration-response relaxation curves to (a and c) adenosine and (b and d) 5′-N-ethylcarboxamidoadenosine (NECA) in pig intravesical ureteral strips contracted to 10^{-7} M U46619, in control conditions and in the presence of the A_1-adenosine receptors inhibitor, 3-dipropyl-8-cyclopentylxanthine (DPCPX), (10^{-8} M) or (10^{-6} M) (a and b) and the A_{2A} selective receptors antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), (3×10^{-8} M) or (10^{-7} M) (c and d). The relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e.mean of 6–9 preparations.

with U46619. The relaxing potency order obtained (NECA = CPCA = 2-CA > adenosine > CPA = IB-MECA = CGS21680) together with the lack of effect of indomethacin and L-NOARG is consistent with a prostanoid- and NO-independent activation of A_{2B} receptors by these agonists. The potentiation induced by 8-PT on the contraction evoked by EFS and exogenous ATP suggests that endogenous adenosine could be modulating the non adrenergic non cholinergic neurotransmission of the pig intravesical ureter.

The relaxation curves to adenosine and NECA were monophasic, suggesting that both agonists activate one receptor site to cause relaxation in the pig intravesical ureter. Removal of the ureteral epithelium did not change the relaxations to adenosine, indicating that these responses are mediated *via* a receptor located on the ureteral smooth muscle. Both prostanoids and NO have been reported to mediate the relaxation induced by adenosine in different tissues. Thus, part of the relaxing effect produced by adenosine in the rabbit isolated heart is mediated through release of prostaglandins (Ciabattoni & Wennmalm, 1985; Karwatowska-Prokopczuk *et al.*, 1988). Adenosine, on the other hand, promotes the release of NO from vascular endothelium (Vials & Burnstock, 1993; Abebe *et al.*, 1995; Li *et al.*, 1995; Danialou *et al.*, 1997) and smooth muscle (Ikeda *et al.*, 1997). However, in the pig intravesical ureter, indomethacin and L-NOARG, which inhibit prostanoid production and NO-synthase activity, respectively, did not modify the relaxations to adenosine, suggesting that adenosine relaxes the ureteral smooth muscle *via* a prostaglandins- and NO-independent mechanism. These results are consistent with those found in the frog aorta, where adenosine induces vasodilatation without involving prostanoid or NO production (Knight & Burnstock, 1996).

The blocker of A_1- and A_2-receptors, 8-PT (10^{-5} M) potently reduced the relaxations induced by adenosine and NECA, suggesting that these relaxations are mediated through P_1-purinoceptors. The fact that 10^{-6} M DPCPX, a concentration high enough to block A_2-receptors, inhibits the relaxations, indicates that they are A_2-receptor-mediated. This hypothesis is in accordance with the general notion that smooth muscle A_2-receptors mediate relaxation, whereas A_1-receptors, which are presynaptically located, operate as a negative feedback mechanism, regulating transmitter release

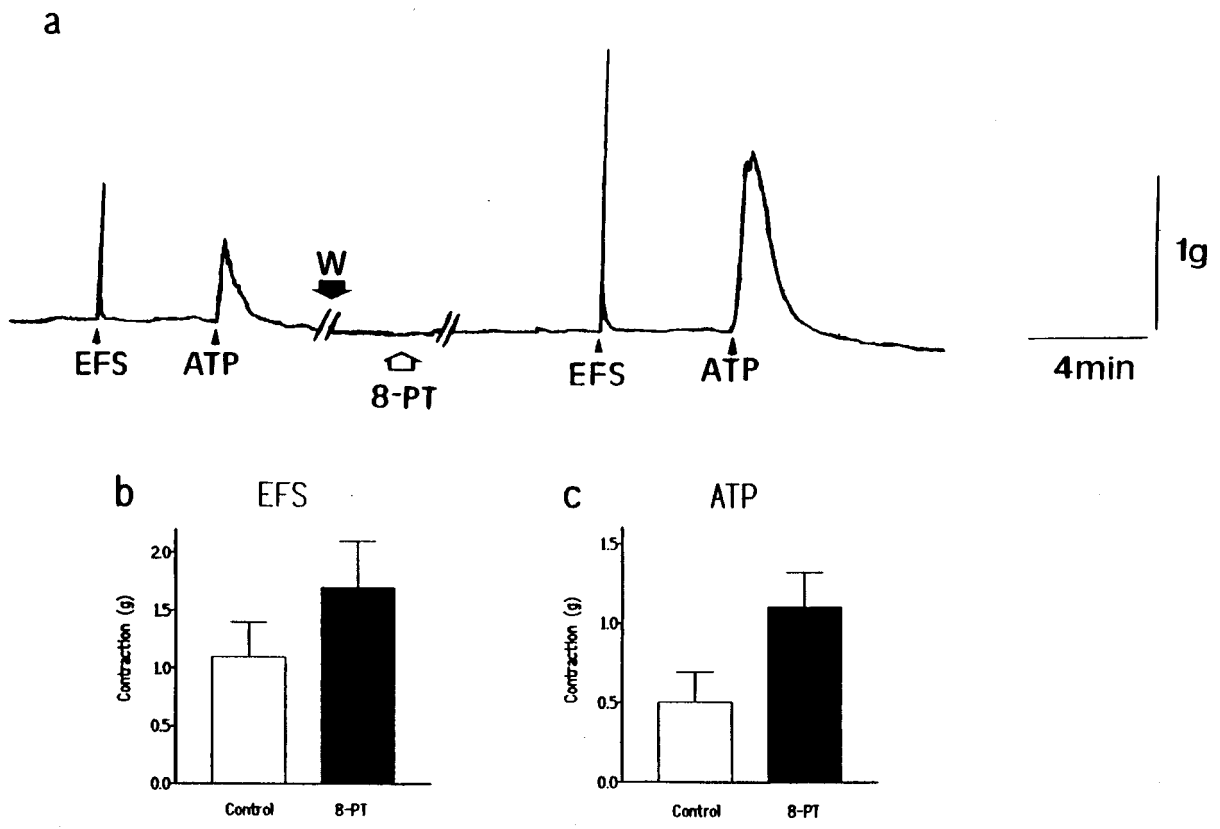

Figure 6 (a) Isometric force recordings showing the effect of 8-phenyltheophylline (8-PT, 10^{-5} M) on contractions induced by both EFS (5 Hz, 1 ms duration, 20 s trains, at 3 min intervals) and exogenous ATP (10^{-4} M), in pig intravesical ureteral strips pretreated with guanethidine (10^{-5} M), atropine (10^{-7} M), L-N^G-nitroarginine (L-NOARG, 3×10^{-5} M) and indomethacin (3×10^{-6} M), to block adrenergic neurotransmission, muscarinic receptors, NO synthase activity and prostaglandins, respectively. Vertical bar shows tension in g and horizontal bar time in min. W: wash out. Contraction (g) induced by (b) EFS (5 Hz) or (c) ATP (10^{-4} M), in the absence or presence of 10^{-5} M 8-phenyltheophylline in the pig intravesical ureter. Results represent means and vertical line s.e.mean of 9–11 preparations.

and/or postsynaptic contractile responses (White, 1988; Huidobro-Toro & Parada, 1989; Acevedo *et al*., 1992). This notion, however, does not unequivocally mean that any adenosine mediated relaxation is exerted through an action at the A_2-receptor, as there are references describing A_1-receptor mediated relaxations. Thus, in the adenosine-mediated relaxation of the rat duodenum (Nicholls *et al*., 1992), in the pig coronary arteries (Merkel *et al*., 1992; Dart & Standen, 1993) and in rat diaphragmatic arterioles (Danialou *et al*., 1997), the involvement of both the A_1- and A_2-receptor subtypes has been suggested. In the present study, the contribution of the A_1-receptor to pig intravesical ureter relaxation has been discarded because of the low potency shown by adenosine and CPA, as well as by the lack of effect of 10^{-8} M DPCPX, a concentration selective for blocking the A_1-receptor, on relaxations to adenosine and NECA.

A_2-receptors are divided into A_{2A}- and A_{2B}-subtypes on the basis of the potency of CGS21680 (15 nM on A_{2A} and practically inactive on A_{2B}) (Jacobson, 1990; Jacobson *et al*., 1995). Thus, the rabbit aorta, mesenteric and coeliac arteries show A_2-receptor mediated vasodilatation, but in those preparations CGS21680 has a relaxing potency which is similar to that exhibited by NECA, so the A_{2A} subtype has been the one implicated in the vasodilatation (Balwierczak *et al*., 1991). However, in vascular tissues such as the dog coronary artery and saphenous vein, guinea-pig aorta (Balwierczak *et al*., 1991), rat mesenteric bed (Rubino *et al*., 1995; Prentice *et al*., 1997) as well as in the rat duodenum and urinary bladder (Nicholls *et al*., 1992) CGS21680 is very weak whereas NECA is a potent agonist, thus suggesting the presence of the A_{2B} subtype in those tissues. In the pig intravesical ureter, the low potency showed by CGS21680, as well as the lack of blocking effect produced by ZM 241385, a selective antagonist of A_{2A} adenosine receptors, on relaxations to adenosine or NECA (Poucher *et al*., 1995), suggest that the adenosine-induced relaxation is mediated through activation of the A_{2B}-subtype. The low potency exhibited by CGS21680 is in accordance with those found in the rat duodenum and urinary bladder (Nicholls *et al*., 1992).

A_3-receptors are clearly different from the A_1- and A_2-receptors, as the A_3-receptors show resistance to methylxanthine blockade. IB-MECA has been reported as a selective A_3-receptors agonist (Jacobson *et al*., 1993; 1995; Gallo-Rodríguez *et al*., 1994). In the pig intravesical ureter, the A_3-receptor subtype does not seem to be involved in the adenosine-mediated relaxation, as this relaxation was potently blocked by 8-PT, whereas the selective A_3-receptor agonist, IB-MECA (Gallo-Rodríguez *et al*., 1994) produced relaxation of the ureteral smooth muscle only at high concentrations.

Adenosine is a regulator at a variety of synapses or neuroeffector junctions where ATP participates in neurotransmission, either as the transmitter itself or as a cotransmitter. Adenosine has also been reported as a paracrine neuromodulator (Acevedo *et al*., 1992). Adenosine exerts its actions through a multiplicity of mechanisms. Thus, A_1-receptor activation may lead to modulation of adenylcyclase activity

and to a negative regulation of Ca^{2+} fluxes (Ribeiro *et al*., 1979; Fredholm & Dunwiddie, 1988), protein kinase C stimulation, opening of K^+ channels (Fredholm *et al*., 1990), as well as to tonic inhibition of the release of ATP *via* presynaptic receptors (Sebastiao & Ribeiro, 1986; Huidobro-Toro & Parada, 1989). A_2-receptors may be concerned with blockade of Ca^{2+} currents or stimulation of adenylcyclase activity (Fredholm *et al*., 1990). The multiciplity of mechanisms operated by adenosine, as well as the pre- and postsynaptic localization of the A_1- and A_2-receptors in the bladder, could explain the lack of selectivity of the adenosine-induced ATP antagonism. Thus, the adenosine analogue, NECA reduced the contractions induced not only by ATP but also those evoked by 5-HT or ACh (Acevedo *et al*., 1992). These authors suggested that endogenous adenosine tone modulates the mouse urinary bladder neurotransmission. This conclusion has been reached on the basis of the potentiation evoked by 8-PT on the contraction induced by electrical stimulation and exogenous ATP (Acevedo *et al*., 1992). In the present study, EFS and exogenous ATP evoked contractions of the intravesical ureteral strips, which were potentiated as a consequence of the blockade of adenosine receptors with 8-PT. These results suggest that adenosine could exert a physiological tonic influence on the ureteral NANC transmission and would thus be consistent with those obtained in the mouse (Acevedo *et al*., 1992) and rat (Parija *et al*., 1991) urinary bladder. The fact that 8-PT increased the contractions induced by two non purinergic agonists (ACh and 5-HT) could indicate that adenosine formed from ATP, either released from nerves or exogenously added, would be diminishing the contractions of the tissue nonspecifically. These results are in accordance with those found in the mouse urinary bladder smooth muscle (Acevedo *et al*., 1992) where NECA reduced the contractions induced by ATP, ACh, 5-HT and prostaglandin $F_{2\alpha}$. The existence of a basal level of adenosine in the pig intravesical ureter does not seem likely as incubation of preparations with 8-PT failed to modify the ureteral basal tone. The lack of effect exhibited by 10^{-8} M DPCPX, on contractions evoked by EFS or exogenous ATP in the pig intravesical ureter, suggests the absence of an A_1-presynaptic receptor that could be inhibiting the release of excitatory transmitters from ureteral nerves. Moreover, the fact that adenosine analogues, NECA, CPCA and 2-CA did not modify the contractions to EFS suggests the absence of inhibitory A_2-receptors presynaptically located, which could be modulating the neurotransmitter release. NECA, however, reduced the contractions evoked by exogenous ATP. All these results suggest that adenosine could be acting *via* postsynaptic A_{2B}-receptors in the porcine ureter.

In summary, the results of the present investigation suggest that the adenosine receptors that cause relaxation of the intravesical ureteral smooth muscle are of the A_{2B}-subtype. Moreover, evidence is presented showing that adenosine could modulate the NANC excitatory neurotransmission of the pig intravesical ureter, possibly through a postsynaptic mechanism. The adenosine-induced modulation could be favouring the dilatation of ureteral lumen, thus contributing to urine boli transport through ureter and its discharge to urinary bladder.

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