Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 460 (2003) 191–199
www.elsevier.com/locate/ejphar

Regional differences in the adenosine A_2 receptor-mediated modulation of contractions in rat vas deferens

Carmen Diniz, Sandra Leal[1], Jorge Gonçalves*

Laboratório de Farmacologia, CEQOFF/FCT, Faculdade de Farmácia, Universidade do Porto, Rua Anibal Cunha, 164, P 4050-047 Porto, Portugal

Received 30 October 2002; received in revised form 9 December 2002; accepted 17 December 2002

Abstract

Adenosine receptors involved in modulation of contractions were characterized in the bisected rat vas deferens by combining pharmacological and immunohistochemical approaches. In both portions, noradrenaline-elicited contractions were enhanced by the adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA), and inhibited by the non-selective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) in the presence of the adenosine A_1 receptor antagonist 1,3-dipropyl-8-cyclopentyl-l,3-dipropylxanthine (DPCPX). The adenosine A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethyl-amino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) also inhibited noradrenaline-elicited contractions but only in the prostatic portion. Contractions elicited by the stable ATP analogue α,β-methyleneATP (α,β-MeATP) were inhibited only by NECA in the presence of DPCPX and only in the prostatic portion. This study provides functional evidence for the presence, in both portions of the rat vas deferens, of an adenosine A_1 receptor-mediated enhancement and of an adenosine A_2 receptor-mediated inhibition of contractions. The latter effect is mediated by both A_{2A} and A_{2B} subtypes in the prostatic portion but only by the A_{2B} subtype in the epididymal portion. This regional variation is supported by the immunohistochemical results that revealed an adenosine A_{2A} receptor immunoreactivity not co-localized with nerve fibres more abundant in the prostatic than in the epididymal portion.

Keywords: Adenosine A_1 receptor; Adenosine A_{2A} receptor; Adenosine A_{2B} receptor; Adenosine A_3 receptor; Postjunctional contraction

1. Introduction

Adenosine exerts multiple modulatory effects through activation of G-protein coupled receptors, known as adenosine or P_1 receptors. Four adenosine receptor subtypes have been recognized (A_1, A_{2A}, A_{2B} and A_3), each presenting a unique pharmacological profile (Fredholm et al., 2001). In addition to the well-known modulation of neurotransmitter release mediated by prejunctional adenosine receptors, adenosine may also influence transmission by modulating postjunctional responses. A direct postjunctional modulation was first reported in rabbit kidney and canine adipose tissue, in situ (Hedqvist and Fredholm, 1976), and later in guinea-pig aorta (Stoggall and Shaw, 1990), rat aorta and bovine coronary artery (Conti et al., 1993), in rat spleen (Fozard and Milavec-Krizman, 1993), guinea-pig pulmonary artery (Szentmiklósi et al., 1995) and rat mesenteric arterial bed (Rubino et al., 1995). In general, contractions are enhanced by activation of adenosine A_1 receptors and inhibited by activation of adenosine A_2 receptors (see Ralevic and Burnstock, 1998).

The rat vas deferens is a tissue with ATP/noradrenaline cotransmission widely used to investigate modulation of postganglionic sympathetic transmission (Allcorn et al., 1986; Gonçalves and Guimarães, 1991; Gonçalves and Queiroz, 1993; Kurz et al., 1993). However, it presents regional differences on the importance of the transmitters involved in the neurogenic contractile response in the bisected rat vas deferens. The prostatic portion shows a more marked purinergic component (mediated by ATP) while the epididymal portion shows a more marked adrenergic component (McGrath, 1978; Sneddon and Machaly, 1992). Therefore, the bisected vas deferens can constitute a model to investigate whether modulation of postjunctional responses (namely by adenosine) is similar on tissues that differ on the importance of ATP as motor transmitter.

* Corresponding author. Tel.: +351-222-078-932; fax: +351-222-078-969.
E-mail address: jorge.goncalves@ff.up.pt (J. Gonçalves).
[1] Present address: Instituto Superior de Ciências da Saúde-Norte, Rua Central de Gandra, 1317, P 4580-117, Gandra, Portugal.

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(02)02926-6

In the rat vas deferens adenosine modulates contractile responses (Hourani and Jones, 1994; Hourani et al., 1993; Brownhill et al., 1996a,b). By using the bisected rat vas deferens, it was suggested the existence of regional differences in this adenosine receptor-mediated modulation: the adenosine A_1 receptor-mediated enhancement of contractions seems to occur in both portions while the adenosine A_2 receptor-mediated inhibition seems to occur only in the prostatic portion (Brownhill et al., 1996a). However, on this study, contractions were elicited by KCl with a likely contribution to the KCl-elicited contractions of the released transmitters, a process influenced by activation of prejunctional adenosine receptors (Ribeiro, 1995). In the present study the adenosine receptor-mediated modulation of contractions were re-examined in the bisected rat vas deferens by comparing the effects of selective adenosine receptor agonists on contractions elicited by exogenous noradrenaline and α,β-methyleneATP (α,β-MeATP), contractions likely mediated by the receptors involved on neurogenic contractions. To support the results obtained with the functional approach, an immunohistochemical study was also carried out on an attempt to confirm the postjunctional adenosine receptor subtypes present in the two portions of the rat vas deferens, particularly the adenosine A_2 receptor subtypes. This aim is justified because it was recently shown that the adenosine A_2 receptor subtypes involved in the facilitation of noradrenaline release are different in the two portions of the bisected rat vas deferens (Queiroz et al., 2002).

2. Materials and methods

2.1. Chemicals

The following drugs were used: 2-*p*-(2-carboxyethyl)-phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21680), N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-l,3-dipropylxanthine (DPCPX), 3,3′-diaminobenzidine (DAB), l-deoxy-l-[6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA), 3,7-dimethyl-l-(2-propynyl)xanthine (DMPX), 5′-*N*-ethylcarboxamidoadenosine (NECA), α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP), L-noradrenaline bitartrate, 3-propylxanthine (enprofylline) and Triton X-100 obtained from Sigma-Aldrich (Alcobendas, Spain); 4-(2[7-amino-2-(2-furyl)[l,2,4]triazolo[2,3-*a*][l,3,5]-triazin-5-ylamino]ethyl)-phenol (ZM 241385) was from Tocris (Bristol, UK). Unless otherwise stated, solutions of drugs were prepared with either distilled water or dimethylsulphoxide (DMSO) and diluted with medium immediately before use (concentration of DMSO in the medium never exceeded 0.01%). Solvents were added to the superfusion medium in parallel control experiments. All other reagents used were of analytical grade.

2.2. Experimental protocols

2.2.1. General

Adult male Wistar rats (230–390 g; CRIFFA, Barcelona, Spain) were kept under standard laboratory conditions: light/dark cycle of 12/12 h, temperature of 20–22 °C, and free access to water and pellet food. The handling and care of all animals were conducted according to the EU guidelines for animal research (86/609/CEE), and adopted by the Portuguese law (Portaria n. 1005/92, n. 1131/97). Animals were killed by cervical dislocation and exsanguination and vasa deferentia (about 55 mg and 2 cm long) removed, dissected free from surrounding tissues and bisected. Prostatic (32.1 ± 1.9 mg; $n = 72$; about 0.9 cm length) and epididymal (17.0 ± 2.3 mg; $n = 57$; about 0.8 cm length) portions were obtained; an intermediate portion of approximately 0.3 cm was rejected.

2.2.2. Contractions

The medium used had the following composition (mM): NaCl 118.6, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.23, $NaHCO_3$ 25.0, glucose 10.0, ascorbic acid 0.3 and dissodium EDTA 0.031. It was saturated with 95% O_2–5% CO_2 and kept at 37 °C. The tissue preparations were suspended in a 20-ml organ bath, the lower end fixed and the upper end attached to an isometric force transducer (MLT050, ADInstruments, East Sussex, UK). The initial tension applied was 17 mN but tissues subsequently relaxed to a resting tension of approximately 8 mN during a 45-min equilibration period. The medium was replaced every 10 min. Contractions were elicited by addition of exogenous 5 μM noradrenaline (in some experiments 10 μM α,β-MeATP was used) measured and stored with a data acquisition system (MacLabsystem: ML740 with ML 110 bridges amplifiers; ADInstruments). Three contractions (C_1, C_2 and C_3) were elicited in each tissue preparation with 25-min intervals. Adenosine receptor agonists were added 5 min before C_2 and C_3, and kept until the contractions had peaked. Adenosine receptor antagonists were added immediately after C_2 and kept for the remaining of the experiment. In control preparations, solvent was added instead of the adenosine receptor agonists or antagonists.

Drug effects were evaluated by ratios of the amplitude of the contractions C_2 and C_3 (C_n) and of C_1; C_n/C_1 ratios obtained in individual experiments in which a test compound A was added before C_2 were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent instead of A). When the interaction of A, added before C_2, and a drug B either added at the beginning of the experiment or after C_2 was studied, the "appropriate control" was a group in which B alone was used.

Results are expressed as mean ± S.E.M.; n denotes the number of tissue preparations. Differences between means were tested for significance using one-way analysis of

variance (ANOVA) followed by Tukey's *t*-test. A *P* value lower than 0.05 was taken to indicate significant differences.

2.2.3. Immunohistochemistry

Four animals were used for immunohistochemical staining procedures. Rat vasa deferentia were dissected free from surrounding tissues, bisected into prostatic and epididymal portions and fixed in formalin (4% formaldehyde, 37 mM of Na_2HPO_4, 29 mM of NaH_2PO_4) overnight. Fixed tissues were extensively rinsed in distilled water for 5 h, dehydrated in graded ethanol solutions and routinely processed to paraffin. Paraffin-embedded tissues were cut using a microtome (Slee HS, Slee Equipment Limited, London, England) into transverse sections of 7 μm thick, and thaw-mounted on poly-L-lysine coated slides (Sigma-Aldrich, Alcobendas, Spain).

For single staining, sections were deparaffinized and rehydrated in graded ethanol solutions and treated with 3% of hydrogen peroxide for 5 min at room temperature, to inactivate endogenous peroxidase. Sections were then incubated with 10% normal horse serum in phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, pH 7.3) for 30 min, at 37 °C. Subsequently, sections were rinsed in PBS and incubated with primary antibodies diluted in phosphate-buffered triton (PBT; 0.3% Triton X-100, in PBS) in a moist chamber for 48 h at 4 °C. To identify receptor subtypes, rabbit polyclonal antibodies adenosine anti-A_1, anti-A_{2A}, anti-A_{2B} and anti-A_3 receptors (Alpha Diagnostics International, San Antonio, TX, USA) were used. The reactivity of these adenosine receptor antibodies has been previously validated (A_1: Nie et al., 1998; A_{2A}: Nie et al., 1999; A_{2B}: Clancy et al., 1999; A_3 Trincavelli et al., 2000). The following dilutions were used—anti-A_1, 1:250; anti-A_{2A}, 1:500; anti-A_{2B}, 1:500 and anti-A_3, 1:100. Thereafter, sections were processed with avidin–biotin–immunoperoxidase method using 3,3′-diaminobenzidine (DAB) as a chromogen. Briefly, immunoperoxidase staining was performed by using Vectastain Elite ABC kit universal (Vector Laboratories, Burlingame, USA). Sections were incubated with biotinylated anti-rabbit IgG (1:125 dilution in PBT) for 1 h at room temperature, washed in PBT and then incubated with avidin–biotin complex reagents (1:250 dilution in PBT) for 1 h at room temperature, using 3,3′-diaminobenzidine/peroxide as substrate.

For double staining, sections previously processed for single staining using one of the adenosine receptor subtype antibodies were further incubated with a neuronal marker (anti-neurofilament antibody; Dako, Glostrup, Denmark; 1:100 dilution in PBT) in a moist chamber for 24 h at 4 °C. The sites of antibody–antigen reaction were detected as above, using Vectastain Elite ABC kit universal (Vector Laboratories). Briefly, sections were incubated with biotinylated anti-mouse IgG (1:125 dilution in PBT) for 1 h at room temperature, washed in PBT and then incubated with avidin–biotin complex reagents (1:250 dilution in PBT). Peroxidase detection was carried out using Vector Vip Kit (Vector Laboratories).

Control tissues were processed similarly, except the primary antibody was replaced by 10% normal horse serum. These conditions resulted in the complete absence of specific immunoreactivity. To stain nerve fibres only, anti-neurofilament antibody was used in sections without previous staining.

Sections submitted to each of the immunohistochemistry protocols were washed with distilled water, dehydrated, cleared in xylene and then coverslipped with Eukitt mounting medium (O. Kindler, Freiburg, Germany). Sections were examined under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan), photographed by a digital camera Cool Pix E950 (1600 × 1200 pixels; Nikon). Image files were opened and the final photomicrographs compo-

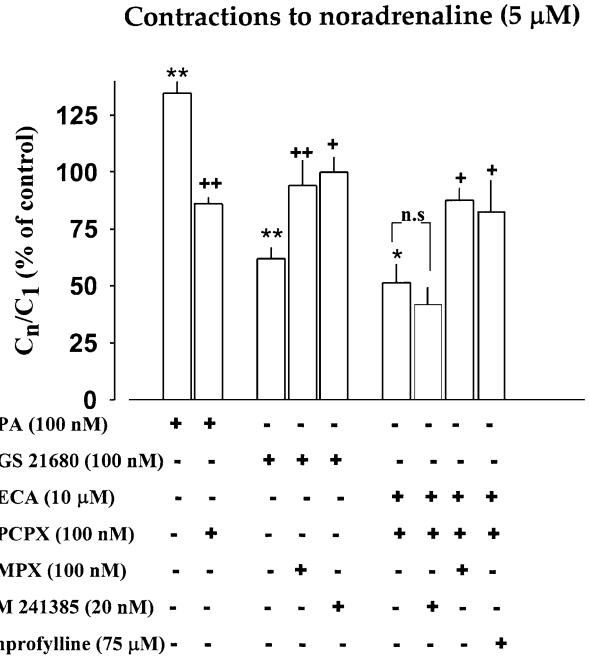

Fig. 1. Prostatic portion of the rat vas deferens: effects of the selective adenosine A_1 receptor agonist CPA (100 nM), of the selective adenosine A_{2A} receptor agonist CGS 21680 (100 nM) and of the adenosine receptor agonist NECA (10 μM) on noradrenaline-elicited contractions, in the absence and in the presence of the selective adenosine A_1 receptor antagonist DPCPX (100 nM), of the adenosine A_2 receptor antagonist DMPX (100 nM), of the selective adenosine A_{2A} receptor antagonist ZM 241385 (20 nM) and of the adenosine A_{2B} receptor antagonist enprofylline (75 μM). C_1 to C_n (C_2 and C_3) are the amplitude of contractions elicited by 5 μM noradrenaline added at 25-min intervals. Adenosine receptor agonists were added 5 min before C_2 and C_3 and kept until contraction to noradrenaline had peaked; antagonists were added after C_2 and kept until the end of the experiment. In experiments with NECA, DPCPX was added at the beginning of the experiment and kept throughout the experiment. *Ordinate*, C_n/C_1 values expressed as percentage of the corresponding average control C_n/C_1 value. Each point is the mean ± S.E.M. of 4–13 experiments. Significant differences from the respective control $^*P<0.05$ and $^{**}P<0.001$; from the effect of the agonist alone, $^{+}P<0.05$ and $^{++}P<0.001$.

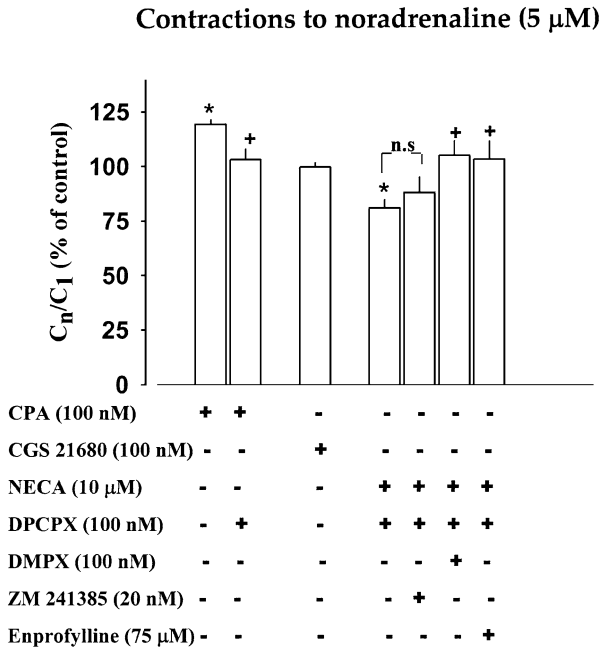

Fig. 2. Epididymal portion of the rat vas deferens: effects of the selective adenosine A_1 receptor agonist CPA (100 nM), of the selective adenosine A_{2A} receptor agonist CGS 21680 (100 nM) and of the adenosine receptor agonist NECA (10 μM) on noradrenaline-elicited contractions, in the absence and in the presence of the selective adenosine A_1 receptor antagonist DPCPX (100 nM), of the adenosine A_2 receptor antagonist DMPX (100 nM), of the selective adenosine A_{2A} receptor antagonist ZM 241385 (20 nM) and of the adenosine A_{2B} receptor antagonist enprofylline (75 μM). C_1 to C_n (C_2 and C_3) are the amplitude of contractions elicited by 5 μM noradrenaline added at 25-min intervals. Adenosine receptor agonists were added 5 min before C_2 and C_3 and kept until contraction to noradrenaline had peaked; antagonists were added after C_2 and kept until the end of the experiment. In experiments with NECA, DPCPX was added at the beginning of the experiment and kept throughout the experiment. *Ordinate*, C_n/C_1 values expressed as percentage of the corresponding average control C_n/C_1 value. Each point is the mean ± S.E.M. of 4–13 experiments. Significant differences from the respective control $^*P<0.05$; from the effect of the agonist alone, $^+P<0.05$.

sites prepared using Photoshop (version 7.0, Adobe Systems, San Jose, USA).

3. Results

3.1. Modulation of contractions by adenosine receptors

Noradrenaline and α,β-MeATP elicited contractions in both portions of the bisected rat vas deferens. Neither the adenosine receptor agonists and antagonists nor their solvent altered the basal tension. The concentration of noradrenaline and α,β-MeATP used to elicit contractions upon which adenosine receptor agonists and antagonists were tested were 5 and 10 μM, respectively. Those concentrations shown to cause comparable responses (about 70% of the maximal response elicited by each agonist). The amplitude of noradrenaline-elicited contractions was higher in the epididymal than in the prostatic portion (7.3 ± 0.6 and 2.2 ± 0.2 mN, respectively, $n=39–55$; $P<0.001$); the amplitude of α,β-MeATP-elicited contractions was similar in the epididymal and in the prostatic portion (8.2 ± 0.8 and 5.6 ± 0.9 mN, respectively, $n=16–17$; non-significant). When 5 μM noradrenaline or 10 μM α,β-MeATP was applied with 25-min intervals ($C_1–C_3$), the amplitude of the contractions remained constant, with C_n/C_1 ratios close to unity (not shown). None of the adenosine receptor antagonists used modified the amplitude of the contractions elicited by 5 μM noradrenaline or 10 μM α,β-MeATP.

Figs. 1 and 2 show the influence of adenosine receptor agonists on the amplitude of noradrenaline-elicited contrac-

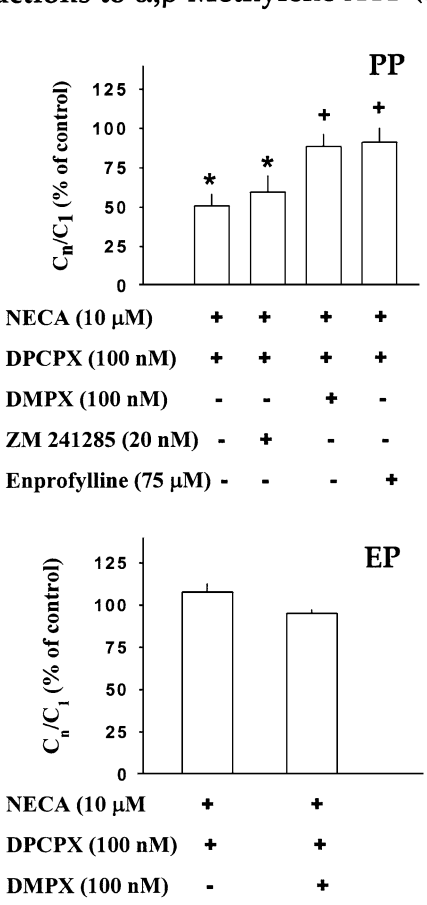

Fig. 3. Bisected rat vas deferens: effects of the adenosine receptor agonist NECA (10 μM) in the presence of selective adenosine A_1 receptor antagonist DPCPX (100 nM) on α,β-MeATP-elicited contractions, in the prostatic (PP) and epididymal (EP) portions, in the presence or in the absence of the adenosine A_2 receptor antagonist DMPX (100 nM), of the adenosine A_{2A} receptor antagonist ZM 241385 (20 nM) or of the A_{2B} antagonist enprofylline (75 μM). C_1 to C_n (C_2 and C_3) are the amplitude of contractions elicited by 10 μM added at 25-min intervals. NECA was added 5 min before C_2 and C_3 and kept until contraction to α,β-MeATP had peaked; DPCPX was added at the beginning and was kept until the end of the experiment; antagonists were added after C_2 and kept until the end of the experiment. *Ordinate*, C_n/C_1 values expressed as percentage of the corresponding average control C_n/C_1 value. Each point is the mean ± S.E.M. of 5–11 experiments. Significant differences from the respective control $^*P<0.05$; from the effect of the agonist alone, $^+P<0.05$.

tions, in the absence and in the presence of adenosine receptor antagonists. CPA (100 nM), a selective adenosine A_1 receptor agonist (Jacobson et al., 1992), increased the amplitude of the contractions, an effect more marked in the prostatic than in the epididymal portion ($P<0.05$; compare Figs. 1 and 2). The CPA-induced enhancement was blocked by DPCPX (100 nM), a selective adenosine A_1 receptor antagonist (Bruns et al., 1987), in both portions of the bisected rat vas deferens.

The selective adenosine A_{2A} receptor agonist CGS 21680 (100 nM; Lupica et al., 1990) reduced the amplitude of noradrenaline-elicited contractions, but only in the prostatic portion (Fig. 1). The inhibition caused by CGS 21680 was prevented by the selective adenosine A_2 receptor antagonist DMPX (100 nM; Daly et al., 1986) and by the selective adenosine A_{2A} receptor antagonist ZM 241385 (20 nM; Poucher et al., 1995).

Due to the lack of selective adenosine A_{2B} receptor agonists, an indirect approach was used to investigate a putative adenosine A_{2B} receptor-mediated influence on contractions (see Figs. 1 and 2): the non-selective adenosine receptor agonist NECA was used (Daly et al., 1986; Klotz et al., 1998) in the presence of DPCPX and its effects challenged by selective adenosine A_2 receptor antagonists. NECA (10 μM) in the presence of 100 nM DPCPX, reduced the amplitude of noradrenaline-elicited contractions in both

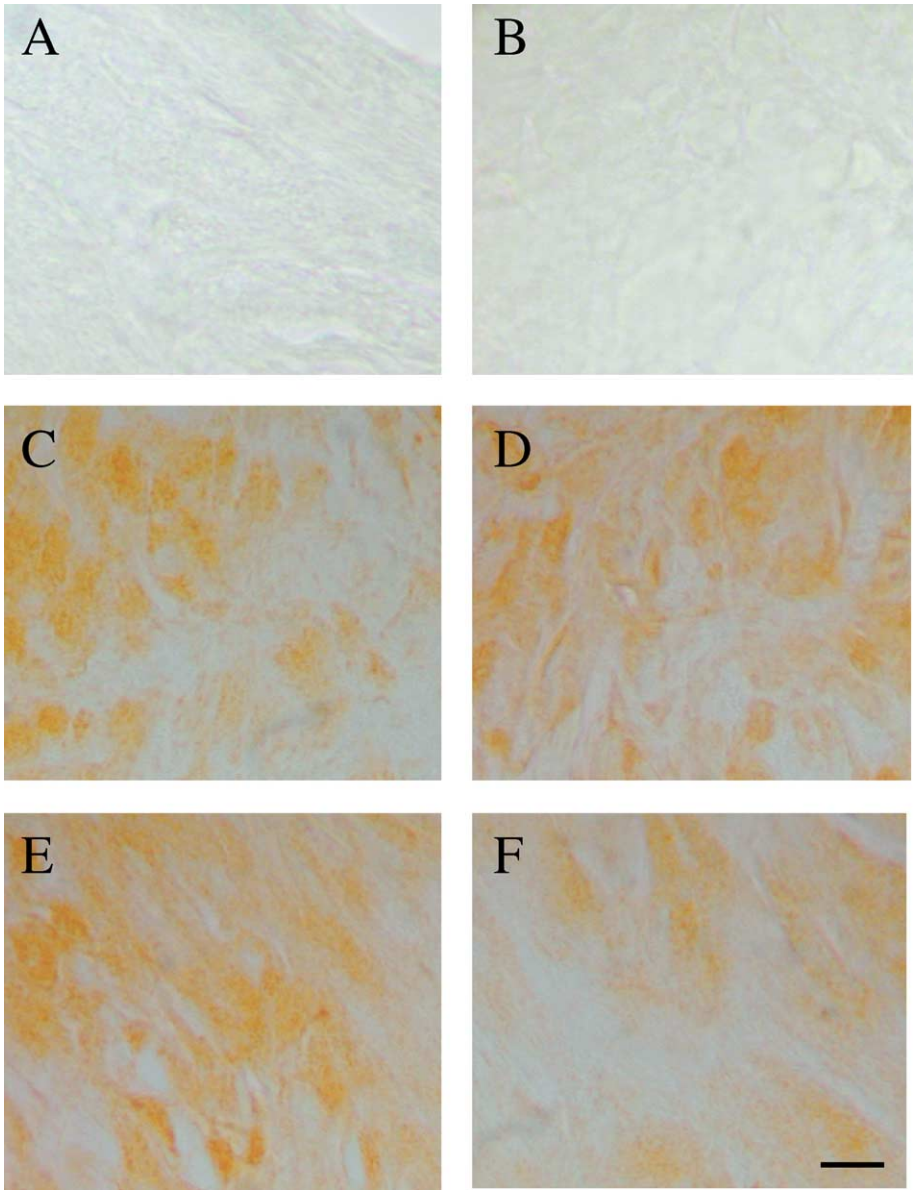

Fig. 4. Representative photomicrographs of transversal sections of the prostatic (left panel) and epididymal (right panel) portions of rat vas deferens showing immunoreactivities to individual adenosine receptors in tissue sections processed in a single batch. Control sections (A and B); and sections treated with adenosine anti-A_{2A} (C and D) or anti-A_{2B} (E and F) receptor antibodies. Bar = 5 μm.

portions of the bisected rat vas deferens. The inhibition caused by NECA in the presence of DPCPX was prevented by DMPX (100 nM), by the adenosine A_{2B} receptor antagonist 3-propylxanthine (enprofylline; 75 μM) but not by ZM 241385 (20 nM; Figs. 1 and 2). NECA-induced inhibition was more marked in the prostatic than in the epididymal portion (maximal reduction reached was $49 \pm 8\%$ and $19 \pm 4\%$, respectively, $n = 7$; $P < 0.01$; see Figs. 1 and 2).

The selective adenosine A_3 receptor agonist IB-MECA (100 nM; Gallo-Rodriguez et al., 1994) did not change contractions elicited by 5 μM noradrenaline either in the prostatic or in the epididymal portion of the rat vas deferens (not shown).

A set of experiments was carried out using the ATP analogue, α,β-MeATP as contractile agent. CPA and CGS 21680 failed to modify the contractile responses elicited by 10 μM α,β-MeATP in both the prostatic and epididymal portions (not shown). NECA, in the presence of DPCPX, reduced contractions elicited by 10 μM α,β-MeATP, only in the prostatic portion, an effect blocked by 100 nM DMPX and 75 μM enprofylline but not by 20 nM ZM 241385 (Fig. 3).

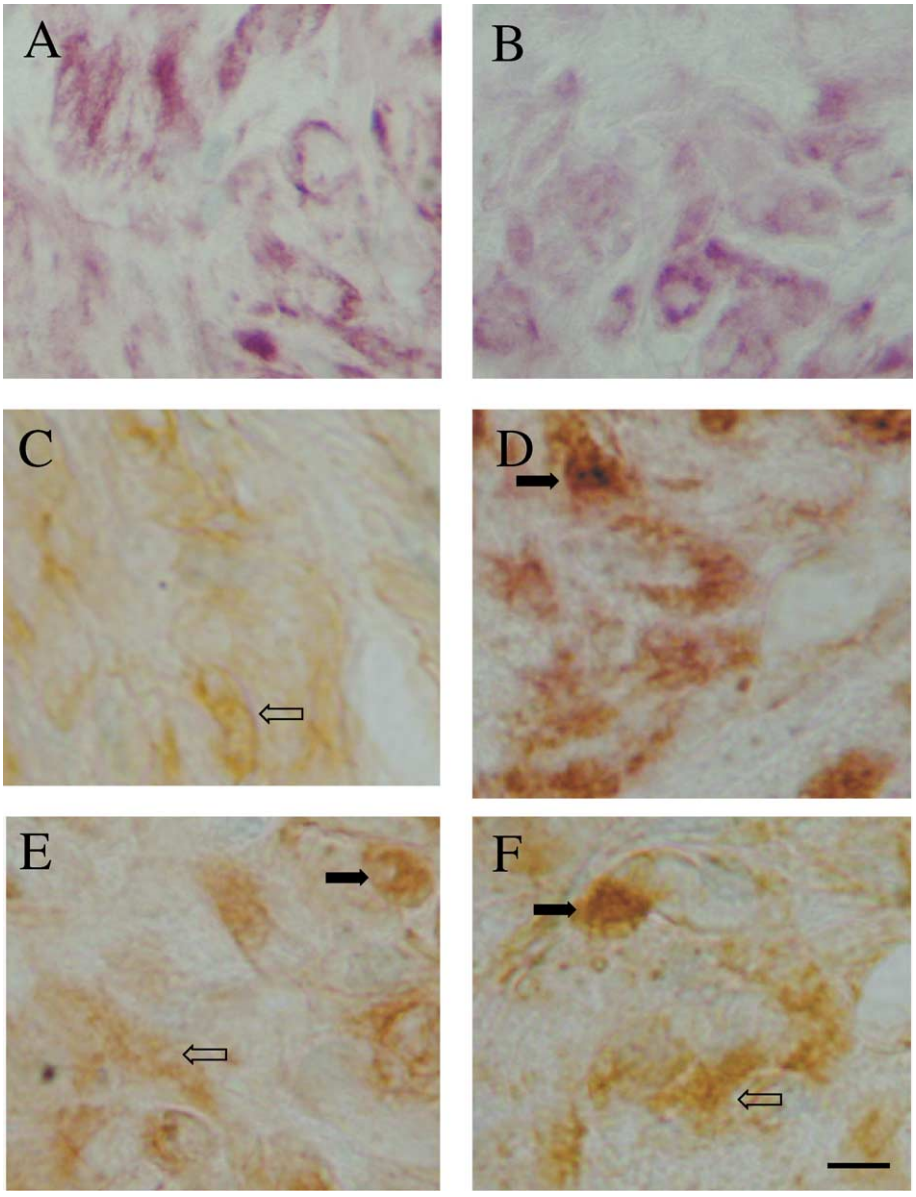

Fig. 5. Transversal sections of the prostatic (left panel) and epididymal (right panel) portions of rat vas deferens showing immunoreactivities to neurofilament alone (A and B), to neurofilament plus adenosine A_{2A} receptors (C and D) or to neurofilament plus adenosine A_{2B} receptors (E and F).Open arrows highlight adenosine receptor immunoreactivity not co-localized with neurofilament (interpreted as an indication of a non-neuronal location of receptor); filled arrows highlight adenosine receptor immunoreactivity co-localized with neurofilament (suggesting a neuronal location of the receptor). Bar = 5 μm.

3.2. Immunohistochemical detection of adenosine receptors

Standard immunohistochemistry techniques were used to characterize the distribution of adenosine receptor subtypes in the bisected rat vas deferens using polyclonal antibodies: adenosine anti-A_1, anti-A_{2A}, anti-A_{2B} and anti-A_3 receptors. Data was obtained from four animals (corresponding to 40 tissue sections per adenosine receptor subtype antibody). Two additional animals were used in preliminary experiments to optimize the staining procedures. Omission of primary antibodies in control incubations resulted, as expected, in the absence of staining.

Single immunohistochemistry data showed intense immunoreactivities for adenosine anti-A_{2A} and anti-A_{2B} both in the prostatic and epididymal portions of the rat vas deferens (Fig. 4). It was also found an intense immunoreactivity for adenosine anti-A_1 receptors in both portions of the rat vas deferens whereas immunoreactivity to adenosine anti-A_3 receptors was not observed (data not shown).

Double immunohistochemistry allowed detection of immunoreactivities to individual adenosine receptor subtypes co-localized with nerve fibres. Neurofilament antibody was used as neuronal fibre marker. Representative photomicrographs from tissue sections of the prostatic and epididymal portions of the rat vas deferens of the same animal, processed by double immunohistochemistry (single batch) show the pattern of distribution immunoreactivities of neurofilament co-localized with individual adenosine A_2 receptors (Fig. 5). Neurofilament immunoreactivity was distributed throughout the prostatic and epididymal portions of the rat vas deferens (Fig. 5A and B, respectively; violet areas) as previously described (Elfvin et al., 1987). In the prostatic portion, an intense adenosine A_{2B} receptor immunoreactivity was co-localized with neurofilament immunoreactivity which was recognized by the presence of darker areas (Fig. 5E; dark purple areas). It was further observed immunoreactivity for adenosine A_{2B} receptors not co-localized with neurofilament immunoreactivity which was recognized by the presence of light brown areas (Fig. 5E; light brown areas). Co-localized areas of adenosine A_{2A} receptor and neurofilament immunoreactivities were rarely found (Fig. 5C). In the epididymal portion, adenosine A_{2A} receptor immunoreactivity was mainly co-localized with neurofilament immunoreactivity (Fig. 5D; dark purple areas) whereas adenosine A_{2B} receptor immunoreactivity was observed either co-localized (see Fig. 5F; dark purple areas) or not co-localized (Fig. 5D; light brown areas) with neurofilament immunoreactivity.

4. Discussion

The present study confirms previous observations that showed, in the rat vas deferens, the occurrence of an adenosine A_1 receptor-mediated enhancement and of an adenosine A_2 receptor-mediated inhibition of contractions (Hourani et al., 1993; Brownhill et al., 1996a) and extends these observations to contractions mediated by receptors likely involved in neurogenic contractions (α adrenoceptors and P2X receptors). These effects of adenosine receptor agonists occur without causing any change on the basal tension of preparations.

It is unlikely that effects of adenosine receptor agonists are due to an interference with noradrenaline and α,β-MeATP inactivation since the effects were not changed by inhibition of noradrenaline uptake with 400 nM desipramine (not shown) and α,β-MeATP is considered to be a stable ATP analogue.

In all likelihood, the enhancement of contractile responses caused by adenosine A_1 receptor agonists and the inhibition caused by the adenosine A_2 receptor agonists are mediated by the respective adenosine receptors. The adenosine A_1 and A_{2A} receptor agonists (CPA and CGS 21680, respectively) were used in concentrations considered to be selective for their respective adenosine receptor. Due to the lack of selective adenosine A_{2B} receptor agonists, the non-selective adenosine receptor agonist NECA was used in the low micromolar range, in the presence of the adenosine A_1 receptor antagonist DPCPX, an approach usually recommended to activate adenosine A_{2B} receptors (Feoktistov and Biaggioni, 1997). DPCPX was used at the concentration of 100 nM to completely prevent adenosine A_1 receptor-mediated responses. Although at this concentration DPCPX has been described to exert some blockade of adenosine A_{2B} receptors (Alexander et al., 1986; Klotz et al., 1998), NECA was still able to inhibit contractions both in the prostatic and in the epididymal portions of the rat vas deferens. Confirmation that effect of NECA is mediated by adenosine A_{2B} receptors was obtained using enprofylline, a compound showed to be 22-fold selective for adenosine A_{2B} receptors versus A_1, 5-fold versus A_{2A} and 6-fold versus adenosine A_3 receptors (Robeva et al., 1996), which blocked the effect of NECA plus DPCPX. Additional evidence were obtained using DMPX, a compound showed to be an antagonist of adenosine A_2 receptors with a K_bA_{2B}/K_bA_{2A} ratio of about 1.3 (Brackett and Daly, 1994), which also blocked the effect of NECA plus DPCPX, and using ZM 241385, a selective adenosine A_{2A} receptor antagonist, 90-fold selective for adenosine A_{2A} receptors versus A_{2B} (Poucher et al., 1995), which failed to block the effect of NECA plus DPCPX. Although the wide distribution of adenosine A_3 receptors (see Ralevic and Burnstock, 1998), the failure of IB-MECA to modify contractions strongly suggest that adenosine A_3 receptors are not involved on postjunctional modulation of contractile responses in the rat vas deferens.

The observation in rat vas deferens, of effects compatible with activation of adenosine A_1, A_{2A} and A_{2B} receptors, and the absence of effects mediated by an adenosine A_3 receptor agonist are in agreement with results obtained by in situ hybridization and RT-PCR (Dixon et al., 1996). They are also compatible with the present immunohistochemical data that revealed immunoreactive areas for antibodies to adeno-

sine A_1 receptor and to both adenosine A_2 receptor subtypes (anti-A_{2A} and anti-A_{2B} receptors) but not to adenosine A_3 receptors.

The two portions of the rat vas deferens present important differences in the role of the purine ATP as motor transmitter (McGrath, 1978; Sneddon and Machaly, 1992) and on the role of the purine adenosine as modulator of noradrenaline release (Queiroz et al., 2002). The present study supports the occurrence of regional differences between the prostatic and epididymal portions on the type of adenosine receptor-mediated modulation of contractile responses: the adenosine A_1 receptor-mediated enhancement occurs in both portions, but seems to be more pronounced in the prostatic than in the epididymal portion whereas the inhibition mediated by adenosine A_2 receptors seems to involve both A_{2A} and A_{2B} subtypes in the prostatic but mainly the A_{2B} subtype in the epididymal portion.

The regional differences in the adenosine A_2 receptor-mediated effects are compatible with the immunohistochemical data that showed, in the prostatic portion, an intense distribution of adenosine A_{2A} and A_{2B} receptor-immunoreactivities not associated with nerve fibres (suggestive of a postjunctional location) whereas, in the epididymal portion, the adenosine A_2 receptor-immunoreactivity not associated with nerve fibres was mainly of the adenosine A_{2B} receptor subtype.

Apparently, the occurrence of adenosine A_2 receptor-mediated inhibition of contractions observed in both portions contrasts with results previously published (Brownhill et al., 1996a) that interpreted the failure of NECA to modify KCl-induced contractions in the epididymal portion as an indication that postjunctional adenosine A_2 receptors would not be involved on modulation of contractions in the epididymal portion. However, NECA was tested in the absence of adenosine A_1 receptor blockade. Therefore, it is conceivable that an adenosine A_1 receptor-mediated enhancement may have masked the adenosine A_2 receptor-mediated inhibition more easily in the epididymal portion (where mainly the adenosine A_{2B} receptor subtype seems to be operating) than in the prostatic portion (where both adenosine A_{2A} and A_{2B} receptor subtypes seem to be operating).

It has been suggested that adenosine A_1 receptor-mediated modulation of contractions may show selectivity for certain contractile agents. In the rat vas deferens, the adenosine A_1 receptor-mediated enhancement was observed on contractions elicited by ATP but not on contractions elicited by α-adrenoceptor agonists (Brownhill et al., 1996b). However, in guinea-pig vas deferens, the opposite (enhancement of contractions elicited by α-adrenoceptor agonists and no influence on contractions elicited by ATP) was observed (Haynes et al., 1998), a pattern of effects identical to those observed in the present study. Therefore, the suggested selectivity is unlikely. The variability on modulation according to the contractile agent used may be due, at least in part, to the amplitude of the induced contractions, since it was observed that contractions induced by a lower concentration of noradrenaline or by tyramine were already potentiated by activation of adenosine A_1 receptors (Brownhill et al., 1996b). The possibility that modulation of contractions elicited by certain contractile agents may be more easily detected cannot be excluded. This could explain why adenosine A_2 receptor-mediated inhibition was more marked on contractions elicited by noradrenaline than on contractions elicited by α,β-MeATP.

In conclusion, this work presents functional and immunohistochemical evidence that supports the presence of postjunctional adenosine A_1 and A_2 (A_{2A} and A_{2B}) receptors, mediating an enhancement and inhibition of contractions without influencing, per se, the basal tonus of the preparation. It further shows that adenosine A_1 receptor-mediated modulation occurs in both prostatic and epididymal portions whereas the adenosine A_2 receptor-mediated modulation seems to involve adenosine A_{2A} and A_{2B} receptor subtypes in the prostatic portion but only the adenosine A_{2B} receptors in the epididymal portion.

Acknowledgements

This work was supported by the Fundação para a Ciência e Tecnologia (FCT: I and D n. 226/94, POCTI-QCA III, POCTI/36545/FCB/2000) and FEDER.

References

Alexander, S.P.H., Cooper, J., Shine, J., Hills, S.J., 1986. Characterization of the human brain putative A_{2B} adenosine receptor expressed in Chinese hamster ovary (CHO·A_{2B4}) cells. Br. J. Pharmacol. 119, 1286–1290.

Allcorn, R.J., Cunnane, T.C., Kirkpatrick, K., 1986. Actions of α,β-methylene ATP and 6-hydroxydopamine on sympathetic neurotransmission in the vas deferens of the guinea-pig, rat and mouse: support for co-transmission. Br. J. Pharmacol. 89, 647–659.

Brackett, L.E., Daly, J.W., 1994. Functional characterization of the A_{2b} adenosine receptor in NIH 3T3 fibroblasts. Biochem. Pharmacol. 47, 801–814.

Brownhill, V.R., Hourani, S.M.O., Kitchen, I., 1996a. Differential distribution of adenosine A_2 receptors in the epididymal and prostatic portions of rat vas deferens. Eur. J. Pharmacol. 303, 87–90.

Brownhill, V.R., Hourani, S.M.O., Kitchen, I., 1996b. Selective enhancement by an adenosine A_1 receptor agonist of agents inducing contraction of the rat vas deferens. Naunyn-Schmiedeberg's Arch. Pharmacol. 335, 499–504.

Bruns, R.F., Fergus, J.H., Badger, E.W., Bristol, J.A., Santay, L.A., Hartman, J.D., Hays, S.J., Huang, C.C., 1987. Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-l,3-dipropylxanthine to rat brain membranes. Naunyn-Schmiedeberg's Arch. Pharmacol. 335, 59–63.

Clancy, J.P., Ruiz, F.E., Sorscher, E.J., 1999. Adenosine and its nucleotides activate wild-type and R117H CFTR through an A_{2B} receptor-coupled pathway. Am. J. Physiol. 276, C361–C369.

Conti, A., Monopoli, A., Gambá, M., Borea, P.A., Ongini, E., 1993. Effects of selective A_1 and A_2 adenosine receptor agonists on cardiovascular tissues. Naunyn-Schmiedeberg's Arch. Pharmacol. 348, 108–112.

Daly, J.W., Padgett, W.L., Shamim, M.T., 1986. Analogues of caffeine and theophylline: effect of structural alterations on affinity at adenosine receptors. J. Med. Chem. 29, 1305–1308.

Dixon, A.K., Gubitz, A.K., Sirinathsinghji, D.J.S., Richardson, P.J., Freeman, T.C., 1996. Tissue distribution of adenosine receptor mRNAs in the rat. Br. J. Pharmacol. 118, 1461–1468.

Elfvin, L.G., Björklund, H., Dahl, D., Seiger, A., 1987. Neurofilament-like and glial fibrillary acidic protein-like immunoreactivities in rat and guinea-pig sympathetic ganglia in I situ and after perturbation. Cell Tissue Res. 250, 79–86.

Feoktistov, I., Biaggioni, I., 1997. Adenosine A_{2B} receptors. Pharmacol. Rev. 49, 381–402.

Fozard, J.R., Milavec-Krizman, M., 1993. Contraction of the rat isolated spleen mediated by adenosine A_1 receptor activation. Br. J. Pharmacol. 109, 1059–1063.

Fredholm, B.B., Ijerman, A.P., Jacobson, K.A., Klotz, K.-N., Linden, J., 2001. International union of pharmacology: XXV. Nomenclature and classification of adenosine receptors. Pharmacol. Rev. 53, 527–552.

Gallo-Rodriguez, C., Ji, X.-D., Melman, N., Siegman, B.D., Sanders, L.H., Orlina, J., Fischer, B., Pu, Q., Olah, M.E., van Galen, P.J.M., Stiles, G.L., Jacobson, K.A., 1994. Structure–activity relationships of N^6-benzyladenosine 5′-uronamides as A_3-selective adenosine agonists. J. Med. Chem. 37, 636–646.

Gonçalves, J., Guimarães, S., 1991. Influence of neuronal uptake on pre- and postjunctional effects of α-adrenoceptor agonists in tissues with noradrenaline–ATP cotransmission. Naunyn-Schmiedeberg's Arch. Pharmacol. 344, 532–537.

Gonçalves, J., Queiroz, G., 1993. Facilitatory and inhibitory modulation by endogenous adenosine of noradrenaline release in the epididymal portion of rat vas deferens. Naunyn-Schmiedeberg's Arch. Pharmacol. 348, 367–371.

Haynes, J.M., Alexander, S.P.H., Hill, S.J., 1998. A_1 adenosine receptor modulation of electrically-evoked contractions in the bisected vas deferens and cauda epididymis of the guinea-pig. Br. J. Pharmacol. 124, 964–970.

Hedqvist, P., Fredholm, B.B., 1976. Effects of adenosine on adrenergic neurotransmission; prejunctional inhibition and postjunctional enhancement. Naunyn-Schmiedeberg's Arch. Pharmacol. 293, 217–223.

Hourani, S.M.O., Jones, D.A.D., 1994. Post-junctional excitatory adenosine A_1 receptors in the rat vas deferens. Gen. Pharmacol. 25, 417–420.

Hourani, S.M.O., Nicholis, J., Lee, B.S.S., Halfhide, E.J., Kitchen, I., 1993. Characterization and ontogeny of P_1-purinoceptors on rat vas deferens. Br. J. Pharmacol. 108, 754–758.

Jacobson, K.A., van Galen, P.J.M., Williams, M., 1992. Adenosine receptors: pharmacology, structure–activity relationships, and therapeutic potential. J. Med. Chem. 35, 407–422.

Klotz, K.-N., Hessling, J., Hegler, J., Owman, C., Kull, B., Fredholm, B.B., Lohse, M.J., 1998. Comparative pharmacology of human adenosine receptor subtypes—characterization of stably transfected receptors in CHO cells. Naunyn-Schmiedeberg's Arch. Pharmacol. 357, 1–9.

Kurz, K., von Kügelgen, I., Starke, K., 1993. Prejunctional modulation of noradrenaline release in mouse and rat vas deferens: contribution of P_1- and P_2-purinoceptors. Br. J. Pharmacol. 110, 1465–1472.

Lupica, C.R., Cass, W.A., Zahniser, N.R., Dunwiddie, T.V., 1990. Effects of the selective adenosine A_2 receptor agonist CGS 21680 on in vitro electrophysiology, cAMP formation and dopamine release m rat hippocampus and striatum. J. Pharmacol. Exp. Ther. 252, 1134–1141.

McGrath, J.C., 1978. Adrenergic and 'non-adrenergic' components in the contractile response of the vas deferens to a single indirect stimulus. J. Physiol. 283, 23–39.

Nie, Z., Mei, Y., Ford, M., Rybak, L., Marcuzzi, A., Ren, H., Stiles, G.L., Ramkumar, V., 1998. Oxidative stress increases A_1 adenosine receptor expression by activating nuclear factor κB. Mol. Pharmacol. 53, 663–669.

Nie, Z., Mei, Y., Malek, R.L., Marcuzzi, A., Lee, N., Ramkumar, V., 1999. A role of p75 in NGF-mediated down-regulation of the A_{2A} adenosine receptors in PC12 cells. Mol. Pharmacol. 56, 947–954.

Poucher, S.M., Keddie, J.R., Singh, P., Stoggall, S.M., Caulkett, P.W.R., Jones, G., Collis, M.G., 1995. The in vitro pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist. Br. J. Pharmacol. 115, 1096–1102.

Queiroz, G., Diniz, C., Gonçalves, J., 2002. Facilitation of noradrenaline release by adenosine A_{2A} receptors in the epididymal portion and adenosine A_{2B} receptors in the prostatic portion of the rat vas deferens. Eur. J. Pharmacol. 448, 45–50.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.

Ribeiro, J.A., 1995. Purinergic inhibition of neurotransmistter release in the central nervous system. Pharmacol. Toxicol. 77, 299–305.

Robeva, A.S., Woodard, R.L., Jin, X., Gao, Z., Bhattacharya, S., Taylor, H.E., Rosin, D.L., Linden, J., 1996. Molecular characterization of recombinant human adenosine receptors. Drug Dev. Res. 39, 243–252.

Rubino, A., Ralevic, V., Burnstock, G., 1995. Contribution of P_1- (A_{2b} subtype) and P_2-purinoceptors to the control of vascular tone in the rat isolated mesenteric arterial bed. Br. J. Pharmacol. 115, 648–652.

Sneddon, P., Machaly, M., 1992. Regional variation in purinergic and adrenergic responses in isolated vas deferens of rat, rabbit and guinea-pig. J. Auton. Pharmacol. 12, 421–428.

Stoggall, S.M., Shaw, J.S., 1990. The coexistence of adenosine A_1 and A_2 receptors in guinea-pig aorta. Eur. J. Pharmacol. 190, 329–335.

Szentmiklósi, A.J., Ujfalusi, A., Cseppentö, Á., Nosztray, K., Kovács, P., Szabó, J.Zs., 1995. Adenosine receptors mediate both contractile and relaxant effects of adenosine in main pulmonary artery of guinea pigs. Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 417–425.

Trincavelli, M.L., Tuscano, D., Cechetti, P., Falleni, A., Benzi, L., Klotz, K.-L., Gremigni, V., Catabenni, F., Lucacchini, A., Martini, C., 2000. Agonist-induced internalization and recycling of the human A_3 adenosine receptors: role in receptor desensitization and resensitization. J. Neurochem. 75, 1493–1501.