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European Journal of Pharmacology 504 (2004) 17–25

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Adenosine receptors involved in modulation of noradrenaline release in isolated rat tail artery

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Received 23 February 2004; received in revised form 16 May 2004; accepted 21 September 2004

Abstract

Adenosine receptors involved in the modulation of noradrenaline release from postganglionic sympathetic nerves in rat tail artery were characterized by studying the effects of adenosine-receptor agonists and antagonists on electrically evoked tritium overflow (100 pulses, 5 Hz) and by immunohistochemistry. The adenosine A_1 receptor-selective agonist N^6-cyclopentyladenosine (CPA; 1–100 nM) and the non-selective adenosine receptor agonist *N*-ethylcarboxamidoadenosine (NECA; 1–10 μM) decreased tritium overflow. These effects were blocked by the adenosine A_1 receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 30 nM). The adenosine A_{2A} receptor-selective agonist 2-*p*–(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamido adenosine (CGS 21680; 1–100 nM) enhanced tritium overflow, an effect blocked by the adenosine A_{2A} receptor-selective antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261; 20 nM) but not changed by the adenosine A_{2B} receptor-selective antagonist *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl) phenoxy]acetamide (MRS 1706; 20 nM). In the presence of DPCPX (30 nM), NECA enhanced tritium overflow, an effect abolished by MRS 1706 but not influenced by SCH 58261. Immunohistochemistry revealed immunoreactivity for all adenosine-receptor subtypes. Areas of co-localization were found for neurofilament with adenosine A_1, A_{2A} and A_{2B} but not A_3 receptors. In conclusion, the present study provides functional and morphological evidence for the occurrence of multiple adenosine receptor-mediated modulation of noradrenaline release in the rat tail: inhibition mediated by adenosine A_1 receptors and facilitation mediated by both adenosine A_{2A} and A_{2B} receptors.

Keywords: Prejunctional adenosine receptor; Adenosine A_{2A} receptor; Adenosine A_{2B} receptor; Immunohistochemistry; Noradrenaline release; Tail artery, rat

1. Introduction

Adenosine plays multiple roles in the modulation of postganglionic sympathetic transmission, acting through activation of G-protein-coupled receptors, known as adenosine receptors. Four subtypes, A_1, A_{2A}, A_{2B} and A_3, have been cloned and characterized by their pharmacological profiles (see Fredholm et al., 2001). Adenosine receptors may be located pre- or postjunctionally, modulating neurotransmitter release or the reactivity of postjunctional cells, respectively (Ralevic and Burnstock, 1998).

Sympathetic transmission is modulated by prejunctional adenosine A_1 receptors that mediate inhibition of noradrenaline release (pulmonary artery: Wiklund et al., 1989; vas deferens: Gonçalves and Queiroz, 1993; Queiroz et al., 2002; mesenteric artery: Ralevic, 2000; Shinozuka et al., 2001; tail artery: Gonçalves and Queiroz, 1996, Fresco et al., 2002) and by prejunctional adenosine A_2 receptors that mediate facilitation of noradrenaline release (vas deferens: Gonçalves and Queiroz, 1993; Queiroz et

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doi:10.1016/j.ejphar.2004.09.048

al., 2002; tail artery: Gonçalves and Queiroz, 1996, Fresco et al., 2002). The available evidence indicates that the receptor involved in this facilitation is of the adenosine A_{2A} receptor subtype (Ralevic and Burnstock, 1998). Recently, evidence was presented suggesting that adenosine A_{2B} receptors may also be involved in the facilitation of noradrenaline release from postganglionic sympathetic nerve terminals in the prostatic portion of the rat vas deferens (Queiroz et al., 2002).

In rat tail artery, noradrenaline release has been shown to be under a dual and opposite adenosine-receptor modulation: inhibition, mediated by adenosine A_1 receptors and facilitation, mediated by adenosine A_2 receptors, likely of the A_{2A} subtype (Gonçalves and Queiroz, 1996; Fresco et al., 2002), although the putative involvement of adenosine A_{2B} receptors has never been excluded, due to the lack of selective agonists and/or antagonists for this subtype of receptor (Feoktistov and Biaggioni, 1997). The aim of the present study was to further characterize the adenosine receptor subtypes involved in the modulation of noradrenaline release in rat tail artery, by combining a functional approach (modulation by selective adenosine receptor agonists in the absence and in the presence of adenosine receptor antagonists of electrically evoked tritium overflow from preparations pre-incubated with [^{3}H]noradrenaline) with an immunohistochemical approach (using antibodies against adenosine A_1, A_{2A}, A_{2B} and A_3 receptors).

2. Materials and methods

2.1. Chemicals

The following drugs were used: levo-[ring-2,5,6-^{3}H]noradrenaline, specific activity 46.8 Ci mmol^{-1} was from DuPont NEN (Garal, Lisboa, Portugal); 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21680), N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 3,3′-diaminobenzidine (DAB), 1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA), desipramine hydrochloride, *N*-ethylcarboxamidoadenosine (NECA) and Triton X-100 were from Sigma Aldrich (Alcobendas, Spain); *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl) phenoxy]acetamide (MRS 1706) was from Tocris (Bristol, UK). 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261) was a kind gift from Dr. Scott Weiss (Vernalis, UK). Unless otherwise stated, solutions of drugs were prepared with either distilled water or dimethylsulphoxide (DMSO) and diluted with medium immediately before use. 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. Animals

Adult male Wistar rats (230–390 g; CRIFFA, Barcelona, Spain) were kept under standard laboratory conditions: light/dark cycles 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 European Union Guidelines for animal research (86/609/CEE) and Portuguese law (Portarias nos. 1005/92 and 1131/97).

2.2.2. Overflow experiments

Animals were killed by cervical dislocation and exsanguination. The ventral tail artery was dissected out and cleaned of connective tissue. Four tissue preparations (endothelium-intact artery segments of approximately 2.5 cm length) were obtained from each animal. No more than two tissue preparations from the same animal were submitted to identical treatments.

Experiments were carried out as previously described (Fresco et al., 2002). Briefly, tissue pieces were incubated in 2 ml medium containing 0.1 μM [^{3}H]noradrenaline, for 40 min at 37 °C. Individual preparations were placed in superfusion chambers, between platinum electrodes, and superfused with [^{3}H]noradrenaline-free medium at a constant rate of 1 ml min^{-1}. Successive 5-min samples of the superfusate were collected from t=55 min onwards (t=0 being the onset of superfusion). At the end of the experiments, tissue preparations were homogenized in 2.5 ml perchloric acid (0.2 M) and the tritium content was determined in superfusate samples and in tissues by liquid scintillation spectrometry (LS 6500, Beckman Instruments, Fullerton, USA).

The medium contained (mM): NaCl 118.6, KCl 4.70, $CaCl_2$ 2.52, $MgSO_4$ 1.23, $NaHCO_3$ 25.0, glucose 10.0, ascorbic acid 0.3 and disodium EDTA 0.031; it was saturated with 95% O_2–5% CO_2 and kept at 37 °C. Desipramine (400 nM; to inhibit neuronal uptake of noradrenaline) and, in some experiments, DPCPX (30 nM; to block adenosine A_1 receptors) were introduced at the beginning of superfusion and were present throughout.

Up to five periods of electrical stimulation were applied (Stimulator II, Hugo Sachs Elektronik, March-Hugstetten, Germany; constant current mode; rectangular pulses; 1 ms width; current strength 50 mA; voltage drop between electrodes 18 V cm^{-1}; 100 pulses at 5 Hz). The first period, starting at t=30 min (S_0), was not used for determination of tritium outflow. The subsequent periods (S_1 up to S_4) started at t=60 min, at 30-min intervals. Concentration–response curves were obtained by adding the agonist at increasing concentrations 5 min before S_2, S_3 and S_4; the agonist was present until the end of each stimulation period. Antagonists were added 20 min before S_2 and were present until the end of the experiment. Tritium outflow is expressed as a fraction of the tritium tissue content at the onset of the respective collection period (fractional rate of outflow; min^{-1}). Electri-

cally evoked overflow of tritium was calculated as the difference between "total tritium outflow during and after stimulation" minus the estimated "basal outflow" and expressed as percentage of the tissue tritium content at the time of stimulation.

Effects of drugs on basal tritium outflow were estimated by ratios b_n/b_1 and expressed as percentage of the mean ratio obtained in the appropriate control; b_n was the fractional rate of outflow in the 5-min period before S_2, S_3 and S_4 (b_2, b_3 and b_4, respectively) and b_1 the fractional rate of outflow in the 5-min period before S_1. Effects of drugs added after S_1 on electrically evoked tritium overflow were evaluated as ratios of the overflow elicited by S_2, S_3 and S_4 (S_n) and the overflow elicited by S_1 (S_n/S_1). S_n/S_1 ratios obtained in individual experiments in which a test compound A was added after S_1 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 after S_1, and a drug B either added after S_1 or at the beginning of superfusion, was studied, the "appropriate control" was a group in which B alone was used (see Von Kügelgen et al., 1995).

Results are expressed as means±S.E.M.; n represents the number of tissue preparations (artery segments). Values from at least $n/2$ animals for each experimental group (identical treatment) are reported in Results. Differences between means were tested for significance using the unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by the multiple comparisons 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. After the induction of deep anaesthesia with sodium pentobarbital (60 mg/kg, intraperitoneally), the rats were killed by intracardiac perfusion with 100 ml of phosphate-buffered saline (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), followed by 400 ml of formalin (4% formaldehyde, 37 mM Na_2HPO_4, 29 mM NaH_2PO_4). Ventral tail arteries were dissected free from surrounding tissues and post-fixed in Bouin's solution (23.8% formalin, 4.7% glacial acetic acid, saturated with picric acid) overnight. Fixed tissues were rinsed with 70% ethanol and then extensively rinsed in distilled water for 5 h, dehydrated in graded ethanol solutions and routinely processed to paraffin. Paraffin block-embedded tissues were cut using a microtome (Slee HS, Slee Equipment, London, England) into transverse sections 7 μm thick and thaw-mounted on poly-L-lysine-coated slides (Sigma-Aldrich, Madrid, Spain).

For single staining, sections were dewaxed, rehydrated in graded ethanol solutions and treated with 3% hydrogen peroxide for 5 min at room temperature, to inactivate endogenous peroxidases. Sections were then incubated with 10% normal horse serum in PBS for 30 min at 37 °C. Subsequently, sections were washed 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 24 h at 4 °C. To identify adenosine receptor subtypes, rabbit polyclonal antibodies anti-A_1, anti-A_{2A}, anti-A_{2B} and anti-A_3 (Alpha Diagnostics International, San Antonio, TX, USA), the reliability of which 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), were used. The following dilutions were used: anti-A_1, 1:500; anti-A_{2A}, 1:500; anti-A_{2B}, 1:500; anti-A_3, 1:250. Thereafter, sections were processed with the avidin–biotin–immunoperoxidase method, using diaminobenzidine (DAB) as a chromogen. Brifley, immunoperoxidase staining was performed by using Vectastain Elite ABC kit universal (Vector Laboratories, Burlingame, USA). Sections were incubated with biotinylated anti-rabbit immunoglobulin G (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 DAB/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 neural marker (anti-neurofilament antibody; Dako, Glostrup, Denmark; 1:100 dilution in PBT) in a moist chamber for 24 h at 4 °C. 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 VectorVip Kit (Vector Laboratories). Control tissues were processed similarly, except that the primary antibody was omitted. 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. Sections were examined under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) and photographed using a digital camera Cool Pix E950 (1600×1200 pixels; Nikon). Image files were opened and final photomicrograph composites were prepared using Photoshop (version 7.0, Adobe Systems, San Jose, USA).

3. Results

3.1. Modulation of noradrenaline release by adenosine receptors

The involvement of adenosine receptors in the modulation of noradrenaline release was studied by testing the effects of adenosine A_1, A_{2A}, A_{2B} and A_3 receptor agonists

Table 1
Effects of adenosine receptor antagonists on electrically evoked tritium overflow from rat tail artery

Drugs added 20 min before S_2	S_2/S_1 % of control (n)
Desipramine (400 nM) throughout	
MRS 1706 (20 nM)	87±16 (4)
SCH 58261 (20 nM)	81±18 (4)
ZM 241385 (20 nM)	112±9 (10)
Desipramine (400 nM)+DPCPX (30 nM) throughout	
MRS 1706 (20 nM)	86±9 (8)
SCH 58261 (20 nM)	93±6 (12)
ZM 241385 (20 nM)	105±6 (10)

After pre-incubation with [^{3}H]-noradrenaline, tissues were superfused throughout with medium containing desipramine (400 nM) or desipramine+DPCPX (30 nM). Tissues were stimulated five times at 30-min intervals (S_0–S_4; 100 pulses at 5 Hz). Antagonists were added 20 min before S_2 and were present throughout the experiments. S_2/S_1 ratios obtained in individual tissue preparations were calculated as a percentage of the appropriate control (solvent). Values presented are means±S.E.M. n denotes the number of tissue preparations.

on electrically evoked tritium overflow from tissue preparations pre-incubated with [^{3}H]noradrenaline. In the absence of drugs (except 400 nM desipramine, which was present in the superfusion medium in all experiments to inhibit the neuronal uptake of noradrenaline), the fractional rate of outflow immediately before S_1 (b_1) was 0.106±0.003% min^{-1} (n=114); tritium overflow elicited by S_1 was 0.499±0.018% (n=114) of the tritium content of the tissue. In experiments in which 30 nM DPCPX (Bruns et al., 1987) was also added at the beginning of superfusion and kept throughout, b_1 was 0.104±0.002% min^{-1} (n=93) and S_1 was 0.397±0.022% (n=93) of the tritium content of the tissue. Basal outflow and electrically evoked tritium overflow remained constant throughout the experiments, with b_n/b_1 and S_n/S_1 values close to unity (not shown). Basal tritium outflow was not changed by any of the drugs added after S_1 (not shown). Adenosine receptor antagonists, which were added 20 min before S_2, also did not influence tritium overflow (Table 1).

In a first series of experiments, the effects of the adenosine A_1 receptor-selective agonist CPA (1–100 nM; Jacobson et al., 1992) and of the non-selective adenosine receptor agonist NECA (1–10 μM; Bruns et al., 1986) on

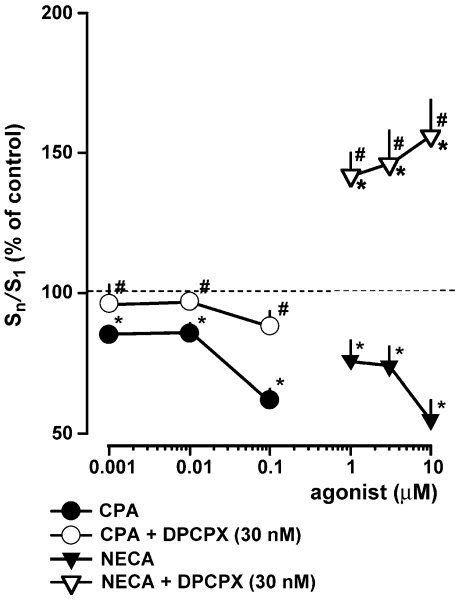

Fig. 1. Effects of the adenosine A_1 receptor-selective agonist CPA (1–100 nM; circles), and the non-selective adenosine receptor agonist NECA (1–10 μM; triangles) in the absence (filled symbols) or in the presence of the adenosine A_1 receptor-selective antagonist DPCPX (30 nM; open symbols) on evoked tritium overflow from isolated rat tail artery. Tissues were electrically stimulated with five trains of 100 pulses at 5 Hz (S_0–S_4). Agonists were added, at increasing concentrations, 5 min before S_2, S_3 and S_4 and were present until the end of the respective stimulation period; DPCPX was added at the beginning of superfusion and was present throughout the experiment. *Ordinates*: S_n/S_1 values obtained in individual tissue preparations, expressed as percentage of the appropriate S_n/S_1 control value. *Abcsissae*: Concentration of the adenosine receptor agonists. Values are means±S.E.M. from 6 to 12 tissue preparations. Significant differences from the appropriate control: *P<0.05; from agonist alone: $^{\#}P$<0.05 (unpaired Student's t-test).

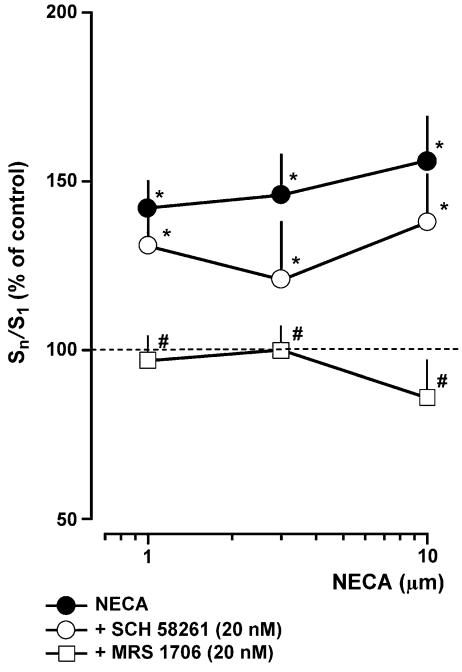

Fig. 2. Effects of the adenosine receptor agonist NECA in the presence of the adenosine A_1 receptor-selective antagonist DPCPX (30 nM) on evoked tritium overflow from isolated rat tail artery in the absence (filled circles) or in the presence of the A_{2A} receptor-selective antagonist SCH 58261 (20 nM; open circles) and of the A_{2B} receptor-selective antagonist MRS 1706 (20 nM; open squares). Tissues were electrically stimulated with five trains of 100 pulses at 5 Hz (S_0–S_4). DPCPX 30 nM was added at the beginning of the experiment and was present throughout superfusion. Agonists were added, at increasing concentrations, 5 min before S_2, S_3 and S_4 and were present until the end of the respective stimulation period; antagonists were added 20 min before S_2 and were present throughout the experiment. *Ordinates*: S_n/S_1 values obtained in individual tissue preparations, expressed as percentage of the appropriate S_n/S_1 control value. *Abscissae*: Concentration of NECA. Values are means±means±S.E.M. from 8 to 21 tissue preparations. Significant differences from solvent: *P<0.05 (unpaired Student's t-test); from agonist alone: $^{\#}P$<0.05 (ANOVA followed by Tukey's t-test).

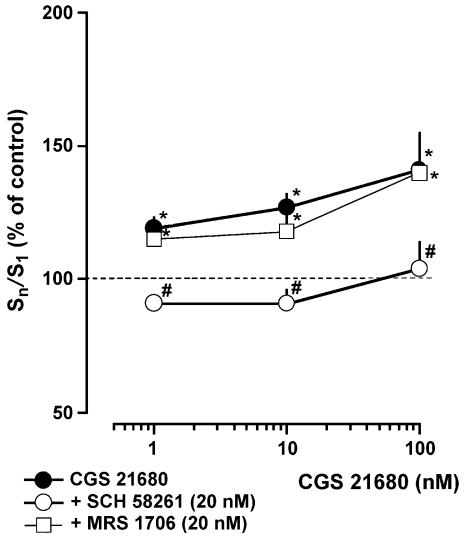

Fig. 3. Effects of the adenosine A_{2A} receptor-selective agonist CGS 21680 (1–100 nM) on evoked tritium overflow from isolated rat tail artery in the absence (filled circles) and in the presence of the adenosine A_{2A} receptor-selective antagonist SCH 58261 (20 nM; open circles) or in the presence of the adenosine A_{2B} receptor-selective antagonist MRS 1706 (20 nM; open squares). Tissues were electrically stimulated with five trains of 100 pulses at 5 Hz (S_0–S_4). Agonists were added, at increasing concentrations, 5 min before S_2, S_3 and S_4 and were present until the end of the respective stimulation period; antagonists were added 20 min before S_2 and were present throughout the experiment. *Ordinates*: S_n/S_1 values obtained in individual tissue preparations, expressed as percentage of the appropriate S_n/S_1 control value. *Abscissae*: Concentration of CGS 21680. Values are means±means±S.E.M. from 7 to 13 tissue preparations. Significant differences from solvent: $^*P<0.05$ (unpaired Student's *t*-test); from agonist alone: $^{\#}P<0.05$ (ANOVA followed by Tukey's *t*-test).

electrically evoked tritium overflow were studied. As shown in Fig. 1, both CPA and NECA decreased tritium overflow in a concentration-dependent manner. The decrease caused by CPA was blocked by the adenosine A_1 receptor-selective antagonist DPCPX (30 nM), which was present in the medium throughout the experiments (Fig. 1). In the presence of 30 nM DPCPX, the effect of NECA was reversed to a facilitation of tritium overflow (Fig. 1), probably reflecting the activation of facilitatory adenosine receptors.

The putative involvement of adenosine A_{2B} receptors in the modulation of noradrenaline release was investigated by studying the effects of the non-selective adenosine receptor agonist NECA (1–10 μM; Feoktistov and Biaggioni, 1997) in the presence of the adenosine A_1 receptor-selective antagonist DPCPX (30 nM; added at the beginning of superfusion and present throughout the experiments). Under these conditions, NECA (1–10 μM) increased the electrically evoked tritium overflow (Fig. 2), an effect blocked by the adenosine A_{2B} receptor-selective antagonist MRS 1706 (20 nM; Kim et al., 2000; Fig. 2) but not by the A_{2A} receptor-selective antagonist SCH 58261 (20 nM; Ongini et al., 1999; Fig. 2).

In another set of experiments, the A_{2A} receptor-selective agonist CGS 21680 (1–100 nM; Lupica et al., 1990) increased tritium overflow in a concentration-dependent manner, an effect that was blocked by the adenosine A_{2A} receptor-selective antagonist SCH 58261 (20 nM) but which was not influenced by the adenosineA_{2B} receptor-selective antagonist MRS 1706 (Fig. 3).

In preliminary experiments, the adenosine A_3 receptor-selective agonist IB-MECA (1, 10 and 100 nM; Gallo-Rodriguez et al., 1994), added 5 min before S_2, S_3 and S_4, did not change tritium overflow (not shown). A modified protocol was also used, in which IB-MECA was added only 1 min before S_2, S_3 and S_4. Under these conditions (1, 10 and 100 nM added 1 min before S_2, S_3 and S_4) IB-MECA again did not modify tritium overflow (S_n/S_1 values of 103±8%, 107±11% and 98±8%, respectively; n=10).

3.2. Immunohistochemical detection of adenosine receptors

Standard immunohistochemistry techniques were used to characterize the adenosine receptors present in rat tail artery, using polyclonal antibodies: adenosine anti-A_1, anti-A_{2A}, anti-A_{2B} and anti-A_3 receptors. Data were 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

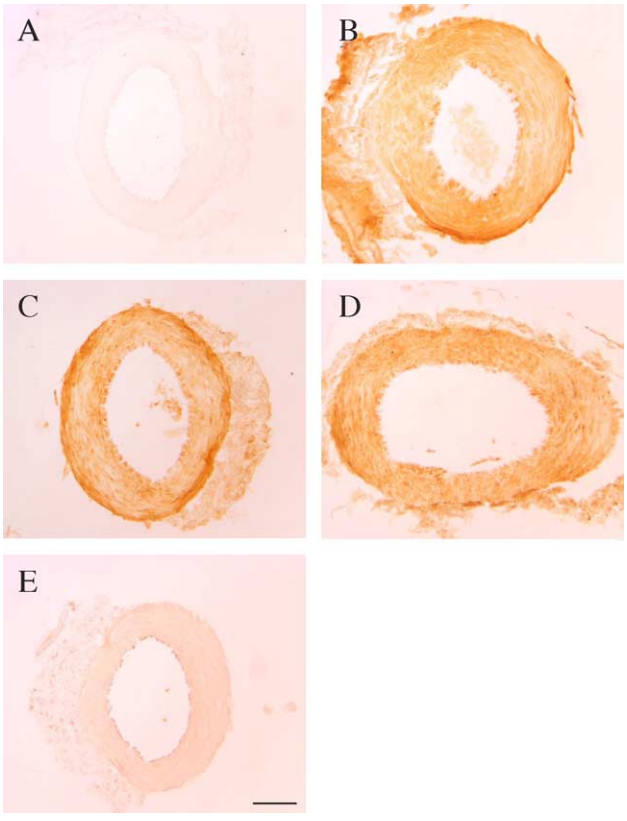

Fig. 4. Representative photomicrographs of transverse sections of the rat tail artery showing the pattern of distribution of individual adenosine receptor immunoreactivity (brown areas) in tissue sections processed in a single batch. Control section (A) reveals no immunoreactivity; sections treated with anti-A_1 (B), anti-A_{2A} (C), anti-A_{2B} (D) or anti-A_3 (E) reveals strong (B, C and D) and sparse (E) immunoreactivity. Bar=313 μm.

control incubations resulted, as expected, in the complete absence of specific immunoreactivity. Representative photomicrographs from sections of the same animal processed by immunohistochemistry (in a single batch) show the pattern of distribution of adenosine A_1, A_{2A}, A_{2B} and A_3 receptor immunoreactivity in the rat tail artery (Fig. 4; brown areas). In general, an intense immunoreactivity for adenosine A_1, A_{2A} and A_{2B} receptors was distributed throughout the adventitia, media and intima tunica (Fig. 4B–D, respectively). Immunoreactivity for adenosine A_3 receptors was distributed throughout the tunica of the artery but sparsely in comparison with that observed with the other adenosine receptor antibodies (see Fig. 4E).

Double immunohistochemistry allowed simultaneous detection of each adenosine receptor subtype and nerve fibres. Neurofilament antibody was used as a marker for nerve fibres. Representative photomicrographs from sections of the same animal processed by double immunohistochemistry (single batch) show the pattern of distribution of neurofilament and adenosine receptor immunoreactivity in the rat tail artery (Fig. 5). Neurofilament immunoreactivity was distributed throughout the tunica (Fig. 5A; violet areas). Adenosine A_1, A_{2A} and A_{2B} receptor immunoreactivity was co-localized with neurofilament immunoreactivity, recognized by the presence of darker areas (Fig. 5B–D, highlighted by filled arrows) while co-localization of adenosine A_3 receptor and neurofilament immunoreactivity was rarely found (Fig. 5E).

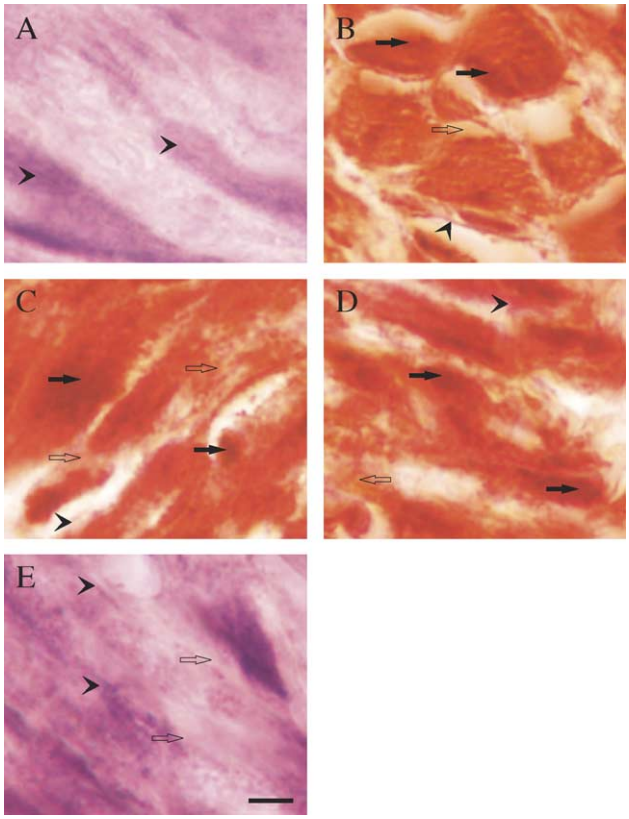

Fig. 5. Double staining of transverse sections from the rat tail artery. Photomicrographs show the pattern of distribution of the neural marker (neurofilament) and individual adenosine receptor immunoreactivity. Sections treated with anti-neurofilament (A); anti-neurofilament plus anti-A_1 (B), anti-neurofilament plus anti-A_{2A} (C) anti-neurofilament plus anti-A_{2B} (D) and anti-neurofilament plus anti-A_3 (E). Head arrows highlight neurofilament immunoreactivity (violet areas); open arrows highlight individual adenosine receptor subtype immunoreactivity (light brown areas) and filled arrows highlight areas of co-localization of adenosine receptor and neurofilament immunoreactivity (dark brown areas). Bar=2.3 μm.

4. Discussion

The involvement of adenosine receptors in the modulation of noradrenaline release in the rat tail artery was investigated by studying the effects of adenosine receptor agonists and antagonists on electrically evoked tritium overflow from tissue preparations of rat tail artery pre-incubated with [^{3}H]noradrenaline, under experimental conditions similar to those previously described (Von Kügelgen et al., 1995; Fresco et al., 2002; Queiroz et al., 2002). As in those studies, electrically evoked tritium overflow was assumed to reflect action potential-evoked neuronal noradrenaline release, and drug-induced changes in evoked tritium overflow were assumed to reflect changes in the neural release of noradrenaline.

The involvement of P1 receptors in the modulation of endogenous noradrenaline release from sympathetic nerves in rat tail artery has been previously described (Shinozuka et al., 1988). Nevertheless, this study did not fully characterize the subtypes of adenosine receptors involved since it used fairly unselective agonists and antagonists. More recent works showed the inhibition of noradrenaline release mediated by adenosine A_1 receptors in the rat tail artery (Gonçalves and Queiroz, 1996; Fresco et al., 2002) as observed in other blood vessels (mesenteric artery: Ralevic, 2000; Shinozuka et al., 2001; pulmonary artery: Vaz-da-Silva et al., 1995). The present study confirmed these observations.

Our study also confirms previous observations (Gonçalves and Queiroz, 1996; Fresco et al., 2002) that revealed an adenosine receptor-mediated facilitation of noradrenaline release in the rat tail artery. The suggested involvement of adenosine A_{2A} receptors was confirmed in the present study by the facilitation of noradrenaline release caused by the adenosine A_{2A} receptor-selective agonist CGS 21680, an effect blocked by the adenosine A_{2A} receptor-selective antagonist SCH 58261 (with a reported selectivity for A_{2A} versus A_{2B} higher than 8000-fold; Ongini et al., 1999) but not by the adenosine A_{2B} receptor-selective antagonist MRS 1706. MRS 1706 has been described as a potent and selective adenosine A_{2B} receptor antagonist (K_i for human adenosine receptors: 1.39 nM for A_{2B}; 112 nM for A_{2A}; 157 nM for A_1; 230 nM for A_3; Kim et al., 2000).

Interest in adenosine A_{2B} receptors was re-awakened after evidence was found for their involvement in the regulation of myocardial contractility (Liang and Haltiwanger, 1995),

vascular tone (Rubino et al., 1995; Shin et al., 2000; Flood and Headrick, 2001) and asthma (Feoktistov and Biaggioni, 1996). Few studies had attempted to investigate the putative involvement of adenosine A_{2B} receptors in the modulation of neurotransmitter release (see, Ralevic and Burnstock, 1998), but recently the involvement of adenosine A_{2B} receptors in the modulation of noradrenaline release was described in rat vas deferens (Queiroz et al., 2002).

Characterization of adenosine A_{2B} receptor-mediated effects has been hampered by the lack of adenosine A_{2B} receptor-selective agonists. This limitation was circumvented in the present study by using a non-selective adenosine receptor agonist combined with adenosine A_1, A_{2A} or A_3 receptor-selective antagonists (Feoktistov and Biaggioni, 1997) and by combining this functional approach with an immunohistochemical study. Adenosine A_{2B} receptors can be activated by NECA in the low micromolar range (Fredholm et al., 1994; Feoktistov and Biaggioni, 1997; Klotz et al., 1998). Therefore, NECA was used in this range of concentrations. The adenosine A_1 receptor-selective antagonist DPCPX was added in order to minimize the NECA-induced inhibition mediated by adenosine A_1 receptors (as shown in Fig. 1).

NECA facilitated noradrenaline release which was not prevented by the selective adenosine A_{2A} receptor antagonist SCH 58261 (20 nM), which strongly suggests the involvement of facilitatory adenosine receptors other than A_{2A} receptors. An indication for the involvement of adenosine A_{2B} receptors in the facilitation of noradrenaline release in rat tail artery was obtained using the selective adenosine A_{2B} receptor-selective antagonist, MRS 1706, which, in a concentration that did not influence the adenosine A_{2A} receptor-mediated facilitation of noradrenaline release, blocked the NECA-elicited facilitation of noradrenaline release.

The involvement of adenosine A_{2B} receptors in the modulation of sympathetic transmission was also proposed in canine pulmonary artery (Tamaoki et al., 1997), where it was reported that the adenosine A_{2B} receptor mediated inhibition of noradrenaline release, an effect opposite to the one observed in the present study. However, in that study, the effects of adenosine agonists were studied on contractile responses elicited by electrical field stimulation and reflected activation of both pre- and postjunctional adenosine receptors. The claimed adenosine A_{2B} receptor-mediated inhibition of sympathetic transmission was, in all likelihood, due mainly to activation of postjunctional adenosine A_{2B} receptors, an effect compatible with the observation that activation of postjunctional adenosine A_{2B} receptors leads to inhibition of postjunctional responses (Shin et al., 2000; Flood and Headrick, 2001; Diniz et al., 2003).

In the rat vas deferens, adenosine A_{2B} receptor-mediated facilitation of noradrenaline release occurred in the prostatic portion, where no adenosine A_{2A} receptor-mediated facilitation was detected (Queiroz et al., 2002), which led to the hypothesis that adenosine A_{2B} receptor-mediated facilitation of noradrenaline release would be an alternative pathway if adenosine A_{2A} receptor-mediated facilitation did not occur. This hypothesis is challenged by the present results because both adenosine A_{2A} and A_{2B} receptor-mediated facilitation seemed to occur in the same tissue and under similar experimental conditions.

In human mast cells co-expressing adenosine A_{2A} and A_{2B} receptor subtypes, activation by NECA of adenosine A_{2A} and A_{2B} receptors revealed an additive effect compared to the effect of CGS 21680 alone (Feoktistov and Biaggioni, 1998). In our study, NECA in the presence of DPCPX (blocking adenosine A_1 receptors) did not cause additive effects, i.e., did not elicit a more marked increase in noradrenaline release relative to that elicited by the activation of A_{2A} receptors only (by CGS 21680), although both adenosine A_{2A} and A_{2B} receptors were detected by immunohistochemistry.

This lack of additive effects could be due to the adenosine receptor subtypes activated by NECA. NECA at the highest concentration used may cause some inhibition of noradrenaline release by displacing the adenosine A_1 receptor antagonist or by activating mainly adenosine A_{2B} receptors, which would explain why the NECA-induced facilitation of noradrenaline release was blocked by the selective adenosine A_{2B} receptor antagonist but not by the selective adenosine A_{2A} receptor antagonist. The lack of additive effects could also be due to factors related to the transduction mechanisms to which adenosine A_2 receptor subtypes are coupled. Adenosine A_{2A} and A_{2B} receptors share the same signal transduction pathway (G_s/adenylyl cyclase/protein kinase A; Ralevic and Burnstock, 1998) and, therefore, the maximal facilitation would be limited by the capacity of the transduction pathway to induce a facilitation of noradrenaline release. Another explanation may be related to the maximal facilitation attainable. The facilitation caused by adenosine A_{2A} receptor activation seems to be due to removal of the influence of release inhibitory receptors and, therefore, the magnitude of the facilitatory effect is limited by the extent of the ongoing release inhibition (Fresco et al., 2002; Queiroz et al., 2003). The same seems to occur with the adenosine A_{2B} receptor-mediated facilitation of noradrenaline release since NECA, in the presence of 30 nM DPCPX, did not cause any enhancement of noradrenaline release when the release inhibitory α_2-autoreceptors were blocked (data not shown). Therefore, it is conceivable that the facilitation caused by activation of adenosine A_{2A} and A_{2B} receptors is not additive because both may be limited by the extent of ongoing inhibition mediated by the release inhibitory receptors.

Evidence for the involvement of adenosine A_{2B} receptors in the modulation of noradrenaline release was provided by immunohistochemical studies: like adenosine A_1 and A_{2A} receptor immunoreactivity, adenosine A_{2B} receptor immunoreactivity was also associated with nerve fibres (visualized as the darker areas highlighted in Fig. 5B–D), suggesting a prejunctional location. In contrast, the adenosine A_3 receptor immunoreactivity was not associated with

nerve fibres (Fig. 5E), indicating that its pre-junctional location is highly unlikely. Furthermore, and confirming the prediction, their involvement in the modulation of noradrenaline release can be excluded because IB-MECA, a selective adenosine A_3 receptor agonist, failed to alter noradrenaline release, even under conditions where the probability of adenosine A_3 desensitization was minimized: IB-MECA was added only 1 min before and removed after the stimulation periods due to a reported agonist-induced desensitization of adenosine A_3 receptor ($t_{1/2}$~1 min; Palmer et al., 1995).

In conclusion, the present study provides functional and morphological evidence for the modulation of noradrenaline release by multiple adenosine receptors in the rat tail artery: inhibition mediated by adenosine A_1 receptors and facilitation mediated by both adenosine A_{2A} and A_{2B} receptors.

Acknowledgements

The authors are deeply grateful for the generous gift of SCH 58261 by Dr. Scott Weiss (Vernalis, UK). The authors also thank Associação Nacional das Farmácias for the scintillation spectrometry equipment and M.C. Pereira for technical assistance. This work was supported by Fundação para a Ciência e Tecnologia (FCT: I&D no. 226/94, POCTI-QCA III, POCTI/36545/FCB/2000) and FEDER.

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