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Involvement of adenosine A_1 receptors in upregulation of nitric oxide by acyclic nucleotide analogues

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Abstract

Acyclic nucleoside phosphonates are a novel class of virostatics effective against replication of both DNA-viruses and retroviruses. They are synthetic analogues of natural nucleotide monophosphates, and purine derivatives thus represent counterparts of AMP. Mono- and di-phosphorylated species are analogues of natural ADP and ATP, respectively. A number of these compounds are endowed with immunostimulatory and immunomodulatory potential. We investigated whether their augmenting effect on the interferon-γ-primed production of nitric oxide (NO) by murine macrophages is mediated by purinoceptors. The test compounds comprise alterations at the N^6-group of the heterocyclic base, i.e., adenine or 2,6-diaminopurine, and at the N^9-side chain, represented by 9-[2-(phosphonomethoxy)ethyl] and 9-[2-(phosphonomethoxy)propyl] moieties: 9-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; *tenofovir*], N^6-cyclopropyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopropyl-(*R*)-PMPDAP], N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)-propyl]2,6-diaminopurine [N^6-cyclopentyl-(*R*)-PMPDAP], N^6-dimethylaminoethyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-dimethylaminoethyl-(*R*)-PMPDAP], N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (N^6-isobutyl-PMEDAP), N^6-cyclopentyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (N^6-cyclopentyl-PMEDAP), N^6-cyclooctyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (N^6-cyclooctyl-PMEDAP), and N^6-cyclohexylmethyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (N^6-cyclohexylmethyl-PMEDAP). The cells were cultured in the presence of interferon-γ (5000 pg/ml) and test compounds (2–50 μM). Formation of nitrites was determined after 24 h using Griess reagent. It was inhibited by specific and nonspecific antagonists of adenosine A_1 receptors (IC_{50} for 8-cyclopentyl-1,3-dipropylxanthine [CPX] was approximately 10 μM), while all other purine P_1 and purine P_2 receptor antagonists remained ineffective to suppress the NO-synergistic effect of acyclic nucleoside phosphonates.

Keywords: Acyclic nucleoside phosphonate; NO (Nitric oxide); Adenosine receptor

1. Introduction

Acyclic nucleotide analogues or acyclic nucleoside phosphonates are a novel class of virostatics effective against replication of both DNA-viruses and retroviruses (De Clercq, 1991). The oral prodrugs of the prototype compounds, i.e., 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA; *adefovir*), and 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; *tenofovir*] were approved by FDA for treatment of hepatitis B (Hepsera), and acquired immunodeficiency syndrome (AIDS) (Viread), respectively. Another important representative of acyclic nucleoside phosphonates is 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)-propyl]cytosine (*cidofovir*) which was approved for treat-

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ment of cytomegalovirus retinitis in patients with AIDS (Rahhal et al., 1996). The major mechanism of antiviral action of acyclic nucleoside phosphonates is the inhibition of virus-induced DNA polymerases (Kramata et al., 1996) or of reverse transcriptases (Crowe, 1999; Holý et al., 1990; Votruba et al., 1990). Besides, they are endowed with immunostimulatory and immunomodulatory potential that may be associated with their beneficial therapeutic effectiveness. Among other effects they upregulate synthesis of nitric oxide (NO) stimulated primarily by interferon-γ (Zídek et al., 2003).

A common characteristics of acyclic nucleoside phosphonates is the lack of intrinsic biological activity, which is believed only to be acquired after their phosphorylation by intracellular kinases (Balzarini et al., 1991; Ho et al., 1992). Some of them are known to be a substrate for phosphorylation by intracellular AMP (dAMP) kinase (Merta et al., 1992). They are considered synthetic analogues of natural nucleotide monophosphates (Hatse et al., 1996), and adenine derivatives thus represent counterparts of AMP. Mono- and noncyclizable di-phosphorylated acyclic nucleoside phosphonates are analogues of ADP and ATP, respectively. All these natural species have been found to influence production of NO via adenosine receptors. It was therefore intriguing to investigate whether the NO-augmenting activity of acyclic nucleoside phosphonates shares similar mode of action.

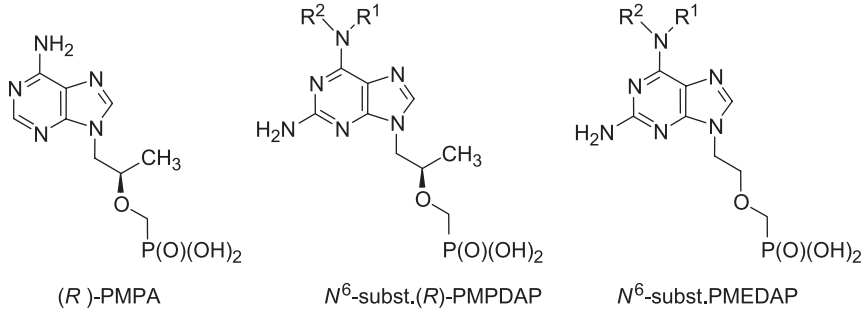
AMP and adenosine exert their biological activities through the signal transduction mediated by the purine P_1 receptor family of cell surface receptors. There are at least four distinct types of adenosine receptors, A_1, A_{2a}, A_{2b}, and A_3 (Fredholm et al., 1994). They are coupled to adenylate cyclase, and their ligation thus controls the synthesis of cyclic AMP (cAMP), either negatively via Gi protein (adenosine A_1 and A_3 receptors) or positively via Gs protein (adenosine A_{2a} and A_{2b} receptors) (Dalziel and Westfall, 1994; Satchell, 1984). The aim of the present experiments was to follow the participation of individual types of adenosine receptors on the NO-enhancing effects of acyclic nucleoside phosphonates. For this purpose, both specific and non-specific antagonists of purine P_1 and P_2 receptors were used.

2. Materials and methods

2.1. Test acyclic nucleoside phosphonates and other chemicals

Acyclic nucleoside phosphonates were synthesized in-house (Institute of Organic Chemistry and Biochemistry) according to the procedures described recently (Holý et al., 2001). They comprise multiple alterations at the N^6-group of the heterocyclic base, i.e., adenine or 2,6-diaminopurine, and at the N^9-side chain, represented by 9-[2-(phosphonomethoxy)ethyl] and 9-[2-(phosphonomethoxy)propyl] moieties: 9-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; *tenofovir*], N^6-cyclopropyl-(*R*)-9-[2-(phosphonomethoxy)-propyl]2,6-diaminopurine [N^6-cyclopropyl-(*R*)-PMPDAP], N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopentyl-(*R*)-PMPDAP], N^6-dimethylaminoethyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-dimethylaminoethyl-(*R*)-PMPDAP], N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (N^6-isobutyl-PMEDAP), N^6-cyclopentyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (N^6-cyclopentyl-PMEDAP), N^6-cyclooctyl-9-[2-(phosphonomethoxy) ethyl]2,6-diaminopurine (N^6-cyclooctyl-PMEDAP), and N^6-cyclohexylmethyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (N^6-cyclohexylmethyl-PMEDAP). All these compounds have previously been found to upregulate the interferon-γ-induced production of NO (Zídek et al., 2003). Their structure is shown in Fig. 1. Stock solutions of acyclic nucleoside phosphonates (5 mM) were prepared in incomplete $NaHCO_3$-containing, phenol red-free RPMI-1640 medium (Sigma-Aldrich, Praha, Czech Republic). They were sterile filtered using non-pyrogenic 0.22-μm filters (Costar, Cambridge, MA), used fresh, or kept no longer than 4 weeks at −20 °C. Required working concentrations were prepared by diluting the stock solution in complete RPMI-1640 culture medium (described below).

All the following compounds were purchased from Sigma-Aldrich: 8-cyclopentyl-1,3-dipropylxanthine (CPX; adenosine A_1 receptor antagonist), 8-(3-chlorostyryl)caffeine (CSC; adenosine A_{2a} receptor antagonist), benzo[*g*]pteridine-2,4(1*H*,3*H*)-dione (adenosine A_{2b} receptor



(*R*)-PMPA N^6-subst.(*R*)-PMPDAP N^6-subst.PMEDAP

Fig. 1. Chemical structure of test compounds. N^6-substituents are specified in Section 2.1.

antagonist; Alloxazine), 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)dihydropyridine-3,5-dicarboxylate (MRS-1191; adenosine A_3 receptor antagonist), 9-chloro-2-(2-furanyl)-[1,2,4]triazolol[1,5-*c*]quinazolin-5-amine (CGS-15943; nonspecific antagonist of adenosine $A_{1/2a/2b/3}$ receptors), xanthine amine congener (XAC; nonspecific antagonist of adenosine $A_{1/2a/2b/3}$ receptors), Suramin (nonspecific purine $P_{2X1/X2/X3/X5/Y2}$ receptor antagonist), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; nonspecific purine $P_{2X3/X5/Y1/Y4}$ receptor antagonist). Stock solutions (100 mM) were prepared either in dimethylsuphoxide (DMSO) or in incomplete RPMI-1640 medium (the latter two antagonists).

Recombinant mouse interferon-γ was purchased from R&D Systems (Minneapolis, MN).

The chromogenic Limulus Amoebocyte Lysate assay (Kinetic-QCL; BioWhittaker, Walkersville, MD) was used to check for possible contamination with lipopolysaccharide. The highest final concentrations of all chemicals used contained <10 pg/ml (i.e., approximately <0.1 endotoxin units/ml), an amount that was previously found (Zídek et al., 2003) biologically virtually inactive.

2.2. Animals

Female mice of the inbred strain C57BL/6, 8–10 weeks old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages in groups of eight, and maintained in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France). Lighting was set at 0600–1800 h, and temperature at 22 °C. All protocols were approved by the institutional ethics committee.

2.3. Isolation and cultivation of macrophages

Animals, killed by cervical dislocation, were i.p. injected with 8 ml of sterile saline. Pooled peritoneal cells collected from mice (n=4–8 in individual experiments) were washed, resuspended in culture medium, and seeded into 96-well round-bottom microplates (Costar) in 100-μl volumes, 2×10^5 cells/well. Adherent cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO_2, and then vigorously shaking the plate and washing the wells three times to remove non-adherent cells. Cultures were maintained at 37 °C, 5% CO_2 in humidified Heraeus incubator for 24 h.

Complete RPMI-1640 culture medium (Sigma-Aldrich, Prague, CR), used throughout the experiments, contained 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma).

2.4. Nitric oxide assay

The concentration of nitrites in cell supernatants was taken as a measure of NO production (Marletta et al., 1988). It was detected in individual, cell-free samples (50 μl) incubated 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphtylendiamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to μM nitrite.

2.5. Analysis of data

Analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test, and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

3. Results

3.1. Pilot screening for intervention of antagonists of purinoceptors

The concentration of nitrites in supernatants of cells stimulated with interferon-γ alone (5000 pg/ml) increased from virtually zero in controls to 16 μM. Although the model acyclic nucleoside phosphonate, i.e, N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine (50 μM) was unable to stimulate NO production on its own, it synergistically augmented secretion of NO triggered by interferon-γ (63 μM). The high output NO production was inhibited by specific adenosine A_1 receptor CPX, and by nonspecific purine P_1 receptor antagonists CGS-15943, and XAC. The formation of NO thus dropped to the level induced by interferon-γ solely. All other antagonists were found ineffective to suppress production of NO (Fig. 2). The antagonists were applied at final concentration of 100 μM, and added to the cells 15 min preceding the administration of the interferon-γ plus test compound. The presence of the solvent, i.e., DMSO, used for preparation of stock solutions of antagonists, did not influence the response of the cells. Its final dilution (0.1%) corresponded to the dilution of antagonists in cell cultures.

3.2. Study of the dose-dependent effects

The inhibitory effects of purine P_1 receptor antagonists were found to be strictly dose-dependent (Fig. 3). The IC_{50}s were identical for the specific adenosine A_1 receptor antagonist CPX: 7.1 μM (95% limits of confidence: 4.5–11.1 μM) and for the nonspecific purine P_1 receptor antagonist CGS-15943: 7.1 μM (4.3–11.8 μM). Another nonspecific purine P_1 receptor antagonist XAC was less potent: 53.4 μM (32.5–88.5 μM).

The inhibitory potential of the adenosine A_1 receptor antagonist CPX was only marginally influenced by the magnitude of the NO stimulatory response evoked by

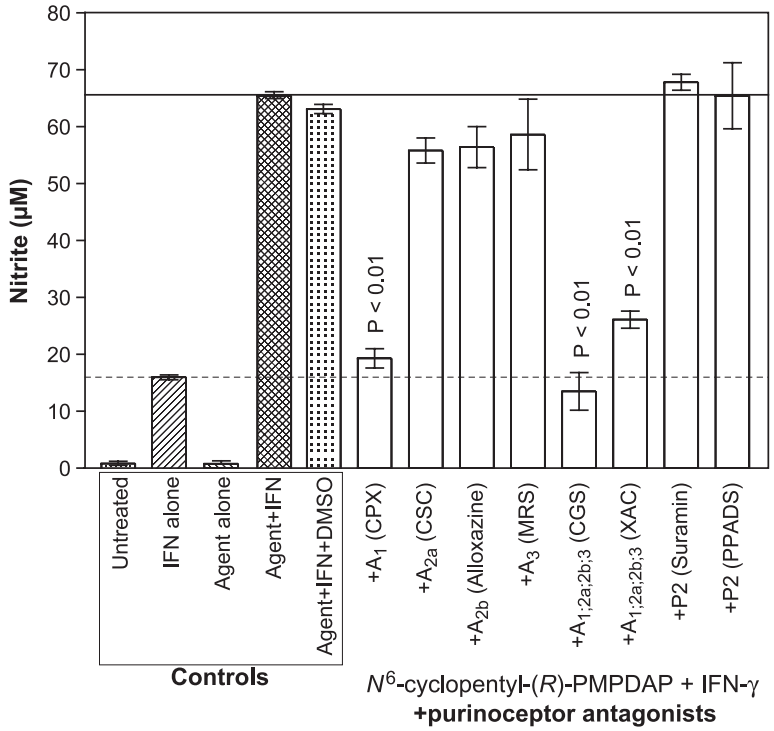


Fig. 2. Effects of purinoceptor antagonists on secretion of NO by murine peritoneal macrophages. The cells (2×10^6/ml) were cultured for 24 h in the presence of mouse interferon-γ (IFN-γ; 5000 pg/ml) and acyclic nucleoside phosphonate N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopentyl-(*R*)-PMPDAP] (50 μM). The antagonists were applied at concentration of 100 μM 15 min before the NO stimulators. The supernatant concentration of nitrite was determined using Griess reagent. Each bar is a mean±S.E.M. for triplicate culture wells. Virtually identical or very similar results have been observed in several other independent experiments.

increasing doses of the test acyclic nucleoside phosphonate N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)-propyl]2,6-diaminopurine (2–25 μM) (Fig. 4). The IC_{50}s were 3.0 μM (0.64–14.0 μM), 3.9 μM (2.0–7.7 μM), 4.8 μM (2.8–8.3 μM), and 6.4 μM (4.5–9.2 μM) for concentrations of 2, 5, 10, and 25 μM of N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine, respectively.

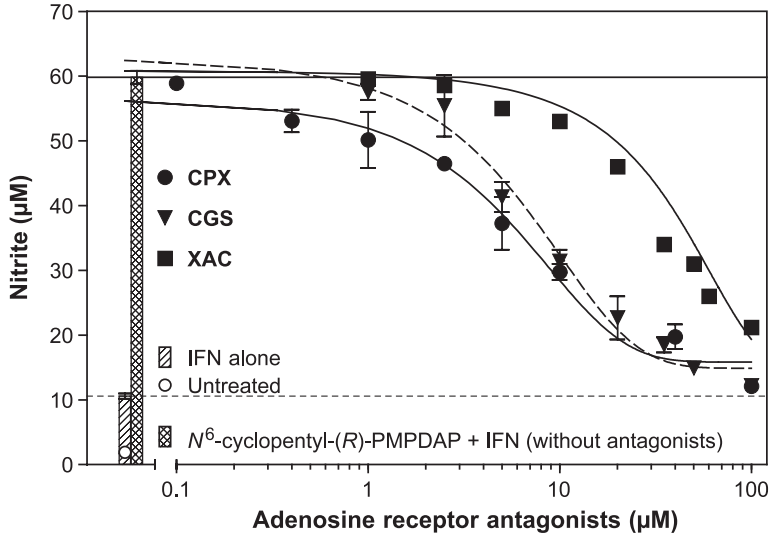


Fig. 3. Dose-dependent inhibitory effects of P1 purinoceptor antagonists on NO production induced by interferon-γ (IFN-γ; 5000 pg/ml) plus acyclic nucleoside phosphonate N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopentyl-(*R*)-PMPDAP] (50 μM) in mouse peritoneal macrophages. Agonists were applied 15 min before the NO stimulators. Specific adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX), and nonspecific adenosine A_{1-3} receptor antagonists, i.e., 9-chloro-2-(2-furanyl)-[1,2,4]triazolol[1,5-*c*]quinazolin-5-amine (CGS-15943) and xanthine amine congener (XAC) were used. The cells (2×10^6/ml) were cultured 24 h in the presence of the agents. The supernatant concentration of nitrite was determined using Griess reagent. Each point is a mean±S.E.M. for duplicate culture wells. The results are representative of two identical experiments.

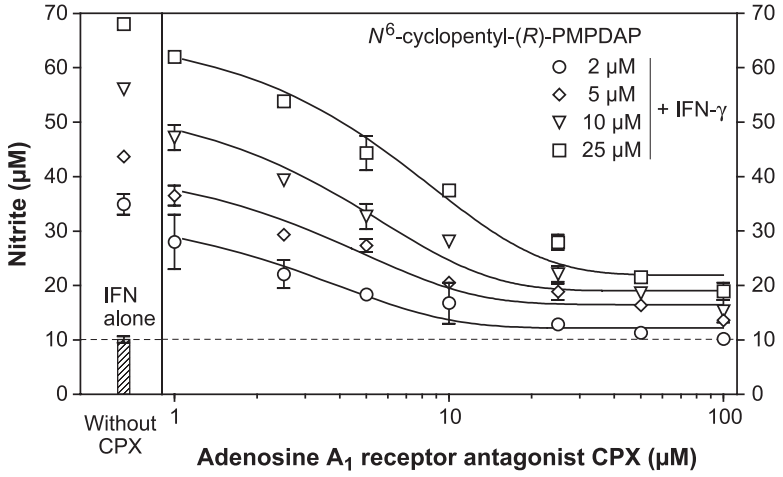


Fig. 4. Inhibition of NO production by specific adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX). The murine macrophages (2×10^6/ml) were cultured 24 h in the presence of interferon-γ (IFN-γ; 5000 pg/ml) and varying doses (2-25 μM) of acyclic nucleoside phosphonate N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopentyl-(*R*)-PMPDAP]. The antagonist was applied 15 min before these agents. The supernatant concentration of nitrite was determined using Griess reagent. Each point is a mean±S.E.M. for triplicate culture wells.

3.3. Dependence on chemical structure of acyclic nucleoside phosphonates

The dose-dependent inhibitory effectiveness of the specific adenosine A_1 receptor antagonist CPX was very similar irrespective of the basic chemical structure of all acyclic nucleoside phosphonates differing by substitution at the 6-amino group and by the type of N^9-side chain (Fig. 5). The IC_{50} estimates for distinct compounds, tested at concentration of 50 μM, ranged from 4.2 μM (N^6-cyclohexylmethyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine) to 16.6 μM (N^6-cyclopropyl-(*R*)-9-[2-(phos-

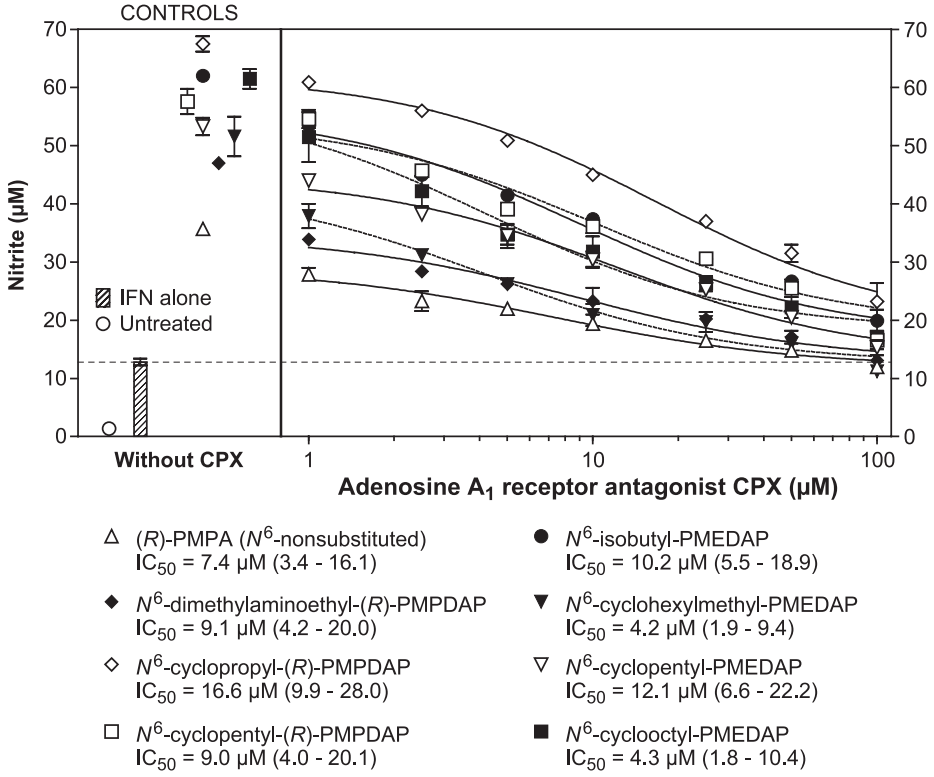


Fig. 5. Dose-dependent inhibitory effect of specific adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX) on production of NO stimulated in murine macrophages in vitro by interferon-γ (IFNγ; 5000 pg/ml) plus 50 μM acyclic nucleoside phosphonates (for full names, see Materials and Methods). The antagonist was applied 15 min before the NO stimulatory agents. The supernatant concentration of nitrite was determined using Griess reagent. Each point is a mean±S.E.M. for triplicate culture wells. The IC_{50}s are shown together with 95% limits of confidence (in parantheses). The results are representative of two identical experiments.

phonomethoxy)propyl]2,6-diaminopurine). The IC_{50}s for other acyclic nucleoside phosphonates are shown in Fig. 5.

4. Discussion

We have demonstrated recently that a number of acyclic nucleoside phosphonates are more or less potent upregulators of NO production by murine macrophages stimulated with interferon-γ (Zídek et al., 2003). Acyclic nucleoside phosphonates themselves are analogues of natural AMP, and their mono- and di-phosphorylated derivatives are analogues of ADP and ATP, respectively. We therefore investigated possible involvement of purinergic receptors in expression of the synergistic effect of acyclic nucleoside phosphonates on biosynthesis of NO. There are a plethora of data showing that activation of purine P_1 and P_2 receptors modulates the NO secretion. The NO-modulating potential of cAMP has been reviewed elsewhere (Zídek, 2001).

Although adenosine per se was reported to be ineffective to interfere with NO production by many cell types including rat cardiac myocytes (Ikeda et al., 1997b), vascular smooth muscle cells from rat thoracic aorta (Ikeda et al., 1997a), and endothelial cells from human saphenous vein (Li et al., 1995), it was able to stimulate activity of NOS in certain human endothelial cells (Li et al., 1998), rat brain astrocytes (Janigro et al., 1996), and cells from porcine carotid artery (Li et al., 1995). NO, stimulated by adenosine through adenosine A_{2a} receptors, has been found to contribute in part to coronary vasodilation in pigs (Hein et al., 1999) and dogs (Parent et al., 1992). Activation of adenosine A_2 receptors by elevated extracellular adenosine per se has been suggested to enhance NO synthesis in rat liver preconditioning (Peralta et al., 1999). Moreover, an increased concentration of NO metabolites has been found in urine of adenosine-treated rats (Franco et al., 1999). High-output lipopolysaccharide-, lipopolysaccharide/interferon-γ-, or interleukin-1β-triggered production of NO was invariably found to be augmented by adenosine in various cell types, such as macrophage cell line RAW 264.7 (Hon et al., 1997), rat cardiac myocytes (Ikeda et al., 1997b), or cells from rat thoracic aorta (Ikeda et al., 1997a).

Conflicting data have been obtained with adenosine A_1 receptor agonists. When applied alone, CCPA (2-chloro-N^6-cyclopentyladenosine) stimulated NO secretion in rat brain astrocytes (Janigro et al., 1996), while it exhibited suppressive activity in both porcine and human endothelial cells (Li et al., 1998). The NO-inhibitory effects of CCPA also were observed in the lipopolysaccharide-activated macrophage cell line RAW 264.7 and in endotoxemic mice (Haskó et al., 1996). Decreased expression of iNOS mRNA was found in endotoxemic animals treated with another adenosine A_1 receptor agonist CHA (N^6-cyclohexyladenosine) (Hon et al., 1995; Moochhala et al., 1996). In contrast, this compound stimulated production of NO in the lipopolysaccharide-stimulated RAW 264.7 macrophages (Hon et al., 1997). Effects of CPA (N^6-cyclopentyladenosine) on biosynthesis of NO by the interleukin-1β-stimulated vascular smooth muscle cells were either up-regulatory or down-regulatory, depending on the dose applied (Ikeda et al., 1997a).

Similar to adenosine A_1 receptor agonists, both stimulatory and inhibitory effects of adenosine A_2 receptor agonists on secretion of NO have been observed. Whereas CPCA/5′ -(*N*-cyclopropyl)-carboxamidoadenosine/augmented NO production in the lipopolysaccharide-activated RAW 264.7 cells (Hon et al., 1997), it inhibited formation of NO metabolism products in the lipopolysaccharide-injected mice (Hon et al., 1995; Moochhala et al., 1996). The lipopolysaccharide-stimulated expression of iNOS mRNA and activity of iNOS protein were also suppressed under both in vivo and in vitro conditions by another adenosine A_2 receptor agonist CGS-21680/2-*p*-(2-carboxyethyl)phenethylamino-5′ -*N*-ethylcarboxamidoadenosine hydrochloride/ (Haskó et al., 1996). Anyhow, this compound, on its own, activated NO formation in porcine and human endothelial cells (Li et al., 1998) and synergised with the NO-up-regulatory effect of interleukin-1β in rat thoracic smooth muscle cells (Ikeda et al., 1997a).

A representative of adenosine A_3 receptor agonists IB-MECA, i.e., N^6-(3-iodobenzyl)-9-(5-(methylcarbamol)-β-D-ribofuranosyl)adenine) marginally inhibited the immune-stimulated (lipopolysaccharide+interferon-γ) expression of NO in RAW macrophages (Szabó et al., 1998). Its inhibitory effect seems to be bound to low concentration, whereas high concentrations of IB-MECA stimulate production of NO in rat aortic muscle cells (Ikeda et al., 1997a).

Non-selective purine P_1 receptor agonists possess predominantly up-regulatory function within the L-arginine/NO metabolism pathway. While being usually ineffective on its own, adenosine A_1/A_2 receptor agonist CADO (2-chloroadenosine) enhanced iNOS mRNA expression and NO production in vascular smooth muscle cells (Ikeda et al., 1997a) and rat cardiac myocytes (Ikeda et al., 1997b) which were treated with interleukin-1β. Similar activity was observed with the adenosine A_1/A_3 receptor agonist R-PIA/*R*(−)-N^6-(2-phenylisopropyl)adenosine)/and adenosine $A_1/A_2/A_3$ receptor agonist NECA (5′ -*N*-ethylcarboxamidoadenosine) in lipopolysaccharide-treated RAW 264.7 macrophages (Hon et al., 1997). NECA was effective not only in combination with lipopolysaccharide or interleukin-1β (Ikeda et al., 1997a) but also on its own in human endothelial cells (Li et al., 1998). On the contrary, both R-PIA and NECA, given prior to lipopolysaccharide, inhibited the systemic production of NO elicited by lipopolysaccharide in mice, and enhanced their survival (Haskó et al., 1996; Hon et al., 1995; Moochhala et al., 1996).

Inconsistent data have been reported regarding the consequences of activation of P_2 purinoceptors with respect to the NO synthesis. Activation of purine P_{2Y} receptor with the partial agonist 2-methylthio-ATP inhibited the lipopolysaccharide/interferon-γ- but not the interferon-γ/tumor necrosis factor-α-induced NO produc-

tion in macrophage cell line (Denlinger et al., 1996). Stimulation of purine P_{2Y2} receptors reduced iNOS expression in rat mesangial cells (Mohaupt et al., 1998). On the other hand, protein kinase C-dependent NO-stimulatory effects of this agonist and less expressed stimulatory effects of UTP (purine P_{2U} receptor agonist) were observed in endothelial cells (Brown et al., 1996). Moreover, P_{2X7}/P_{2Z} purinergic receptor was found to possess a significant role in the lipopolysaccharide activation of iNOS in RAW 264.7 cells: the purine P_{2X} receptor antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid suppressed NO production (Sommer et al., 1999). ATP itself, released by noradrenaline, stimulated production of NO in endothelial cells from rat caudal artery, and was thus suggested to participate in shear stress-associated synthesis and release of NO (Kwon et al., 1999).

From the point of view of NO production, these data document ambiguity of activation of purinergic receptors. Our findings support those data demonstrating the up-regulatory function of especially adenosine A_1 receptors. It should be stressed that in contrast to a plethora of reported experiments, using lipopolysaccharide or cytokines (mostly interleukin-1β and tumor necrosis factor-α) as primary NO stimulators, we used interferon-γ as a priming NO activation signal. The present data indicate that the NO-augmenting action of acyclic nucleoside phosphonates is mediated by the P_1 receptor purinergic system. Obviously, the activation of adenosine A_1 receptors plays the major role, because the specific and non-specific antagonists of adenosine A_1 receptors solely, but not those of adenosine A_{2a}, A_{2b}, A_3 receptors and of certain purine P_2 receptors, have been found to suppress production of NO. Noteworthy, the antagonists blocked the synergistic signal provided by acyclic nucleoside phosphonates, but remained ineffective to suppress NO production induced by interferon-γ per se. Although not specifically investigated, it may be suggested that acyclic nucleoside phosphonates are ligands for adenosine A_1 receptors. Since the test compounds are predominantly the N^6-substituted derivatives of adenine, the finding is in consonance with a general notion that the N^6-substituted derivatives of adenosine are the most potent selective agonists of adenosine A_1 receptors (Müller, 2000). Interestingly, certain types of acyclic phosphonate analogues of adenosine diphosphates target the purine P_{2Y} receptors in platelets and exhibit platelet antiaggregatory activity (Xu et al., 2002).

The ligation of adenosine A_1 receptors would presume consequent inhibition adenylyl cyclase activity resulting in decreased cAMP formation. Recently published data support this possibility. A series of derivatives of 9-[2-(phosphonylmethoxy)ethyl]adenine (PMEA) have been found to inhibit a preparation of adenylyl cyclase derived from rat brain (Shoshani et al., 1999). Not only adenine derivatives but also 2,6-diaminopurine derivatives of acyclic nucleoside phosphonates have been found to inhibit adenylyl cyclase activity in murine macrophages (Shen et al., 2004). It might be speculated whether this novel mode of action of acyclic nucleoside phosphonates could be considered as a significant contribution to their antiviral effectiveness. Interestingly, the cAMP-enhancers dibutyryl-cAMP and forskolin enhance replication of human immunodeficiency virus (HIV) in MT-4 cells (Nokta and Pollard, 1992). Furthermore, the expression of a chemokine receptor CXCR4, an important co-receptor for HIV entry into cells (Feng et al., 1996), is increased by cAMP (Rola-Plesczynski et al., 1999).

In conclusion, the synergistic effect of acyclic nucleoside phosphonates on interferon-γ-primed NO production by murine macrophages can be blocked by antagonists of P_1 purinergic receptors. The compounds can be presumed to be ligands of adenosine A_1 receptors.

Acknowledgements

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