Phosphorylated adenosine derivatives as low-affinity adenosine-receptor agonists

Methodological implications for the adenylate cyclase assay

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(Received 17 November 1983/Accepted 31 January 1984)

In cellular systems provided with activatory (R_a -site) receptors for adenosine, such as rat cerebral microvessels and rat liver plasma membranes, the adenosine-receptor antagonist 8-phenyltheophylline (10 μ M) significantly decreased adenylate cyclase activity if ATP was the substrate and only if GTP was present. With dATP as substrate, adenylate cyclase activities in both preparations remained unaffected by 8-phenyltheophylline. In rat cerebral-cortical membranes, with inhibitory (R_i -site) receptors for adenosine, 8-phenyltheophylline significantly enhanced adenylate cyclase activity only in the presence of GTP and if ATP was the substrate. In rat cardiac ventricular membranes, which are devoid of any adenylate cyclase-coupled adenosine receptor, the methylxanthine had no GTP-dependent effect, irrespective of the substrate used. All assay systems contained sufficiently high amounts of adenosine deaminase (2.5 units/ml), since no endogenous adenosine, formed from ATP, was found chromatographically. In order to demonstrate a direct influence of phosphorylated adenosine derivatives on adenylate cyclase activity, we investigated AMP in a dATP assay system. AMP was verified chromatographically to remain reasonably stable under the adenylate cyclase assay conditions. In the microvessels, AMP increased enzyme activity in the range 0.03–1.0 mM, an effect competitively antagonized by 8-phenyltheophylline. In the cortical membranes, 0.1 mM-AMP inhibited adenylate cyclase, which was partially reversed by the methylxanthine. The presence of GTP was again necessary for all observations. In the ventricular membranes, AMP had no effect. Since the efficacy of adenosine-receptor agonists and, probably, that of other hormones on adenylate cyclase activity can be more efficiently measured with dATP as the enzyme substrate, this nucleotide seems preferable for adenylate cyclase measurements in systems susceptible to modulation by adenosine.

Adenosine acts as stimulator of adenylate cyclase in a wide variety of tissues, an action which is mediated via external cell-membrane receptors classified as R_a -sites (Londos *et al.*, 1980, 1983). On the other hand, receptor-mediated inhibition by

Abbreviations used: NECA, 5'-(*N*-ethylcarboxamido)adenosine; PIA, *N*⁶-(1-2-phenylisopropyl)adenosine; p[CH]pA, adenosine 5'-[α,β -methylene]diphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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adenosine has been observed in brain and fat-cells. These receptors, designated as R_i -sites, have structural requirements for adenosine analogues different from those of the R_a -sites. In many cases, distinct methodological considerations are essential to the measurement of adenosine effects on adenylate cyclase, owing to high background concentrations of adenosine in the assay medium, resulting from (i) metabolism of the substrate ATP by membrane-bound nucleotide phosphohydrolases, (ii) metabolism of cyclic AMP, which is frequently included in the assay to trap radio-labelled cyclic AMP, and (iii) endogenous contaminating adenosine. This problem could be

largely eliminated by adding adenosine deaminase to the medium, thereby catalysing the conversion of adenosine into inactive inosine, and/or by the use of dATP as the adenylate cyclase substrate, since its nucleoside metabolite, 2'-deoxyadenosine, does not display agonism at adenosine receptors (Cooper & Londos, 1979). Since background adenosine also represents a problem in the measurement of the effects of other hormones on adenylate cyclase, the use of adenosine deaminase in the assay medium has been generally recommended (Johnson, 1980).

A further, as yet ignored, difficulty in the determination of adenylate cyclase activity would arise if the enzyme substrate ATP were shown to possess agonistic properties at adenosine receptors. Though there has been no investigation of the effect of phosphorylated adenosine derivatives on adenylate cyclase, it is evident from binding studies that the affinity of AMP, ADP and ATP for R_1 -site adenosine receptors is rather low, with an IC_{50} (concentration causing half-maximal inhibition of radioligand binding) of about 0.1 mM (Schwabe & Trost, 1980; Trost & Schwabe, 1981). In contrast with conditions *in vivo*, adenosine receptors are actually exposed to such high concentrations under the conditions of the adenylate cyclase assay, where substrate concentrations of ATP of 0.1–1.0 mM are generally used. The present study demonstrates that phosphorylated adenosines are indeed low-affinity agonists at adenylate cyclase-coupled adenosine receptors. In adenosine-responsive cellular systems, such confusing effects of ATP on adenylate cyclase activity can again be avoided if dATP is the enzyme substrate.

Materials and methods

$[\alpha\text{-}^{32}\text{P}]\text{ATP}$, $[\alpha\text{-}^{32}\text{P}]\text{dATP}$, cyclic $[2,8\text{-}^3\text{H}]\text{AMP}$, cyclic $[2,8\text{-}^3\text{H}]\text{dAMP}$ and $[8\text{-}^{14}\text{C}]\text{AMP}$ were obtained from New England Nuclear, Boston, MA, U.S.A. PIA and the other nucleosides, adenine, ATP, dATP, ADP, AMP, cyclic AMP, GTP, Hepes, phosphocreatine, creatine kinase (EC 2.7.3.2), and adenosine deaminase (EC 3.5.4.4) were purchased from Boehringer, Mannheim, Germany; cyclic dAMP, $p[\text{CH}_2]\text{pA}$ and dithiothreitol were from Sigma, St. Louis, MO, U.S.A.; 8-phenyltheophylline was from Calbiochem, La Jolla, CA, U.S.A. NECA was kindly supplied by Byk Gulden Lomberg, Konstanz, Germany. All other compounds were analytical grade or the best commercially available product.

Tissue preparations

Previously described methods were used for the preparations of rat cardiac ventricular membranes (Schütz & Tüisl, 1981), rat liver plasma mem-

branes (Schütz *et al.*, 1982a) and rat cerebral microvessels (Schütz *et al.*, 1982b). Brain membranes were obtained from the cortex of freshly decapitated rats, the organ being sliced, transferred to ice-cold 10 mM-Hepes (pH 7.5)/1 mM-MgCl₂/1 mM-2-mercaptoethanol, homogenized in a glass homogenizer with a Teflon pestle, and centrifuged at 3000g for 10 min. The resulting pellet was resuspended and washed three times. All fractions were finally resuspended in the above medium at a protein concentration of 2 mg/ml and used immediately or stored under liquid N₂. Protein was determined by dye-binding (Coomassie Blue G-250) by using the Bio-Rad (Richmond, CA, U.S.A.) assay kit.

Adenylate cyclase assay

Enzyme activity was determined in 200 μl reaction mixtures containing 0.1 mM- $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ or $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (1 μCi), 0.1 mM-cyclic AMP or -cyclic dAMP, 40 mM-Hepes (pH 7.5), 1 mM-dithiothreitol, 5 mM-phosphocreatine, 5 units of creatine kinase, 0.5 unit of adenosine deaminase and 5 mM-MgCl₂. For the brain and ventricular membranes, the MgCl₂ concentration was 1 mM and the medium also contained 80 mM-NaCl. Reaction was started by adding the respective membrane or microvessel fraction at a protein content of 40 μg of microvessels, liver and ventricular membranes, or 4 μg for brain membranes. Incubations were carried out at 30°C for 15 min (6 min for hepatic adenylate cyclase activity). Cyclic $[\text{}^{32}\text{P}]\text{AMP}$ or cyclic $[\text{}^{32}\text{P}]\text{dAMP}$ was quantified as described by Cooper & Londos (1979), with use of cyclic $[\text{}^3\text{H}]\text{AMP}$ or cyclic $[\text{}^3\text{H}]\text{dAMP}$ for recovery monitoring. Each value was determined in triplicate.

Chromatography experiments

To determine the extent of AMP degradation and phosphorylation under the adenylate cyclase assay conditions, 0.1 mM- $[\text{}^{14}\text{C}]\text{AMP}$ was added to the identical adenylate cyclase assay mixture (with unlabelled dATP), followed by the appropriate incubation period. After heat inactivation, the denatured protein was removed by centrifugation. For separation of ^{14}C -labelled nucleosides and adenine, 100 μl of the supernatant were applied on silica-gel thin-layer plates (60 F₂₅₄; Merck, Darmstadt, Germany), together with carrier amounts of adenosine, inosine, hypoxanthine, adenine and AMP. The plates were developed once with butanol/ethyl acetate/methanol/25% (w/v) NH₄Cl (7:4:3:4, by vol.) as described by Shimizu *et al.* (1969). After separation of the different purine compounds, the u.v.-absorbing bands were scraped off the plates and the radioactivity was counted. The ^{14}C -labelled adenine nucleotides

were separated by reversed-phase high-performance liquid chromatography as described by Taylor *et al.* (1981), by using a Milton-Roy minipump, a column (0.4 cm $\times$ 25 cm) packed with Lichrosorb RP18 (Merck), and a 0.2M-(NH₄)₂-HPO₄/(NH₄)₃PO₄ elution system, pH 5.1 (flow rate 1 ml/min); 20 μ l of the supernatant described above was applied on to the column with a Rheodyne 7125 injector. Successive 0.5 ml fractions were sampled and counted for radioactivity. Nucleotide standards were monitored spectrophotometrically at 245 nm (Gilson Holochrom). Their respective retention times are indicated in Fig. 2.

Results

Effects of 8-phenyltheophylline

Tables 1 and 2 show adenylate cyclase activity in a microvessel preparation, as well as in membrane preparations derived from rat liver, cortex and heart ventricles, assayed with ATP (Table 1) or dATP (Table 2) as substrate. A high concentration of adenosine deaminase (2.5 units/ml) was added to each enzyme assay, to eliminate background adenosine totally. 8-Phenyltheophylline (10 μ M), a high-affinity antagonist at R_a-site as well as at R_i-site adenosine receptors (Bruns *et al.*, 1980), had no effect on the basal enzyme activity of either

preparation, irrespective of the substrate used. In the presence of GTP, however, 8-phenyltheophylline significantly diminished the activity of the microvessel and the hepatic adenylate cyclase, an effect observed only with ATP as substrate. Both the brain microvessels and the hepatic membranes contain an R_a-site adenosine receptor, since the specific agonist NECA stimulated adenylate cyclase activities; stimulation was more pronounced in the assay with dATP than in that with ATP (205% versus 84% and 109% versus 72% in the microvessel and hepatic adenylate cyclase respectively). On the other hand, the methylxanthine significantly enhanced cerebral-cortical adenylate cyclase when GTP was present, an effect also limited to the assay with ATP. Cortical membranes contain an R_i-site adenosine receptor, since the specific agonist PIA inhibited cortical adenylate cyclase activity. Inhibition by the adenosine analogue was doubled (22% versus 11%) when dATP was substituted for ATP as substrate. Finally, the cardiac ventricular adenylate cyclase was unresponsive to 8-phenyltheophylline in both the presence and the absence of GTP, irrespective of the assay type. In this preparation, no adenosine-receptor site was detectable, since NECA was neither stimulatory nor inhibitory. Integrity of the cardiac adenylate cyclase system was verified by the use of (-)-isoprenaline and

Table 1. Rat brain microvessel, hepatic, cerebral-cortical and cardiac-ventricular adenylate cyclase activity with [³²P]ATP as substrate

The concentrations of reagents were: 8-phenyltheophylline (8-PT) 10 μ M, GTP 10 μ M, NECA 5 μ M and PIA 1 μ M. The means $\pm$ S.E.M. for four to seven different experiments are shown. Significant effects of 8-PT were assessed by Student's *t* test for paired data: **P* < 0.05, †*P* < 0.01.

	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)			
	Microvessels	Liver	Brain	Heart
No addition	12.1 $\pm$ 2.8	3.3 $\pm$ 0.5	173 $\pm$ 29	21.4 $\pm$ 3.6
8-PT	11.2 $\pm$ 2.8	3.9 $\pm$ 0.7	162 $\pm$ 31	21.4 $\pm$ 2.7
GTP	29.3 $\pm$ 2.2	13.9 $\pm$ 3.5	307 $\pm$ 35	55.5 $\pm$ 3.3
GTP, 8-PT	19.8 $\pm$ 2.1†	7.1 $\pm$ 2.3*	336 $\pm$ 38*	58.4 $\pm$ 13.6
GTP, NECA	53.8 $\pm$ 6.9	23.9 $\pm$ 3.0	—	54.7 $\pm$ 6.3
GTP, PIA	—	—	271 $\pm$ 27	—

Table 2. Rat brain microvessel, hepatic, cerebral-cortical and cardiac-ventricular adenylate cyclase activity with [³²P]dATP as substrate

For concentrations of reagents see Table 1.

	Adenylate cyclase activity (pmol of cyclic dAMP/min per mg of protein)			
	Microvessels	Liver	Brain	Heart
GTP	8.1 $\pm$ 1.3	16.5 $\pm$ 3.9	214 $\pm$ 30	45.3 $\pm$ 6.7
GTP, 8-PT	7.7 $\pm$ 1.8	17.7 $\pm$ 6.3	217 $\pm$ 23	44.9 $\pm$ 8.3
GTP, NECA	26.4 $\pm$ 3.2	34.4 $\pm$ 6.6	—	49.3 $\pm$ 4.7
GTP, PIA	—	—	168 $\pm$ 22	—

prostaglandin E_1 , which acted as potent stimulators (results not shown).

Effects of AMP

Owing to the sufficiently high concentration of adenosine deaminase used in the above experiments (see Table 4), background adenosine can in all probability be excluded as the agent responsible for the observed effects, and a potential adenosine-like action of the substrate ATP would appear to provide a more appropriate explanation. Since it is difficult to demonstrate such an action directly and, moreover, the use of ADP is ruled out owing

to its rapid phosphorylation by the substrate-regenerating system of the assay medium, we investigated 5'-AMP as a representative phosphorylated adenosine for a potential adenosine-receptor-mediated effect on adenylate cyclase. dATP served as substrate for these experiments.

As illustrated in Fig. 1, AMP stimulated the adenylate cyclase of the microvessels, which, as reported above, contain an R_a -site adenosine receptor. The AMP effect was strictly dependent on the presence of GTP in the assay (Table 3). The threshold concentration for significant stimulation was $30\text{ }\mu\text{M}$. Since maximum stimulation was not obtained even at the highest concentration used (1 mM), the ED_{50} of AMP seems to be well above 0.1 mM . In the presence of 8-phenyltheophylline, the effect-concentration curve of AMP was markedly shifted to the right. By contrast, the adenylate cyclase activity of cerebral-cortical membranes, a tissue containing inhibitory R_i -site adenosine receptors, was decreased by 0.1 mM -AMP (Table 3). Though this effect was only slight (10% inhibition), it was significant, depended on the presence of GTP, and appeared to be partially reversed by 8-phenyltheophylline. In cardiac ventricular membranes, which are devoid of any adenosine receptor, AMP obviously did not display GTP-dependent alterations.

Fig. 1. Stimulation of rat brain microvessel adenylate cyclase in the absence (○) and presence (●) of $10\text{ }\mu\text{M}$ -8-phenyltheophylline

The adenylate cyclase assay was performed with [^{32}P]dATP as the substrate and in the presence of $10\text{ }\mu\text{M}$ -GTP. The means of three triplicate determinations that varied by less than 15% are shown.

Extent of AMP metabolism

Possible degradation or phosphorylation of AMP in the adenylate cyclase assay medium was investigated by incubating [^{14}C]AMP under the individual conditions chosen for each tissue fraction. As indicated by t.l.c. (Table 4), degradation of AMP was minimal: 88.6–94.6% of the ^{14}C label recovered was identified in the phosphorylated form. The small amount of remaining radioactivity in excess of the respective blank values appeared in the inosine fraction. In the presence of $20\text{ }\mu\text{M}$ -

Table 3. Influence of 5'-AMP (0.1 mM) on microvessel, cerebral-cortical and cardiac ventricular adenylate cyclase in the absence and presence of GTP ($10\text{ }\mu\text{M}$), and in the presence of GTP and 8-phenylthiophylline (8-PT, $10\text{ }\mu\text{M}$) [$\alpha\text{-}^{32}\text{P}$]dATP was the enzyme substrate. Assay conditions are described more fully in the Materials and methods section. The means $\pm$ S.E.M. for five different experiments are shown. Significance was assessed by Student's *t* test for paired data: *significantly different ($P < 0.05$) from the values obtained in the presence of GTP alone; †significantly different ($P < 0.01$) from the respective value obtained in the absence of 8-PT.

	Adenylate cyclase activity (pmol of cyclic dAMP/min per mg of protein)		
	Microvessels	Brain	Heart
No addition	6.75 ± 0.25	175 ± 67	19.1 ± 0.58
AMP	7.19 ± 0.20	178 ± 58	20.2 ± 0.79
GTP	11.9 ± 3.36	244 ± 50	44.0 ± 3.35
GTP, AMP	$25.0 \pm 7.51^*$	$219 \pm 50^*$	42.7 ± 3.32
GTP, AMP, 8-PT	$12.9 \pm 3.04^\dagger$	227 ± 49	—

Table 4. Extent of degradation of [^{14}C]AMP (0.1 mM) under the conditions used for the adenylate cyclase assay in Table 3, but with unlabelled dATP

In particular, the same protein concentrations were used (40 $\mu\text{g}/\text{tube}$ for the microvessels and for the cardiac-ventricular membranes and 4 $\mu\text{g}/\text{tube}$ for the cerebral-cortical membranes). Values in parentheses were obtained in the presence of 20 μM -p[CH₂]pA. Incubations were terminated by heating (95° C for 1 min), and the ^{14}C -labelled purine compounds were identified by t.l.c., as described in the Materials and methods section. Blank values were obtained in the absence of membrane protein. The total radioactivities recovered ranged from 84 to 93%. Mean values (triplicate determinations) of a single experiment are shown.

	Radioactivity recovered (%)			
	Microvessels	Heart	Brain	Blanks
AMP, ADP, ATP	94.6 (96.3)	88.6 (90.8)	89.7 (91.5)	97.5 (96.8)
Adenosine	0.5 (0.4)	0.5 (0.4)	0.5 (0.4)	0.5 (0.7)
Inosine	3.4 (1.9)	9.2 (7.6)	8.9 (6.9)	0.8 (0.8)
Hypoxanthine	0.5 (0.5)	0.5 (0.3)	0.4 (0.4)	0.4 (0.6)
Adenine	0.5 (0.5)	0.7 (0.5)	0.4 (0.4)	0.4 (0.6)

Fig. 2. Elution of adenine nucleotides by high-performance liquid chromatography

For details see the Materials and methods section: 0.1 mM-[^{14}C]AMP was added to the adenylate cyclase assay medium (with unlabelled dATP) and incubated under the conditions chosen for the microvessel (●), cerebral-cortical (△) and cardiac (■) adenylate cyclase. Blank values (○) were obtained in the absence of protein. The total radioactivity recovered was 85%. Mean values (triplicate determinations) from a single experiment are shown. Arrows indicate the elution positions of nucleotide standards.

p[CH₂]pA, an inhibitor of 5'-nucleotidase (Gentry & Olsson, 1975), only a slightly smaller amount of the ^{14}C label was identified as inosine. Hence inclusion of a 5'-nucleotidase inhibitor was unnecessary in the adenylate cyclase determinations with AMP. The radioactivities identified as adenosine, hypoxanthine and adenine did not differ from the respective blank values, indicating that sufficient amounts of adenosine deaminase had been added to the different adenylate cyclase assay media. Separation of adenine nucleotides by high-performance liquid chromatography (Fig. 2) almost excluded any phosphorylation of AMP to ATP by

the microvessels and the cerebral-cortical membranes, since the respective radioactivities were equal to the blank values. Only in the assay medium incubated with the cardiac ventricular membranes was any appreciable phosphorylation noted. In summary, the AMP concentration in the adenylate cyclase assay medium showed only a very small decrease during incubation with the adenosine-sensitive tissue preparations and, most importantly, adenosine was not detected as a degradation product in any of the tissue preparations used in the adenylate cyclase assay.

Discussion

The methodological drawbacks encountered in investigations on adenosine modulation of adenylate cyclase were first discovered by Cooper & Londos (1979), who introduced dATP as the adenylate cyclase substrate. The resulting 'adenosine-free' system has permitted direct measurements of adenosine effects on adenylate cyclase. Moreover, adenosine deaminase, which traps background adenosine generated from ATP and cyclic AMP in the conventional assay system, can now be omitted in the investigation of adenosine deaminase-resistant adenosine analogues. Hence a potential indirect action of adenosine analogues via adenosine deaminase blockade and a consequent increase in endogenous adenosine can be ruled out by the use of the dATP assay.

We now describe a third advantage of the assay system with dATP, namely that dATP is not an adenosine-receptor agonist. In the present study, several lines of evidence suggest that ATP is a low-affinity adenosine-receptor agonist: in the presence of adenosine deaminase and with ATP as the substrate, the activity of the microvessel and hepatic adenylate cyclases, both of which are sensitive to activatory (R_a -site selective) adenosine

analogues such as NECA, was significantly diminished by 8-phenyltheophylline, whereby the presence of a guanine nucleotide proved essential for these observations. This methylxanthine represents a high-affinity adenosine-receptor antagonist. On the other hand, the activity of the cerebral-cortical adenylate cyclase, which is sensitive to inhibitory (R_i -site-selective) adenosine analogues such as PIA, was GTP-dependently enhanced by the methylxanthine. All these responses with the adenosine-receptor antagonist were no longer observed when dATP replaced ATP as enzyme substrate. Finally, in the cardiac ventricular preparation, which is devoid of either adenosine receptor, adenylate cyclase activity was unaltered by 8-phenyltheophylline, in the assays with both ATP and dATP.

An alternate explanation for these findings would be the accumulation of endogenous adenosine in the ATP assay in spite of the high adenosine deaminase concentration used. This possibility is, however, unlikely, in view of the fact that no radioactive adenosine was detected on chromatography when [^{14}C]AMP was added to the microvessel, cerebral-cortical or cardiac-ventricular adenylate cyclase assay medium. Hence the chosen amount of adenosine deaminase sufficed for those systems.

Moreover, AMP acted as stimulator of the microvessel adenylate cyclase and inhibitor of the cerebral-cortical enzyme, but had no effect on the cardiac ventricular enzyme. These actions of AMP were strictly dependent on the presence of a guanine nucleotide, were at least partly inhibited by 8-phenyltheophylline, and cannot, furthermore, be attributed to an AMP metabolite, as revealed by high-performance liquid chromatography and t.l.c. Although the effect of AMP on the microvessel adenylate cyclase was not yet maximal even at a concentration of 1 mM, the intrinsic activity of the nucleotide appears considerably higher than that of NECA at the concentration of 5 μ M (see Fig. 1), which represents a maximal stimulatory concentration (Schütz *et al.*, 1982b). At present we have no explanation for this observation.

In accordance with these results, the effects of adenosine analogues on adenylate cyclase activity were more pronounced in the presence of dATP as the substrate. Stimulation by NECA and inhibition by PIA were greater in the assay with dATP. In the assay with ATP, quantitatively comparable efficacies were achieved only when the control adenylate cyclase activity was measured in the presence of 8-phenyltheophylline. A similar finding has been reported in a study on cerebral-

cortical membranes (Cooper *et al.*, 1980): in that preparation, adenylate cyclase inhibition by PIA was also substantially higher in the assay with dATP than in that with ATP, and here also a sufficiently high adenosine deaminase concentration was apparently used. Hence, owing to the confusing effects so far observed with the conventional ATP assay, i.e. generation of background adenosine and adenosine-like effects of ATP, dATP may be recommended as the preferred substrate for adenylate cyclase measurements in systems susceptible to modulation by adenosine.

It must be emphasized that the observed adenosine-receptor-mediated effects of phosphorylated adenosine derivatives on adenylate cyclase probably have no physiological relevance. The ED_{50} of AMP on cerebral microvessel adenylate cyclase activity, as determined in the present study, is far above 0.1 mM, a nucleotide concentration that is not assumed to occur extracellularly. However, ATP concentrations of this order must be taken into account under the conditions of the adenylate cyclase assay carried out with broken-cell preparations.

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