Adenosine-receptor-mediated stimulation of low- K_m GTPase in guinea-pig cerebral cortex

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Inhibition of receptor-coupled adenylate cyclase by hormones is proposed to be associated with GTP hydrolysis. Since adenosine inhibits cerebral-cortical adenylate cyclase via A_1 adenosine receptors, the present study attempts to verify this mechanism for A_1 -selective adenosine derivatives. In guinea-pig cortical membranes N^6 -(phenylisopropyl)adenosine (PIA) increased the V_{max} of the low- K_m GTPase, with an EC_{50} (concentration causing 50% of maximal stimulation) of about 0.1 μM , and the stimulatory effect was competitively antagonized by 5 μM -8-phenyltheophylline. The rank order of potency of the stereoisomers of PIA and of 5-(N -ethylcarboxamido)adenosine (NECA) to stimulate GTPase correlated with their ability to inhibit adenylate cyclase activity (R -PIA > NECA > S -PIA). Competition binding studies with (–)- N^6 -([125 I]iodo-4-hydroxyphenylisopropyl)adenosine suggest that adenylyl imidodiphosphate (p[NH]ppA), an essential component of the GTPase assay system, is a more potent A_1 -receptor agonist than ATP, with an IC_{50} (concentration giving half-maximal displacement of radioligand binding) of 7.9 μM . On the basis of the p[NH]ppA concentration used in the GTPase assay (1.25 mM), enzyme stimulation by adenosine seems to be highly underestimated. Nevertheless, adenosine-induced GTP hydrolysis reflects an increased turnover of guanine nucleotides at the N_1 regulatory site and appears to be a crucial step in the sequence of events processing the inhibitory signal to adenylate cyclase.

INTRODUCTION

Stimulation and inhibition of adenylate cyclase by hormones or transmitters are GTP-dependent processes, and both are associated with stimulation of a low- K_m GTPase [1–3]. Ample evidence has been presented that the responsible guanine-nucleotide-binding proteins, N_s and N_i , are GTPases [4, 5]. Receptors for adenosine have been identified in the rat cerebral cortex [6, 7]; these are coupled as inhibitors to adenylate cyclase [8] and regulated by guanine nucleotides [9, 10]. Thus receptor-mediated inhibition of adenylate cyclase may be an expression of a fundamental action at the level of an associated GTPase. The present paper demonstrates that typical adenosine-receptor agonists stimulate cerebral-cortical GTP hydrolysis in a similar manner to that shown to occur in fat-cell membranes [11].

Preliminary results of this paper were presented at the 26th Spring Meeting of the German Pharmacological Society [12].

MATERIALS AND METHODS

Materials

[γ - 32 P]GTP (10–40 Ci/mmol) and [α - 32 P]dATP (800 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). [125 I]HPIA was from Amersham International (Amersham, Bucks., U.K.). Na_2 ATP (grade I) and p[NH]ppA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 8-Phenyltheophylline was bought from Calbiochem (La Jolla, CA, U.S.A.). NECA

was generously given by Byk Gulden Lomberg (Konstanz, Germany). R -PIA, S -PIA, CHA, creatine kinase (EC 2.7.3.2), adenosine deaminase (EC 3.5.4.4), phosphocreatine, 2'-dATP, cyclic 2'-dAMP and Hepes were purchased from Boehringer (Mannheim, Germany). All other reagents and compounds were analytical grade or the best commercially available products.

Cerebral membrane preparation

The cerebral cortex from freshly decapitated guinea pigs was freed from white matter and pial vessels and homogenized in 5 vol. of 10 mM-Hepes (pH 7.5)/1 mM- $MgCl_2$ /0.25 M-sucrose in a glass homogenizer with a Teflon pestle (ten strokes, setting 6). The homogenate was centrifuged at 9000 g for 10 min. The supernatant was centrifuged at 37000 g for 10 min and the resulting pellet was washed three times in the above-mentioned medium without sucrose, and then taken up at a protein concentration of about 2 mg/ml and stored in liquid N_2 . Protein was determined by dye-binding (Coomassie Blue G 250) by using the Bio-Rad assay kit.

GTPase assay

The GTPase assay was carried out as described by Cassel & Selinger [2], as modified by Aktories *et al.* [1], on samples (100 μ l final volume) containing 50 mM-triethanolamine/HCl (pH 7.4), 2 mM- $MgCl_2$, 0.2 mM-EGTA, 1 mM-dithiothreitol, 0.1 mM-ATP (with GTP impurities of 0.1%, as determined by h.p.l.c.), 5 mM-phosphocreatine, 1.25 mM-p[NH]ppA, 100 mM-NaCl, 1 mg of creatine kinase/ml, 2.5 units of adenosine

Abbreviations used: [125 I]HPIA, (–)- N^6 -([125 I]iodo-4-hydroxyphenylisopropyl)adenosine; R -PIA, N^6 -(R -phenylisopropyl)adenosine; S -PIA, N^6 -(S -phenylisopropyl)adenosine; CHA, N^6 -cyclohexyladenosine; NECA, 5-(N -ethylcarboxamido)adenosine; p[NH]ppA, adenylyl imidodiphosphate; EC_{50} , concentration of agonist giving 50% of maximal stimulation; IC_{50} , concentration giving half-maximal displacement of radioligand binding.

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deaminase/ml, 0.2% bovine serum albumin, [γ - 32 P]GTP ($\sim 0.1 \mu\text{Ci}$) and unlabelled GTP at the indicated concentrations. The reaction was started by the addition of a diluted prewarmed suspension of the cortical membranes ($5\text{--}10 \mu\text{g}/50 \mu\text{l}$). After incubation for 5 min at 25°C , the reaction was stopped by the addition of $700 \mu\text{l}$ of ice-cold sodium phosphate buffer (pH 6.9; 20 mM) containing 5% (w/v) activated charcoal. After centrifugation ($1400 g$ for 10 min), the radioactivity in a $500 \mu\text{l}$ sample of the supernatant was determined by Čerenkov radiation.

The data obtained in the isotope-dilution experiments were analysed by non-linear least-squares curve fitting, assuming the presence of two independent enzymic activities. A weighting subroutine was included in order to correct for the non-homogeneity of the variance. All calculations were carried out on a PDP 11/10 computer.

Adenylate cyclase

Enzyme activity in the guinea-pig cerebral cortex was determined as previously described [13], with dATP as enzyme substrate. All assays were performed in triplicate.

[125 I]HPIA binding

Competition binding experiments were conducted with $50 \mu\text{l}$ of cerebral-cortical membrane suspension (approx. $15 \mu\text{g}$ of protein/assay) incubated in 300 pM -[125 I]HPIA, 40 mM-Hepes and 5 mM-MgCl₂ to give a total volume of $200 \mu\text{l}$. Concentrations of the competitive agents are indicated in Fig. 5. The membranes were preincubated with 1 unit of adenosine deaminase/ml for 20 min at 37°C to remove endogenous adenosine. After incubation for 2 h at 37°C the mixture was diluted with 2.5 ml of the ice-cold buffer mentioned above, and immediately poured on to Whatman GF/C filters. The filters were washed with $2 \times 5 \text{ ml}$ of ice-cold buffer and transferred to scintillation vials. Non-specific binding was defined as binding not displaced by $10 \mu\text{M}$ -CHA.

RESULTS

Adenylate cyclase activity

As described for the rat cerebral cortex [8], agonists selective for the adenosine A₁-receptor also inhibited adenylate cyclase activity in guinea-pig cortical membranes. Basal activity in the presence of $10 \mu\text{M}$ -GTP was $318 \pm 4.26 \text{ pmol/min per mg}$ of protein (mean $\pm$ S.D., quadruplicate determination) and decreased to $250 \pm 6.01 \text{ pmol/min per mg}$ on addition of $3 \mu\text{M}$ -CHA.

GTPase activity

[γ - 32 P]GTP dilution curves in the presence and absence of $3 \mu\text{M}$ -R-PIA are shown in Fig. 1. All assay tubes contained 20 nM -[γ - 32 P]GTP, 10 nM unlabelled GTP (as in 0.1% impurity of the Sigma ATP), and increasing concentrations of added GTP as detailed in the Figure. At all GTP concentrations below $100 \mu\text{M}$, release of [32 P]P_i was greater in the presence than in the absence of R-PIA (the significance of this effect is shown in detail in Fig. 2). A plateau was reached at GTP concentrations of about $10 \mu\text{M}$. Computerized curve fitting revealed the presence of a low- and a high- K_m GTPase.

The apparently low- K_m PIA-sensitive enzyme activity accounted for $68.1 \pm 5.9\%$ (mean $\pm$ S.E.M.) of total [γ - 32 P]GTP hydrolysis. This value remained virtually

Fig. 1. [γ - 32 P]GTP hydrolysis by guinea-pig cerebral-cortical membranes at various concentrations of unlabelled GTP

Computerized curve fitting of the isotope-dilution curves was performed. $\circ$, Control; $\bullet$, $+ 3 \mu\text{M}$ -R-PIA. Results are means $\pm$ S.E.M. ($n = 3$).

Fig. 2. Computerized curve fitting of the saturation isotherm of the low- K_m GTPase activity (v) in the absence ($\circ$) and presence ($\bullet$) of $3 \mu\text{M}$ -R-PIA

Enzyme activity is calculated by the difference between [γ - 32 P]GTP hydrolysis measured at the GTP concentrations indicated and that at $400 \mu\text{M}$ -GTP. Results are means $\pm$ S.E.M. ($n = 4$). Significance: $*P < 0.05$, $**P < 0.01$ (t test for paired data).

unaltered in the presence of R-PIA ($66.4 \pm 5.0\%$). The contribution of the high- K_m PIA-insensitive component of total GTPase activity (K_m approx. $700 \mu\text{M}$) is routinely corrected by subtraction of the amount of [32 P]P_i released

Fig. 3. Concentration-response curves for the stimulatory effect of adenosine-receptor agonists on the low- K_m GTPase

The substrate concentration of GTP was $2 \mu\text{M}$. ●, *R*-PIA; ▲, NECA; ○, *S*-PIA. The means of three experiments are shown.

in the presence of $400 \mu\text{M}$ -GTP, as described by others [1, 2]. By this procedure the kinetics of the stimulatory effect of *R*-PIA on the low- K_m GTPase were evaluated, as illustrated in Fig. 2. Enzyme activity increased as a function of GTP concentration and appeared to reach saturation at approx. $2 \mu\text{M}$ -GTP. However, full saturation was obviously not obtained. This might be due to increasing influence of the high- K_m GTPase at the higher GTP concentrations. *R*-PIA significantly increased the rate of GTP hydrolysis at each GTP concentration used. Non-linear least-squares curve fitting of the saturation isotherms revealed that GTP hydrolysis was characterized by an apparent K_m of $0.45 \pm 0.01 \mu\text{M}$ and an apparent V_{max} of $78.6 \pm 9.2 \text{ pmol/min per mg}$ (means $\pm$ S.E.M.), and that addition of $3 \mu\text{M}$ -*R*-PIA significantly increased the V_{max} to $133.6 \pm 17.5 \text{ pmol/min per mg}$ ($P < 0.01$), whereas the K_m ($0.53 \pm 0.02 \mu\text{M}$) was not significantly altered.

In the concentration-response curves, the stimulatory effect of *R*-PIA on the low- K_m GTPase was compared with the effects of NECA and *S*-PIA, further typical agonists at the adenosine receptor (Fig. 3). A constant GTP concentration of $2 \mu\text{M}$ was used in these experiments. Calculation of the apparent EC_{50} showed that these compounds stimulated the enzyme with a rank order of potency *R*-PIA ($0.1 \mu\text{M}$) > NECA ($1 \mu\text{M}$) > *S*-PIA ($10 \mu\text{M}$). Fig. 4 illustrates the antagonism of $5 \mu\text{M}$ -8-phenyltheophylline to CHA, an adenosine-receptor agonist as potent as *R*-PIA. Inhibition appeared to be competitive, with a methylxanthine-induced shift in the EC_{50} of CHA from $0.1 \mu\text{M}$ to approx. $30 \mu\text{M}$. Basal low- K_m GTPase, however, remained unchanged after addition of 8-phenyltheophylline.

Effect of p[NH]ppA and ATP on [125]HPIA binding

In order to test the possibility that GTPase stimulation might be underestimated, owing to the presence of the phosphorylated adenosine derivatives involved in the GTPase assay system, the affinity of ATP and p[NH]ppA to brain A_1 -adenosine receptors was investigated. As shown in Fig. 5, p[NH]ppA was 10 times more potent

Fig. 4. Competitive antagonism by 8-phenyltheophylline of CHA stimulation of low- K_m GTPase

●, Control; ▲, $+ 5 \mu\text{M}$ -8-phenyltheophylline. The means of three experiments are shown.

Fig. 5. Competition of p[NH]ppA (▲) with cerebral-cortical [125]HPIA binding as compared with CHA (●) and ATP (○)

Means of three experiments are shown.

than ATP in displacing [125]HPIA from its binding sites, and an IC_{50} of $7.9 \mu\text{M}$. At the concentration of p[NH]ppA used in the GTPase assay (1.25 mM), more than 90% of the available binding sites are occupied. The high affinity of CHA (0.7 nM) confirms that p[NH]ppA and ATP interact with A_1 -adenosine receptors.

DISCUSSION

The results presented here demonstrate that adenosine stimulates a high-affinity low- K_m GTPase in cerebral-cortical membranes obtained from guinea pigs. Stimulation of GTP hydrolysis appeared to result from the interaction of adenosine with its receptors. The typical adenosine-receptor agonists CHA, *R*-PIA and NECA caused half-maximal stimulation of the low- K_m GTPase at concentrations of $0.1 \mu\text{M}$ (CHA, *R*-PIA) and $1.0 \mu\text{M}$ (NECA) and are competitively antagonized by the methylxanthine 8-phenyltheophylline, findings in excellent agreement with the potency displaced by these compounds in experiments on rat cerebral-cortical adenylate cyclase [8]. Furthermore, a similar rank order of potency, PIA stereoselectivity and methylxanthine antagonism are reported in several adenosine-receptor-binding studies

carried out on rat and guinea-pig brain membranes [6, 7]. Hence adenosine stimulation of GTP hydrolysis appears to be tightly coupled to receptor occupancy, a suggestion put forward by Aktories *et al.* [11] in studies on fat-cell membranes, a preparation likewise containing A₁-adenosine receptors. It is probable that the stimulatory effect of adenosine analogues on low-*K_m* GTPase and the guanine nucleotide effect on radioactive PIA binding [9, 10] result from the interaction of the adenosine receptor with the same guanine-nucleotide-binding moiety in brain membranes. PIA stimulation of GTPase occurred at GTP concentrations (with a *K_m* of 0.7 μ M) which are similar to the respective IC₅₀ of GTP in inhibiting [³H]PIA binding [10], as well as with the half-maximal GTP concentration necessary for PIA inhibition of adenylate cyclase [8].

The increase in low-*K_m* GTPase in response to the adenosine analogues is relatively small, at most 50%, which is in the range reported by other authors for hormone-stimulated GTPase in different membrane fractions [1–3, 11, 14, 15]. On the basis of the affinity of adenine nucleotides to adenosine receptors [13], we propose that stimulation of low-*K_m* GTPase is underestimated, since the GTPase assay system contained high concentrations of ATP and p[NH]ppA. The latter compound acts as an inhibitor of non-specific GTPases [2]. Our hypothesis would imply a decrease in basal enzyme activity on addition of the adenosine-receptor antagonist 8-phenyltheophylline, which, however, was not the case in the present experiments. On the other hand, it becomes evident from the competition-binding studies that the IC₅₀ of p[NH]ppA to [¹²⁵I]HPIA-labelled adenosine receptors is rather low (7.9 μ M), being one order of magnitude below that of ATP (see Fig. 5). At 0.1 mM-p[NH]ppA, the decrease in [¹²⁵I]HPIA binding was almost maximal (decreased to about 10% of the initial values), and the p[NH]ppA concentration used in the GTPase assay (1.25 mM) would even suffice to overcome competitive inhibition by 8-phenyltheophylline. Hence, under the conditions of the GTPase assay adenosine analogues can presumably bind only to the

residual 10% of adenosine receptors, and, owing to the relatively high affinity of p[NH]ppA to cerebral adenosine receptors, any stimulation of the low-*K_m* GTPase in that tissue appears to be highly underestimated. Owing to successive decreases in the low-*K_m* high-affinity component of overall GTPase activity, lowering of the p[NH]ppA concentration was not possible in the present study.

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