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Functional coupling between A_1 adenosine receptors and G-proteins in rat hippocampal membranes assessed by high-affinity GTPase activity

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1 In order to investigate the functional interaction between adenosine receptors and G-proteins in native brain membranes, stimulation of high-affinity GTPase activity by adenosine receptor agonists was characterized in rat hippocampal membranes.

2 Addition of 1 μM **R**-N^6-phenylisopropyladenosine (**R**-PIA), a selective A_1 adenosine receptor agonist, augmented the V_{max} of the low-K_M GTPase by 51%, with a slight increase in the K_M value.

3 Several adenosine receptor agonists stimulated the high-affinity GTPase activity in a concentration-dependent manner, with a rank order of potency indicative of the involvement of A_1 adenosine receptor subtype as follows: **R**-PIA > N^6-cyclohexyladenosine > 5'-N-ethylcarboxamidoadenosine $\geq$ 2-chloroadenosine > **S**-PIA > CGS 21680, 2-phenylaminoadenosine.

4 The selective A_1 adenosine receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine, inhibited **R**-PIA-stimulated high-affinity GTPase activity in a competitive manner, with a K_B value of 2.5 nM.

5 The activating effects on high-affinity GTPase of **R**-PIA (via A_1 adenosine receptors) and of 5-HT (via 5-HT_{1A} receptors) were completely additive, indicating that A_1 adenosine and 5-HT_{1A} receptors were coupled to distinct pools of G-proteins in hippocampus.

6 Stimulation of high-affinity GTPase activity by adenosine receptor agonists can be used as a valuable measure for the investigation of the functional coupling between A_1 adenosine receptors and G-proteins associated with adenylyl cyclase inhibition.

Keywords: A_1 adenosine receptor; G-protein; rat hippocampal membranes

Introduction

Adenosine evokes many biological responses in mammalian species by activating the adenosine receptors on the cellular membranes (Collis & Hourani, 1993; Fredholm *et al.*, 1994). It is now widely accepted that adenosine receptors are composed of pharmacologically heterogeneous subtypes as in many other transmitter receptors coupled with G-proteins. At least four subtypes of the receptor, designated A_1, A_{2a}, A_{2b}, and A_3 (Collis & Hourani, 1993; Fredholm *et al.*, 1994), have been recognized so far. Of these, the A_1 adenosine receptors have been characterized extensively and it is known that this receptor subtype is coupled, via pertussis toxin (islet-activating protein, IAP)-sensitive G-proteins, to several intracellular effector systems, e.g., inhibition of adenylyl cyclase (EC 4.6.1.1), potassium channels, calcium channels, phospholipases A_2 or C, and guanylyl cyclase (Olsson & Pearson, 1990; Linden, 1991).

In the central nervous systems, the interaction between A_1 adenosine receptors and G-proteins has been investigated mainly by radioligand binding methods (Gavish *et al.*, 1982; Goodman *et al.*, 1982; Yeung & Green, 1983; Lohse *et al.*, 1984; Klotz *et al.*, 1986; 1990; Stiles, 1988). Another line of study on A_1 receptor-G-protein coupling has dealt with the modulation of G-protein functional activity subsequent to an activation of the A_1 adenosine receptors by agonists. [^{35}S]-GTPγS binding to G-proteins has been shown to be stimulated by A_1 receptor agonists in bovine cerebral cortical membranes (Lorenzen *et al.*, 1993) as well as in experiments using purified A_1 adenosine receptors and purified G-proteins (Freissmuth *et al.*, 1991; Munshi *et al.*, 1991). Stimulation of high-affinity GTPase (EC 3.6.1.-) activity of G-proteins by adenosine agonists has been reported in adipocyte membranes (Aktories *et al.*, 1982), in guinea-pig cerebral cortical membranes (Hausleithner *et al.*, 1985), and in rat cerebellum membranes (Wojcik *et al.*, 1985).

The authors have recently developed a method for detecting agonist-induced high-affinity GTPase activity through dopamine D_2, muscarinic, and $GABA_B$ receptors in striatum (Odagaki & Fuxe, 1995a) and 5-HT_{1A} receptors in hippocampus (Odagaki & Fuxe, 1995c,d) of rats. In this study, we adapted this method with minor modifications to rat hippocampal membranes to investigate the functional coupling between A_1 adenosine receptors and G-proteins. In addition to the pharmacological characterization of this response, additivity between high-affinity GTPase activities stimulated via A_1 adenosine receptors and 5-HT_{1A} receptors, both of which were reported to couple to the same effector systems in a previous article (Zgombick *et al.*, 1989), was studied.

Methods

Animals

Male Sprague-Dawley rats (Alab, Stockholm) weighing 200-250 g were used. The animals were kept under regular lighting conditions (lights on at 06 h 00 min and off at 20 h 00 min) in a temperature-controlled environment with free access to food pellets and tap water.

Membrane preparation

Rats were decapitated and their brains were quickly removed. The hippocampi dissected from each rat were homogenized in 5 ml of ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA,

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1 mM dithiothreitol, pH 7.4) containing 10% (w/v) sucrose with a motor-driven Teflon/glass tissue grinder (20 strokes). The supernatant, derived from a centrifugation of the homogenate at 1,000 *g* for 10 min, was kept on ice while the pellet was resuspended in 5 ml of TED/sucrose buffer and was centrifuged again at 1,000 *g* for 10 min. The combined supernatant was centrifuged at 9,000 *g* for 20 min, and the resulting pellet was resuspended in 10 ml of TED buffer and centrifuged again at 9,000 *g* for 20 min. The pellet was resuspended in 10 ml of TED buffer and maintained on ice for 30 min. After a centrifugation of the suspension at 35,000 *g* for 10 min, the pellet was resuspended in 1.5 ml of 50 mM Tris-HCl buffer (pH 7.4). This homogenate (1.6–3.2 mg protein ml^{-1}) was frozen on powdered dry ice and stored at −70°C for no longer than five weeks without deterioration in GTPase activity.

The protein concentration was measured by the method of Lowry *et al.* (1951) with BSA used as a standard.

GTPase assay

GTP hydrolyzing activity was assayed in hippocampal membrane preparation by the method previously described by the authors (Odagaki & Fuxe, 1995d) with some modifications, which measures $^{32}P_i$ released from [γ-^{32}P]-GTP by an enzymatic conversion of GTP to GDP and P_i. The modified points as compared to our previous method were as follows: (i) addition of adenosine deaminase, to exclude the likely effect of endogenous adenosine, as in the previous reports dealing with adenosine receptor-mediated GTPase activity (Aktories *et al.*, 1982; Hausleithner *et al.*, 1985; Wojcik *et al.*, 1985), (ii) omission of 3-isobutyl-1-methylxanthine, in order to avoid a possible interferring effect of the xanthine derivative as an adenosine receptor antagonist (see Bruns *et al.*, 1986). The reaction mixture (final volume 100 μl) contained the following constituents: 50 mM Tris-HCl (pH 7.4), 0.3 μM GTP ([γ-^{32}P]-GTP plus unlabelled GTP), 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM adenylylimidodiphosphate, 5 mM phosphocreatine, creatine phosphokinase (50 u ml^{-1}), BSA (50 μg), 0.1 mM EDTA, 0.2 mM EGTA, 0.2 mM dithiothreitol, 0.5 mM cyclic AMP, 100 mM NaCl, adenosine deaminase (2 u ml^{-1}), and membrane protein (4–8 μg). The hippocampal membranes (25 μl) and drug(s) (agonist and/or antagonist; 25 μl) were put into 1.5 ml microcentrifuge tubes (Eppendorf) placed in ice-cold water bath. The enzyme reaction was initiated by addition of assay mixture (50 μl) containing [γ-^{32}P]-GTP and other reagents and subsequent immersion of the tubes in a 30°C water bath. When the tubes contained both agonist and antagonist, they were kept in ice-cold water for 60 min before initiation of the reaction. The enzyme reaction was terminated after incubation of the assay mixture at 30°C for 15 min by transferring the tubes to an ice bath followed by the addition of 500 μl of 20 mM phosphoric acid (pH 2.5) containing 5% (w/v) activated charcoal. The tubes were kept chilled for about 30 min and centrifuged at 11,000 *g* for 10 min. An aliquot (200 μl) from the supernatant fraction was mixed with 5 ml of scintillation cocktail, and the radioactivity was counted in a liquid scintillation spectrometer. The high-affinity GTPase activity was routinely calculated by subtracting the amount of $^{32}P_i$ released from [γ-^{32}P]-GTP in the presence of 100 μM unlabelled GTP from the total activity.

Data analysis

Unless otherwise described, all results were presented as mean ± s.e.mean of the indicated number of separate experiments, each performed at least in duplicate. Agonist-induced stimulation of high-affinity GTPase activity was expressed as percentage increase above unstimulated basal value, and the concentration-response curves were analyzed by single factor repeated measures ANOVA followed by Scheffe *F*-test. Maximal percentage stimulation above basal activity ($\%E_{max}$) and EC_{50} were calculated by means of computer-assisted non-linear regression analysis, according to a one site model. For inhibition by antagonists, IC_{50} was also determined by using computer-assisted non-linear regression analysis assuming a one site model. Because of the limited number of concentrations used and the variability of values, analysis using a two site model was not tested in either case. EC_{50} and IC_{50} values were converted to the negative logarithmic values (pD_2 and pIC_{50}, respectively). The dissociation constant (K_B) for 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was calculated by means of Schild plot analysis (Arunlakshana & Schild, 1959) of the data on concentration-response curves of **R**-N^6-phenylisopropyladenosine (**R**-PIA) in the absence and presence of increasing concentrations of DPCPX. Statistical significance was defined as $P < 0.05$.

Chemicals

[γ-^{32}P]-GTP (30 Ci $mmol^{-1}$) was purchased from New England Nuclear (Boston, MA, U.S.A.). **R**-PIA, S-PIA, and adenosine deaminase were obtained from Boehringer (Mannheim, Germany). 2-Phenylaminoadenosine, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680) HCl, and DPCPX were from Research Biochemical Inc. (Natick, MA, U.S.A.). N^6-cyclohexyladenosine (CHA), 5'-N-ethylcarboxamidoadenosine (NECA), 2-chloroadenosine, 5-HT creatine sulphate, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Isotopic dilution experiment

The isotope dilution curves of [γ-^{32}P]-GTP hydrolysis by rat hippocampal membranes measured in the absence and presence of 1 μM **R**-PIA are shown in Figure 1a. Low concentrations of GTP potently decreased the hydrolysis of 0.1 μM [γ-^{32}P]-GTP with a plateau reached at GTP concentrations of about 10 μM, indicating the existence of high- and low-affinity GTPase activities. The addition of 1 μM **R**-PIA increased the [γ-^{32}P]-GTP hydrolysis significantly at GTP concentrations below 3 μM, but not at 100 μM.

The high-affinity GTPase activity was estimated by subtracting the amount of [γ-^{32}P]-GTP hydrolyzed in the presence of 100 μM unlabelled GTP from the total amount of $^{32}P_i$ released, as routinely utilized in many previous reports as to high-affinity GTP hydrolyzing activity. The kinetic analysis of the high-affinity GTPase activity was performed by employing Eadie-Hofstee plots (Figure 1b). The unstimulated, basal high-affinity GTPase was characterized by an apparent K_M of 0.30 μM [the negative logarithm of K_M (pK_M) = 6.53 ± 0.02, $n = 4$] and an apparent V_{max} of 1552 ± 95 pmol mg^{-1} protein 15 min^{-1}. Addition of 1 μM **R**-PIA significantly increased the V_{max} to 2342 ± 136 pmol mg^{-1} protein 15 min^{-1} ($P < 0.001$, Student's paired two-tailed *t* test), with a slight increase in the apparent K_M to 0.47 μM ($pK_M = 6.33 \pm 0.01$, $n = 4$; $P < 0.01$, Student's paired two-tailed *t* test).

Stimulation of high-affinity GTPase activity by adenosine receptor agonists

Examples of concentration-response curves of high-affinity GTPase activity in rat hippocampal membranes elicited by **R**-PIA, S-PIA, and CGS 21680 are shown in Figure 2. The selective and potent A_1 adenosine agonist, **R**-PIA, stimulated the high-affinity GTPase activity in a concentration-dependent manner with an EC_{50} value of 7.5 nM ($pD_2 = 8.12 \pm 0.16$, $n = 8$) and a $\%E_{max}$ of 11.9 ± 0.6. S-PIA, a less active enantiomer, also stimulated the enzyme activity to the same extent ($\%E_{max} = 11.1 \pm 0.9$, $n = 8$), but with an approximately 30 fold less potent affinity than **R**-PIA (mean $EC_{50} = 230$ nM; $pD_2 = 6.65 \pm 0.07$, $n = 8$), indicating a stereoselectivity of the

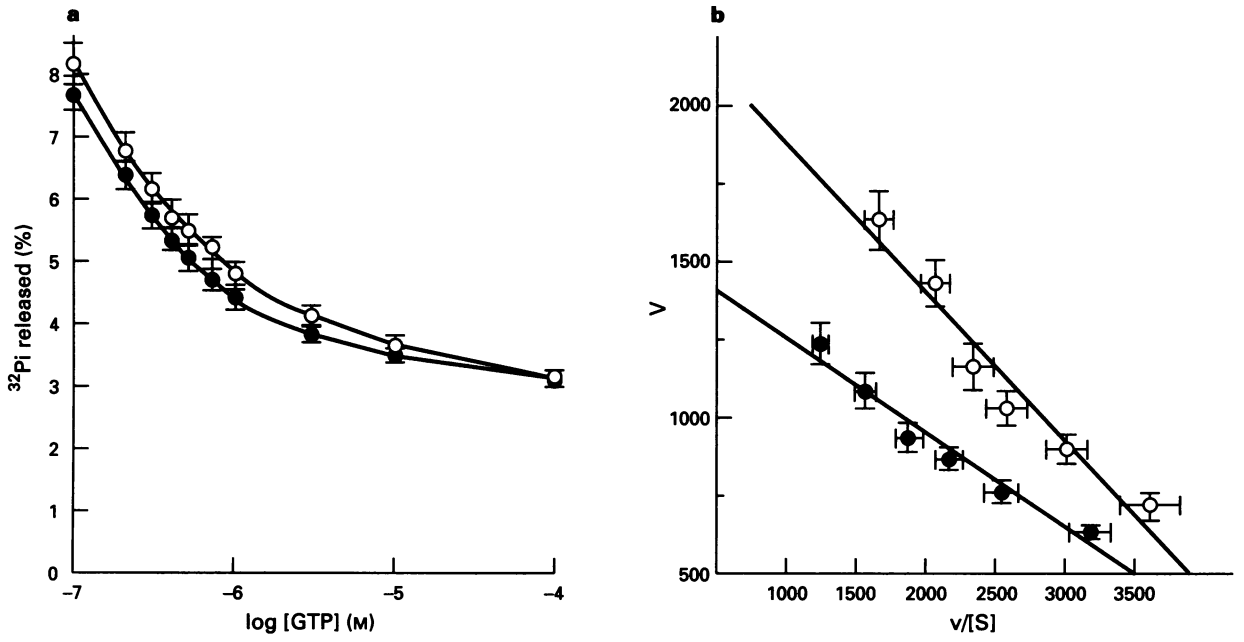

Figure 1 Hydrolysis of [γ-^{32}P]-GTP (0.1 μM) by rat hippocampal membranes in the presence of different concentrations of unlabelled GTP. (a) The radioactivity of $^{32}P_i$ released from 0.1 μM [γ-^{32}P] GTP in the absence of agonist (●) and in the presence of 1 μM **R**-PIA (○) is expressed as percentage of radioactivity of added [γ-^{32}P]-GTP per μg of membrane protein. The abscissa scale indicates the concentration of GTP (0.1 μM [γ-^{32}P]-GTP plus unlabelled GTP). The values are mean ± s.e.mean of four separate experiments, each performed in duplicate. The values in the presence of **R**-PIA are significantly different from those in the absence of **R**-PIA at lower concentrations of GTP ($P < 0.05$ at 0.1, 0.4, 0.5, 1.0, and 10 μM, $P < 0.01$ at 0.3 and 0.7 μM, Student's paired two-tailed t test adjusted by the Bonferroni procedure). (b) The high-affinity GTPase activity was calculated by subtracting the amount of [γ-^{32}P]-GTP hydrolyzed in the presence of 100 μM unlabelled GTP from the total amount of $^{32}P_i$ released at different GTP concentrations and analyzed by means of Eadie-Hofstee transformation. The points represent the values in the absence of agonist (●) and in the presence of 1 μM **R**-PIA (○) at 0.2, 0.3, 0.4, 0.5, 0.7 and 1.0 μM GTP. Values are mean ± s.e.mean of four separate experiments.

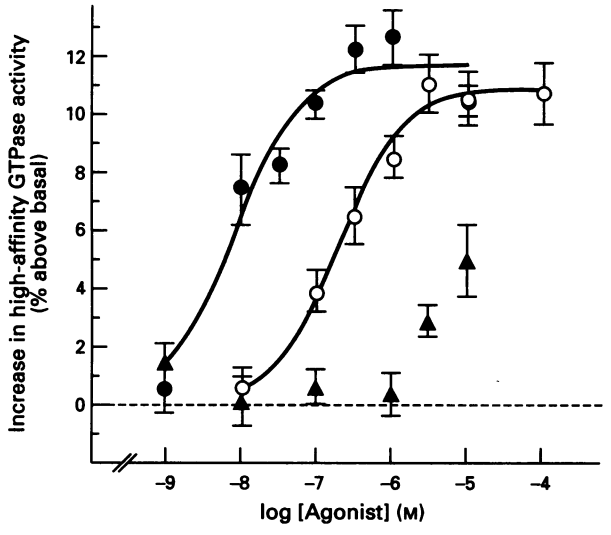

Figure 2 Stimulation of high-affinity GTPase activity by several adenosine receptor agonists in rat hippocampal membranes. Hydrolysis of [γ-^{32}P]-GTP (0.3 μM) was measured after incubation of hippocampal membranes at 30°C for 15 min in the absence and presence of increasing concentrations of **R**-PIA (●), S-PIA (○), and CGS 21680 (▲). Nonspecific, low-affinity GTP hydrolyzing activity was defined in the presence of 100 μM unlabelled GTP and was subtracted from total activity to define high-affinity GTPase activity. Stimulation was expressed as percentage increase above basal high-affinity GTPase activity. Basal high-affinity GTPase activities in the absence of agonist were 480 ± 40 ($n = 8$), 478 ± 39 ($n = 8$), and 517 ± 21 ($n = 7$) pmol mg^{-1} protein 15 min^{-1} for **R**-PIA-, S-PIA-, and CGS 21680-stimulation, respectively. Values are mean ± s.e.mean of the indicated number of separate experiments, each performed in duplicate.

PIA-induced response. The selective and potent A_{2a} adenosine receptor agonist, CGS 21680, failed to alter the activity at least up to 1 μM, suggesting that this compound was an agonist with a very low potency.

The results on several adenosine receptor agonists including these three compounds are summarized in Table 1. The maximal responses to all compounds except for the last two (CGS 21680 and 2-phenylaminoadenosine) were of the same magnitude (mean $\%E_{max}$, 11.1–14.1%). The rank order of potency of these compounds was **R**-PIA > CHA > NECA ≥ 2-chloroadenosine > S-PIA > CGS 21680, 2-phenylaminoadenosine. Although it is impossible to determine the exact EC_{50} value for CGS 21680 because of a lack of saturability in the concentrations used (~10 μM), the expected EC_{50} value falls around 10-30 μM assuming that the maximal response by CGS 21680 is the same position as in the other agonist-induced concentration-response curves. 2-Phenylaminoadenosine did not stimulate the high-affinity GTPase activity up to 1 μM, and then reduced the activity at and above 10 μM. This inhibitory effect of 2-phenylaminoadenosine is probably due to some nonspecific, receptor-unrelated mechanism considering the high concentrations necessary for the inhibition. The high concentrations of ethanol [12.5 and 1.25% (v/v) at 10 and 1 μM 2-phenylaminoadenosine], which were needed to dissolve this compound, were probably the cause of this inhibition.

Figure 3 shows the relationship between the pD_2 values of agonists obtained in the present study and the pD_2 values of the same compounds as agonists for the adenylyl cyclase inhibiting response in hippocampal membranes reported previously by Zgombick *et al.* (1989). There was a highly significant correlation between the mean pD_2 values determined in the two different biochemical experiments ($r = 0.98$, $n = 5$, $P < 0.01$), suggesting that these two responses were mediated through the same type of receptors.

Table 1 Effects of several adenosine receptor agonists on high-affinity GTPase activity in rat hippocampal membranes

Agonist	n[a]	pD_2	*Mean* EC_{50} (nM)	$\%E_{max}$
R-PIA	8	8.12±0.16	7.5	11.9±0.6
CHA	6	7.70±0.07	20	14.1±0.4
NECA	6	7.15±0.17	71	13.9±0.8
2-Chloroadenosine	7	7.04±0.10	92	11.9±1.0
S-PIA	8	6.65±0.07	230	11.1±0.9
CGS 21680	7	–	>3000	inactive up to 3 μM[b]
2-Phenylaminoadenosine	4	–	–	inactive up to 1 μM[c]

Rat hippocampal membranes were incubated with 0.3 μM [γ-^{32}P]-GTP for 15 min at 30°C in the absence and presence of increasing concentrations of agonists as described in Methods. The concentration-response curves were analyzed by single factor repeated measures ANOVA followed by Scheffe *F*-test.
[a] Number of experiments.
[b] Stimulated by 5.0±1.3% at 10 μM (see Figure 2).
[c] Inhibited nonspecifically at and above 10 μM.

Competitive antagonism of R-PIA-stimulated high-affinity GTPase activity by DPCPX

DPCPX is the only currently available antagonist that shows a consistent and marked selectivity for the A_1 adenosine receptor (Collis & Hourani, 1993). The above-described pharmacological profile of adenosine receptor agonist-stimulated high-affinity GTPase activity indicated the involvement of the A_1 adenosine receptor subtype and prompted us to investigate the inhibitory action of the selective A_1 adenosine receptor antagonist, DPCPX. The addition of 3 nM, 10 nM, and 100 nM DPCPX progressively shifted the concentration-response curve elicited by **R**-PIA in the absence of DPCPX to the right in a parallel manner (Figure 4a). The $\%E_{max}$ value in the absence of DPCPX (12.1±1.1%, $n=4$) was not affected by the addition of DPCPX [13.5±1.9 ($n=3$), 11.7±1.7 ($n=4$), and 11.0±1.4 ($n=4$) % for the responses in the presence of 3, 10 and 100 nM DPCPX, respectively].

Schild analysis of the four independent experiments gave a mean apparent K_B value for DPCPX of 2.5 nM ($pK_B=8.6\pm0.10$), with a slope of 1.05±0.14 (Figure 4b). Thus, **R**-PIA-stimulated high-affinity GTPase activity was potently inhibited by DPCPX in a competitive manner.

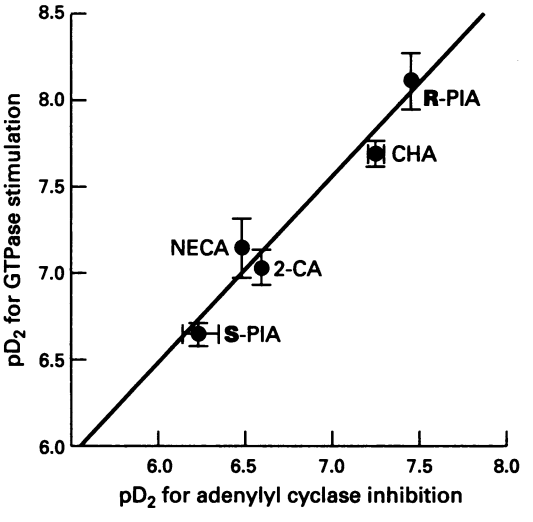

Figure 3 Relationship between the pD_2 values of adenosine receptor agonists for stimulation of high-affinity GTPase activity obtained in the present study (Table 1) and their pD_2 values for inhibition of adenylyl cyclase activity in hippocampal membranes reported by Zgombick *et al.* (1989). The correlation coefficient (*r*) of the fit of the regression line to the data was 0.98 ($P<0.01$), with a slope of 1.09. Values are mean±s.e.mean of 6–8 experiments in the present study and of 3–11 experiments in the paper by Zgombick *et al.* (1989).

Additivity between R-PIA- and 5-HT-stimulated high-affinity GTPase activities

Since A_1 adenosine and 5-HT_{1A} receptors have been shown to be coupled to the same effector systems by IAP-sensitive G-proteins in the rat hippocampus (Zgombick *et al.*, 1989), we then determined whether or not the increase in high-affinity GTPase activity following full activation of both receptors is additive (Figure 5). High-affinity GTPase activity was stimulated by 5-HT in a concentration-dependent manner. In the presence of maximally effective concentrations of **R**-PIA (1 μM, see Figure 2), under which conditions the activity was already stimulated by 14.3±1.0% ($n=8$), 5-HT was still able to increase further the enzyme activity to the same extent as in the absence of **R**-PIA. The addition of 1 μM **R**-PIA resulted in a parallel shift upward of the concentration-response curve of 5-HT, obtained in the absence of **R**-PIA, indicating that the responses elicited by these two agonists were completely additive.

Discussion

In the present investigation, the authors have provided a method for detecting the adenosine receptor-mediated high-affinity GTPase activity in the native rat hippocampal membranes and pharmacologically characterized the receptor subtype involved. The results presented here clearly demonstrate that the receptor subtype responsible for the response belongs to A_1 adenosine receptors. The comparison of EC_{50} values for **R**- and S-PIA indicates a 30 fold stereoselectivity of the response. The rank order of agonist potency, namely: **R**-PIA > CHA > NECA ≥ 2-chloroadenosine > S-PIA > CGS 21680, 2-phenylaminoadenosine, is consistent with the pharmacological profile of A_1 adenosine receptors (Collis & Hourani, 1993). Additionally, the selective and potent A_1 adenosine receptor antagonist, DPCPX, antagonized the effect of **R**-PIA in a competitive manner with a potent K_B of 2.5 nM.

The EC_{50} values for adenosine receptor agonists obtained in this study are around 10 fold greater than the respective K_i values reported previously using binding studies (Bruns *et al.*, 1986). This is probably because of the existence of the two or more interconvertible affinity states of agonist binding sites reciprocally modulated by divalent cations and guanine nucleotides (Goodman *et al.*, 1982; Yeung & Green, 1983; Lohse *et al.*, 1984; Ramkumar & Stiles, 1988; Stiles, 1988), as reported for many other transmitter receptors coupled with G-proteins. Guanine nucleotides including GTP and GTP analogues convert the high affinity state to the low affinity state, which can explain the lower affinities of agonists seen in the functional biochemical studies assessing adenylyl cyclase activity or GTP hydrolyzing activity such as the present investigation, in which GTP or its analogues were essentially necessary to be included for the purpose of measurement itself.

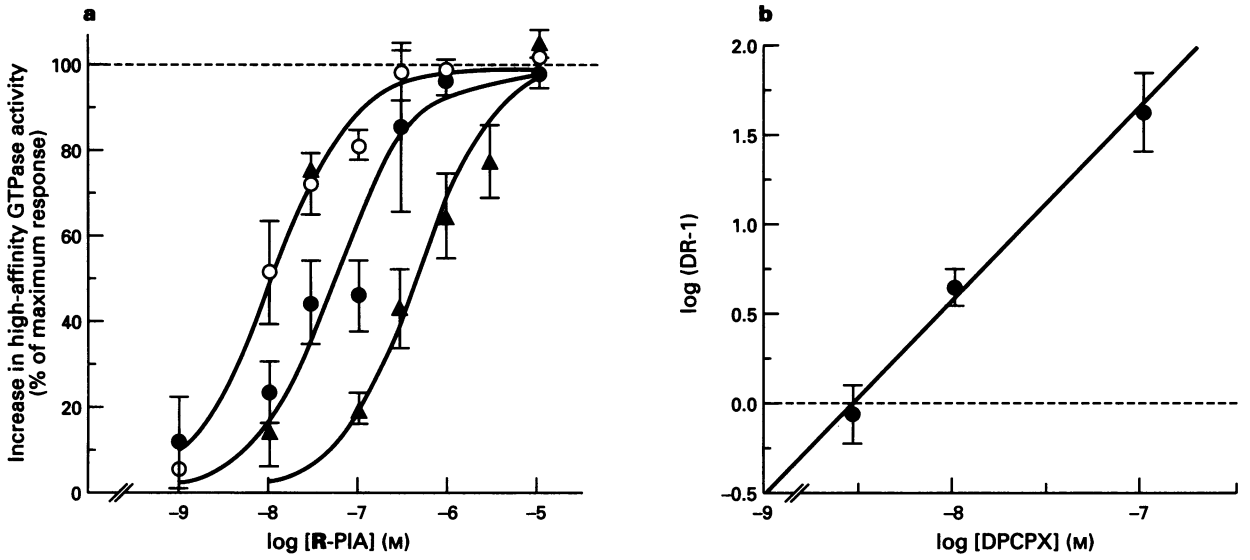

Figure 4 Antagonism of R-PIA-stimulated high-affinity GTPase activity by DPCPX. (a) Concentration-response curves for the stimulatory effect of R-PIA on the high-affinity GTPase activity were drawn in the absence (○) and presence of 10 nM (●) and 100 nM (▲) DPCPX. The data using 3 nM DPCPX were omitted from the figure for the sake of clarity. The high-affinity GTPase activity was calculated by subtracting the nonspecific, low-affinity GTP hydrolyzing activity defined as $^{32}P_i$ released from 0.3 μM [γ-^{32}]-GTP in the presence of 100 μM unlabelled GTP from total activity. The $\%E_{max}$ values of the respective concentration-response curves were normalized to 100 and the stimulatory effects of different concentrations of R-PIA were expressed as percent. Values are mean ± s.e.mean of four experiments, each performed in duplicate. Mean EC_{50} values for R-PIA were 10.0 nM ($pD_2 = 8.00 \pm 0.22$, $n=4$) in the absence of DPCPX and 15.3 nM ($pD_2 = 7.82 \pm 0.25$, $n=3$), 56.0 nM ($pD_2 = 7.25 \pm 0.23$, $n=4$), and 459 nM ($pD_2 = 6.34 \pm 0.14$, $n=4$) in the presence of 3, 10, and 100 nM DPCPX, respectively. Basal high-affinity GTPase activities were 890 ± 80 pmol mg^{-1} protein 15 min^{-1} in the absence of DPCPX, 851 ± 81 and 822 ± 89 pmol mg^{-1} protein 15 min^{-1} in the presence of 10 and 100 nM DPCPX, respectively ($n=4$). (b) Schild regression analysis of the data. The dose ratio (DR) refers to the ratio between the EC_{50} values of R-PIA in the presence and absence of a given concentration of DPCPX. Values are mean ± s.e.mean of 3–4 experiments, each performed in duplicate.

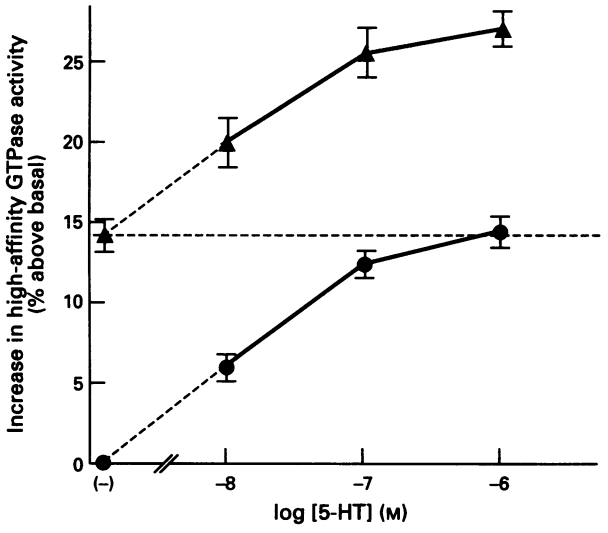

Figure 5 Additivity between stimulatory effects of R-PIA and 5-HT on high-affinity GTPase activity in rat hippocampal membranes. Stimulatory effects of different concentrations of 5-HT on the high-affinity GTPase activity were determined in the absence (●) and presence (▲) of 1 μM R-PIA. The high-affinity GTPase activity was calculated by subtracting the nonspecific, low-affinity GTP hydrolyzing activity defined as $^{32}P_i$ released from 0.3 μM [γ-^{32}P]-GTP in the presence of 100 μM unlabelled GTP from total activity. Stimulation is expressed as percentage increase above basal high-affinity GTPase activity in the absence of agonist (500 ± 23 pmol mg^{-1} protein 15 min^{-1}, $n=8$). Values are mean ± s.e.mean of 8 experiments, each performed in duplicate.

Although our assay system includes adenylylimidodiphosphate which is known to have a relatively high affinity for the A_1 adenosine receptors (Williams & Braunwalder, 1986), the possibility that the measured EC_{50} values are modified by this compound as compared with the true respective EC_{50} values has been ruled out. Thus, omission of adenylylimidodiphosphate from the assay mixture did not influence the EC_{50} values for R-PIA-stimulated high-affinity GTPase activity (pD_2, 8.30 ± 0.08 in the absence of adenylylimidodiphosphate; 8.16 ± 0.11 in the presence of 0.5 mM adenylylimidodiphosphate, $n=6$; $P > 0.05$, Student's paired two-tailed t test).

All functional studies on the coupling between adenosine receptors and G-proteins by means of measuring functional activity of G-proteins using brain membranes reported thus far indicate that the receptor subtype involved in the response is an A_1 adenosine receptor (Hausleithner *et al.*, 1985; Wojcik *et al.*, 1985; Lorenzen *et al.*, 1993). Our results obtained in the present study have added the new findings that functional coupling between A_1 adenosine receptors and G-proteins can be detected by utilizing the high-affinity GTPase activity in rat hippocampal membranes. In spite of the existence of the A_2 adenosine receptors in rat hippocampus, which are well known to stimulate adenylyl cyclase activity probably through G_s proteins (Fredholm *et al.*, 1983; Dunwiddie & Fredholm, 1985; Fredholm *et al.*, 1986), we could not detect the G_s-mediated activity stimulated via A_2 adenosine receptors. Although the reason for the failure to detect the involvement of A_2 adenosine receptors in the present study is uncertain, it can probably be accounted for by the notion that our assay systems seem to favour the G_i-mediated GTP hydrolysis associated with adenylyl cyclase inhibition more than the GTPase activity derived from an activation of G_s proteins, which are supposed to couple the A_2 adenosine receptors to adenylyl cyclase. Indeed, all of the receptors mediating agonist-induced high-affinity GTPase activity in our experiments have been shown to be pharmacologically defined as receptors coupled to adenylyl cyclase inhibition via IAP-sensitive G-proteins (dopamine D_2, pirenzepine-insensitive muscarinic, and $GABA_B$ receptors in striatum, Odagaki & Fuxe, 1995a; $5\text{-}HT_{1A}$ receptors, Odagaki & Fuxe, 1995c,d, and A_1 adenosine receptors (present study) in hippocampus). This fact that IAP-sensitive G-proteins are

favoured in our assay system may be accounted for by some factors, e.g., the experimental conditions used in the assay such as an inclusion of NaCl in an assay mixture and the fact that in the brain IAP-sensitive G-proteins ($G_{i/o}$) are far more abundant than G_s (Hepler & Gilman, 1992).

It is not certain from which subclass(es) of G-proteins the A_1 adenosine receptor-mediated high-affinity GTPase activity derives. To date, cDNAs encoding at least 21 different G-protein α subunits have been cloned (Hepler & Gilman, 1992). Of these, the G_i subfamily is most likely responsible for the coupling of the A_1 adenosine receptors to intracellular effectors, since the A_1 adenosine receptor-mediated responses have been shown to be sensitive to IAP treatment (Dolphin & Prestwich, 1985; Zgombick *et al.*, 1989). When A_1 adenosine receptors were purified from bovine cerebral cortex by affinity chromatography, IAP-sensitive G-proteins were co-purified, which were identified as G_{i1}, G_{i2}, and G_O by immunoblotting (Munshi *et al.*, 1991). Additionally, it has been demonstrated that purified A_1 adenosine receptors are able to interact functionally with IAP-sensitive platelet G-proteins (Munshi & Linden, 1990) and with $G_{i/O}$ purified from bovine brain (Freissmuth *et al.*, 1991; Munshi *et al.*, 1991) in reconstitutional experiments. With regard to the selectivity of the coupling between A_1 adenosine receptors and subclasses of the IAP-sensitive G-proteins, Munshi *et al.* (1991) reported that A_1 adenosine receptors could interact with G_{i1}, G_{i2}, and G_O rather indiscriminately, though with a slight preferential coupling to G_{i2} compared with the other two G-proteins. Taken together with the highly significant correlation observed between the high-affinity GTPase activity and adenylyl cyclase inhibition mediated by IAP-sensitive G-proteins (Zgombick *et al.*, 1989), it can be assumed with certainty that high-affinity GTPase activity determined in the present study derives from IAP-sensitive G-proteins (G_i subfamily) which couple A_1 adenosine receptors negatively to adenylyl cyclase.

One of the advantageous points of the experimental systems where the functional receptor-G-protein coupling can be investigated using native membranes is that they may reflect a more realistic feature of the protein-protein interaction occurring in a natural milieu than reconstitution experiments suggesting solely a functional potentiality in an artificial environment. Following pharmacological characterization of the adenosine receptor-mediated high-affinity GTPase activity and concluding that this response reflects the functional coupling between A_1 adenosine receptors and $G_{i/O}$ proteins in the native rat hippocampal membranes, we examined the additivity between R-PIA- and 5-HT-stimulated activities. High-affinity GTPase activity stimulated by 5-HT in rat hippocampal membranes has been shown to result from the functional coupling between the 5-HT_{1A} receptors and probable IAP-sensitive G-proteins, namely $G_{i/O}$, associated with adenylyl cyclase inhibition, although the assay condition was somewhat different from that in the present study (Odagaki & Fuxe, 1995c,d). In a previous report (Zgombick *et al.*, 1989), it was shown that A_1 adenosine receptors and 5-HT_{1A} receptors in rat hippocampus were coupled to the same effector systems through IAP-sensitive G-proteins in biochemical as well as electrophysiological studies. By means of investigating whether or not the two receptor-mediated high-affinity GTPase activities are additive, it can be determined at which level the convergence of the signalling processes originally derived from the two distinct receptors takes place. Thus, the additive relationship following full activation of the two receptors means the coupling of both receptors independently to the distinct pools of G-proteins which share the common effectors whereas the non-additivity indicates that the two receptors share the same G-protein pool (see Milligan, 1988). Our experimental results presented in this paper clearly exclude the latter possibility and favour the former case. A similar situation has been reported as to the relationship between A_1 adenosine receptors and $GABA_B$ receptors in rat cerebellum, both of which are coupled to the common adenylyl cyclase catalytic units (Wojcik *et al.*, 1985). Despite the non-additivity between the two receptor-mediated inhibitory effects of adenylyl cyclase activity, there was a clear-cut additive relationship between the high-affinity GTPase activities mediated through A_1 adenosine and $GABA_B$ receptors. Although the independence of each receptor-mediated signalling is strictly conserved to the step of receptor-G-protein coupling, convergence may occur at the subsequent step, namely the interaction between G-proteins and effector molecules in some cases. In rat hippocampal membranes, there has also been a simple additivity among high-affinity GTPase responses stimulated by full activation of 5-HT_{1A}, muscarinic, and $GABA_B$ receptors (Odagaki & Fuxe, 1995b).

In summary, the measurement of high-affinity GTPase activity stimulated by adenosine receptor agonists provides a useful tool for investigating a functional coupling between adenosine receptors and G-proteins in the native rat hippocampal membranes. The pharmacological profile of this response clearly indicates the involvement of the A_1 adenosine receptor subtype. Along with several lines of evidence reported previously, it can be concluded that this method reflects A_1 adenosine receptor-mediated activation of the $G_{i/O}$ protein function associated with adenylyl cyclase inhibition. Additionally, the data regarding the additivity between high-affinity GTPase activities mediated via A_1 adenosine and 5-HT_{1A} receptors indicate that the two receptors are coupled independently to distinct pools of G-proteins, which may share the same adenylyl cyclase catalytic units with each other.

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