0026-895X/91/040517-07$03.00/0

MOLECULAR PHARMACOLOGY, 39:517-523

Mechanism of A_2 Adenosine Receptor Activation. I. Blockade of A_2 Adenosine Receptors by Photoaffinity Labeling

MARTIN J. LOHSE, KARL-NORBERT KLOTZ, and ULRICH SCHWABE

Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, D-6900 Heidelberg, (M.J.L., K.-N.K., U.S.), and Laboratorium für Molekulare Biologie, Universität München, Max Planck Institut für Biochemie, D-8033 Martinsried, (M.J.L.)

Received June 29, 1990; Accepted January 22, 1991

SUMMARY

It has previously been shown that covalent incorporation of the photoreactive adenosine derivative (*R*)-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine [(*R*)-AHPIA] into the A_1 adenosine receptor of intact fat cells leads to a persistent activation of this receptor, resulting in a reduction of cellular cAMP levels [*Mol. Pharmacol.* **30**:403–409 (1986)]. In contrast, covalent incorporation of (*R*)-AHPIA into human platelet membranes, which contain only stimulatory A_2 adenosine receptors, reduces adenylate cyclase stimulation via these receptors. This effect of (*R*)-AHPIA is specific for the A_2 receptor and can be prevented by the adenosine receptor antagonist theophylline. Binding studies indicate that up to 90% of A_2 receptors can be blocked by photoincorporation of (*R*)-AHPIA. However, the remaining 10–20% of A_2 receptors are sufficient to mediate an adenylate cyclase stimulation of up to 50% of the control value. Similarly, the activation via these 10–20% of receptors occurs with a half-life that is only 2 times longer than that in control membranes. This indicates the presence of a receptor reserve, with respect to both the extent and the rate of adenylate cyclase stimulation. These observations require a modification of the models of receptor-adenylate cyclase coupling, which is described in the accompanying paper [*Mol. Pharmacol.* **39**:524–530 (1991)].

Adenosine is a modulator of many physiological functions, including the nervous and cardiovascular systems, metabolism, and immune responses. Most of its effects appear to be mediated via membrane-bound receptors (for a review, see Refs. 1–3). On the basis of biochemical and pharmacological experiments, these receptors have been subdivided into the A_1 (or R_i) and the A_2 (or R_a) subtypes (4, 5). According to the classical concept, the A_1 receptor mediates an inhibition and the A_2 receptor a stimulation of adenylate cyclase. Pharmacologically, the A_2 receptor is characterized by a high affinity for 5′-substituted adenosine analogues, such as NECA, and the A_1 receptor by a high affinity for N^6-substituted adenosine analogues, such as (*R*)-PIA.

We have recently used an agonist photoaffinity label, (*R*)-AHPIA (6), for the investigation of the activation mechanism of the A_1 adenosine receptor (7). Covalent labeling of the receptor of intact fat cells resulted in its persistent activation, as judged from a persistent reduction of cAMP levels. These data were interpreted as providing evidence for the validity of the occupancy theory of receptor activation. The occupancy theory of receptor activation (8) predicts that a receptor is activated as long as it is occupied by an agonist, whereas the rate theory (9) assumes that receptor activation is proportional to the rate of receptor occupation.

Although (*R*)-AHPIA is about 60-fold A_1 selective, it is also an agonist at the A_2 receptor (6). Therefore, it might also be used as a tool to investigate the activation mechanism of the A_2 receptor. In the present study we report that, in contrast to the persistent activation of A_1 receptors, covalent labeling of the A_2 receptor with (*R*)-AHPIA results in a persistent blockade. This irreversible blockade can be used to investigate the coupling mechanism between the A_2 receptor and adenylate cyclase. To this end, we have applied the classical analyses of concentration-response curves following receptor inactivation that have led to the concepts of spare receptors or receptor reserve (10) and to models of receptor-effector coupling (11, 12).

Experimental Procedures

Materials

(*R*)-AHPIA was synthesized as described earlier (13). [^{32}P]Orthophosphate and [^{3}H]NECA were obtained from Amersham-Buchler (Braunschweig, West Germany) and New England Nuclear (Dreieich, West Germany), respectively. Sepharose CL6B and PD10 columns were purchased from Pharmacia (Freiburg, West Germany). All other materials were from sources described previously (14).

ABBREVIATIONS: NECA, 5′-*N*-ethylcarboxamidoadenosine; (*R*)-PIA, (*R*)-N^6-phenylisopropyladenosine; (*R*)-AHPIA, (*R*)-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; XAC, xanthine amine congener; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Methods

Membrane preparations. Outdated platelet-rich plasma (5–6 days after preparation) was obtained from a local blood bank, and membranes were prepared as described earlier (6). In several instances, experiments were also done with membranes from fresh platelets; the results were essentially the same, although adenylate cyclase activity was somewhat higher in membranes from fresh platelets. Membranes from isolated rat epididymal fat cells were prepared as described (7). The protein content was determined according to the method of Peterson (15).

Adenylate cyclase assay. The activity of adenylate cyclase of human platelet and rat fat cell membranes was determined as described earlier (6). To detect receptor-mediated stimulation, GTP was present at 1 μM, whereas 10 μM GTP and 10 μM forskolin were used to best detect receptor-mediated inhibition (6). Unless stated otherwise, the incubation temperature was 37°. [α-^{32}P]ATP was synthesized according to the method of Walseth and Johnson (16).

In some experiments, GTP (1 μM) was replaced by Gpp(NH)p (1 μM) to cause quasiirreversible activation of the enzyme (17). These experiments were done at 25° to slow down the rate of activation.

Photoaffinity labeling. Platelet membranes were incubated with different concentrations of (*R*)-AHPIA in 50 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$ (TM buffer), at 30°. The same concentrations of the nonphotolabile parent compound (*R*)-PIA were used for controls. After 5 min, the samples were put on ice and irradiated for 3 min with UV light, using a Mineralight TM 15 lamp at a distance of 15 cm. The membranes were then diluted 10-fold with the same buffer and centrifuged at 39,000 × *g* for 5 min. This washing step was repeated twice, and the final pellet was resuspended in 50 mM Tris·HCl, pH 7.4, containing 1 mM $MgCl_2$.

In some experiments, 8-azidoadenosine was used as the photolabile compound. The treatment of samples was exactly as described for (*R*)-AHPIA.

Rat fat cell membranes were labeled by the same procedure. In order to stabilize the adenylate cyclase activity (18) and to promote dissociation of (*R*)-AHPIA that was noncovalently bound to A_1 receptors (6), a different washing buffer was used, 50 mM Tris·HCl, pH 7.4, 10 μM forskolin, 100 μM GTP. The final pellet was resuspended in 50 mM Tris·HCl, pH 7.4, containing forskolin and GTP, to give final concentrations of 10 μM of both compounds in the adenylate cyclase assay.

Solubilized A_2 receptors, eluted as the peak of specific [^{3}H]NECA binding from the Sepharose column (see below), were incubated with 10 μM (*R*)-AHPIA [or (*R*)-PIA for controls] for 60 min at 0° and then UV irradiated, as described above. Separation of noncovalently bound (*R*)-AHPIA from the solubilized receptor was achieved by three dialysis steps against 50 volumes of elution buffer, followed by "desalting" over PD 10 columns.

Solubilization and gel filtration of A_2 adenosine receptors. A_2 adenosine receptors of human platelet membranes were solubilized as recently described (19). In brief, platelet membranes were centrifuged at 13,000 × *g* for 5 min, and the pellet was resuspended at a protein concentration of 10 mg/ml in TM buffer containing 1% CHAPS. After 30 min on ice, the solution was diluted with 4 volumes of TM buffer and then centrifuged at 100,000 × *g* for 30 min. The supernatant was dialyzed at 4° against four successive portions of TM buffer containing 0.02% CHAPS. In order to separate the A_2 receptors from non-receptor binding sites for [^{3}H]NECA (19), the supernatant was subsequently loaded onto a 60- × 1.6-cm Sepharose CL6B column, which was eluted with the dialysis buffer at 4°. Fractions of 2 ml were collected and tested for binding activity.

Binding assay. The binding of [^{3}H]NECA to the fractions was measured in a total volume of 300 μl, containing 10 nM radioligand and 200-μl aliquots of the eluate fractions. (*R*)-PIA (100 μM) was used to define nonspecific binding (19). The incubation lasted for 60 min at 0° and was terminated by filtration through Whatman GF/B glass fiber filters that had been presoaked in 0.3% polyethyleneimine, as described for solubilized A_1 receptors (20).

Data analysis. Concentration-response curves were fitted as described earlier (7), with the general equation:

$$E = E_0 + E_m \frac{[L]^n}{EC_{50}^n + [L]^n} \quad \text{(I)}$$

with E being effect, E_0 being basal effect, E_m being maximal effects of the individual curve, n being the slope factor, EC_{50} being the EC_{50} value, and L being the ligand concentration. For the experiment shown in Fig. 4, the data were fitted to a model of pharmacological agonism (21):

$$E = E_{max} \frac{\tau^n[L]^n}{(K_D + [L])^n + \tau^n[L]^n} \quad \text{(II)}$$

with E_{max} being the absolute maximal effect, and K_D being the dissociation constant of the ligand-receptor interaction; the "transducer ratio" τ denotes the ratio of total receptor concentration, R_T, to K_E, the concentration of receptor-ligand complexes eliciting a half-maximal effect ($0.5 \times E_m$): $\tau = R_T/K_E$. τ_1 and τ_2 denote the transducer ratios without and with receptor blockade, respectively.

This algorithm is based upon a two-step model of agonist-effect coupling (21), which includes (a) agonist-receptor interaction, governed by a dissociation constant K_D, and (b) a similar receptor-effect relationship, with the corresponding constant K_E. Because both R_T and K_E determine the efficiency of the receptor-effect relationship, these two terms are combined to give the transducer ratio τ.

The accumulation of cAMP in kinetic adenylate cyclase experiments was fitted to the integrated rate equation:

$$[\text{cAMP}] = \Delta V_{eq}\left(t - \frac{1}{k}(1 - e^{-k_{on}t})\right) + V_b \times t \quad \text{(III)}$$

where V_b denotes the basal enzyme activity, ΔV_{eq} the activity increase induced by NECA at equilibrium, and k_{on} the observed rate constant of activation. In addition, the measured values were numerically differentiated with a three-point formula, in order to plot ΔV as a function of time.

The parameters of these equations were estimated from the data by nonweighted nonlinear curve fitting.

Results

We have previously shown that covalent labeling of A_1 adenosine receptors of intact fat cells causes a persistent reduction of intracellular cAMP levels (7). Similar results can be obtained with fat cell membranes (Fig. 1); covalent labeling with 100 nM (*R*)-AHPIA, followed by extensive washing, reduced the activity by more than 30%, compared with controls. The effect of covalently bound (*R*)-AHPIA corresponded to about 80% of the maximal inhibition obtained with reversibly bound (*R*)-PIA. The maximal inhibition of adenylate cyclase activity via A_1 receptors [10 μM (*R*)-PIA] was not affected by the pretreatment with (*R*)-AHPIA. This suggests that the receptors that were not covalently labeled with (*R*)-AHPIA remained accessible for activation by (*R*)-PIA.

In contrast, pretreatment of platelet membranes with (*R*)-AHPIA (up to 100 μM) did not result in a measurable activation of A_2 adenosine receptors (Fig. 1). However, pretreatment with (*R*)-AHPIA reduced the stimulatory effect of the A_2 receptor agonist NECA; using 10 μM (*R*)-AHPIA for the covalent labeling, 10 μM NECA stimulated adenylate cyclase activity by only 70%, compared with 190% in (*R*)-PIA-pretreated control membranes. It appears that covalent labeling of the membranes with (*R*)-AHPIA resulted in an irreversible blockade of A_2 receptors, because (a) this blockade could not be washed out with even more extensive washing procedures and (b) the

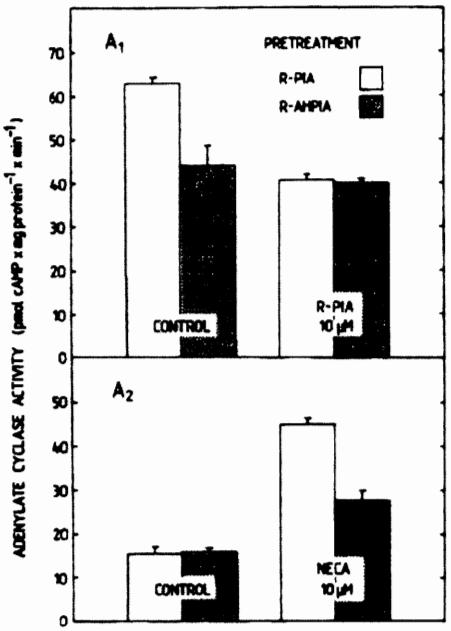

Fig. 1. Effects of covalent incorporation of (*R*)-AHPIA into membranes of rat fat cells (A_1 receptor) and human platelets (A_2 receptor) on adenylate cyclase activity. Fat cell membranes were pretreated with 100 nM (*R*)-AHPIA or (*R*)-PIA, and platelet membranes with 10 μM (*R*)-AHPIA or (*R*)-PIA (according to the approximately 100-fold lower affinity of the ligands for the A_2 receptor), as described in Experimental Procedures. After removal of noncovalently bound ligand, the adenylate cyclase activity was measured under basal conditions (control) or with maximal stimulation of A_1 receptors [10 μM (*R*)-PIA, in the presence of 10 μM GTP and 10 μM forskolin] or A_2 receptor [10 μM NECA, in the presence of 1 μM GTP].

TABLE 1

Effects of pretreatment with adenosine derivatives on adenylate cyclase activity of human platelet membranes

After preincubation with the adenosine derivatives, UV irradiation, and washing, as detailed in Experimental Procedures, the adenylate cyclase activity of the pretreated membranes was assayed under basal conditions and with maximal stimulation by NECA and prostaglandin E_1. Data are means ± standard errors of three independent experiments, with triplicate samples.

Pretreatment	Adenylate cyclase assay conditions		
	Basal	NECA (10 μM)	Prostaglandin E_1 (10 μM)
	pmol of cAMP/mg of protein × min		
(*R*)-PIA (10 μM)	15.5 ± 1.5	45.0 ± 1.5	170 ± 10
(*R*)-AHPIA (10 μM)	16.0 ± 0.5	27.5 ± 2.5	176 ± 4
8-Azidoadenosine (10 μM)	15.0 ± 1.6	42.4 ± 4.4	168 ± 12

products resulting from UV irradiation of (*R*)-AHPIA in TM buffer retained the agonist activity of (*R*)-AHPIA, although with a somewhat lower EC_{50} value [5.5 μM versus 2.3 μM (*R*)-AHPIA].

The specificity of this effect was confirmed along three lines. Firstly, pretreatment with (*R*)-AHPIA did not affect stimulation of adenylate cyclase by other hormones, such as prostaglandin E_1 (Table 1). Secondly, pretreatment of the membranes with 100 μM 8-azidoadenosine, a compound that proved to be devoid of A_2 receptor activity, did not affect the NECA-stimulated activity. And thirdly, the effect of (*R*)-AHPIA could be prevented by the adenosine receptor antagonist theophylline (Fig. 2); increasing concentrations of (*R*)-AHPIA during the

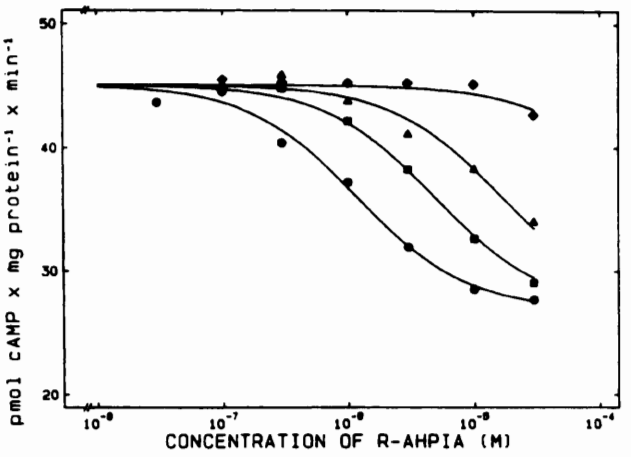

Fig. 2. Effects of theophylline on the inactivation of A_2 receptors by covalent incorporation of (*R*)-AHPIA. Human platelet membranes were pretreated with different concentrations of (*R*)-AHPIA, washed, and assayed for adenylate cyclase activity in the presence of 10 μM NECA, as described in the legend to Fig. 1. Theophylline concentrations during the pretreatment were 0 (●), 3 (■), 10 (▲), and 100 μM (◆). IC_{50} values of (*R*)-AHPIA pretreatment for the reduction of stimulated adenylate cyclase activity were 1.2, 4.8, 16.8, and >100 μM, respectively.

pretreatment produced a concentration-dependent reduction of NECA-stimulated activity, with an IC_{50} value of 1.2 μM. Theophylline shifted this curve in a parallel manner to higher concentrations, suggesting a competitive antagonism between theophylline and (*R*)-AHPIA. From the data, an apparent pA_2 value for theophylline of 5.77 can be calculated; however, for reasons outlined below, this is an overestimate of the affinity of theophylline.

We have interpreted the persistent activation of A_1 receptors by covalently bound (*R*)-AHPIA as evidence for the occupancy theory of receptor activation (7, 8). The blockade of A_2 receptors by covalent labeling with (*R*)-AHPIA would be compatible with the rate theory of receptor activation, as developed by Paton (9). However, kinetic experiments on the activation of adenylate cyclase by NECA argue against this (Fig. 3); activation by both submaximal (300 nM) and maximal (10 μM) concentrations of NECA results in an exponential activation time course, with a faster apparent activation by the higher concentration of NECA. This is compatible only with models based on the occupancy theory (22). Consequently, it must be assumed that, although the receptor is activated according to the occupancy theory, covalent occupancy of the A_2 receptor with (*R*)-AHPIA leads to inactivation of the receptor.

The extent of such an irreversible blockade is classically estimated by measuring the effects of an agonist with and without receptor blockade (10). Fig. 4 shows the stimulation of adenylate cyclase activity by NECA after blockade of the A_2 receptors by pretreatment with (*R*)-AHPIA, as well as that in (*R*)-PIA-pretreated control membranes. Pretreatment with (*R*)-AHPIA markedly reduced the efficacy and the potency of NECA; the maximal stimulation decreased from 160% to 90%, and the EC_{50} value increased from 280 nM to 900 nM. Simultaneous fitting of the curves to Eq. II estimates a K_D value for NECA (under the conditions of the adenylate cyclase assay) of 2.3 μM and a maximal stimulation (in pretreated membranes) of almost 200%. The transducer ratio, r, is 5.8 in (*R*)-PIA-pretreated and 0.98 in (*R*)-AHPIA-pretreated membranes. Be-

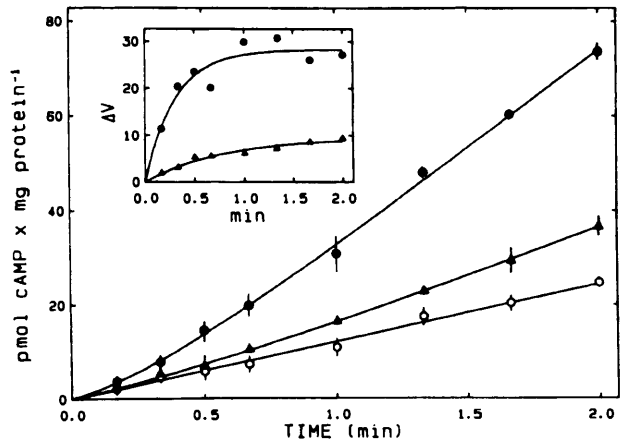

Fig. 3. Kinetics of the stimulation of adenylate cyclase in human platelet membranes by NECA. The assay was started by the addition of membranes and was conducted for the indicated times at 37°, under basal conditions (○) and in the presence of 300 nM (▲) or 10 μM (●) NECA. The data (pmol of cAMP produced/mg of protein) were fitted by the integrated exponential Eq. III. *Inset*, NECA-stimulated activity, ΔV, in pmol of cAMP/mg of protein × min (numerically differentiated data, fitted by a simple exponential curve using the parameters obtained from the integrated curve). The estimated parameters are: maximal stimulation, ΔV_{eq}, by 300 nM NECA and 10 μM NECA, 8.9 and 28.2 pmol of cAMP/mg of protein × min, respectively; and rate constants, k_{on}, 1.1 (300 nM NECA) and 3.1 min^{-1} (10 μM NECA).

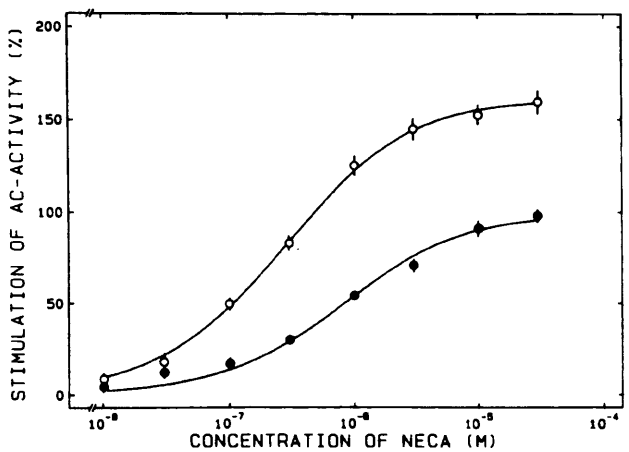

Fig. 4. Concentration-dependent stimulation of adenylate cyclase by NECA, in human platelets after photoincorporation using 10 μM (*R*)-AHPIA (●) and in control membranes pretreated with 10 μM (*R*)-PIA (○). After the pretreatment, the adenylate cyclase (AC) activity was measured at 37° in the presence of different concentrations of NECA. The GTP concentration was 1 μM. The concentration-response curves were fitted with Eq. II, giving the following parameter estimates: maximal stimulation, E_{max}, 200%, affinity of NECA, K_D, 2.3 μM; slope factor, n, 0.8; transducer ratios, τ_1 (control membranes), 5.8, and τ_2 [(*R*)-AHPIA-pretreated membranes], 1.8.

cause τ is proportional to the number of receptors ($\tau = R_T/K_E$), this indicates that the proportion of covalently blocked receptors is $(\tau_1 - \tau_2)/\tau_1 = 83\%$. This is a surprisingly high yield, compared with photoincorporation of (*R*)-AHPIA into A_1 receptors of about 40% (6).

Therefore, we attempted to measure the blockade of A_2 receptors with radioligand binding techniques. Due to their high nonspecific binding, neither [^{3}H]NECA (90% nonspecific binding) (23) nor [^{3}H]XAC (80–90% nonspecific binding) (24) gave conclusive results in binding assays with platelet membranes. In order to overcome the high nonspecific binding, we have recently described the separation of non-receptor [^{3}H]NECA binding sites from A_2 adenosine receptors by gel filtration (19). This technique allows binding assays with [^{3}H]NECA to the solubilized A_2 receptor, which elutes with the void volume. Fig. 5 shows the elution profile of (*R*)-AHPIA- and (*R*)-PIA-pretreated and subsequently solubilized membranes, as determined by [^{3}H]NECA binding. A peak of specific [^{3}H]NECA binding, defined by the presence of 100 μM (*R*)-PIA, eluted with the void volume of solubilized control membranes, followed by a larger peak of nonspecific [^{3}H]NECA binding. Pretreatment with (*R*)-AHPIA drastically reduced the amount of specific [^{3}H]NECA binding; the total specific binding (*shaded areas* in Fig. 5) was reduced by 80%. This value is similar to the estimate of irreversibly blocked A_2 receptors from adenylate cyclase experiments (Fig. 4). Thus, it must be concluded that covalent labeling of A_2 receptors with (*R*)-AHPIA results in a blockade of a large proportion (80–85%) of receptors.

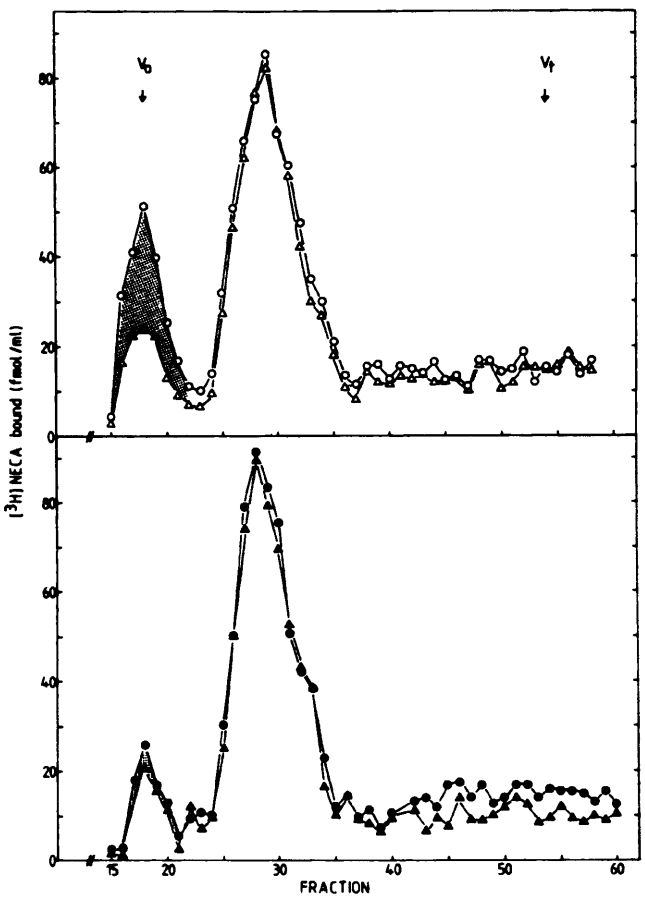

Fig. 5. Gel filtration of solubilized platelet membranes after pretreatment with (*R*)-PIA (*top*) and (*R*)-AHPIA (*bottom*). After photoincorporation of (*R*)-AHPIA (10 μM) or control treatment with (*R*)-PIA (10 μM), membranes were solubilized with 1% CHAPS, and the extract was applied to a Sepharose CL6B column. The eluted fractions were tested for [^{3}H]NECA binding. ○, Total binding; △, nonspecific binding [plus 100 μM (*R*)-PIA]. Specific binding (*shaded areas*) was 219 ± 15 fmol/mg of protein after (*R*)-PIA pretreatment and 43 ± 7 fmol/mg of protein after (*R*)-AHPIA pretreatment (means ± standard errors of three independent experiments).

Similar results were obtained when A_2 receptors were first solubilized and subjected to gel filtration and the pooled peak containing the receptors was then incubated with (*R*)-AHPIA (10 μM, 0°, 30 min) and UV irradiated. After removal of noncovalently bound ligand by dialysis and desalting, the (*R*)-PIA- and the vehicle- (1% dimethyl sulfoxide in TM buffer) pretreated membranes bound 31 and 37 fmol of [^{3}H]NECA/mg of protein, respectively. The (*R*)-AHPIA-pretreated samples bound only 2 fmol/mg of protein. This corresponds to a photoincorporation of (*R*)-AHPIA of more than 90%.

Irreversible blockade of receptors has been used as a tool for the investigation of receptor-effector coupling by many authors. In particular, this method enables a differentiation between catalytic and noncatalytic activation of adenylate cyclase by receptors (12). Such an investigation is facilitated if the activation of the enzyme by the receptor is essentially irreversible. An activation that appears to be permanent within the time scale of the present experiments can be obtained by substituting for GTP in the adenylate cyclase assays with the hydrolysis-resistant analogue Gpp(NH)p (17) and by lowering the incubation temperature from 37° to 25°; Fig. 6 shows that, in the presence of GTP, the addition of the adenosine receptor antagonist XAC (10 μM) causes reversion of the NECA stimulation. In the presence of Gpp(NH)p, the adenylate cyclase remains in the NECA-stimulated state after addition of XAC.

These conditions were used to determine the characteristics of adenylate cyclase stimulation by NECA in (*R*)-AHPIA-pretreated versus (*R*)-PIA-pretreated membranes (Fig. 7). In (*R*)-PIA-pretreated control membranes, 10 μM NECA stimulated adenylate cyclase with an apparent rate constant $k = 0.19$ min^{-1}, to a maximal extent $\Delta V_{eq} = 5.3$ pmol of cAMP/mg of protein × min. In (*R*)-AHPIA-pretreated membranes, the stimulation occurred more slowly ($k = 0.11$ min^{-1}) and to a smaller extent ($\Delta V_{eq} = 2.7$ pmol of cAMP/mg of protein × min). Thus, the receptors remaining after blockade with (*R*)-AHPIA are not capable of activating all adenylate cyclase molecules. In addition, the reduction of the receptor number by about 80% reduces the rate of adenylate cyclase activation by only 40%. Thus, the reduction of the receptor number resulted in a proportional reduction of neither ΔV_{eq} nor k_{on}, i.e., both parameters are not linearly related to receptor number.

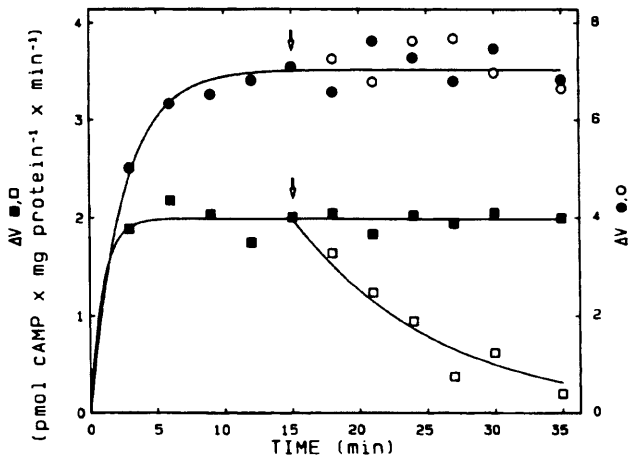

Fig. 6. Irreversible stimulation by NECA of human platelet adenylate cyclase in the presence of Gpp(NH)p (*circles*) and reversible stimulation in the presence of GTP (*squares*). Basal and NECA (10 μM)-stimulated adenylate cyclase activity were each assayed at 25°, in the presence of either 1 μM Gpp(NH)p or 1 μM GTP. Shown is the NECA-stimulated activity increase, ΔV (as in the *inset* of Fig. 3). After 15 min, A_2 receptors were blocked by addition of 100 μM XAC (*arrow*) (*open symbols*). Buffer containing the solvent (1% dimethyl sulfoxide) was added at the same time to control samples (*filled symbols*).

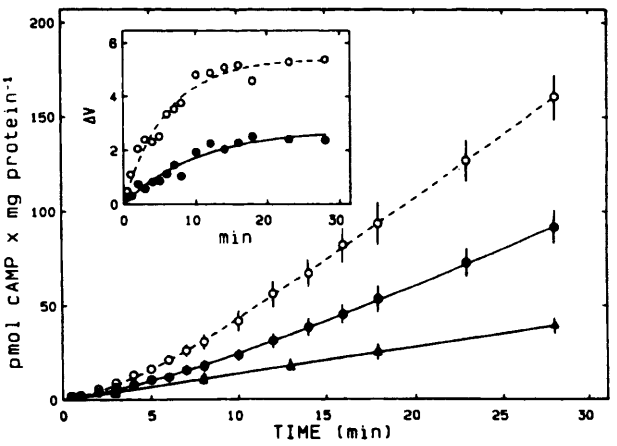

Fig. 7. Kinetics of the irreversible stimulation of adenylate cyclase by 10 μM NECA in human platelets after photoincorporation using 10 μM (*R*)-AHPIA (*filled symbols*) and in control membranes pretreated with 10 μM (*R*)-PIA (*open symbols*). After the pretreatment, the adenylate cyclase activity was measured at 25°, using 1 μM Gpp(NH)p under basal conditions (*triangles*) or in the presence of 10 μM NECA (*circles*). The data (pmol of cAMP produced/mg of protein) were fitted by the integrated exponential Eq. III. *Inset*, NECA-stimulated activity, ΔV, as in Fig. 3 (pmol of cAMP/mg of protein × min). Curve fitting gives the following parameter estimates: maximal stimulation, ΔV_{eq}, after control and (*R*)-AHPIA pretreatment, 5.3 and 2.7 pmol of cAMP/mg of protein × min, respectively; and rate constants, k_{on}, 0.19 min^{-1} (control) and 0.11 min^{-1} [(*R*)-AHPIA pretreatment].

Discussion

The photolabile adenosine analogue (*R*)-AHPIA is a full agonist at A_1 and a partial agonist at A_2 adenosine receptors (6). Covalent incorporation of (*R*)-AHPIA into A_1 receptors leads to a persistent activation of the receptors both in intact fat cells (7) and in fat cell membranes (Fig. 1). In contrast, covalent labeling of A_2 receptors with (*R*)-AHPIA does not activate the receptors but results in a persistent blockade. The available data clearly indicate that this blockade can only be due to covalent occupation of the binding site by (*R*)-AHPIA.

Although the covalent occupation of A_2 receptors with the agonist (*R*)-AHPIA leads to receptor blockade, kinetic experiments on the activation of adenylate cyclase via A_2 receptors show that the receptor is not activated according to the rate theory (9); the time course of activation can be adequately fitted by a monoexponential curve, i.e., it appears to be first order. Such first-order kinetics are predicted only by models based on the occupancy theory (8, 11, 12, 22, 25). Therefore, models based on the validity of the occupancy theory can be used to analyze the effects of covalent labeling of A_2 receptors with (*R*)-AHPIA.

First, the activation of adenylate cyclase by NECA after blockade with (*R*)-AHPIA allows an estimate of the transducer ratio τ (21) of A_2 receptors in platelet membranes. This ratio is 5.8 in control membranes, i.e., there are almost 6 times as many A_2 receptors as needed for a half-maximal activation of adenylate cyclase. Consequently, the EC_{50} value of NECA in

stimulating adenylate cyclase is about 10 times lower than its K_D value. Because the transducer ratio is not only tissue specific but also agonist specific, this difference will be less for partial agonists. For example, whereas NECA stimulated the adenylate cyclase in control pretreated membranes by 170% (Fig. 4), (*R*)-PIA maximally stimulated the enzyme in the same membranes by 90% (data not shown). The maximal effect α and the EC_{50} value of a given compound can be derived from Eq. II (21):

$$\alpha = E_m\tau/(1 + \tau)$$

$$EC_{50} = K_D/(1 + \tau)$$

for the case of slope factors $n = 1$. It follows from $E_m = 200\%$ (Fig. 4) and $\alpha = 90\%$ that $\tau = 0.8$ for (*R*)-PIA, compared with $\tau = 5.8$ for NECA. Consequently, the EC_{50} value of adenylate cyclase activation overestimates the affinity of NECA for the A_2 receptor by a factor of 6.8, but that of (*R*)-PIA by a factor of only 1.8. Together with the demonstration of spare A_1 receptors in fat cells (7), this shows that the simple determination of agonist affinities and selectivities from adenylate cyclase data gives data that differ from the true affinities, as seen in radioligand binding experiments.

Second, a number of mechanistic models based on the occupancy theory have been developed for receptor-effector coupling in general and for receptor-adenylate cyclase coupling in particular. Of these, only a few can account for the relatively simple kinetic and steady state properties of adenylate cyclase activation (11, 25). The characteristic features observed in the present study are (a) first-order kinetics of adenylate cyclase activation by different concentrations of NECA, (b) a nonlinear relationship between extent of stimulation and receptor number, (c) a nonlinear relationship between the rate of stimulation and receptor number, and (d), as a consequence of these nonlinear relationships (which correspond to the classical concept of a "receptor reserve"), a difference between the EC_{50} value of NECA and the K_D value of about 10-fold.

The simple first-order kinetics can be accomodated by two general models, described by Levitzki and colleagues (11, 12, 25) as "precoupled" and "collision-coupling" models. The precoupled model assumes a preexisting receptor-effector complex, which upon agonist binding becomes activated. The collision-coupling model assumes that the agonist-occupied receptor catalytically activates many effectors. Although the adenosine receptor of turkey erythrocytes has been reported to be precoupled to adenylate cyclase (12), the differences between K_D and EC_{50} (feature d) clearly dismiss the precoupled model for the human platelet A_2 receptor. In addition, the precoupled model predicts that a reduction of receptor number must result in a proportional decrease of irreversibly activated effectors; this contrasts with feature b. Although the collision-coupling model can accomodate differences between EC_{50} and K_D (26), it would predict that reduction of receptor number results in a proportional reduction in activation rate but no alteration of the maximal effect if activation is irreversible (11). This is because it is assumed that the receptors can catalytically activate all adenylate cyclase molecules. In our experiments, reduction of the receptor number reduced both the maximal effect and the rate of activation (features b and c). However, it is likely that receptors are capable of activating only a limited number of adenylate cyclase molecules, which are in their neighborhood.

In the accompanying paper, we propose a restricted collision-coupling model, which combines features of the collision-coupling model proposed by Levitzki and co-workers (11, 25) and the random hit matrix model developed by Bergman and Hechter (27). This model appears to account for all the observations (a to d) of the present study.

The reasons why covalent incorporation of (*R*)-AHPIA activates the A_1 and blocks the A_2 receptor, although both receptors are activated according the occupancy theory, are not clear. A possible explanation is that the covalent bond is formed at the 2-position of (*R*)-AHPIA. It has been proposed that the 2-position of adenosine is important for A_2 receptor binding, and a 2-phenylamino substituent confers some A_2 selectivity to adenosine (28). The idea that the 2-position interacts with the A_2 receptor is supported by the high percentage of photoincorporation of (*R*)-AHPIA, via its 2-azido substituent, into A_2 receptor compared with A_1 receptors. The A_2 receptor may consequently be more sensitive to the formation of a covalent bond at this position than the A_1 receptor.

Although the photoincorporation of (*R*)-AHPIA into A_2 receptors was very high, we have been unable to obtain a specifically labeled band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a variety of membranes, using ^{125}I-AHPIA. Membranes used for this purpose were prepared from human platelets, N1E 115 neuroblastoma cells, and rat lung and liver. This failure is probably due to the low affinity of (*R*)-AHPIA for A_2 receptors, which is in the micromolar range.

In summary, we have shown that (*R*)-AHPIA can be very effectively photoincorporated into A_2 adenosine receptors. The resulting blockade of the receptor provides a tool for the study of the activation mechanism of the receptor. This reveals the presence of spare A_2 receptors in human platelet membranes and calls for a modification of the classical models of A_2 receptor-adenylate cyclase coupling. Such a model will be proposed in the accompanying paper.

Acknowledgments

The excellent technical assistance of Ms. Heidrun Vogt and Mr. Hans-Peter Gensheimer is gratefully acknowledged. [^{3}H]XAC was kindly provided by Dr. Ken Jacobson, National Institutes of Health.

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Send reprint requests to: M. J. Lohse, Laboratorium für Molekulare Biologie, Universität München, Max Planck Institute für Biochemie, D-8033 Martinsried, FRG.