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Agonist Photoaffinity Labeling of A_1 Adenosine Receptors: Persistent Activation Reveals Spare Receptors

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SUMMARY

This study describes experiments investigating the mechanism of activation of A_1 adenosine receptors. Isolated rat fat cells were used as a cellular model. The A_1 receptors of these cells were covalently labeled with the agonist photoaffinity label *R*-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine. The covalent incorporation of the label into the binding subunit of the receptor was verified by demonstration of specific labeling of a peptide with M_r=35,000 by the radioiodinated label. Such covalent labeling followed by removal of label not covalently bound led to a concentration-dependent reduction of cellular cAMP levels. This persistent effect of covalent labeling occurred with an IC_{50} value of 9 nM compared to an IC_{50} value of 0.9 nM for the direct reduction of cAMP levels by the label. The affinity of the label was determined in binding experiments. The K_i value of 19 nM was about 20 times higher than the corresponding IC_{50} value of cAMP reduction. Finally, the comparison between covalent binding and its effects suggests that covalently labeled receptors were fully activated. The data are interpreted as evidence for a receptor activation according to the occupancy theory. The analysis of the various concentration-response curves reveals the presence of spare receptors, which can be demonstrated by the method of agonist photoaffinity labeling.

A great variety of hormones, transmitters, and drugs exert their effects via membrane-bound receptors. Two basic theories have been developed to explain how these receptors are activated by their respective agonists (1): the occupancy theory (2) predicts that a receptor is activated as long as it is occupied by the agonist, whereas the rate theory (3) predicts that receptor activation is proportional to the rate of agonist-receptor interaction. Both theories have subsequently been modified to accommodate various experimental findings (1). We have studied this question of receptor activation for the A_1 adenosine receptor with the technique of labeling with an agonist photoaffinity label (4).

Photoaffinity ligands can be covalently attached to their binding sites by UV irradiation (5). They have been widely used in the biochemical characterization of membrane-bound receptors. However, only little use has been made of them to investigate receptor function. Galardy *et al.* (6) have defined the criteria which should be applied to demonstrate functionally the covalent labeling of a receptor: 1) a persistent effect; 2) the protection against this effect by a specific competitor; and 3) the correlation between the persistent effect and the incorporation of the label.

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The receptors investigated in the present study were A_1 adenosine receptors. Membrane-bound receptors for adenosine occur in many tissues (see Ref. 7 for a review). They have been subdivided into two types, of which the A_1 (or R_i) receptor mediates an inhibition of adenylate cyclase activity (8, 9). These A_1 receptors have been characterized by radioligand binding studies in brain, fat cells, and myocardium (10, 11). Recently, we have reported the development of an agonist photoaffinity label for the A_1 receptor, R-APHIA. Photoaffinity labeling with the radioiodinated compound identified the binding subunit of the receptor as a peptide of M_r=35,000 (4). In order to investigate the functional effects of such covalent binding we used isolated epididymal rat fat cells. Activation of A_1 adenosine receptors in these cells in the presence of β-adrenoceptor agonists causes a 90% reduction of intracellular cAMP levels and, thus, gives a response which can easily be measured.

We report here that covalent labeling of A_1 receptors gives a persistent response which meets all three criteria defined for the functional demonstration of covalent receptor labeling.

Experimental Procedures

Materials

Carrier-free $Na^{125}I$ was purchased from Amersham-Buchler, Braunschweig, West Germany. ^{125}I-CYP and [^{3}H]PIA were from New Eng-

ABBREVIATIONS: *R*-AHPIA, *R*-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine; *R*–/*S*-PIA, *R*–/*S*-N^6-phenylisopropyladenosine; [^{3}H]PIA, [^{3}H]N^6-phenylisopropyladenosine; ^{125}I-HPIA, *R*-N^6-^{125}I-*p*-hydroxyphenylisopropyladenosine; ^{125}I-AHPIA, *R*-2-azido-N^6-^{125}I-*p*-hydroxyphenylisopropyladenosine; ^{125}I-CYP, (–)^{125}I-cyanopindolol; ScAMPTME, 2′-*O*-monosuccinyladenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester; KRBG buffer, Krebs-Ringer bicarbonate-glucose buffer.

land Nuclear, Dreieich, West Germany. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from British Drug House, Poole, United Kingdom. ScAMPTME and Triton X-100 were from Sigma, Taufkirchen, West Germany, and bovine serum albumin, fraction V, was from Serva, Heidelberg, West Germany. All other materials were from sources described previously (12).

Methods

Preparation of ligands. The synthesis of *R*-AHPIA has been described in detail elsewhere (13). The iodination of the compound to give ^{125}I-AHPIA was done as previously described (4). Likewise, ^{125}I-HPIA and ^{125}I-ScAMPTME were iodinated by the chloramine T method (14) and the reaction products were separated by gel filtration on Sephadex G-25 SF.

Preparation of isolated fat cells and fat cell membranes. Rat epididymal fat cells were isolated as described by Rodbell (15). A Krebs-Ringer-bicarbonate buffer containing 10 mM glucose and 1% bovine serum albumin, pH 7.4 at 37°, was used (KRBG buffer). Membranes from these cells were prepared by the method of McKeel and Jarett (16) with omission of the sucrose density gradient centrifugation step. The protein content of the membrane preparation was measured according to the method of Lowry *et al.* (17).

Photoincorporation. The isolated cells (3000 in 1 ml) were incubated with different concentrations (0.1–100 nM) of *R*-AHPIA for 10 min at 37° in a shaking water bath. Adenosine deaminase (2 units/ml) was present to remove endogenous adenosine (18) to below the detection limit (5 nM) of a determination method adapted from Ontyd and Schrader (19). The samples were then irradiated for 3 min at 25° in a water bath with a Mineralight TM 15 UV lamp at a distance of 15 cm. The reduced temperature was used to prevent cell damage by heating. The cells were then washed three times in 10 times the original volume of KRBG buffer (37°) with intervening centrifugations at 200×*g* for 90 sec. As an alternative to the washing procedure, 1 mM theophylline (final concentration) was added in some experiments after the UV irradiation in order to occupy all A_1 receptors not covalently labeled.

Photoincorporation of ^{125}I-AHPIA was achieved with intact cells after incubation of 600,000 cells in 2 ml with approximately 1 nM ^{125}I-AHPIA. After the irradiation the cells were lysed by addition of 8 ml of 0.05% Triton X-100 in water and sonication for 1 min in an ultrasound bath. Crude membranes were obtained by centrifugation of 6,000×*g* for 60 min, followed by resuspension in 50 mM Tris-HCl, pH 7.4, and the same centrifugation step.

Photoincorporation of ^{125}I-AHPIA into fat cell membranes was achieved as described for brain membranes (4).

Measurement of cAMP production. After the pretreatment described above, the cells were incubated at 37° with 1 μM isoprenaline in the presence of adenosine deaminase (2 units/ml). The incubation volume was 1 ml containing approximately 3000 cells. Other compounds were added as indicated. After 10 min the incubation was stopped by addition of 200 μl of 3.9 M $HClO_4$. cAMP was determined by radioimmunoassay using ^{125}I-ScAMPTME as labeled tracer (20).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples prepared after photoincorporation of ^{125}I-AHPIA were electrophoresed under reducing conditions as described (4). Gels were stained with Coomassie blue and then dried for autoradiography.

Binding assays. Binding of [^{3}H]PIA to intact cells was done with some modifications of the method described (21) using 200,000 cells in 250 μl of KRBG buffer. One μM nitrobenzylthioinosine was used to inhibit possible uptake of the radioligand by the nucleoside carrier. Nonspecific binding was defined by the presence of 1 mM theophylline. The incubation was carried out for 20 min at 37°, a time sufficient to reach equilibrium even at low ligand concentrations, and was terminated by rapid filtration of the samples through glass fiber filters. Binding of ^{125}I-AHPIA to isolated cells was done in the same way, but with a radioligand concentration of 200 pM.

Radioligand binding was also measured with the fat cell membranes prepared after the pretreatments described above. Saturation experiments were carried out using 10 μg of membrane protein in a total volume of 250 μl. Binding of ^{125}I-HPIA (0.1–10 nM) to A_1 receptors was done as described by Ukena *et al.* (22), but in the presence of 2 mM $MgCl_2$. Binding of ^{125}I-CYP (2–200 pM) to β-adrenergic receptors was measured according to the method of Engel *et al.* (23).

Data analysis. Radioligand binding data were analyzed with the aid of the nonlinear curve-fitting program SCTFIT (24), providing estimates of affinity (K_D) and binding capacity (B_{max}) for various models.

As described by Black *et al.* (25), concentration-response curves were first fitted with the general equation:

$$E = E_0 \frac{[L]^n}{EC_{50}^n + [L]^n} \quad (1)$$

with E, effect; E_0, maximal effect of the individual curve; n, slope factor; and L, ligand concentration. For the experiment shown in Fig. 2, the data were subsequently fitted to a model of pharmacological agonism (25):

$$E = E_m \frac{\tau^n[L]^n}{(K_D + [L])^n + \tau^n[L]^n} \quad (2)$$

with E_m, theoretical maximal effect (i.e., effect if all receptors are fully activated); K_D, dissociation constant of the ligand-receptor interaction; τ, "transducer ratio," denoting the ratio of total receptor concentration R_T to the concentration of receptor-ligand complexes eliciting a half-maximal effect (0.5 × E_m); and K_E: $\tau = R_T/K_E$. This equation differs from the one used by Furchgott (26) to describe the concept of spare receptors only by the introduction of the slope factor, n, which can account for receptor-occupation/effect relationships which are not rectangular hyperbolic (25). The parameters of these equations were estimated from the data by nonweighted nonlinear curve fitting.

Both for the program SCTFIT and for the fitting to Eq. 2, improvement of a fit by assuming another model was tested with an F test. The more complex model was accepted if it gave a significantly ($p<0.01$) better fit.

Results

We have previously shown that *R*-AHPIA can be used to covalently label the A_1 adenosine receptor of membranes; the ligand is specifically incorporated into a single peptide of M_r=35,000 (4). Fig. 1 shows that the same specific labeling of a peptide with M_r=35,000 can be obtained in intact cells. However, high concentrations of the ligand have to be used in order to achieve a relatively modest incorporation: the samples from intact cells (Fig. 1, *lanes 1* and *2*) and membranes (Fig. 1, *lanes 3* and *4*) contained roughly similar amounts of membrane protein (approximately 100 μg), but the cells had been incubated with 10 times higher concentrations of ^{125}I-AHPIA (1 nM versus 0.1 nM). In order to elucidate the reason for this difference, we determined the yield of the photoincorporation. This was done by measuring the specific binding of ^{125}I-AHPIA to isolated cells which could not be dissociated after UV irradiation (Table 1). Under control conditions the plateau at the end of the dissociation reaction amounted to about 5% of the initial value, whereas after UV irradiation, 24% of the specific binding remained. The difference of 19% indicates the photoincorporation of the label, compared to 30–40% in membranes (4). Therefore, the differences between the autoradiograms after labeling of membranes might be due to a lower affinity for *R*-AHPIA in intact cells but not to a particularly low yield of the photoincorporation. These experiments demonstrate that covalent labeling of the receptor can also be produced in intact cells.

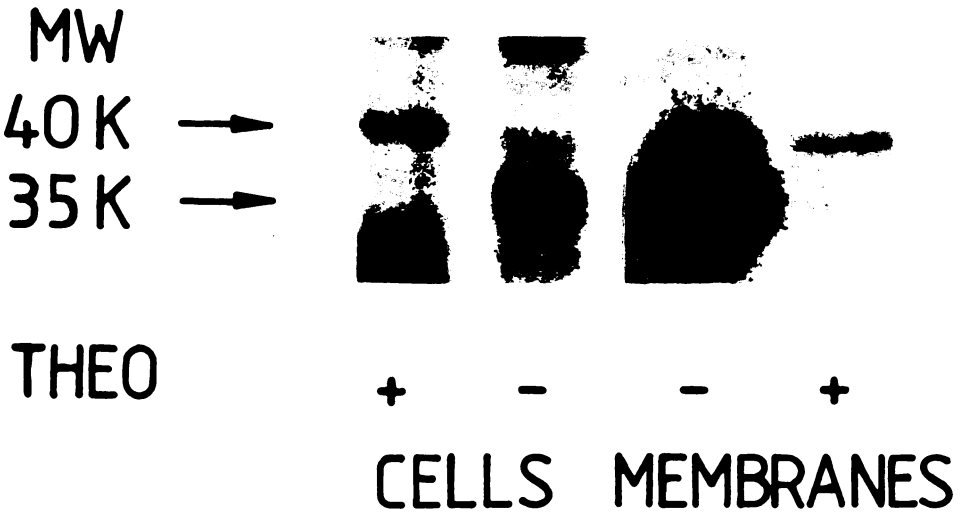

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis after photoaffinity labeling of A_1 adenosine receptors of isolated fat cells or fat cell membranes with ^{125}I-AHPIA. Shown is an autoradiogram of the gel after 14 (membranes) or 28 (cells) days of development using an SR 342 intensifying screen (DuPont, Frankfurt, West Germany). Incubation of membranes and cells with ^{125}I-AHPIA and UV irradiation, the subsequent preparation of the samples and electrophoresis were done as described under Experimental Procedures. *THEO*, the presence of 1 mM theophylline during the incubation.

TABLE 1

Photoincorporation of ^{125}I-AHPIA into isolated fat cells

Isolated cells were incubated with 200 pM ^{125}I-AHPIA as described under Experimental Procedures and then either UV irradiated (UV light) or kept in the dark (Control). Dissociation of the radioligand not covalently bound was initiated by the addition of theophylline (1 mM final concentration), and the residual binding was measured after 15 min, a time sufficient to reach a plateau under both conditions. Data are means ± standard errors of three experiments with triplicate samples.

Dissociation condition	^{125}I-AHPIA bound	
	Control	+UV light
	fmol/10^6 cells	
Before	1.62 ± 0.23	1.69 ± 0.17
After	0.19 ± 0.02	0.54 ± 0.10[a]

[a] $p < 0.01$ vs. control (paired t test).

TABLE 2

Effects of pretreatment with *R*-AHPIA or *R*-PIA on cAMP levels of isolated fat cells

Isolated cells were incubated with 10 nM *R*-AHPIA or 10 nM *R*-PIA and either UV irradiated (+UV light) as described under Exprimental Procedures or kept in the dark (Control). The ligand not covalently bound was then removed by extensive washing and the cAMP production was measured as outlined under Experimental Procedures. Data are means ± standard errors of three experiments with triplicate samples.

Pretreatment	Control	+UV light
	fmol cAMP/cell	
R-PIA (10 nM)	5.3 ± 0.1	5.7 ± 0.8
R-AHPIA (10 nM)	5.3 ± 0.1	3.3 ± 0.7[a]
+ theophylline (1 mM)	5.9 ± 0.7	5.8 ± 0.6

[a] $p < 0.01$ vs. control (paired t test).

Incubation of isolated fat cells with A_1 receptor agonists is known to cause a concentration-dependent reduction of cAMP levels after stimulation with isoprenaline. The reduction had the same concentration-response curve in the absence and the presence of phosphodiesterase inhibitors such as rolipram (100 μM) and Ro 20-1724 (500 μM), with IC_{50} values of about 1 nM (data not shown). This suggests that the reduction was mainly due to the inhibition of adenylate cyclase.

When cells were preincubated with 10 nM *R*-AHPIA and UV irradiated, and the *R*-AHPIA not covalently bound was removed by extensive washing, then the cAMP levels were still lower than those in controls that had been preincubated with 10 nM *R*-PIA (Table 2). Such a reduction was not seen without UV irradiation. The addition of a saturating concentration of theophylline (1 mM) together with *R*-AHPIA prevented the reduction, suggesting that it was mediated via A_1 adenosine receptors. Instead of removing the *R*-AHPIA not covalently bound by the washing procedure, in some experiments a high concentration (1 mM) of the adenosine receptor antagonist theophylline was added after the UV irradiation to displace the *R*-AHPIA not covalently bound. This resulted in a reduction of cAMP levels by the covalent labeling similar to that seen with the washing procedure (5.8±0.7 fmol/cell, control, versus 3.8±0.2 fmol/cell, +UV light). These results indicate that A_1 adenosine receptors can be persistently activated by covalent labeling with *R*-AHPIA and suggest that the receptor is activated according to the occupancy theory.

The persistent effect of *R*-AHPIA was concentration dependent (Fig. 2). Pretreatment with the parent compound *R*-PIA in concentrations up to 100 nM followed by addition of 1 mM theophylline was without any effect, indicating that theophylline effectively displaced the agonist from the receptor. However, compared with the direct effect of *R*-AHPIA, the

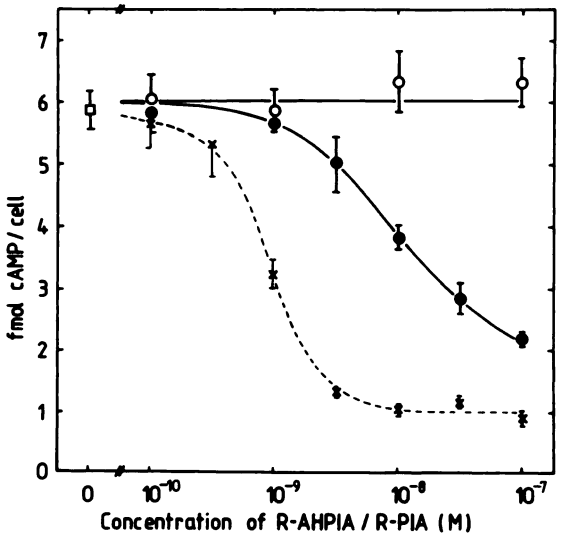

Fig. 2. Effects of pretreatment with *R*-AHPIA or *R*-PIA and UV irradiation on cAMP levels of isolated rat fat cells. After incubation of the cells with various concentrations of *R*-AHPIA (●) or *R*-PIA (○) and UV irradiation as described under Experimental Procedures, 1 mM theophylline was added to occupy all receptors not covalently labeled. The production of cAMP was measured as described under Experimental Procedures. x---x, effect of *R*-AHPIA added directly to untreated cells together with isoprenaline. Data are means ± standard errors of three experiments with triplicate samples.

curve of the persistent effect was shifted to 10 times higher concentrations and at the same time flattened. A fit with the general equation (1) gives IC_{50} values of 0.9 nM for the direct and 9 nM for the persistent effect of *R*-AHPIA, and slope factors of 1.9 and 1.2, respectively. The shift of the IC_{50} value is best explained by assuming the existence of spare receptors (26). When less receptors can be occupied by an agonist, the classical theory of spare receptors (26, 27) predicts first a shift of the concentration-response curve to higher concentrations and only later a reduction of the maximal response. It must be assumed that the covalent labeling procedure results in such a reduced proportion of receptors that can be activated, since the yield of covalent labeling with *R*-AHPIA is only 30–40% in membranes (4) and about 20% in intact cells. However, according to these theories the slope factors of all curves should be 1 (i.e., a midpoint slope of 0.576 in a normalized lg concentration curve). This is evidently not the case in our experiment.

Black *et al.* (25) have proposed an interpretation of such observations by assuming that the relationship between receptor occupation and final effect need not be rectangular hyperbolic. Using their equation (2), our data of Fig. 2 can be fitted very well if the slope factor is allowed to differ from 1 (Fig. 3). The fit gives a ratio τ_1 (direct):τ_2 (persistent) of 13.4:1.8. Taking this as an indicator of the activated receptors, this means that the covalent labeling results in an activation of 15% of the receptors. Assuming that the covalently activated receptors are fully active (i.e., K_E identical for directly and covalently activated receptors), then these 15% would represent the yield of the covalent reaction. This agrees relatively well with the directly determined value of 19% (see Table 1). The theoretical maximal effect, E_m, is estimated at about 85% and the slope factor close to 2; the binding is estimated to occur with a K_D value of about 11 nM.

These estimations would indicate that less than 10% of the receptors have to be occupied by the agonist in order to produce a half-maximal effect, and that the binding responsible for the effect occurs with a low affinity. Therefore, we determined the affinity of the A_1 receptors of intact cells for the radioligand [^{3}H]PIA. Saturation experiments (Fig. 4) demonstrated binding which was clearly of low affinity with a K_D of 18.8 nM. No high affinity component was detectable with nonlinear curve fitting. Neither could we detect a high affinity component with the radioligand ^{125}I-HPIA which, due to its higher specific activity, can be used at lower concentrations, but which has a 2 times higher nonspecific binding in intact cells. In order to verify that the binding sites labeled by [^{3}H]PIA represent the low affinity state of the A_1 receptor, competition for these binding sites by different agonists and antagonists was examined (Table 3). The K_i values calculated from these experiments are in good agreement with those reported for the low affinity state of the A_1 adenosine receptor in membranes (12, 22). For *R*-AHPIA we measured a K_i value of 18.7 nM which is in reasonable agreement with the K_D estimated from the concentration-response curves.

From these data the proportion of covalently occupied recep-

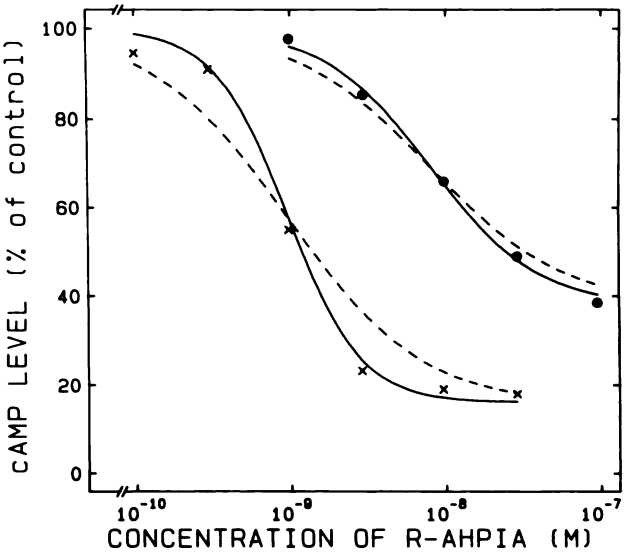

Fig. 3. Curve fitting to the data of Fig. 2. The data points of the direct (×) and persistent (●) effects of *R*-AHPIA were fitted simultaneously to Eq 2. E_m, K_D, and n were shared for both data sets, whereas τ_1 and τ_2 were estimated independently for the direct or persistent effect, respectively. The best fit (—) was obtained with the following parameters: E_m=85%, K_D=11 nM, n=1.9, τ_1=13.4, and τ_2=1.8. – – –, the best fit obtained with n=1, in which case Eq. 2 simplifies to the equation used by Furchgott (26); this results in a significantly worse fit.

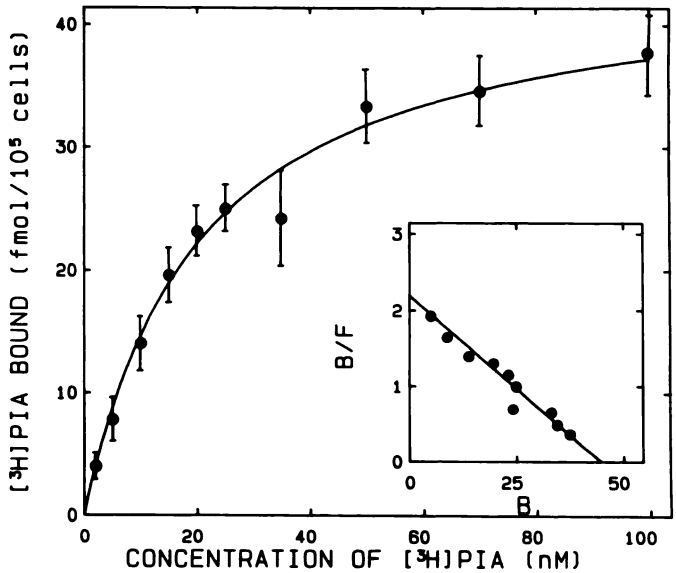

Fig. 4. Saturation of [^{3}H]PIA binding to isolated fat cells. The cells were incubated with 2–100 nM concentrations of the radioligand as described under Experimental Procedures. Nonspecific binding was linear and amounted to 60% of total binding at a radioligand concentration of 100 nM. Nonlinear curve fitting gave a B_{max} value of 45 fmol/10^5 cells and a K_D value of 18.8 nM. The *inset* shows the Scatchard plot of the data with bound (*B*) radioligand (fmol/10^5 cells) and free (*F*) radioligand (nM). Data represent means ± standard errors of four experiments with duplicate samples.

TABLE 3

Competition for [^{3}H]PIA binding to isolated fat cells

The competition curves were done with eight different concentrations of the competing compounds at a radioligand concentration of 5 nM. Data are the geometric means and 95% confidence limits calculated from three experiments.

Compound	K_i	(95% Confidence limits)
	nM	nM
R-PIA	14.7	(10.2–21.3)
R-AHPIA	18.7	(16.2–21.6)
NECA[a]	71.6	(65.8–78.0)
S-PIA	1,010	(660–1,820)
IBMX[a]	3,090	(2,800–3,400)
Theophylline	14,900	(9,000–24,700)

[a] NECA, 5′-*N*-ethylcarboxamidoadenosine; IBMX, 3-isobutyl-1-methylxanthine.

tors after pretreatment with 10 nM *R*-AHPIA can be estimated at 15% × 10/(18.7+10) = 5%. Given the high nonspecific binding and the low affinity of [^{3}H]PIA in the experiments with intact cells, such a small proportion can hardly be determined from binding experiments using intact cells. Indeed, the difference of the B_{max} values which we observed after pretreatment with 10 nM *R*-AHPIA compared with controls (10 nM *R*-PIA) was not significant due to a relatively high standard error (38±7 versus 41±6 fmol/10^5 cells). However, in membranes in the presence of mM concentrations of magnesium, most A_1 receptors are in the high affinity state and, at the same time, nonspecific binding is low (12, 22). Therefore, we measured binding to membranes from cells pretreated with either 10 nM *R*-AHPIA or 10 nM *R*-PIA.

Because of its high specific activity, ^{125}I-HPIA was used to quantitate the A_1 adenosine receptors; for control purposes we also measured binding of ^{125}I-CYP to β-adrenergic receptors (Table 4). Nonlinear curve fitting gave only one component of ^{125}I-HPIA binding, indicating that practically all A_1 receptors were in the high affinity state. The B_{max} value of ^{125}I-HPIA binding after covalent labeling with 10 nM *R*-AHPIA was reduced by a small but significant amount compared to controls. There was no change in the affinity for the radioligand. No alterations were seen in the binding of ^{125}I-CYP. The small decrease of the B_{max}value for ^{125}I-HPIA after labeling of the cells with 10 nM *R*-AHPIA is in agreement with the predictions of the model outlined above. It shows that occupation of less than 10% of the receptors with the covalent label results in an almost half-maximal effect. The same is predicted by the model for the direct activation by *R*-AHPIA. This would suggest that the persistently activated and the directly activated receptor have the same full activity.

However, the exact extent of covalent labeling is difficult to determine from these experiments both due to experimental errors and also because not all receptors found in the membrane fraction need to be at the cell surface. Therefore, we used another approach and tested the effect of *R*-PIA on the cAMP levels of fat cells after persistent activation with 10 nM *R*-AHPIA and washing. If covalent labeling with *R*-AHPIA led to full activation of the receptors, then, at a given effect of the covalent label, as many receptors should be occupied as would be occupied at the concentration of *R*-PIA (L_b), leading to the same effect (E_b). Then, the concentration-response curve of *R*-PIA after the covalent labeling would be equivalent to the segment of the concentration-response curve of *R*-PIA starting at the ligand concentration, L_b, and the effect, E_b. If the curve follows Eq. 1, then the occupancy theory predicts that this new curve has an EC_{50}' which differs from the original EC_{50} by a factor of: $[\sqrt[n]{(1 + E_b)/(1 - E_b)} - \sqrt[n]{E_b/(1-E_b)}]$ (1). An EC_{50}'—or, in our case, IC_{50}'—significantly above this value would indicate that more receptors were occupied by *R*-AHPIA than were predicted from the activation, and, hence, that the covalent labeling did not fully activate the receptors. Fig. 5 gives an IC_{50} value for the control cells of 1.24 nM (pA_2 8.90±0.06); after pretreatment with *R*-AHPIA, the IC_{50}' value is 1.43 nM (pA_2 8.85±0.03). With E_b = 54% and n = 1.6 (control curve), the expected pA_2' value would be 8.90±0.06. This is not significantly different from the measured value. Therefore, it must be assumed that the receptors covalently labeled with *R*-AHPIA were fully activated.

TABLE 4

Effects of pretreatment of fat cells with 10 nM *R*-AHPIA or 10 nM *R*-PIA on radioligand binding to the membrane fraction

Isolated fat cells were preincubated with the respective ligand in batches (~2 × 10^5 cells/ml) and UV irradiated, and membranes of the cells were prepared as described under Experimental Procedures. The B_{max} and K_D values of ^{125}I-HPIA binding to the A_1 adenosine receptor and of ^{125}I-CYP binding were estimated from nonlinear curve fitting of saturation experiments as described under Experimental Procedures. For both ligands the data were adequately fitted by assuming one class of homogeneous binding sites, indicating that practically all A_1 receptors were in the high affinity state. Data are means and standard errors or 95% confidence intervals of three experiments.

^{125}I-HPIA binding	B_{max}	K_D
	fmol/mg protein	*nM*
Pretreatment		
R-PIA	890 ± 40	1.37 (1.12–1.68)
R-AHPIA	833 ± 24[a]	1.31 (1.12–1.52)
^{125}I-CYP binding		
		pM
Pretreatment		
R-PIA	65 ± 3	13.4 (11.5–15.6)
R-AHPIA	64 ± 4	12.9 (11.8–14.1)

[a] $p < 0.01$ (paired t test) vs. *R*-PIA pretreatment.

Discussion

The present study investigates the functional effects of covalent labeling of A_1 adenosine receptors with an agonist in intact fat cells. Both in membranes and in intact cells ^{125}I-AHPIA covalently and specifically labeled a peptide with M_r=35,000, which has previously been identified in studies with membranes as the binding subunit of the A_1 adenosine receptor (4, 28, 29). Higher concentrations of the ligand were needed to demonstrate specific labeling in intact cells in spite of a com-

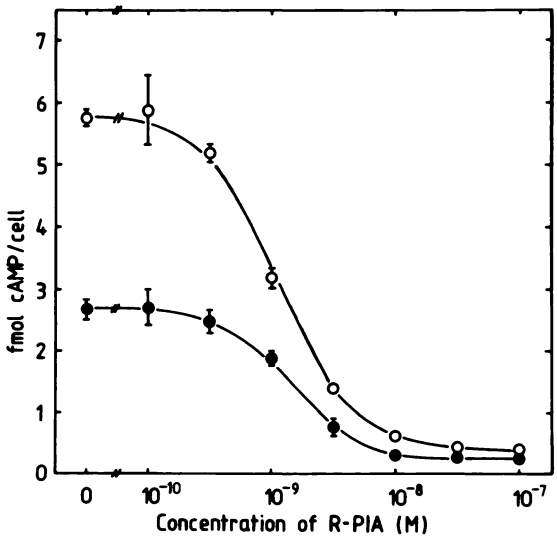

Fig. 5. Effects of *R*-PIA on cAMP levels in isolated fat cells pretreated with 10 nM *R*-AHPIA (●) or 10 nM *R*-PIA (○) and UV irradiation. Isolated fat cells were pretreated in batches (approximately 2×10^5 cells/ml) and, after the UV radiation, *R*-AHPIA or *R*-PIA was removed by extensive washing. The production of cAMP was then measured in the presence of different concentrations of *R*-PIA as described under Experimental Procedures. Curve fitting using Eq. 1 gives, for the control curve, an IC_{50} value of 1.25 nM and a slope factor of 1.6, and an IC_{50}' value of 1.43 after *R*-AHPIA pretreatment. It should be noted that the latter values are only approximations as, for n>1, the curve after pretreatment with *R*-AHPIA will not be symmetrical. Data are means ± standard errors of three experiments with triplicate samples. Standard errors are not shown if smaller than the corresponding *symbol*.

parable yield of the covalent reaction, suggesting already a lower affinity of the receptor compared to membranes.

The covalent labeling with the agonist was sufficient to produce a response of up to 75% of the maximal effect obtained by direct activation. The specificity of this response was shown by its suppression with theophylline. Thus, the first two criteria for the functional demonstration of covalent receptor labeling, as described by Galardy *et al.* (6; see introduction), are satisfied. These observations indicate the validity of the occupancy theory for this receptor, because no such response would be anticipated if the rate theory were true. It appears that the occupation of the receptor by the agonist is sufficient to activate the receptor and that *R*-AHPIA retains its agonistic activity after photoincorporation.

A persistent agonistic response has also been shown with photolabile analogues of the peptide hormone cholecystokinin (6), insulin (30), and adrenocorticotropin (31). These results suggest the general validity of the occupancy theory for the activation of receptors.

In addition, our experiments can be analyzed in a quantitative manner to meet criterion 3. This analysis yields several interesting points.

First, our data strongly suggest the presence of spare receptors. This is indicated in the first place by the different IC_{50} values of the direct and the persistent effect without a proportional decrease of the maximal effect. These curves could be fitted well with a model of pharmacological agonism. The τ value of about 13 for the control cells indicates that there are 13 times more receptors than needed to produce a half-maximal effect; this means that occupation of less than 10% of the receptors by an agonist is sufficient to produce a half-maximal effect. The predictions of this model were confirmed by the observation that pretreatment with 10 nM *R*-AHPIA and UV irradiation, which produces about 40% of the theoretical maximal effect, occupies less than 10% of the receptors.

Second, the model predicts that the binding of *R*-AHPIA to the receptors of intact cells occurs with a K_D value of 11 nM. This corresponds to the low affinity state observed in fat cell membranes (22). Binding studies using [^{3}H]PIA confirmed this prediction. In contrast to the nonspecific uptake of [^{3}H]PIA in hepatoma cells (32), competition for these binding sites clearly identified them as a low affinity state of the A_1 adenosine receptor. Neither with [^{3}H]PIA nor with ^{125}I-HPIA did we detect significant amounts of high affinity agonist binding. This suggests that the vast majority of the receptors of intact cells are in the low affinity state.

It has been suggested (33, 34) that the high affinity state of stimulatory adenylate cyclase-coupled receptors is responsible for the response. This does not appear to be the case in our experiments, where an inhibitory response was measured. Instead, the low IC_{50} value of the direct response to receptor agonists seems to be due to the presence of spare receptors. These spare receptors shift the concentration-response curve to the left and, thus, allow both a rapid response (as low affinity reflects fast kinetics) and a high sensitivity of the system. It may be speculated that the high affinity state is a transitory state occurring between receptor occupation by an agonist and signal transduction. In membranes washed free from GTP, a large proportion of the receptors stays in this high affinity state.

Third, fitting of the concentration-response curves of the direct effect gives slope factors above 1. This means that the response is very sensitive to small changes in the agonist concentration. The binding of agonists to receptors in membranes occurs with slope factors below 1 if both high and low affinity states are present, and with slope factors of about 1 if only the low affinity state is present (12, 22). Thus, the reason for a slope factor above 1 must lie in one of the steps subsequent to receptor occupation. The localization of this step, which causes the deviation from a rectangular hyperbolic concentration-response curve, remains to be identified.

Finally, the photoincorporation of *R*-AHPIA does not seem to affect those receptors which are not covalently labeled and activated. This follows from the concentration-response curves after photoincorporation of *R*-AHPIA and in control cells. Thus, upon UV irradiation, *R*-AHPIA can either be covalently linked to the binding site and then retain its fully agonistic activity, or it can leave the binding site free.

The concept of spare receptors was originally developed from experiments using irreversible inactivation of receptors and determining, then, the response to agonists. In this study we have shown that spare receptors can also be demonstrated by use of an irreversible agonist. This method allows the direct correlation between receptor occupation and response. At the same time these experiments provide direct evidence for the validity of the occupancy theory in this system.

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