Functional Characterization of A_1 Adenosine Receptors by Photoaffinity Labelling

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Summary

The ligand-binding subunit of the A_1 adenosine receptor has been identified in membranes with the photoaffinity label *R*-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine (*R*-AHPIA). Covalent labelling of the A_1 receptor can also be achieved in intact cells. The dissociation of the radioiodinated label (^{125}I-AHPIA) from isolated rat fat cells was incomplete after UV irradiation, leaving about 20% of irreversible specific binding. Such covalent labelling of the receptor led to a concentration-dependent reduction of cellular cyclic AMP levels. This persistent effect of covalent labelling occurred with an IC_{50} value of 9 n*M*, as compared to an IC_{50} value of 0.9 n*M* for the direct reduction of cyclic AMP levels by the ligand. The difference in the IC_{50} values can be explained by assuming spare receptors. This hypothesis was verified in binding studies using [^{3}H]PIA as a radioligand. *R*-AHPIA inhibited binding of [^{3}H]PIA to intact fat cells with a K_i value of about 20 n*M*, which is about 20 times higher than the corresponding IC_{50} value of cyclic AMP reduction. These data show that the A_1 receptor is activated according to the occupancy theory. The high sensitivity of the activation in intact cells is due to a large number of spare receptors.

Introduction

Various physiological effects of adenosine seem to be mediated by membrane-bound receptors. Biochemical and pharmacological studies have led to their subdivision into the A_1 (or R_i) and the A_2 (or R_a) subtype [4, 8]. The A_1 receptor is coupled in an inhibitory and the A_2 receptor in a stimulatory manner to adenylate cyclase.

The A_1 receptor has been extensively characterized in radioligand-binding studies using both agonist and antagonist radioligands [2, 6]. The aim of our recent studies was to improve this characterization by the development of a photoaffinity label for the A_1 receptor. *R*-AHPIA was synthesized as a covalent specific ligand for the A_1 receptor. Its structure is shown in Fig. 1. It differs from *R*-PIA in a *p*-hydroxy group on the phenyl ring which allows iodination, and, more importantly, an azido group in the 2 position of the purine. This azido group confers photoreactivity to the ligand. *R*-AHPIA can be covalently introduced into the ligand-binding subunit of the A_1 adenosine receptor of different membranes [3] and identifies it as a peptide with M_r = 35 000.

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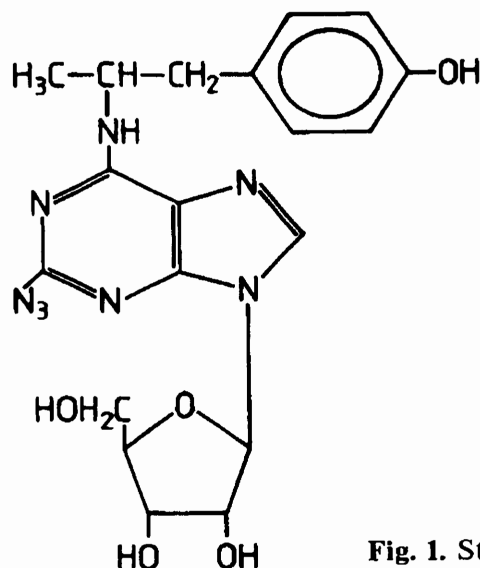

Fig. 1. Structure formula of *R*-AHPIA

Methods

Photoaffinity Labelling of A_1 Receptors in Intact Cells

Using the radioiodinated ^{125}I-AHPIA, a small amount of specific binding can be detected in intact isolated rat fat cells. As has been described for [^{3}H]PIA, this binding is accompanied by a relatively high nonspecific binding. The dissociation of the radioligand after addition of a saturating concentration of theophylline was almost complete within 20 min. However, if the cells were UV-irradiated before the addition of theophylline, then the dissociation was incomplete, leaving 15%–20% of irreversible specific binding. This indicates the covalent incorporation of 15%–20% of the reversibly bound photoaffinity label (Fig. 2). When the membranes of cells labelled by this procedure were subjected to SDS-PAGE specific labelling of a band corresponding to the molecular weight of 35 000 was detected. This band has previously been identified as the ligand-binding subunit of the A_1 adenosine receptor [3]. Compared with labelling of membranes the band was rather weak, suggesting a low affinity of the receptor in intact cells. Thus, it appears that photoaffinity labelling of the A_1 receptor with *R*-AHPIA is also possible in intact cells.

Persistent Activation of A_1 Receptors by Photoaffinity Labelling

We then examined the functional effects of the covalent binding of an agonist to the A_1 receptor. This was done by measuring cyclic AMP levels in cells pretreated with different concentrations of *R*-AHPIA and UV irradiation, followed by blockade of all receptors not covalently labelled with *R*-AHPIA by addition of theophylline (1 m*M*). Subsequently, the production of cyclic AMP was stimulated by the addition of 1 μM isoprenaline, the reaction stopped after 10 min, and the cyclic AMP levels determined by radioimmunoassay.

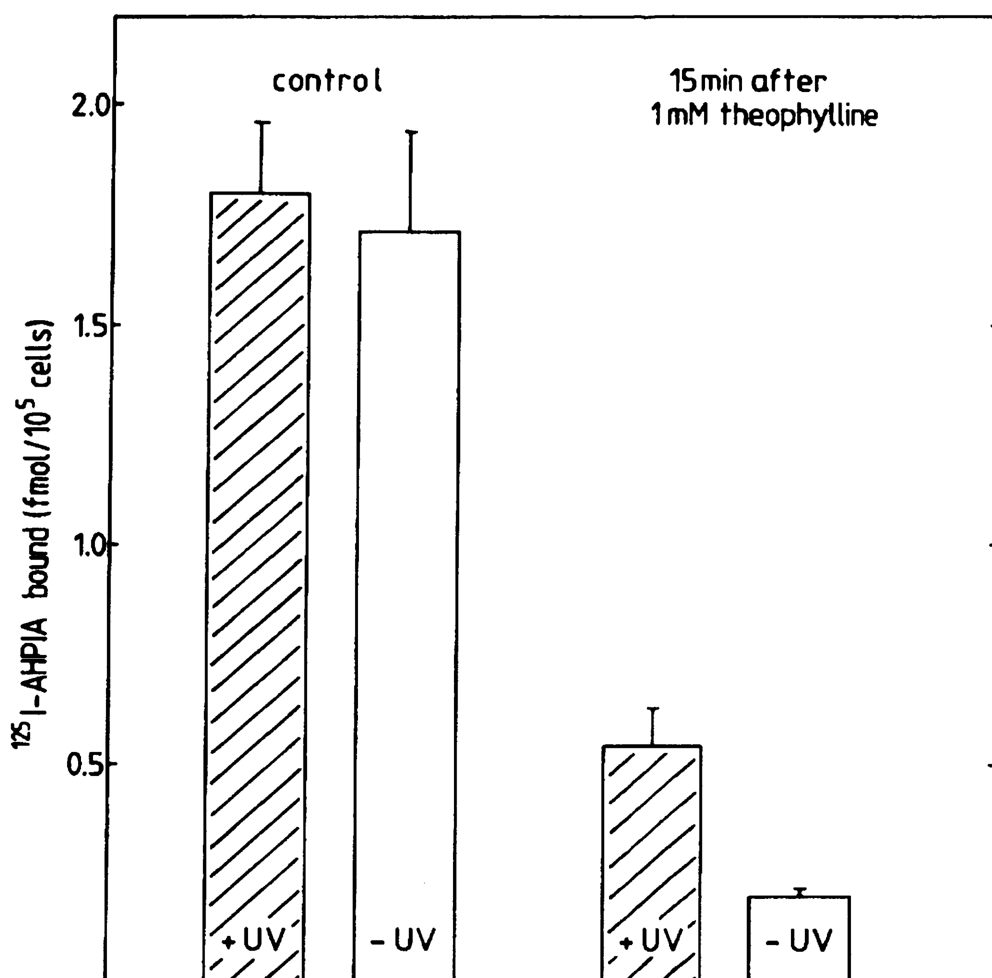

Fig. 2. Photoincorporation of ^{125}I-AHPIA into A_1 receptors of intact fat cells. After equilibration of ^{125}I-AHPIA binding (100 p*M*) at 37° C to 300 000 cells in 1 ml, cells were either UV-irradiated for 3 min at 20° C or kept in the dark. Dissociation of the radioligand was initiated by addition of theophylline (1 m*M*), and residual binding was measured after 20 min. Data shown are means and SEMs from three experiments

The top panel in Fig. 3 shows the direct, reversible effect of *R*-AHPIA, i.e., added together with isoprenaline. *R*-AHPIA lowered the cyclic AMP levels, with an IC_{50} value of 0.9 n*M* and a maximal reduction by about 80%. The bottom panel of Fig. 3 shows the effects of covalently bound *R*-AHPIA. Photoaffinity labelling with different concentrations of *R*-AHPIA led to a concentration-dependent reduction of cellular cyclic AMP levels. The IC_{50} value of this effect was about 9 n*M*, with a maximal reduction of cyclic AMP levels by about 65%. Thus, *R*-AHPIA led to a persistent inhibition of cyclic AMP production after photoaffinity labelling, but 10 times higher concentrations were needed to produce a half-maximal effect. The maximal effect however, was not markedly different. These results are somewhat unexpected if we assume a 17% yield of the covalent reaction as determined above (Fig. 2). If it is assumed that 17% of the label reversibly bound can be covalently incorporated, then we would expect a concentration-

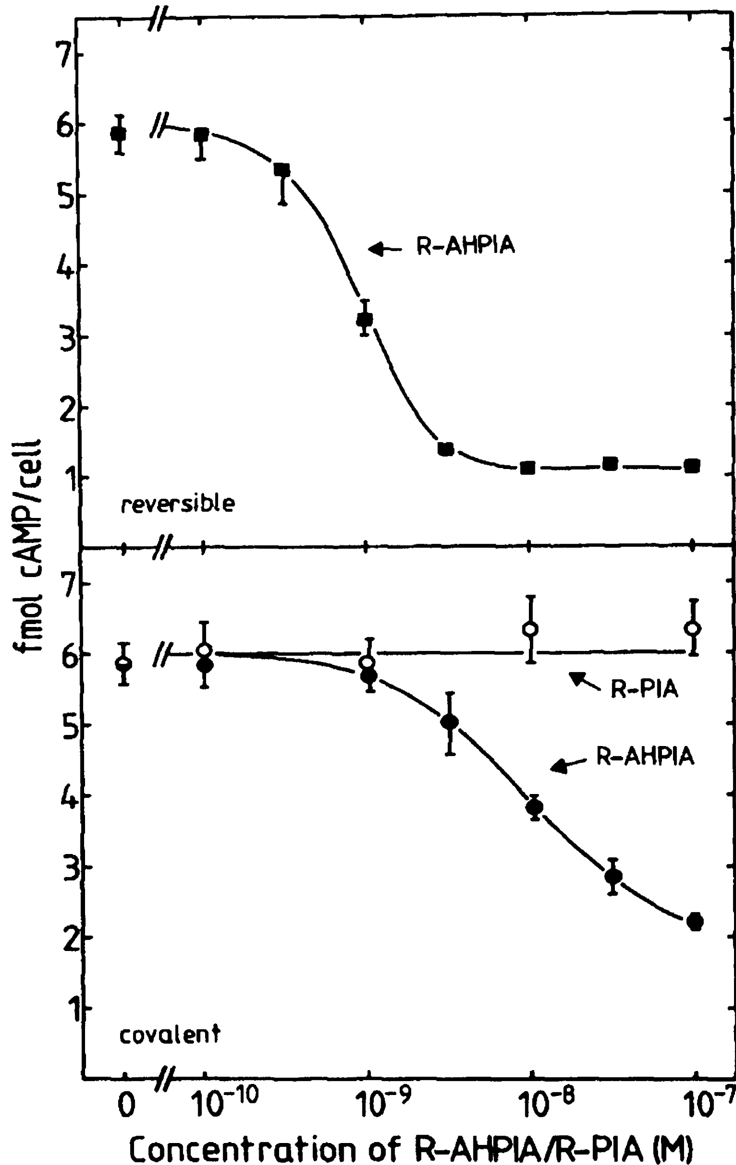

Fig. 3. Reduction of cyclic AMP levels in isolated rat fat cells by *R*-AHPIA. The *upper graph* shows the direct reduction caused by the presence of the indicated concentrations of *R*-AHPIA. The *lower graph* shows the reduction caused by pretreatment with the indicated concentrations of either *R*-AHPIA or *R*-PIA and UV irradiation, followed by blockade with 1 m*M* theophylline of all A_1 receptors not covalently labelled. In both experiments cyclic AMP levels were measured in the presence of 1 μM isoprenaline and incubation was at 37° C for 10 min

response curve for the persistent effect with the same IC_{50} value, but only 17% of the maximal effect compared to the reversible receptor activation.

Demonstration of Spare A_1 Receptors

The difficulties in the interpretation of the concentration dependence of the persistent effect can be overcome by assuming spare receptors. This assumption

would predict that the activation of only part of the receptors (which must be expected after the covalent labelling) led first to a shift of the concentration–response curve to higher concentrations and only then to a reduction of the maximal effect. If this hypothesis were correct there would be a dissociation between binding and response so that occupation of a minor proportion of receptors led to a significant effect. In order to study this question we performed binding experiments with intact cells, using [^{3}H]PIA as radioligand. The binding of [^{3}H]PIA to isolated fat cells was saturable with a K_D value of about 20 n*M*. *R*-AHPIA competed for these binding sites with a K_i value of 19 n*M*. This value is 20 times higher than the corresponding IC_{50} value for cyclic AMP reduction. The relationship between binding – measured as inhibition of [^{3}H]PIA binding – and response – measured as reduction of cyclic AMP – is shown in Fig. 4 both for the reversible, direct and for the covalent, persistent activation of the receptor. It can be seen from Fig. 4 that the reversible response is almost maximal at a concentration of 3 n*M* *R*-AHPIA; the same concentration of *R*-AHPIA leads to an occupation of about 20% of the receptors. Similarly, a half-maximal response is achieved with occupation of only 5% of the receptors. This does indeed indicate the presence of a large proportion of spare receptors. The same calculations can be done for the covalent, persistent activation of the receptor, even though the inhibition of binding cannot be reliably measured. However, if the covalent reaction is assumed to occur with a yield of 17% (see Fig. 2) a binding curve can be constructed by multiplying the values of the reversible binding by 0.17. A compari-

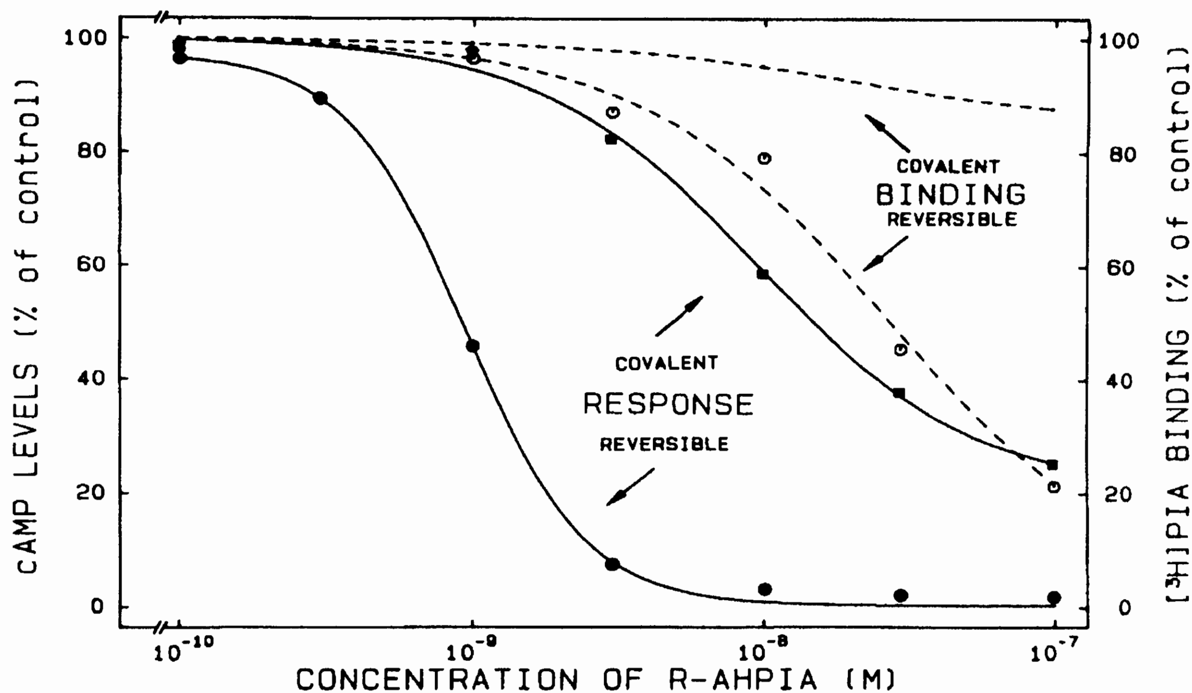

Fig. 4. Comparison of binding and response to *R*-AHPIA. For both the reversible and the covalent occupation of A_1 receptors by *R*-AHPIA the response is expressed as the reduction of cyclic AMP levels (0%: basal level = 1.1 fmol/cell; 100%: level in the presence of 1 μM isoprenaline = 5.9 fmol/cell) and the binding as the percentage inhibition of [^{3}H]PIA binding (5 n*M*). The response data are from Fig. 3. The curve for covalent binding was computed by assuming a 17% yield of photoincorporation and using the data for the reversible binding

son of this constructed curve with the measured response of the covalent binding leads to similar conclusions to those obtained for the reversible activation (Fig. 4): Again a reduction of cyclic AMP levels by 50% of the maximal reduction, which is achieved at about 20 n*M*, corresponds to a receptor occupation of about 5%. The covalent activation of the receptor does not lead to the same maximal reduction in cyclic AMP levels as does the reversible activation. This is because the yield of the covalent reaction is less than 20%. Therefore, receptor occupation with the covalent label never exceeds this level of 20%, which is just not sufficient to produce the maximal response.

The binding curve in Fig. 4 for covalent binding has only been calculated, as its determination by binding studies using intact cells appears impossible in view of the minor effects and the relative inaccuracy of binding studies with [^{3}H]PIA in intact cells. However, in order to verify the assumptions made for the calculation of this curve, we determined the proportion of occupied receptors by preparing membranes from the cells covalently labelled with *R*-AHPIA. The use of these membranes allows accurate saturation studies for the determination of the unoccupied receptors. After covalent labelling with 10 n*M* *R*-AHPIA or, for control purposes, with 10 n*M* *R*-PIA, the parent compound, we determined the number of A_1 receptors with the radioligand ^{125}I-HPIA. Table 1 shows that the covalent labelling with 10 n*M* *R*-AHPIA led to a minor loss of the B_{max} value for ^{125}I-HPIA binding with no change in the K_D value. This loss of about 5% of the binding sites indicates the proportion of the receptors covalently labelled. These 5% of occupied receptors are sufficient to produce almost a 50% response, which agrees well with the results obtained for the reversible activation.

Table 1. Estimation of covalent receptor occupation after labelling of isolated rat fat cells with 10 n*M* *R*-AHPIA

Pretreatment	*R*-PIA	*R*-AHPIA
B_{max} (fmol/mg protein)	890 ± 40	833 ± 24
K_D (n*M*)	1.37 (1.12–1.68)	1.31 (1.12–1.52)

The cells were pretreated with either 10 n*M* *R*-AHPIA or 10 n*M* *R*-PIA (control), and membranes were prepared as described by McKeel and Jarett [5]. Binding parameters for the high-affinity binding to A_1 adenosine receptor were obtained from saturation experiments with ^{125}I-HPIA, as described by Ukena et al. [7]. Data displayed are means and SEMs or 95% confidence intervals from three experiments

Conclusion

The data indicate that covalent labelling of A_1 receptors leads to their persistent activation. This observation agrees with the predictions of the occupancy theory of receptor activation [1]. It indicates that the receptor is activated as long as it is

occupied by an agonist. The correlation between binding and response measurements shows that only a minor proportion of receptors need be occupied to produce a half-maximal response. This is true for both the reversible and the covalent activation of the receptor and suggests a large proportion of spare receptors. At the same time it appears that covalent labelling leads to the same full activation as does reversible binding.

References

1. Clark AJ (1937) General pharmacology. Springer, Berlin Handbook of experimental pharmacology, vol 4
2. Daly JW (1982) Adenosine receptors: targets for future drugs. J Med Chem 25:197–207
3. Klotz K-N, Cristalli G, Grifantini M, Vittori S, Lohse MJ (1985) Photoaffinity labeling of A_1-adenosine receptors. J Biol Chem 260:14659–14664
4. Londos C, Cooper DMF, Wolff J (1980) Subclasses of external adenosine receptors. Proc Natl Acad Sci USA 77:2551–2554
5. McKeel DW, Jarett L (1970) Preparation and characterization of a plasma membrane fraction from isolated fat cells. J Cell Biol 44:417–432
6. Schwabe U (1981) Direct binding studies of adenosine receptors. Trends Pharmacol Sci 2:299–303
7. Ukena D, Furler R, Lohse MJ, Engel G, Schwabe U (1984) Labelling of R_i adenosine receptors in rat fat cell membranes with (–)[^{125}iodo]-N^6-hydroxyphenylisopropyladenosine. Naunyn Schmiedebergs Arch Pharmacol 317:277–285
8. Van Calker D, Müller M, Hamprecht B (1978) Adenosine inhibits the accumulation of cyclic AMP in cultured brain cells. Nature 276:839–841

Discussion

Linden: Do you see any evidence of desensitization of the adenosine response after persistent covalent occupancy of the receptors with an agonist?

Lohse: We have not observed any desensitization of the response to covalent A_1 receptor occupancy over several hours. However, we have not observed responses for any periods of time longer than that.

Baer: I was surprised to hear that you retained a functioning receptor after a photoaffinity labelling reaction – which I would expect to result in chemical destruction of a binding protein or receptor. Are there examples in the literature where after photoaffinity labelling reactions the function of such proteins has been retained?

Lohse: As we see from the persistent receptor activation, the functional integrity of the A_1 receptor was maintained after the photoincorporation of *R*-AHPIA. It is possible that we were just lucky, but there are examples in the literature of photoreactive peptide hormones which retained agonist activity after photoincorporation. This has been described for insulin, ACTH, and vasopressin. Of course, these molecules are considerably larger than the *R*-AHPIA molecule, so that we might have anticipated difficulties.

Daly: The maximal irreversible binding you can attain is only 10% of the total number of A_1 receptors as measured by reversible binding. Is this correct?

Lohse: When we use membranes from rat brain for example, or adipocytes, the photoincorporation is 30–40%; that is to say, 30–40% of the reversible specific binding is covalent. This is achieved at ligand concentrations of about 70 p*M*, but we have no evidence that it varies with ligand concentration. In intact adipocytes the yield of this reaction was lower, ranging from 10% to 20% at different ligand concentrations. This is possibly due to the lower affinity of the receptor in intact cells to agonists, so that at the moment of UV irradiation it is not fixed as well as it is in membranes.

Londos: Was "spareness" determined in the presence of a stimulatory hormone and, if so, how does this affect the apparent number of spare receptors?

Lohse: In all these experiments the cyclic AMP response was measured in the presence of 1 μM isoprenaline, which produced a maximal stimulation of cyclic AMP production. Of course the law of mass action applies not only to hormone-receptor binding but also to the steps subsequent to receptor occupation. So the number of spare receptors will depend on the presence of opposing factors. In my opinion our experiments show that even in the presence of a maximal β-adrenergic stimulation there is a large number of spare receptors.