Photochemical cross-linking of ^{125}I-hydroxyphenylisopropyl adenosine to the A_1 adenosine receptor of rat adipocytes

Allan Green, Charles A. Stuart, Robert A. Pietrzyk and Maristella Partin

Division of Endocrinology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77550, USA

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Rat adipocyte plasma membranes were incubated with the A_1 adenosine receptor agonist, ^{125}I-hydroxyphenylisopropyl adenosine (1 nM) and then treated with the photoactive cross-linking agent, ANB-NOS. The membranes were solubilized and analyzed by SDS-PAGE and autoradiography. A single protein, M_r approx. 38 000, was specifically labeled. Reduction with 2-mercaptoethanol did not affect the apparent M_r of the labeled protein. Labeling was inhibited by the adenosine receptor agonists, HPIA, PIA and NECA, and by the antagonist, theophylline, but was unaffected by inosine. We conclude that the A_1 adenosine receptor is a single protein of M_r approx. 38 000.

(Rat) Adipocyte Adenosine receptor Adenosine Photochemical cross-linking

1. INTRODUCTION

Adenosine, which is found at low concentrations in all mammalian tissues, has a wide range of biological actions. These actions include effects on electrical activity in the CNS, hemodynamic effects, inhibition of lipolysis in adipose tissue, and modulation of the actions of various hormones (review [1]). Many of the actions of adenosine are thought to be mediated via specific cell-surface receptors, which can be subdivided into A_1 adenosine receptors (which are inhibitory to adenylate cyclase) and A_2 receptors (stimulatory to adenylate cyclase) (reviews [2,3]).

Recently, much attention has been focused on the A_1 adenosine receptor. This receptor has been characterized mainly in rat brain and adipose tissue. Several techniques for covalently labeling the receptor have recently been reported [4–6], and demonstrate that it has an M_r of 35 000–38 000. Such techniques should prove valuable tools in studies of adenosine actions and receptors. However, each of the techniques reported to date involves complex organic synthesis of radiolabeled ligands. Here, we describe a simple technique for covalently radiolabeling A_1 adenosine receptors in rat adipocyte plasma membranes, using a commercially available, thoroughly characterized ligand, ^{125}I-HPIA [7–9].

Abbreviations: ^{125}I-HPIA, (−)-N^6-^{125}I-*p*-hydroxyphenylisopropyladenosine; HPIA, (−)-N^6-*p*-hydroxyphenylisopropyladenosine; PIA, (−)-N^6-phenylisopropyladenosine; NECA, 5′-(*N*-ethyl)carboxamidoadenosine; ANB-NOS, *N*-5-azido-2-nitrobenzoyloxysuccinimide; SANPAH, *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate; sulfo-SANPAH, sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Bovine serum albumin (type CRG-7) was purchased from Armour Pharmaceuticals (Kankakee, IL). Collagenase (type CLS) was from Worthington Biochemical (Freehold, NJ). ^{125}I-HPIA was from Amersham (Arlington Heights, IL), adenosine deaminase, PIA, theophylline, NECA and PMSF were from Sigma (St. Louis, MO). HPIA was from Boehringer Mannheim (Indianapolis, IN). ANB-NOS, SANPAH and sulfo-SANPAH were purchased from Pierce (Rockford, IL). All reagents for SDS-PAGE were from Bio-Rad (Richmond, CA).

2.2. *Isolation of adipocyte plasma membranes*

Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats weighing 200–250 g, by a modification of the method of Rodbell [10] as previously described [11]. The liberated cells were washed three times in a buffer consisting of 137 mM NaCl, 5 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5 mM glucose, 20 mM Hepes (pH 7.4) plus bovine serum albumin (10 mg/ml). The cells were then washed once in homogenizing buffer (250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 20 mM Hepes, pH 7.4) and homogenized in a volume of buffer equal to twice the packed cell volume, using 8 strokes of a Potter-Elvehjem homogenizer fitted with a teflon pestle. The homogenate was centrifuged for 5 min at $1000 \times g$, and the supernatant for 15 min at $16000 \times g$. The resulting pellet was resuspended and layered onto a 'cushion' of 35% (w/w) sucrose, and centrifuged at $108000 \times g$ for 1 h in a Sorvall SW-28 rotor. The band of membranes at the interface of the 35% sucrose was collected, diluted with homogenizing buffer and centrifuged at $16000 \times g$ for 30 min. The resulting pellet was resuspended in 154 mM NaCl, 10 mM $MgCl_2$, 50 mM Hepes, pH 7.6. The protein concentration was measured by the method of Bradford [12] using bovine γ-globin as a standard, and adjusted to 1–2 mg/ml.

2.3. *Photochemical cross-linking of ^{125}I-HPIA to adipocyte membranes*

Adipocyte plasma membranes (50–80 μg protein) were incubated with 1 nM ^{125}I-HPIA in a total volume of 70 μl of 154 mM NaCl, 10 mM $MgCl_2$, 50 mM Hepes, pH 7.6, plus adenosine deaminase (10 μg/ml) to remove endogenous adenosine. After 2.5 h incubation at 37°C, the tubes were cooled on ice, and 430 μl ice-cold buffer (without adenosine deaminase) was added. This was followed by 25 μl of a 10 mM solution of the cross-linking agents. ANB-NOS and SANPAH were dissolved in dimethyl sulfoxide. Sulfo-SANPAH was dissolved in water. Photolysis was initiated immediately with a 200 W incandescent lamp positioned approx. 5 cm above the samples. The tubes were kept in an ice bath during photolysis. After photolysis the membranes were centrifuged (5 min in a Beckman microfuge), washed once in buffer and dissolved in sample buffer for analysis by SDS-PAGE and autoradiography.

2.4. *SDS-PAGE*

SDS-PAGE was performed by the method of Laemmli [13]. Samples were heated at 95°C with or without 5% (v/v) 2-mercaptoethanol, and then run on 10% polyacrylamide gels with 4% stacking gels, at 25 mA per gel for the stacking gel and 35 mA for the resolving gel. The gels were stained with Coomassie blue and dried. Autoradiography of the dried gels was performed in X-ray cassettes containing intensifying screens [2] at −70°C for 3–14 days. X-ray film was developed in a Kodak RP X-Omat processor and the bands were quantitated with a Quick Scan Jr. TLC photodensitometer (Helena Laboratories, Beaumont, TX).

3. RESULTS

Adipocyte plasma membranes were equilibrated with 1 nM ^{125}I-HPIA in the absence or presence of excess unlabeled HPIA (10 μM). ANB-NOS (0.5 mM) was then added and the samples were exposed to light for 0–60 min and then analysed by SDS-PAGE and autoradiography (fig.1). Specific labeling of a single peptide, apparent M_r approx. 38000, can be seen. The degree of labeling of the receptor is dependent on photolysis time. There was virtually no labeling in the absence of light, and labeling increased continuously over the 1 h photolysis period. Subsequent experiments therefore employed a 1 h photolysis step.

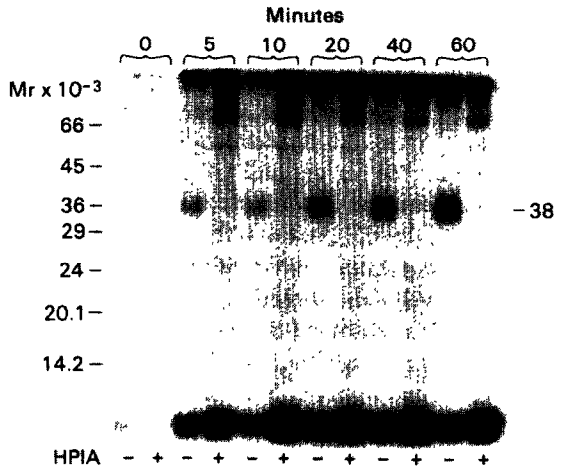

Fig.1. Time course of photolabeling of adenosine receptors. Adipocyte plasma membranes were incubated with ^{125}I-HPIA (1 nM) for 2.5 h at 37°C. The membranes were cooled on ice. ANB-NOS was added and the samples were exposed to light for 0–60 min as indicated. Each time point was obtained in the absence or presence of an excess of unlabeled HPIA (10 μM) as indicated. The samples were analysed by SDS-PAGE and autoradiography as described in section 2. The lines on the left-hand side of the figure show the position of standard M_r markers, as revealed by Coomassie blue staining of the gel. The line on the right-hand side shows the position and approximate M_r of the specifically labeled protein.

In addition to the specifically labeled protein, two bands were labeled non-specifically. In fact, labeling of these proteins was actually increased in the presence of unlabeled HPIA. The increased labeling was probably due to the fact that, in the absence of unlabeled HPIA, approx. 60% of the ^{125}I-HPIA was receptor-bound in these experiments (not shown), and therefore not available for non-specific labeling. The apparent M_r values of these proteins were approx. 55000 and 68000. Furthermore, they corresponded to the two major proteins visualized on the gels with Coomassie blue staining.

To determine whether the specifically labeled peptide contains disulfide bonds, membranes were

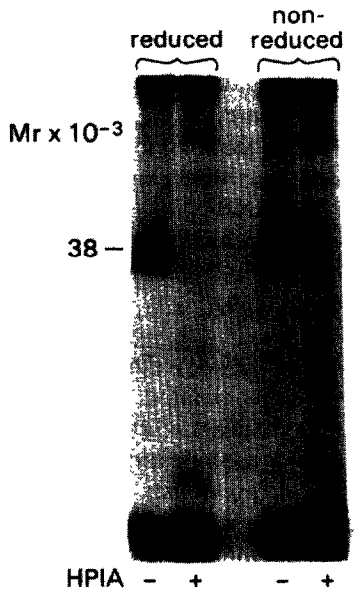

Fig.2. Effect of reduction on adenosine receptors. Membranes were photochemically cross-linked with ^{125}I-HPIA and then either reduced with 5% mercaptoethanol, or left untreated, and analysed by SDS-PAGE and autoradiography.

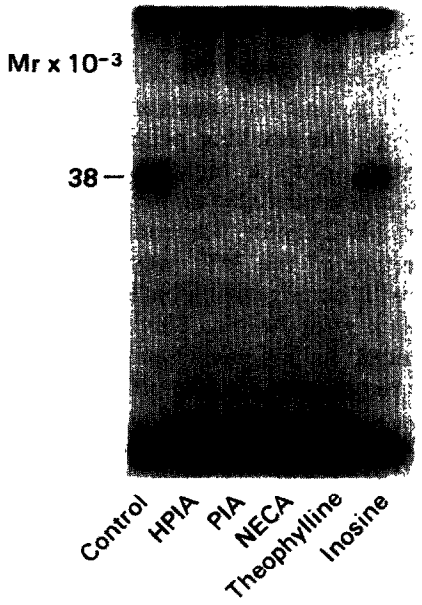

Fig.3. Effects of potential inhibitors on labeling of adenosine receptors. Membranes were incubated with ^{125}I-HPIA plus other compounds as indicated, and then cross-linked with ANB-NOS and analysed as described in section 2. The concentration of PIA, HPIA, NECA and inosine was 100 nM, and that of theophylline was 1 mM.

cross-linked with ^{125}I-HPIA and then reduced with 5% (v/v) 2-mercaptoethanol, or left untreated. The autoradiogram in fig.2 demonstrates that reduction did not affect migration of the labeled protein, suggesting that it consists of a single subunit.

Fig.3 demonstrates that labeling of the 38 kDa protein is inhibited by the adenosine analogs PIA, HPIA and NECA. Similarly, labeling is completely inhibited by 1 mM theophylline. A lower concentration of theophylline (50 μM) caused approx. 60% inhibition of labeling (not shown). Inosine, which is structurally very similar to adenosine but is not an adenosine receptor agonist [14], did not inhibit labeling.

In addition to ANB-NOS, two other commonly used photoactive cross-linking agents (SANPAH and sulfo-SANPAH) were tested for their ability to cross-link ^{125}I-HPIA to the adenosine receptor (fig.4). These compounds did result in some specific labeling, but they were much less effective than ANB-NOS.

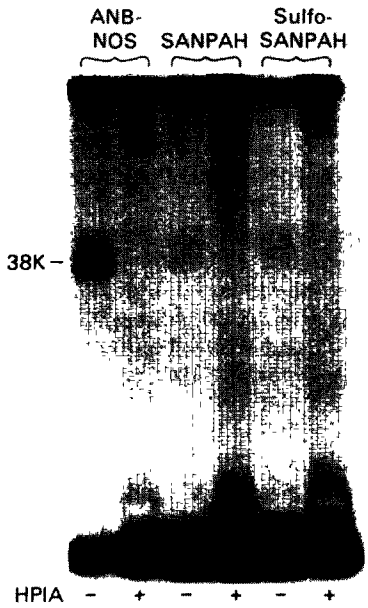

Fig.4. Effects of different cross-linking agents. Membranes were incubated with ^{125}I-HPIA with or without HPIA as described in section 2. ANB-NOS, SANPAH or sulfo-SANPAH (0.5 mM) was then added and the samples were photolyzed for 1 h and analysed by SDS-PAGE and autoradiography.

4. DISCUSSION

We have demonstrated photochemical cross-linking of ^{125}I-HPIA to a rat adipocyte membrane protein. The apparent M_r of this protein is approx. 38000. Cross-linking was inhibited by adenosine receptor agonists (PIA, HPIA and NECA) and by theophylline (an adenosine receptor antagonist) but not by inosine. Furthermore, ^{125}I-HPIA has been thoroughly characterized as a ligand for studies of the A_1 adenosine receptor in fat cell and brain membranes [7–9]. We conclude, therefore, that the labeled protein is the A_1 adenosine receptor, or at least the ligand-binding subunit of the receptor.

Neither the mobility of the labeled protein nor the number of labeled bands observed was altered following reduction of the samples with 2-mercaptoethanol. This finding suggests that the adenosine receptor consists of a single peptide, rather than several subunits joined by disulfide bonds.

The cross-linking agent utilized successfully in these studies was ANB-NOS. This is a heterobifunctional compound containing a hydroxysuccinimide active ester coupled to an azidophenyl group. The hydroxysuccinimide group enables the compound to couple spontaneously to primary amines, while the azidophenyl group, when activated with light, inserts into carbon-carbon or carbon-hydrogen bonds. Since the ligand utilized in these studies (^{125}I-HPIA) does not possess a primary amine group, we conclude that the mechanism of cross-linking involves reaction of the hydroxysuccinimide group with a primary amine on the adenosine receptor (probably a lysine) and insertion of the azidophenyl group into the ligand molecule. Thus, it is probable that the adenosine receptor has a lysine very close to the binding site. This is supported by the finding that SANPAH and sulfo-SANPAH, which are very similar to ANB-NOS but contain a six-carbon spacer between the active groups, were much less effective as cross-linkers.

Two other techniques for labeling A_1 adenosine receptors have recently been published. Klotz et al.

[4] and Choca et al. [5] synthesized 2-azido-^{125}I-HPIA and ^{125}I-azidobenzyladenosine, respectively. These compounds were reported to be photoaffinity probes for the adenosine receptor, and labeled a 35–36 kDa protein in adipocyte and brain membranes. Secondly, Stiles et al. [6] synthesized a compound (^{125}I-labeled N^6-2-(4-aminophenyl)-ethyladenosine) that could be cross-linked to the adenosine receptor with SANPAH. Their compound labeled a 38 kDa protein in rat brain and fat cell membranes. Interestingly, the compound could be cross-linked with SANPAH but not with ANB-NOS. This is the reverse of what we have found with ^{125}I-HPIA. Since their compound does possess a primary amine it is possible that the cross-linking mechanism involved reaction of the hydroxysuccinimide group with the ligand rather than the receptor.

The current studies demonstrate that ^{125}I-HPIA, which has been thoroughly characterized by several groups as a ligand for the A_1 adenosine receptor [7–9], can be covalently cross-linked to rat adipocyte plasma membranes. The findings confirm that the adenosine receptor is a single membrane protein with an apparent M_r of approx. 38000. Furthermore, our technique is simple, and utilizes reagents which are readily available from commercial sources. In view of the simplicity of the technique, and the rapidly increasing interest in adenosine as a regulatory molecule, this technique should prove valuable in studies of the structure and function of the A_1 adenosine receptor.

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