Demonstration of Distinct Agonist and Antagonist Conformations of the A₁ Adenosine Receptor*

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Abstract

A₁ adenosine receptor-binding subunits can be visualized using high affinity antagonist and agonist photoaffinity radioligands. In the present study, we examined whether agonists and antagonists bind to the same receptor-binding subunit and if agonists and antagonists induce different conformational states of the receptor in intact membranes. It was demonstrated that several agonist and antagonist photoaffinity probes all labeled a Mᵋ 38,000 protein which is the A₁ receptor-binding subunit. When the agonist and antagonist photoaffinity labeled peptides were denatured and subjected to partial peptide map analysis using a two-dimensional gel electrophoresis system similar peptide fragments were generated from each specifically labeled protein. This suggests that both classes of ligand label and incorporate into the same binding subunit.

Proteolytic digestions of agonist- and antagonist-occupied receptors in native intact membranes revealed distinct and different peptide fragments depending on whether the ligand was an agonist or an antagonist. Manipulation of incubation conditions to perturb ligand-receptor interactions alter the pattern of peptide fragments generated with each specific protease.

These data suggest that agonist and antagonist photoaffinity probes interact with and incorporate into the same binding subunit but that agonist binding is associated with a unique and detectable receptor conformation.

The A₁ receptor is the subtype of adenosine receptor that is inhibitory to adenylate cyclase and exhibits an agonist potency order of R-PIA¹ > N₆-ethyladenosine-5'-uronic acid > N₆-S-phenyl-2-propyladenosine. This receptor is found in a wide range of tissues and exerts a variety of physiologic effects including the suppression of cardiac contractility and atrial ventricular nodal conduction, central nervous system sedation, and the inhibition of lipolysis (1,2).

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¹The abbreviations used are: R-PIA, N₆-R-phenyl-2-propyladenosine; AZPNEA, N₆-azidophenyl-ethyladenosine; CHAPS, 3-[{(3-cholamidopropyl)dimethylammonio]-l-propanesulfonate; G protein, guanine nucleotide-binding protein; SANPAH, N-succinimidyl 6-(4'-azido-2'-nitrophenyl)amino)hexanate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAPAXAC, 8-[4-(4-aminophenyl acetylmino)ethyl][carbonyl]-methyl][oxy][ phenyl]-1,3-dipropylxanthine; HPLC, high performance liquid chromatography.
Radioligand binding studies have demonstrated a dichotomy in the binding properties of $A_1$ adenosine receptor ligands. Agonist ligands have been clearly shown to recognize two affinity states with the number of high affinity binding sites being dramatically decreased by guanine nucleotides or sodium chloride and increased by the addition of divalent cations such as magnesium. In contrast, antagonist ligands recognize a single affinity state and in most tissues, binding appears to be unaffected by either guanine nucleotides or divalent cations (3–6). Interestingly, the addition of high concentrations of salt has even been reported to significantly increase antagonist binding in some instances (7). These observations all lead to the conclusion that agonist and antagonist ligands functionally interact with the $A_1$ adenosine receptor in a very different manner.

Studies in a variety of receptor systems, including the $A_1$ adenosine receptor, have suggested that agonists alone, have the unique ability to induce a conformational change in the receptor that permits it to interact with its $G$ protein (8). This interaction results in the formation of a functionally important high affinity state which is thought to represent the association of agonist ligand, receptor, and $G$ protein, the so called “ternary complex.” The second or low affinity agonist binding state is thought to represent a binary complex of agonist ligand and receptor (9, 10). The single low affinity state that is recognized by antagonist ligands, therefore, suggests that only agonists are capable of inducing the conformational change in the receptor that leads to the formation of the agonist specific high affinity state.

The recent synthesis of high affinity agonist and antagonist photoaffinity probes for the $A_1$ adenosine receptor (11, 12) has permitted the protein structure of the $A_1$-binding subunit to be studied. It appears that agonist and antagonist photoaffinity probes incorporate into similar if not identical proteins of 38–40 kDa apparent molecular mass.

We now report on the application of a high affinity agonist ($^{125}$I-AZPNEA) and two high affinity antagonists ($^{125}$I-PA-PAXAC-SANPAH and azido-$^{125}$I-PAPAXAC) photoaffinity probes to the study of two questions: 1) Do agonists and antagonists bind to the same binding subunit of the $A_1$ adenosine receptor? and 2) Do agonists induce a detectable, unique conformation in the $A_1$ adenosine receptor (in membrane preparations) by the act of binding to the subunit?

Using the technique of partial peptide mapping, we demonstrate that agonist and antagonist photoaffinity probes label the same molecular weight polypeptide (the $A_1$ receptor) at nearly, if not exactly, the same location. In addition, we provide the first physical evidence suggesting that agonist binding to the $A_1$ receptor in membranes induces a unique conformational change in the labeled $A_1$ receptor-binding subunit that is reflected in a distinct pattern of partial digestion products that are not duplicated by an antagonist probe. These findings have important implications for the understanding of $A_1$ receptor agonist and antagonist binding behavior.

**EXPERIMENTAL PROCEDURES**

**Materials**

Adenosine deaminase, guanine triphosphate (GTP), *Staphylococcus aureus* V8 protease, elastase, chymotrypsin, chloramine T, and agarose were all obtained from Sigma. R-PIA was purchased from Boehringer Mannheim. PAPAXAC was synthesized as previously described (12). $N^\alpha$-2-(4-Aminophenyl)ethyladenosine was provided by Dr. R. A. Olsson, University of South Florida, Tampa, FL. SANPAH was purchased from Pierce Chemical Co., and Na$^{125}$ I (carrier-free) was obtained from Amersham Corp. All other reagents were of the highest available grade and were purchased from standard sources.
Radioiodination of PAPAXAC

PAPAXAC was radioiodinated by the chloramine-T method and purified as previously described (12).

Synthesis of Azido-\[^{125}\text{I}\]-PAPAXAC

The conversion of \[^{125}\text{I}\]-PA-PAXAC to azido-\[^{125}\text{I}\]-PAPAXAC was performed using a modification of the technique of Lavin et al. (13) and Stiles et al. (11).

The purified \[^{125}\text{I}\]-PAPAXAC was collected after HPLC separation, placed in a plastic microcentrifuge tube, and dried completely in a vacuum centrifuge (Speed Vac Concentrator, Savant Instruments Inc., Farmingdale, NY). The residue was then dissolved in 10 \(\mu\)l of 6 N acetic acid, diluted with 10 \(\mu\)l of H\(_2\)O, and cooled to 0 °C in a melting ice bath.

Ten \(\mu\)l of sodium nitrite (5 mg/ml H\(_2\)O) was added, and the mixture was maintained in the ice bath for 2 min. The solution was then removed from the bath, warmed to room temperature, and placed in subdued lighting. Ten \(\mu\)l of sodium azide (5 mg/ml H\(_2\)O) was added and allowed to react for 5 min before 8 \(\mu\)l of ammonium hydroxide was added to quench the reaction by raising the pH.

The azido-\[^{125}\text{I}\]-PAPAXAC was purified by HPLC using a Waters C\(_{18}\) Bondpack column with a mobile phase consisting of 75% methanol and 25% 20 mM ammonium formate, pH 8.0, at a flow rate of 1.0 ml/ min. Fractions were collected at 30-s intervals, and 5-\(\mu\)l aliquots from each fraction were counted in a Packard Gamma counter to determine the completeness of separation. A single radioactive peak emerged at approximately 9 min consisting of the azido-\[^{125}\text{I}\]-PAPAXAC with an assumed specific activity of 2175 Ci/mmol. Essentially none of the original \[^{125}\text{I}\]-PAPAXAC remained since a radioactive peak was not detected at 6.5 min (the time when the \[^{125}\text{I}\]\text{PAPAXAC emerged in the original purification}).

Synthesis of \[^{125}\text{I}\]-PAPAXAC-SANPAH

Using a modification of the approach outlined previously (12), 40 \(\mu\)l of the \[^{125}\text{I}\]-PAPAXAC solution was placed into a plastic microcentrifuge tube and alkalinized with 5 \(\mu\)l of 1 N NaOH. Five \(\mu\)l of SANPAH (0.5 mg/ml dimethyl sulfoxide) was then added, mixed well, and allowed to react for 5 min. At the end of that time a 5-\(\mu\)l aliquot was counted in a gamma counter and the remaining solution was used for photoaffinity labeling.

Membrane Preparation

Fresh bovine brain was obtained from a local abattoir and prepared as previously outlined (12). Briefly, cerebral cortex was excised and placed in a buffer composed of 50 mM Tris-HCl, 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride adjusted to pH 7.4 at 5 °C. The cortex was then minced and homogenized with 10 strokes of a motor driven nylon pestle before being filtered through four layers of cheese cloth and centrifuged at 40,000 X g for 10 min. This pellet was resuspended in the above buffer and recentrifuged at 40,000 X g for an additional 10 min. The final pellet was then suspended in a buffer composed of 50 mM Tris-HCl, 10 mM MgCl\(_2\), and 1 mM EDTA adjusted to pH 7.4 at 37 °C. Adenosine deaminase (0.3 units/ml) was added and the suspension was incubated at 37 °C for 20 min to remove endogenous adenosine. The crude membranes (~4,500 mg protein/ml) were then divided into 2-ml aliquots, frozen in liquid nitrogen, and stored at −80 °C. These membranes remained stable for at least 4 weeks when prepared and stored in this manner.
Photoaffinity Labeling

Bovine brain membranes were prepared for photoaffinity labeling by suspending 0.4 ml of the frozen membranes in 25 ml of 50 mM Tris with 10 mM MgCl₂ and 1mM EDTA (this is hereafter referred to as 50/10/1 buffer). Adenosine deaminase was added (2.5 units/ml), and the membranes were incubated in a shaking waterbath at 37 °C for 30 min. At the end of this time, the membranes were centrifuged at 43,000 X g for 5 min and then resuspended in 18 ml of 50/10/1 buffer with 0.9 units/ml of adenosine deaminase.

Labeling was then performed in the manner previously described for¹²⁵I-N⁶-2-(4-aminophenyl) ethyladenosine (11) since these conditions proved to be optimal for all the ligands utilized in this study. Sufficient photoaffinity ligand was added to 4-ml aliquots of the above membrane suspension to yield a 0.5 nM concentration of ligand in the final reaction volume. This concentration was sufficient to label the single affinity state recognized by antagonists in membrane preparations as well as the high affinity agonist state. (The concentration of radioligand required to label the low affinity agonist state would have been prohibitively high both in terms of¹²⁵I radioactivity and nonspecific binding.)

The membrane/ligand suspension was then incubated in foil-wrapped tubes at 37 °C for 1 h. While maintaining subdued lighting, the suspension was diluted with 10 volumes of ice-cold 50/10/1 buffer containing 0.05% CHAPS and sedimented at 43,000 X g for 5 min. The remaining pellet was suspended in 4 ml of 50/10/1 buffer and either underwent proteolytic digestion (as outlined in a later section) or photoincorporation.

Photoincorporation was begun by pouring the membrane/ligand suspension into an iced Petri dish and exposing this suspension to UV light from a model UVCG-25 mineral light at a distance of 2 cm for 4 min. The photolysed membranes were then washed once with 0.05% CHAPS in 10 volumes of 50/10/1, centrifuged at 43,000 X g for 5 min, washed against with 10 volumes of plain 50/10/1 buffer, and sedimented a final time at 43,000 X g for 5 min. The remaining pellet was then solubilized and prepared for SDS-PAGE.

SDS-PAGE

Electrophoresis was performed according to the method of Laemmli (14) in homogeneous slabs of 12, 16, or 18%.

Samples were solubilized in 10% SDS, 10% glycerol, 25 mM Tris-HCl, and 5% β-mercaptoethanol, pH 6.8, at 25 °C for 45 min.

After electrophoresis, the gels were dried and exposed to Kodak XAR5 film with dual intensifying screens. Films were typically developed after 24–72 h.

Limited Proteolysis in SDS-Polyacrylamide Gels (Two-dimensional Peptide Maps)

Limited proteolysis was performed as previously outlined (15–17).

Briefly, the labeled receptor was subjected to electrophoresis on a 12% gel. The region of the wet gel containing the receptor was excised and electrophoresed in the second dimension on a higher percentage 105-mm separating gel with a 35-mm stacking gel. The excised gel section was bonded to the second dimension stacking gel with agarose, and the entire well was filled with sample buffer containing the appropriate enzyme, 2% SDS, 10% glycerol, and 50 mM Tris-HCl adjusted to pH 6.8 at room temperature.
Limited Proteolysis in Membranes

Digestions of the ligand-occupied receptor were performed both before and after photoincorporation.

Digestions prior to photoincorporation were referred to as bound digestions. In this instance the membranes were incubated with ligand as outlined above but at the end of the 1-h incubation period, the appropriate amount of enzyme (chymotrypsin or elastase) was added to the reaction mixture. After 20 min of digestion, the membrane/ligand solution was exposed to UV light for 4 min and then washed with buffer and solubilized as above.

Digestions after photoincorporation began with the same incubation. The membrane/ligand solution then underwent photoincorporation, but instead of proceeding with a series of washes, the labeled receptors were washed only once with 50/10/1 buffer, centrifuged at 43,000 X g for 5 min, and then resuspended in 4 ml of 50/10/1 buffer. At this point, the labeled receptor suspension was treated in one of two ways.

The appropriate enzyme was added to one set of the labeled receptor suspension and was incubated at 37 °C for 20 min. The enzymatic digestion was stopped by cooling the mixture on ice and diluting it with ice-cold 50/10/1 buffer containing 0.05% CHAPS. The mixture was washed again with the CHAPS solution and finally with plain 50/10/1 buffer before being prepared for SDS-PAGE. These samples were referred to as the incorporated digestions.

SDS was added to the other set of labeled receptor suspensions resulting in a final SDS concentration of 1%. This suspension was first incubated at 37 °C for 10 min (to allow the proteins to fully denature), then the appropriate enzyme was added and a second 20-min incubation at 37 °C was performed. At the end of this time, the suspension was heated to 60 °C for 5 min (to inactivate the enzyme), dried completely in a vacuum centrifuge, and the pellet was then prepared for SDS-PAGE in the previously described buffer without additional SDS. These samples were referred to as the denatured digestions.

Protein Determinations

Protein contents were determined by the method of Bradford (18).

RESULTS

Agonist and Antagonist Photolabeling

Fig. 1 illustrates the photoaffinity labeling of the bovine brain A₁ adenosine receptor. Each photoaffinity probe is displayed as a control lane on the left and a nonspecific lane (10⁻⁵ M R-PIA) on the right. The first four lanes show antagonist photolabeling with lanes 1 and 2 using the ¹²⁵I-PAPAXAC-SANPAH probe and lanes 3 and 4 the azido-¹²⁵I-PAPAXAC probe. Lanes 5 and 6 are labeled with the agonist probe ¹²⁵I-AZPNEA. Each pair of lanes shows a specifically labeled 38-kDa protein. The higher molecular mass band (50 kDa) seen in the AZPNEA labeled lanes is a nonspecific band and has been inconsistently seen in a variety of tissues (data not shown). In each instance, however, the intensity of the band changes with the intensity of the background in that lane and not as a function of various competitor concentrations as a specifically labeled protein would.

Two-dimensional Partial Digestions

Partial peptide maps of the denatured ¹²⁵I-labeled binding subunits were constructed to determine if the agonist and antagonist photoaffinity probes incorporated into similar polypeptides. Figs. 2 and 3 show the results of the two-dimensional digestions using either
Staphylococcus V8 protease or chymotrypsin. Both figures are organized in the same manner with the antagonists $^{125}$I-PAPAXAC-SANPAH on the far left, azido-$^{125}$I-PAPAXAC in the middle, and the agonist $^{125}$I-AZPNEA on the right.

Following Staphylococcus V8 digestion (Fig. 2), multiple fragments are apparent and are labeled A to F from top (heavier fragments) to bottom. Band A (the undigested receptor) is common to all three lanes as is the fragment labeled B. The third fragment (labeled C) appears to have an identical apparent molecular weight in lanes 1 and 3, but the relative proportion is much less in the AZPNEA lane. Fragment C in lane 2 may have a slightly smaller apparent molecular weight than the corresponding fragment in the other lanes and appears to be more diffuse in nature although the possibility of an additional band between fragments C and D cannot be excluded. A doublet (bands D and E) is again seen in all lanes while band F appears only in lane 2 with the azido-$^{125}$I-PAPAXAC probe. Peptides labeled with a second agonist photoaffinity probe $^{125}$I-R-2-azido-N$^6$-p-hydroxyphenylisopropyl adenosine (19) also underwent two-dimensional partial digestion with Staphylococcus V8 protease (results not shown). That partial peptide map was identical to the map for $^{125}$I-AZPNEA but the percentage of incorporation of this probe into the binding subunit was so low that it was impossible to distinctly see all four peptide maps on a film developed from a single polyacrylamide gel.

While Staphylococcus V8 protease cleaves a limited number of peptide bonds (specifically those on the carboxylic side of glutamic and aspartic acid residues), chymotrypsin has a much broader range of action (being able to hydrolyze peptide bonds involving any of the aromatic amino acids as well as certain amines and esters) (20,21). Therefore, following chymotrypsin digestion a greater number of labeled peptide fragments should be found, and this is apparent in Fig. 3. At least 12 labeled fragments can be seen by examining all three lanes. Nine of these fragments (those that appear in at least two lanes) have been labeled A through I to facilitate their discussion. Again, band A represents the undigested receptor and is common to all three lanes.

Looking first at the two antagonist labeled lanes (lanes 1 and 2), we find they share at least seven common fragments (bands A-E, G, and I) and possibly two intermediate fragments located between bands C and D and band D and E. Additionally, band F appears to be unique to lane 1, and lane 2 exhibits a fragment between bands G and H that appears to be unique to the azido-$^{125}$I-PAPAXAC-labeled receptor. Although the relative abundance may vary, the two antagonist probes have in common seven (possibly as many as nine) of the digestion products out of the eight (possibly 10 fragments seen in each of lanes 1 and 2).

The agonist (AZPNEA)-labeled receptor (lane 3) shares bands A through E (band E is very faint in lane 3) and I with the antagonist-labeled lanes. Lane 3 also has two labeled fragments located between bands E and I that appear to be in the same region as bands G and H in the antagonist lanes but have a slightly smaller apparent molecular weight and do not exactly correspond to the antagonist-labeled fragments (total of eight bands in lane 3). While differences clearly exist between the agonist- and antagonist-labeled lanes the agonist-labeled receptor does exhibit six of the seven fragments that are common to both antagonist-labeled lanes. The finding that six of seven fragments common to both antagonist probes are found in the digested agonist-labeled receptor indicates that these peptide maps are more similar than different and suggests that they are derived from a common polypeptide.

Native Digestions

Agonist specific conformational changes in the A$_1$-binding subunit were sought by examining peptide maps produced by digesting native membrane-bound receptor/ligand...
complex under conditions that promote the agonist-specific high affinity state. These maps were contrasted to those generated when the digestions were performed following incorporation of the ligand in both the native and denatured states.

The results of these digestions are displayed in Figs. 4 and 5. Both figures are organized in the same format. The lanes are grouped into three pairs with the antagonist ligand (\( ^{125}\text{I}-\text{PAPAXAC-SANPAH} \)) on the left and the agonist ligand (\( ^{125}\text{I}-\text{AZPNEA} \)) on the right in each pair. The first pair of lanes (lanes 1 and 2) are the bound digestions. These are the result of first allowing the ligand to bind to the receptor, then digesting it with the appropriate enzyme, and finally initiating photoincorporation by exposing the entire suspension to UV light. The incorporated digestions are shown in the second pair of lanes (lanes 3 and 4). In this instance, the ligand binds to the membrane receptor, is photoincorporated, washed with buffer, and then the ligand/receptor complex is digested with the indicated protease. The final pair of lanes (lanes 5 and 6) represent the denatured digestions where the ligand is first bound to the receptor then photoincorporated and finally denatured (but not fully solubilized) with SDS before being digested.

These denatured digestions differ from the prior two-dimensional partial digestions in several important ways. In the former, the labeled receptor was fully solubilized in 10% SDS and then subjected to a first dimension electrophoresis before being digested with the appropriate enzyme during a second dimension electrophoresis. This procedure should make all the appropriate cleavage sites available to the enzyme and produce a number of partial digestion fragments. In the latter case, the labeled membranes are placed in 1% SDS for 10 min, an amount of time likely to disrupt functional receptor/ligand interactions but not sufficient to fully solubilize the receptor. Since it is reasonable that some sites would be protected by the cell membrane, it is not surprising that the denatured digestion peptide maps tend to show fewer labeled bands than the two-dimensional peptide maps.

Fig. 4 shows the chymotrypsin digestion products from the labeled A1 receptor-binding subunit. The most obvious and expected finding is that the number of labeled fragments generated by the digestions in intact membranes are much fewer than those generated following the two-dimensional electrophoretic digestion of the denatured receptor as seen in Figs. 2 and 3. The bound digestions (lanes 1 and 2) show a prominent fragment at 38 kDa (band A representing the undigested receptor) that is found in both the antagonist and agonist-labeled lanes. The 29-kDa fragment (band B) is also common to both lanes, in slightly different proportions, but the 15-kDa fragment (band C) is much more prominent with the agonist \( ^{125}\text{I}-\text{AZPNEA} \)-labeled receptor (lane 2). The digestions following ligand incorporation and washing (lanes 3 and 4) demonstrate the same fragments at 38 and 29 kDa but the agonist-specific 15-kDa fragment has now been markedly decreased, suggesting that an agonist-induced conformational change was necessary to promote the generation of this fragment (discussed more fully below). The denatured digestions in lanes 5 and 6 are very different from the other four lanes. In this instance, the 38-kDa fragment is absent, indicating complete digestion of the intact receptor. A new 21-kDa fragment (band D) has appeared in the antagonist-labeled lane (lane 5), and now both the antagonist and agonist lanes show prominent 15-kDa fragments.

The prominence of the 15-kDa fragment in lanes 2, 5, and 6 suggests that this fragment is produced only when the appropriate digestion site(s) are available. The availability of these sites appears to be increased in two circumstances: 1) when either an agonist- or antagonist-labeled receptor is denatured before digestion or 2) when an agonist is coupled to the membrane-bound receptor and induces a conformation that is not associated with antagonist binding.
The bound, incorporated and denatured digestion pairs for elastase are shown in Fig. 5 and are quite different from those seen with chymotrypsin. This difference is not surprising since elastase has specificity for peptide bonds on the carboxyl-terminal side of uncharged nonaromatic amino acids (21). Again, the bound digestions (lanes 1 and 2) show a prominent 38-kDa band of undigested receptor in both lanes but the intermediate molecular mass fragment at 26 kDa (band B) is found only in the antagonist-labeled lane. The 15-kDa fragments are seen in both lanes 1 and 2, but this time they are more prominent with the antagonist-labeled receptor. The undigested receptor at 38 kDa is seen in both lanes of the incorporated digestions (lanes 3 and 4) and the 26-kDa fragment still occurs in only the antagonist lane (lane 3) but now the 15-kDa fragment (band C) is well defined in both lanes 3 and 4 being slightly more prominent in the agonist-labeled lane in this case.

One interpretation of the increased prominence of the 15-kDa fragment in lanes 1, 3, and 4 (compared with lane 2) is that an agonist-specific conformational change, reflected in lane 2, decreases the availability of an elastase digestion site that produces the 15-kDa fragment. If photoincorporation now changes the availability of this site, we could see an increase in the agonist-labeled 15-kDa fragment as is displayed in lane 4. The process of incorporation and washing seems to have had more of an effect on the agonist-labeled receptor since the 15-kDa band is more prominent in this lane now. Denaturing the receptor, as seen in lanes 5 and 6, seems to accentuate the differences seen in lanes 3 and 4 since the agonist-labeled band is now more prominent still.

The validity of the comparisons we have made with regard to the prominence of various fragments in the peptide maps requires that the comparisons are made between similar populations of receptors. Using the radioactivity contained in each of the specifically labeled bands as an indicator of the number of receptors displayed in each lane, we found that the population of receptor labeled by the agonist probe differed by less than 3% between lanes, while those labeled with the antagonist probe differed by less than 12%. These small differences in total receptor population indicate that the observed differences in digestion patterns are not attributable to changes in the magnitude of the receptor population. This finding is in clear contrast to our attempts to predigest the membrane-bound receptor in the absence of any ligand. Under these conditions we found a 70% decrease in the agonist-bound receptor population and a 36% decrease in the antagonist-bound population, thus making it impossible to draw any meaningful conclusions.2

The time course of the bound digestions were studied to determine the behavior of the uniquely labeled fragments. The results of the bound digestion time course studies are shown for chymotrypsin with the 125I-AZPNEA-labeled receptor in Fig. 6 and for elastase with the 125I-PAPAXAC-SAN-PAH-labeled receptor in Fig. 7. The figures are labeled in a similar manner with the fragments labeled A-C on the left, the relative molecular weights on the right and each lane from left to right depicting longer digestion times before photoincorporation. A control lane of undigested receptor (time 0) is included on Fig. 6 for completeness.

In the chymotrypsin digestion time course for 125I-AZPNEA (Fig. 6), the intensity of each band (A-C) clearly decreases with increasing digestion time, but the relative intensity of the bands in each lane is unchanged. The ratio of cpm in band C to cpm in band A remains constant in each lane with a ratio of 1.1 to 1, implying that the fragments are not interconverted. The same study was performed with chymotrypsin and the antagonist-labeled receptor (results not shown). This demonstrated the same progressive decrease in the

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38, 29, and a faint 15-kDa fragment with increasing digestion times without any evidence of interconversion or new digestion products.

Fig. 7 shows the elastase digestion time course of the $^{125}$I-PAPAXAC-SANPAH-bound receptor. Again, the intensity of each band decreases with increasing digestion times. The relative intensity of band C compared with that of band A continues to remain constant with a ratio of cpm in band C to cpm in band A of 0.6 to 1. The time course for the elastase digestion of the $^{125}$I-AZPNEA-bound receptor (results not shown) demonstrated a progressive decrease in the intensity of the 38 and a faint 15-kDa band again without the generation of any new products or evidence of interconversions. These time course studies demonstrate that the patterns we observed in the bound, incorporated, and denatured digestions for 20 min of digestion are representative of the patterns over a wide range of digestion times and thus are not serendipitous.

**DISCUSSION**

Our ability to synthesize high affinity, radiolabeled agonist and antagonist probes has made the A$_1$ adenosine receptor an ideal prototype for studying receptor conformational changes.

A variety of approaches have been taken in an effort to understand the nature of A$_1$ receptor binding. These range from attempts to perturb receptor binding with guanine nucleotides, sodium chloride, and divalent cations (6) to sophisticated studies of structure activity relationships (22, 23). While we now realize the necessity of the ribose moiety for agonist action and the effect of various xanthine ring substitutions on antagonist potency, we still do not have any physical evidence to distinguish agonist from antagonist binding at the molecular level. This study provides the first such evidence.

The belief that agonist and antagonist photoaffinity probes labeled similar molecular weight polypeptides was first suggested in a recent publication (12). The present study provides further evidence that agonists and antagonists do label the same binding subunit by demonstrating that the agonist probe $^{125}$I-AZPNEA specifically labels the same molecular weight polypeptide as the two antagonist probes azido-$^{125}$I-PAPAXAC and $^{125}$I-PAPAXAC-SANPAH when displayed on a single polyacrylamide gel (Fig. 1).

The belief that these labeled peptides are all the same protein is strengthened by the *Staphylococcus* V8 digestions shown in Fig. 2. The most straightforward comparison involves the $^{125}$I-PAPAXAC-SANPAH-labeled receptor (lane 1) and the $^{125}$I-AZPNEA-labeled receptor (lane 3). The similarity of bands A-E in both of these lanes strongly suggests that they are fragments of the same labeled protein and even though the azido-$^{125}$I-PAPAXAC-labeled receptor in lane 2 differs from the other two lanes in bands C and F, it is still quite similar to the peptide maps seen in lanes 1 and 3.

The chymotrypsin digestion shown in Fig. 3 resulted in a greater number of labeled fragments making the comparisons more complex. If we begin the examination by looking at the fragments common to both the antagonist labeled lanes (lanes 1 and 2), we will see a pattern of similarity develop.

The antagonist-labeled receptors each generate seven fragments denoted by A-G and I in Fig. 3. While the relative intensities may vary between lanes, this concordance of fragments suggests that these two peptide maps were the result of digesting a very similar protein. The fact then, that a majority (six of seven) of the labeled fragments (bands A-E and I) common to both antagonists correspond to an agonist-labeled fragment suggests that the agonist-labeled peptide map was also derived from the same peptide that produced the antagonist-labeled peptide maps.
Clearly, this reasoning does not prove that all three peptide maps were the result of digesting the exact same labeled receptor (that will have to await sequencing of the receptor protein) but the combined physical evidence presented in Figs. 1–3 is very suggestive that the same protein (which we believe is the A1-binding subunit) is labeled by the agonist and both antagonist photoaffinity probes.

We next approached the question of how these ligands could induce different physiologic and biochemical responses upon binding to the same receptor-binding subunit. One postulated mechanism is that agonists and antagonists have different effects on the conformation of the receptor protein. By this hypothesis, the agonist conformation is the only conformation which is favorable for an effective interaction with the appropriate G protein (2, 9). This postulate has become dogma for most receptor systems but up to now, has had very little experimental support.

The chymotrypsin native digestion (Fig. 4) suggests that agonist-specific conformational changes do occur when 125I-AZPNEA binds to the A1-binding subunit. Since the binding and digestion conditions were identical for both ligands and chosen to promote functional ligand/receptor interactions, (i.e. formation of the agonist high affinity state) any differences between the peptide maps generated from antagonist-labeled receptors (lane 1) and high affinity agonist labeled receptors (lane 2) should be a reflection of differences in receptor conformation. The presence of a very prominent 15-kDa band in lane 2 suggests that significant differences in the agonist high affinity and antagonist-bound receptor conformation do exist.

The prominence of the 15-kDa band in lane 2 can be interpreted as showing that the high affinity agonist state increased the availability (compared with the antagonist state) of the chymotrypsin digestion site(s) that leads to the formation of that fragment.

Washing the bound receptor membranes followed by photoincorporating the ligand into the receptor protein could reasonably be expected to alter the receptor/ligand interaction. If this alteration does occur, then lanes 3 and 4 suggest that the receptor/agonist ligand complex is sufficiently altered (loss of agonist high affinity state) to dramatically decrease the availability of the site responsible for the 15-kDa fragment.

The denaturation of the receptor with SDS appears to make the digestion site, which produces the 15-kDa fragment, available regardless of whether the receptor is labeled with an agonist or antagonist (lanes 5 and 6). The 21-kDa fragment seen in lane 5 occurs only with the antagonist-labeled receptor and may be the result of less complete digestion or small differences in the site of ligand incorporation.

This change in the pattern of proteolytic fragments is the first physical evidence suggesting that antagonist and high affinity agonist binding to the A1 receptor-binding subunit result in distinct receptor conformations that are reflected in changing proteolytic site availability.

The same analysis can be applied to the peptide maps generated with elastase (Fig. 5). In this case, the appearance of two unique bands (bands B and C) in the antagonist lane (lane 1) indicate, by analogy, that either antagonist binding makes two elastase proteolytic sites available or agonist binding suppresses the availability of these sites. The presence of a faint 15-kDa fragment in lane 2 suggests that agonist binding may incompletely suppress the availability of that elastase cleavage site. The 26-kDa fragment in the incorporated antagonist-labeled lane (lane 3) appears unchanged from lane 1. The 15-kDa band may be slightly decreased in lane 3, but is clearly present in the agonist-labeled lane (lane 4) suggesting that washing and photoincorporation increased the availability of the agonist-labeled site more than the antagonist-labeled site. These findings complement the results of
the chymotrypsin digestion in that the former suggested an agonist-induced conformational increase in the availability of a proteolytic site, while the latter suggests an agonist-induced conformational decrease in a proteolytic site’s availability. The denatured digestions in lanes 5 and 6 show a loss of the 26-kDa fragment (possibly due to a more complete digestion) but the 15-kDa fragments show the same relative intensities seen in lanes 3 and 4. It appears then, that denaturing the labeled receptor does not have a significant detectable effect on the availability of the elastase cleavage sites responsible for the production of the 15-kDa fragments.

The time course studies provide further information on receptor binding. It can be appreciated in Fig. 6 that the intensity (and consequently the amount) of all three labeled fragments decreases with increasing digestion times. As the 38-kDa band decreases, we do not observe an increase in either the 28- or 15-kDa fragments and both these fragments are present at the earliest time point. If we consider the fact that enzymes such as chymotrypsin and elastase can cleave several different combinations of amino acid pairs, it follows that the enzymes may have different efficiencies at each amino acid pair. The chymotrypsin digestion results (Fig. 6) can be interpreted as demonstrating that the agonist induces a conformational state such that two sites are rapidly and efficiently cleaved by chymotrypsin resulting in the rapid formation of the major 28- and 15-kDa fragments. These fragments (as well as the remaining 38-kDa band) are then degraded by cleavage at other “less efficient” sites to yield fragments that are too small to be resolved from the ion front and resulting in the observed parallel decrease in the 28- and 15-kDa fragments. It should be remembered also that we are only detecting the peptide fragments that contain the incorporated radioactive probe with this technique.

Can we go on to localize the site of ligand binding and incorporation into the receptor? At this point, it is not possible, but since the two-dimensional peptide maps for Staphylococcus V8 and chymotrypsin are similar for all three probes, it seems likely that the site(s) of covalent photoincorporation of the nitrenes are similar for all three ligands used in this study. Furthermore, since the distance between the nitrene and the presumed binding region (adenine ring for agonists and xanthine ring for antagonists) are small in comparison to the length of the polypeptide chain we can argue that they all may bind in the same location or “pocket.”

In conclusion, we have demonstrated that these agonist and antagonist photoaffinity ligands label the same molecular weight polypeptide of the A<sub>1</sub> receptor and appear to incorporate into the receptor in similar if not identical domains. Furthermore, the simple act of binding of a ligand to the A<sub>1</sub> adenosine receptor appears to induce conformational changes that are different for agonists and antagonists as shown by peptide mapping. This is the first physical evidence suggesting that agonists and antagonists are associated with distinct conformations of the membrane bound A<sub>1</sub> receptor and may serve as a basis for understanding the differences in agonist and antagonist binding behavior.

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FIG. 1. Autoradiograph of photoaffinity labeled A₁ adenosine receptor-binding subunit

Four-ml aliquots of the bovine brain membranes were photoaffinity labeled as described under “Experimental Procedures” section in both the presence (denoted by + in lanes 2, 4, and 6) and absence (denoted by - in lanes 1, 3, and 5) of $10^{-5}$ M R-PIA. The suspension was then photolysed, solubilized, and subjected to electrophoresis on a 16% polyacrylamide gel. Approximately 130 μg of protein was loaded in each lane. Both antagonist probes ($^{125}$I-PAPAXAC-SANPAH and azido-$^{125}$I-PAPAXAC) as well as the agonist probe ($^{125}$I- AZPNEA) labeled the same specific band with an apparent molecular mass of 38 kDa. The higher molecular mass band (~50 kDa) seen in the AZPNEA lanes is a nonspecific band that is discussed more fully in the text. This labeling is representative of multiple experiments.
FIG. 2. Partial peptide map of the $\alpha_1$ adenosine receptor binding subunit following proteolysis with *Staphylococcus* V8 protease

Approximately 250 $\mu$g of protein was used in each lane of the first dimension SDS-PAGE. The specifically labeled 38-kDa band was excised from the 12% separating gel, digested with 100 $\mu$g of *Staphylococcus* V8 protease and subjected to electrophoresis in a second dimension on an 18% polyacrylamide gel. The peptide fragments generated from the antagonist ($^{125}$I-PAPAXAC-SANPAH, azido-$^{125}$I-PAPAXAC) and agonist ($^{125}$I-AZPNEA) probes are labeled A-F and were typical of the results seen in four experiments.
FIG. 3. Partial peptide map of the A<sub>1</sub> adenosine receptor binding subunit after digestion with chymotrypsin

Approximately 250 μg of protein were used in each lane of the first dimension SDS-PAGE. The specifically labeled 38-kDa band was then excised from that gel, digested with 12 mg of chymotrypsin, and subjected to electrophoresis in a second dimension on an 18% polyacrylamide gel. The peptide fragments generated from the antagonist (¹²⁵I-PAPAXAC-SANPAH, azido-¹²⁵I-PAPAXAC) and agonist (¹²⁵I-AZPNEA) probes are labeled A-I and were typical of the results seen in three experiments.
FIG. 4. Autoradiograph of the native digestion products following digestion of the A1 adenosine receptor-binding subunit with chymotrypsin

Lanes 1, 3, and 5 are labeled with the antagonist ligand ¹²⁵I-PAPAXAC-SANPAH while lanes 2, 4, and 6 are labeled with the agonist ligand ¹²⁵I-AZPNEA. The samples were prepared as outlined under “Experimental Procedures” for the bound, incorporated, and denatured digestions using 1.5 mg of chymotrypsin and contain approximately 130 μg of protein/lane. Electrophoresis was performed in a 16% polyacrylamide separating gel. The digestion products are labeled A-D on the left and the relative molecular weight markers (M_r) are shown on the right. These results are representative of four experiments.
FIG. 5. Autoradiograph of the native digestion products following digestion of the A1 adenosine receptor-binding subunit with elastase

Lanes 1, 3, and 5 are labeled with the antagonist ligand $^{125}$I-PAPAXAC-SANPAH while lanes 2, 4, and 6 are labeled with the agonist ligand $^{125}$I-AZPNEA. The samples were prepared as outlined under “Experimental Procedures” for the bound, incorporated, and denatured digestions using 1.5 mg of elastase and contain approximately 130 μg of protein/lane. Electrophoresis was performed in a 16% polyacrylamide separating gel. The digestion products are labeled A-C on the left and the relative molecular weight markers ($M_r$) are shown on the right. These results are representative of three experiments.
FIG. 6. Autoradiograph of the time course of chymotrypsin proteolysis of the agonist (¹²⁵I-AZPNEA)-labeled A₁-binding subunit
Membranes were labeled as outlined for bound digestions, and 4-ml aliquots were incubated with 1.5 mg of chymotrypsin for 5, 10, 20, 45, and 60 min. The digestion products are labeled A-C on the left with the relative molecular weights (Mr) indicated on the right. Lane 1 shows the undigested receptor (time 0). The dye front (DF) is shown at the bottom of the figure. This experiment was repeated twice with similar results.
FIG. 7. Autoradiograph of the time course of elastase proteolysis of the agonist $^{125}$I-PAPAXACSANPAH labeled $A_1$-binding subunit
Membranes were labeled as outlined for bound digestions and 4-ml aliquots were incubated with 1.5 mg of elastase for 5, 10, 20, 45, and 60 min. The digestion products are labeled A-C on the left with the relative molecular weights ($M_r$) indicated on the right. The dye front ($DF$) is shown at the bottom of the figure. These results are typical of two experiments.