0026-895X/88/06724-05$02.00/0

MOLECULAR PHARMACOLOGY, 34:724-728

ACCELERATED COMMUNICATION

High Affinity Acylating Antagonists for the A_1 Adenosine Receptor: Identification of Binding Subunit

GARY L. STILES and KENNETH A. JACOBSON

Departments of Biochemistry and Medicine, Duke University Medical Center, Durham, North Carolina 27710 (G.L.S.) and Laboratory of Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 (K.A.J.)

Received August 5, 1988; Accepted September 7, 1988

SUMMARY

Two isomeric isothiocyanate derivatives of the A_1 adenosine receptor antagonist xanthine amine cogener (XAC) have been synthesized and found to be potent affinity labels (irreversibly bound ligands) for A_1 adenosine receptors. The interaction of *m*- and *p*-isomers of 1,3-dipropyl-8-isothiocyanatophenyl(aminothiocarbonyl(2-aminoethylaminocarbonyl(4-methyloxy(phenyl)))))-xanthine (DITC-XAC) with rat brain A_1 receptors is of high affinity (EC_{50} = 27 and 52 nM, respectively) as determined by radioligand competition curves. These compounds reduced the number of A_1 receptors (>90% at 500 nM *m*-DITC-XAC) in brain membranes, without any change in the affinity of the remaining receptors for [^{125}I]N^6-2-(4-aminophenyl)ethyladenosine. Prior reaction of the isothiocyanate moiety with ethylenediamine did not alter the affinity of the XAC derivative for the A_1 receptor but eliminated its ability to covalently incorporate into the receptor. Incubation of brain membranes with radiolabeled *p*- and *m*-DITC-XAC results in the specific labeling of a M_r 38,000 peptide. This labeling can be blocked with both an A_1 adenosine receptor-specific agonist and an antagonist. This specific protein has the same molecular weight as the protein labeled with A_1-selective photoaffinity probes. The much higher efficiency of incorporation of these affinity probes compared with photoaffinity probes should make them extremely useful for structural studies of A_1 adenosine receptors.

In the last several years, the development of reversible and photoaffinity radioligands of high affinity for the A_1AR has advanced knowledge concerning the structure and function of these physiologically important receptors. Photoaffinity probes have been utilized to define (a) the receptor binding subunit using both agonist and antagonist ligands (1, 2); (b) the glycoprotein nature of the A_1AR (3); (c) the peptide structure of A_1AR from a variety of tissues in direct comparisons by partial peptide mapping techniques (3); (d) the presence of spare A_1ARs (4); and (e) potential differences in receptors following pathophysiological regulation of the A_1AR (5, 6).

A major drawback to photoaffinity/photoaffinity cross-linking radioligands is the relative inefficiency of their incorporation into the receptor. For the cross-linking process, the efficiency is ~0.1–3.0% (7) whereas for direct photoaffinity probes using aryl azides, incorporation in the range of ~20% is achievable (7). However, for an agent to be useful for complete structural studies, it is necessary to use direct affinity probes containing chemically reactive groups, such as a bromoacetyl moiety or an isothiocyanate group, which can directly couple covalently with a nucleophilic residue such as an amino or thiol group of a protein. A useful affinity probe must contain a highly reactive chemical group (while maintaining high affinity and selectivity) and be amenable to radiolabeling to high specific activity with either tritium or radioiodine. A very useful approach to the design of adenosine receptor ligands has been that of "functionalized congeners" such as XAC, which can serve as intermediates for the synthesis of biologically active conjugates (8, 9).

In this manuscript, we report the successful synthesis and utility of a prototypical affinity label for the A_1AR. The non-radiolabeled compound can covalently incorporate into the

G.L.S. is an Established Investigator of the American Heart Association and is supported by National Heart, Lung and Blood Institute Grant RO1HL35134 and Supplement and Grant-in-Aid 880662 from the American Heart Association, with funds contributed in part by the North Carolina Affiliate.

ABBREVIATIONS: A_1AR; A_1 adenosine receptor; XAC, xanthine amine cogener 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; DITC-XAC, 1,3-dipropyl-8-(isothiocyanatophenyl(aminothiocarbonyl(2-aminoethylaminocarbonyl(4-methyloxy(phenyl)))))xanthine; APNEA, N^6-aminophenylethyladenosine; IBMX, 3-isobutyl-1-methylxanthine; CHAPS, 3-[(3-cholamide propyl)dimethylammonio]-1-propane sulfonate; *R*-PIA, *R*-N^6-phenylisopropyladenosine; DMSO, dimethyl sulfoxide; PAPAXAC, 8-[4-[[[[2-(4-aminophenyl acetylamino)ethyl]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ED-*p*-DITC-XAC, 1,3-dipropyl-8-(2-aminoethylaminothiocarbonyl(4-aminophenyl)(aminothiocarbonyl(2-aminoethylaminocarbonyl(4-methyloxy(phenyl)))))(xanthine; *p*-DITC, 1,4-phenylenediisothiocyanate; *m*-DITC, 1,3-phenylenedisothiiocyanate; [^{125}I] AZPNEA, [^{125}I]-N^6-2-(4-azidophenyl)ethyladenosine.

A_1AR with high efficiency (>75%) to block subsequent radioligand binding. In addition, the tritiated form can be used to identify the binding subunit of the A_1AR in membranes by fluororadiography.

Methods

^{125}I-APNEA was synthesized as described previously (1). Na^{125}I was purchased from Amersham (Arlington Heights, IL). *p*-DITC was purchased from Fluka (Buchs, Switzerland). Sprague-Dawley rats were from Charles River (Wilmington, MA) and bovine brain was from a local abattoir. [^{3}H]XAC (8) and 2,5-diphenyloxazole were from NEN Research Products (Boston, MA). Electrophoresis reagents were from BioRad (Richmond, CA).

Synthesis of *m*-DITC. A modification of the procedure of Newman *et al.* (10) was used. 1,3-Phenylenediamine (4.0 g, 37 mmol; Fluka) was dissolved in 300 ml of chloroform. Water (100 ml) and sodium bicarbonate (8.0 g, 95 mmol) were added and the mixture was stirred vigorously. After 10 min, 8.0 ml (104 mmol) of freshly distilled thiophosgene was added. After 1 hr the phases were separated, and the organic layer was dried (Na_2SO_4) and evaporated. The residue was recrystallized from petroleum ether and then from acetonitrile. The product was isolated as a white solid (5.6 g, 79% yield), melting at 50.5–51°.

Preparation of *m*-DITC-XAC. 1,3-Phenylenediisothiocyanate (0.2 g, 1.04 mmol) was dissolved in 15 ml of dimethylformamide in a glass container. XAC (8) (0.2 g, 47 mmol) was added in portions while the mixture was stirred. After 1 hr the solution that formed was filtered through neutral alumina. Dry ether (50 ml) and petroleum ether (50 ml) were added, and two layers formed. The upper layer was removed and ether and petroleum ether were added to the lower layer. After the glass was scratched with a glass rod, a precipitate was formed slowly. The solid was filtered, washed with ether, and dried under a vacuum, providing 0.18 g (62% yield) of product, *m*-DITC-XAC, m.p. 186–188°. UV absorption peaks in methanol were at 248, 272, and 310 nm. Analysis for $C_{29}H_{32}N_8O_4S_2 \cdot 0.5\ H_2O$: calculated, 55.31% C, 5.28% H, 17.79% N; found, 55.06% C, 5.50% H, 18.16% N. A similar procedure was used for the synthesis of *p*-DITC-XAC, obtained in 56% yield.

The isomers of DITC-XAC were shown by thin layer chromatography (silica, chloroform/methanol/acetic acid, 85:10:5) to be quantitatively reactive towards primary amines, such as ethylenediamine, in molar excess (for which R_F-values of 0.87 and 0.33 were obtained for *p*-DITC-XAC and product, respectively). The isomers of DITC-XAC are stable to storage at −20°.

NMR spectrum of *p*-DITC-XAC in DMSO-d_6:δ 9.79 (NH), 8.31 (t, 1H, NH), 8.07 (d, 2H, 8-Ar, C-2 and C-6, J = 8.8 Hz), 7.95 (1H, NH), 7.50 (d, 2H, NH-Ar, C-3 and C-5, J = 8.8 Hz), 7.33 (d, 2H, NH-Ar, C-2 and C-6, J = 8.7 Hz), 7.10 (d, 2H, 8-Ar, C-3 and C-5, J = 8.8 Hz), 4.57 (s, 2H, CH_2O), 4.02 and 3.87 (each t, C-1 Pr, 3.62 (m, 2H, C-2 Et), 3.4 (2H, C-1 Et), 1.74 and 1.58 (each m, 2H, C-2 Pr), 0.89 (q, 6H, C-3 Pr).

Synthesis of the tritiated forms of DITC-XAC was using a similar procedure, scaled down to a solution of 25 μCi of [^{3}H]XAC in 50 μl of ethanol/DMSO (50:50) to which was added a 1000-fold excess of *m*- or *p*-DITC in DMSO. The mixture was incubated at 25° for 2 hr and used directly for labeling studies. For all labeling experiments, the tritiated forms of *p*- and *m*-DITC-XAC were made immediately before use.

Synthesis of ED-*p*-DITC-XAC. *p*-DITC-XAC (22 mg, 35 μmol) was dissolved in a minimum of dimethylformamide and treated with ethylenediamine (24 μl, 0.35 mmol). The solvent was evaporated under a stream of nitrogen with gentle heating on a steam bath, and ether was added to form a solid. The residue was recrystallized from dimethylformamide/ether. The solid was filtered, washed with ether, and dried *in vacuo*, providing 18 mg (75% yield) of product, ED-*p*-DITC-XAC, which melted at 148°. The NMR spectrum was consistent with the assigned structure.

Membrane preparation. Rat cerebral cortex and bovine cerebral cortex membranes were prepared as previously described (1, 7). Membranes were treated with adenosine deaminase (0.5 units/ml) for 20 min at 37° before radioligand binding assays or incorporation studies.

Radioligand binding assay. Membranes (40 μg of protein, 150 μl) were incubated for 1 hr at 37° in a total volume of 250 μl, containing 50 μl of radioligand at the indicated concentration and 50 μl of competing ligand. *p*-DITC-XAC and *m*-DITC-XAC were weighed out just before use and dissolved in DMSO and then diluted in H_2O. Bound and free radioligand were separated by addition of 4 ml of 50 mM Tris/10 mM $MgCl_2$/1 mM EDTA, pH 8.26 at 5° (buffer A) with 0.02% CHAPS, followed by vacuum filtration on glass filters with additional washes totaling 12 ml of buffer. Filters were counted in a γ counter at an efficiency of 75%. Nonspecific binding was defined with 10^{-5} M *R*-PIA.

Saturation and competition binding data were analyzed using computer modeling programs as previously described (11, 12).

Protocols for incorporation of nonradioactive compounds. Membranes were prepared as described above and then incubated with the indicated concentration of ligands for 45 min at 37°. Initial experiments demonstrated that complete incorporation had occurred by 30 min (data not shown). Membranes were then washed three times by sequential resuspension and centrifugation with buffer A containing 0.02% CHAPS. Membranes were then suspended in buffer A, containing 10^{-4} M IBMX and incubated at 25° for 18 hr. This prolonged incubation is not associated with a decrease in A_1AR binding. Membranes were then washed with buffer A twice, treated with adenosine deaminase as described above, and used in radioligand binding assays as described above.

The overnight treatment with IBMX was found to be necessary to remove all the noncovalently bound *p*- and *m*-DITC-XAC from the A_1ARS. It was found that multiple washes (up to eight) alone were insufficient to remove all the noncovalently bound compounds.

Incorporation of radiolabeled ligands. Membranes prepared as above were incubated with the indicated concentration of radiolabeled *p*- and *m*-DITC-XAC with and without competitors for 1 hr at 37°. Membranes were then washed twice with buffer A containing 0.03% CHAPS. Membranes were solubilized in 8% SDS, 25 mM Tris, pH 6.8 at 25°, 10% glycerol, 0.01% bromophenyl blue, and 5% β-mercaptoethanol. Proteins were resolved on 11% polyacrylamide gels as described by Laemmli (13). Gels were then fixed in 10% trichloroacetic acid and subjected to fluororadiography using the method described by Bonner and Laskey (14). Dried gels were then exposed to Kodak XAR-5 film at −80° for 2–7 days.

Results

Both *p*-DITC-XAC and *m*-DITC-XAC and their radioactive analogs were synthesized as potential affinity probes capable of interacting directly with the A_1AR in an irreversible covalent manner. As demonstrated in Fig. 1, these two compounds competed for specific A_1AR binding sites, as assessed by ^{125}I-APNEA binding [an A_1AR-selective radioligand (3)] to rat brain membranes. IC_{50} values of 51.8 ± 2.1 nM for *p*-DITC-XAC and 26.6 ± 0.5 nM for *m*-DITC were obtained. Also shown in Fig. 1 is the competition curve for ED-*p*-DITC-XAC, which is a nonreactive derivative of *p*-DITC-XAC in which the isothiocyanate group has been inactivated by reaction with ethylenediamine. This compound demonstrates an IC_{50} of 37.1 ± 5.5 nM. For the first two ligands, the IC_{50} values represent essentially nonequilibrium conditions, because the ligands interact with receptor in a nonreversible manner and the values are for the specific conditions described. The apparent potency of an irreversible ligand will increase with time (15).

In order to demonstrate the covalent incorporation of these putative affinity probes into the rat brain A_1AR, membranes were incubated with a range of concentrations of these ligands.

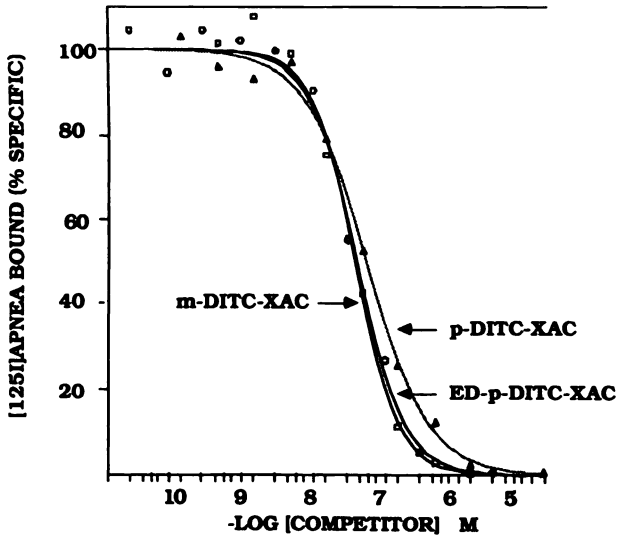

Fig. 1. ^{125}I-APNEA competition curves of XAC derivatives for A_1AR. Rat brain membranes were prepared as described in Methods. Radioligand binding was performed using 0.2 nM ^{125}I-APNEA and the indicated concentration of competing ligand for 1 hr at 37°. Binding in the absence of competitor was 0.02 nM. Each curve was replicated two to five times. The curves shown are representative. The curves were modeled using a four-parameter logistic equation (11)

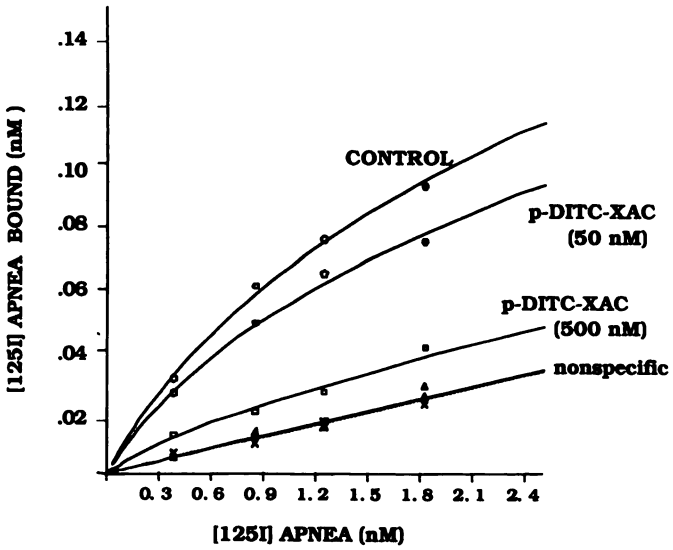

Fig. 2. Inactivation of A_1AR by *p*-DITC-XAC. Rat cerebral cortex membranes were incubated with the indicated concentration of *p*-DITC-XAC or vehicle (*CONTROL*) for 45 min at 37° and then washed extensively as described in Methods. Membranes were subjected to saturation curve analysis with increasing concentrations of ^{125}I-APNEA (similar results were obtained with [^{3}H]XAC as the radioligand). Nonspecific binding was determined with 10^{-6} M *R*-PIA and was identical in control and *p*-DITC-XAC-treated membranes. These are representative curves, which were replicated three or four times. Data were analyzed using a nonlinear least squares curve fitting program (12).

The membranes were then washed extensively, as described in Methods, and subjected to radioligand binding. As shown in Fig. 2, control membranes bound ^{125}I-APNEA with a K_D of 0.7 ± 0.3 nM and specific binding of 0.12 ± 0.02 nM. Membranes incubated with 50 nM and 500 nM *p*-DITC-XAC demonstrated a decrease in specific binding (B_{max}) by 26% and 85%, respectively. There was no change in the K_D for ^{125}I-APNEA under these conditions, suggesting that these ligands are interacting in an irreversible manner.

As further controls, separate aliquots of membranes were incubated with 100 nM XAC or 100 nM ED-*p*-DITC-XAC under exactly the same conditions and subjected to the same wash procedures. Under these conditions, there was no decrease in specific binding compared with membranes incubated in the absence of XAC derivatives (data not shown).

In a similar manner, the effects of *m*-DITC-XAC on A_1AR specific binding are shown in Fig. 3. Under the conditions described, ^{125}I-APNEA bound with a K_D of 1.5 ± 0.3 nM in control membranes, with specific binding of 0.16 ± 0.02 nM. At a concentration of 500 nM, *m*-DITC-XAC specific binding decreased by 92%, with no change in the K_D for ^{125}I-APNEA. A summary of the inactivation of the A_1AR by these compounds at different concentrations is shown in Table 1.

As further confirmation that these A_1AR affinity ligands were interacting with the same A_1AR binding subunit previously identified by photoaffinity labeling using iodinated agonist and antagonist photoaffinity probes (1, 2, 4, 7), we prepared tritiated analogs of *p*- and *m*-DITC-XAC and incorporated them into the A_1AR of cerebral cortex membranes. We have previously demonstrated that the A_1AR binding subunit resides on a M_r 38,000 protein (1, 2). After incubation of membranes with the ^{3}H-labeled affinity probe either in the absence or presence of competing ligand for 1 hr at 37°, the membranes were washed, solubilized, and separated by SDS-PAGE. Covalently labeled proteins were then detected by fluororadiography. As can be seen in Fig. 4, the radioactive affinity ligand covalently incorporated into a large number of proteins, as would be expected from its ability to react chemically with amino or thiol groups. However, of great importance is the fact that incorporation into a M_r 38,000 protein is specifically blocked by inclusion of either XAC at 10^{-6} M or (*R*)-PIA at 10^{-6} M. This specifically labeled protein is precisely the same molecular weight protein labeled by both ^{125}I-AZPNEA and ^{125}I-PAPAXAC (2, 7). These data, therefore, confirm that these two affinity probes not only inactivate the receptor, as determined by the inability of an A_1AR-specific radioligand to bind to the A_1AR after exposure to these affinity probes, but also interact with the same A_1AR binding subunit as previously described by photoaffinity/photoaffinity cross-linking probes.

Discussion

This paper describes the pharmacological and biochemical properties of two potent affinity ligands (*p*- and *m*-DITC-XAC) for the A_1AR. These xanthine isothiocyanates interact with the A_1AR of rat brain membranes with affinities in the 20–50 nM range and with the A_1AR of bovine brain membranes with approximately 10 times higher affinity (data not shown). This interaction is nonreversible and covalent in nature. It is of interest that *m*-DITC-XAC is of slightly higher affinity and incorporates with greater efficiency than *p*-DITC-XAC (see Table 1 and Fig. 2 and 3).

The inactivation of the A_1AR by covalent incorporation of these ligands is supported by many findings presented here. First, incubation of rat cerebral cortex membranes with increasing concentrations of xanthine isothiocyanates followed by extensive washing and subsequent radioligand binding demonstrates a dose-dependent decrease in A_1AR binding with no alteration in the affinity of the remaining receptors for the

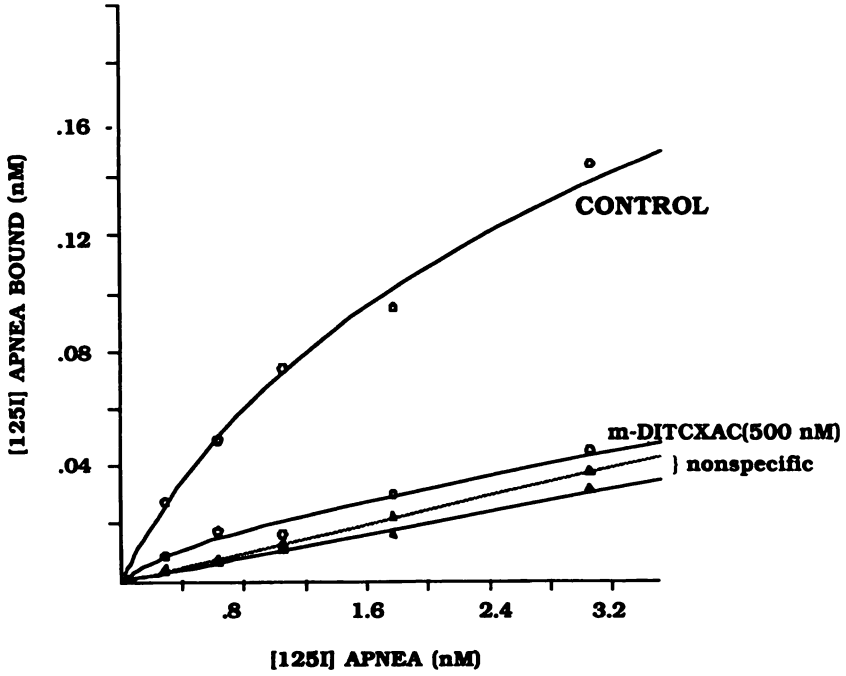

Fig. 3. Inactivation of A_1AR by *m*-DITC-XAC. Cerebral cortex membrane preparation, incubation conditions, and radioligand binding were exactly as described in the legend to Fig. 2. This is a representative experiment of three similar experiments. The lower nonspecific curve is paired with the *m*-DITC-XAC treated membranes.

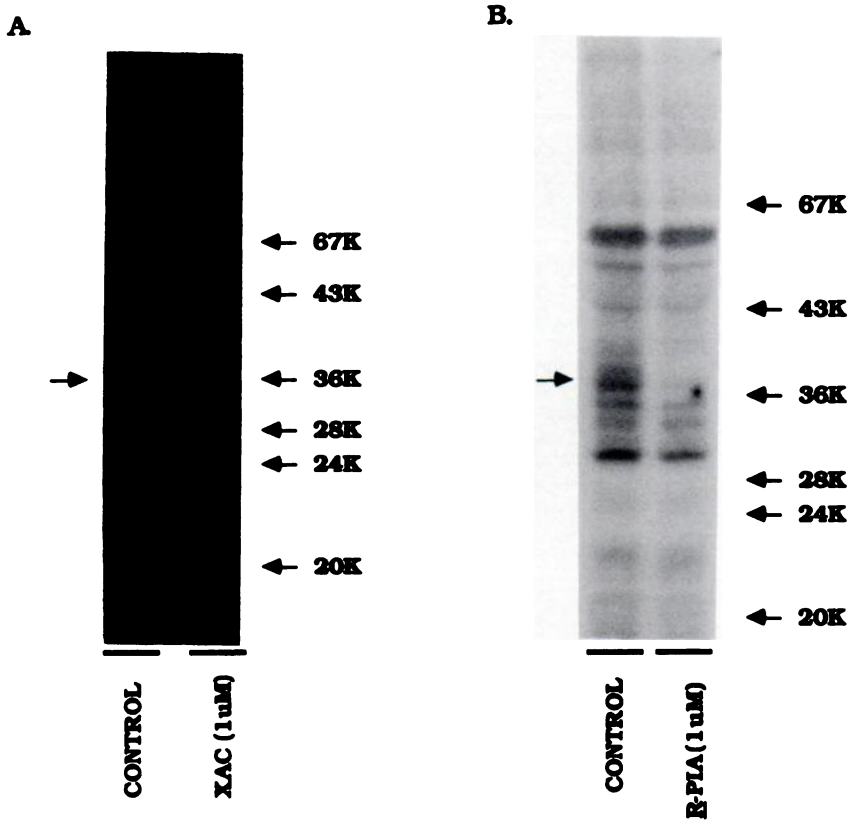

Fig. 4. Incorporation of [^{3}H]*p*- and *m*-DITC-XAC in the A_1AR of cerebral cortex membranes. Cerebral cortex membranes were incubated with ~20 nM [^{3}H]*p*- or *m*-DITC-XAC alone or with the indicated concentration of *R*-PIA or XAC for 45 min at 37°. The membranes were then washed twice with buffer A containing 0.03% CHAPS with intervening centrifugations. The membranes were then solubilized in SDS buffer and subjected to PAGE and fluororadiography as described by Bonner and Laskey (14). A, Membranes labeled with [^{3}H]*p*-DITC-XAC in the presence and absence of XAC (10^{-6} M). B, Membranes labeled with [^{3}H]*m*-DITC-XAC in the presence and absence of 10^{-6} M *R*-PIA. Covalent labeling experiments were repeated two to five times.

radioligand. The prolonged washing procedure and incubation with a noncovalent A_1AR antagonist such as IBMX was necessitated because it is difficult to remove high affinity, hydrophobic ligands from the membranes. This has also been demonstrated in adrenergic receptor systems (15, 16). Second, incubation of membranes with ED-*p*-DITC-XAC, in which the isothiocyanate moiety has been rendered inactive, results in no loss of receptor number after washing and subsequent radioligand binding. It should be noted that inactivation of the isothiocyanate group does not appreciably alter the affinity of the ligand for the A_1AR. Similarly, the incubation of membranes with the high affinity antagonist XAC does not reduce A_1AR binding after similar wash procedures.

Third, and most importantly, using the tritiated form of these ligands we could demonstrate the covalent interaction of these ligands with the A_1AR binding subunit after SDS-PAGE

TABLE 1
Inactivation of A_1AR

Drug	Decrease in specific binding (B_{max})				N*
	5 nM	50 nM	100 nM	500 nM	
	%				
p-DITC-XAC	9 ± 4	29 ± 3	63 ± 2	77 ± 4	4
m-DITC-XAC	48 ± 5	67 ± 1		91 ± 5	3
ED-*p*-DITC			0		2
XAC			0		2

* Number of experiments.

and fluororadiography. We have previously demonstrated that the A_1AR of rat and bovine cerebral cortex resides on a M_r 38,000–40,000 protein (1, 2). In this study, *p*- and *m*-DITC-XAC specifically incorporated into a M_r 38,000 peptide (see Fig. 4). As would be expected with a ligand containing a highly reactive chemical group, a large number of membrane proteins are nonspecifically labeled with these compounds. This phenomenon has been seen with a variety of tritiated affinity probes such as [^{3}H]bromacetylalprenololmenthine and [^{3}H] phenoxybenzamine (16, 17). However, with the A_1AR ligands reported here only a single protein is specifically labeled, as determined with the A_1-selective antagonist XAC or the A_1-selective agonist *R*-PIA. Thus, incubation of membranes with XAC (10^{-6} M) or *R*-PIA (10^{-6} M) prevents the incorporation of *p*- and *m*-DITC-XAC into the M_r 38,000 peptide while not affecting the labeling of all the nonspecifically labeled proteins (Fig.4).

The covalent coupling of both *p*- and *m*-DITC-XAC to the A_1AR appears to be efficient because, at xanthine concentrations of 500 nM, approximately 70% and greater than 90%, respectively, of the receptors are inactivated. This highly efficient incorporation process should make these compounds exceptionally useful in studies of receptor turnover and structure-function relationships and for use in the purification of the receptor.

Acknowledgments

The authors would like to express their thanks for the technical skills of Mary Pound and to Dr. Vickram Ramkumar for his help and to Linda Scherich for her excellent secretarial skills.

References

1. Stiles, G. L., D. T. Daly, and R. A. Olsson. The A_1 adenosine receptor: identification of the binding subunit by photoaffinity crosslinking. *J. Biol. Chem.* **260**:10806–10811 (1985).
2. Stiles, G. L., and K. A. Jacobson. A new high affinity iodinated adenosine receptor antagonist as a radioligand/photoaffinity crosslinking probe. *Mol. Pharmacol.* **32**:184–188 (1987).
3. Stiles, G. L. Photoaffinity crosslinked A_1 adenosine receptor binding subunits: homologous glycoprotein expression by different tissues. *J. Biol. Chem.* **261**:10839–10843 (1986).
4. Lohse, M. J., K. Klotz, and U. Schwabe. Agonist photoaffinity labeling of A_1 adenosine receptors: persistent activation reveals spare receptors. *Mol. Pharmacol.* **30**:403–409 (1986).
5. Green, R. M., and G. L. Stiles. Chronic caffeine ingestion sensitizes the A_1 adenosine receptor-adenylate cyclase systems in rat cerebral cortex. *J. Clin. Invest.* **77**:222–227 (1986).
6. Parsons, W. J., and G. L. Stiles. Heterologous desensitization of the inhibitory A_1 adenosine receptor-adenylate cyclase system in rat adipocytes: regulation of N_s and N_i. *J. Biol. Chem.* **262**:841–847 (1987).
7. Stiles, G. L., D. T. Daly, and R. A. Olsson. Characterization of the A_1 adenosine receptor-adenylate cyclase system of cerebral cortex using an agonist photoaffinity probe. *J. Neurochem.* **47**:1020–1025 (1986).
8. Jacobson, K. A., D. Ukena, K. L. Kirk, and J. W. Daly. A [^{3}H]xanthine amine congener of 1,3-dipropyl-8-phenylxanthine: an antagonist radioligand for adenosine receptors. *Proc. Natl. Acad. Sci. USA* **83**:4067–4093 (1986).
9. Jacobson, K. A., D. Ukena, W. Padgett, K. L. Kirk, and J. W. Daly. Molecular probes for extracellular adenosine receptors. *Biochem. Pharmacol.* **36**:1697–1708 (1987).
10. Newman, A. H., H. W. M. Lueddens, P. Skolnick, and K. C. Rice. Novel irreversible ligands specific for "peripheral" type benzodiazepine receptors. *J. Med. Chem.* **30**:1901–1904 (1987).
11. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E106 (1978).
12. DeLean, A., A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modelling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–13 (1982).
13. Laemlli, U. K. Cleavage of structural protein, during the assembly of the head of bacteria phase T_4. *Nature (Lond.)* **277**:680–682 (1970).
14. Bonner, W. M., and R. A. Laskey. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83–88 (1974).
15. Dickinson, K. E. J., S. L. Heald, P. W. Jeffs, R. J. Lefkowitz, and M. G. Caron. Covalent labeling of the β-adrenergic ligand-binding site with *para*-(bromoacetylamidyl)benzylcarazolol: a highly potent β-adrenergic affinity label. *Mol. Pharmacol.* **27**:499–506 (1985).
16. Pitha, J., J. Zjawlony, N. Nasria, R. J. Lefkowitz, and M. G. Caron. Potent *beta*-adrenergic antagonist possessing chemically reactive group. *Life Sci.* **27**:1791–1798 (1980).
17. Regan, J. W., R. M. DeMarinis, M. G. Caron, and R. J. Lefkowitz. Identification of the subunit binding site of α_2-adrenergic receptors using [^{3}H] phenoxybenzamine. *J. Biol. Chem.* **259**:7864–7869 (1984).

Send reprint requests to: Gary L. Stiles, Box 3444, Departments of Biochemistry and Medicine, Duke University Medical Center, Durham, NC 27710.