Photoaffinity labelling of dopamine D_2 receptors by [^{3}H]azidomethylspiperone

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We have characterized the dopamine D_2 receptor photaffinity probe, [^{3}H]azido-*N*-methylspiperone ([^{3}H]AMS). In the absence of light, [^{3}H]AMS bound reversibly and with high affinity (K_d 70 pM) to sites in canine striatal membranes and was competitively inhibited by dopaminergic agonists and antagonists with an appropriate D_2 receptor specificity. Upon photolysis, [^{3}H]AMS covalently incorporated into a peptide of M_r 92000 as assessed by fluorography following SDS-polyacrylamide gel electrophoresis. Labelling of this peptide was specifically and stereoselectively blocked by D_2 antagonists and agonists. Minor specifically labelled peptides of M_r 70000–55000 were observed under some conditions and were the result of proteolytic degradation of the peptide at M_r 92000.

D_2 dopamine receptor *Photoaffinity labeling* *Lectin affinity chromatography* *SDS-PAGE* *Fluorography*

1. INTRODUCTION

Dopamine can stimulate (D_1 receptors) or inhibit (D_2 receptors) adenylate cyclase [1,2] and induce many psychomotor responses [3–5]. A number of specific D_2 receptor photoaffinity probes have been developed [6–8], but only recently has the ligand binding subunit of the D_2 receptor been identified [9,10]. These photoaffinity ligands, however, are not commercially available. To probe the structure of the dopamine D_2 receptor, we have employed a novel, high-affinity, commercially available, photoreactive derivative of spiperone: azido-*N*-methylspiperone [11]. We now report here that (i) [^{3}H]AMS exhibits reversible, saturable and stereospecific binding to striatal D_2 dopamine receptors and (ii) upon photolysis [^{3}H]AMS covalently labels a peptide of M_r 92000 as the ligand binding subunit of the D_2 receptor.

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Abbreviations: [^{3}H]AMS, [^{3}H]azido-*N*-methylspiperone; WGA, wheat-germ agglutinin

2. MATERIALS AND METHODS

[^{3}H]AMS (78 Ci/mmol) was synthesized by New England Nuclear (Boston, MA). M_r standards, SDS and electrophoresis reagents were obtained from Bio-Rad; protease inhibitors from Sigma [6,12,13].

2.1. *Membrane preparation and reversible binding of [^{3}H]AMS*

Canine striatal membranes were prepared and assayed for [^{3}H]AMS binding [6] at 22°C. Specific [^{3}H]AMS binding was defined as that inhibited by 10 μM *S*-sulpiride or 1 μM (+) butaclamol.

2.2. *Photoaffinity labelling and electrophoresis*

Striata were homogenized (Polytron, setting 5, 15 s) in 50 mM Tris-HCl buffer (pH 7.4) containing 4 mM $MgCl_2$, 120 mM NaCl in the absence or presence of the following protease inhibitors: 10 mM EDTA, 15 μg/ml benzamidine, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl

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fluoride at 4°C. Homogenates were centrifuged twice for 15 min at 48000 × *g* and membrane pellets resuspended in the above buffer. Membranes (1 mg protein/ml) were incubated in the dark in a total volume of 15 ml at a D_2 receptor concentration of 300 pM with 0.8–1.0 nM [^{3}H]AMS for 60 min at 22°C in the absence or presence of various agents and photolyzed [6]. Irradiated samples were pelleted by centrifugation (48000 × *g*; for 10 min), washed once, solubilized in Tris-buffer containing 1% digitonin (at 100 mg tissue/ml), and partially purified by WGA-Sepharose affinity chromatography [13]. Aliquots (0.5 ml) of specifically eluted receptor were desalted over Sephadex G-50 columns at 22°C to exchange the buffer to 0.2% SDS, 10 mM Tris-HCl, pH 6.8, lyophilized overnight and brought up to volume with sample buffer (50 mM Tris, 10% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue, pH 6.8).

Aliquots (300 μg protein) were allowed to stand for 60 min at 22°C before being electrophoresed on 3 mm-thick slab gels containing 6 and 10% acrylamide in the stacking and separating gel, respectively [14]. Gels used for fluorography were fixed, treated with Enlightning (NEN) prior to drying using a Bio-Rad (model 224) gel dryer, and then exposed (2–6 weeks) at −70°C to Kodak XAR-5 film. Fluorographs were routinely scanned using a Bio-Rad scanning densitometer (model 1650). Protein standards were visualized by Coomassie blue staining. Protein concentrations were determined as described [13].

3. RESULTS AND DISCUSSION

3.1. *[^{3}H]AMS binding to canine striatal preparations*

In the absence of light, the binding of [^{3}H]AMS (70 pM) reached equilibrium within 120 min at 22°C; the K_d from the on/off rates [12] was 16 pM (fig.1). [^{3}H]AMS binding displayed in 120 mM NaCl a K_d of 59 ± 4 pM and a B_{max} of 21 ± 2 pmol/g (fig.2). [^{3}H]AMS binding was not significantly altered in the absence of NaCl (K_d 77 ± 8 pM; B_{max} 21 ± 1 pmol/g) or in the presence of multiple protease inhibitors (K_d 56 ± 6 pM; B_{max}, 22 ± 1 pmol/g). The specific binding of [^{3}H]AMS accounted for 70–80% of total binding. Dopaminergic compounds specifically inhibited the binding of [^{3}H]AMS (70 pM) (fig.3). Agonist/[^{3}H]AMS competition curves had a Hill slope less than one and were best described by computer-assisted analysis [15] as comprising two

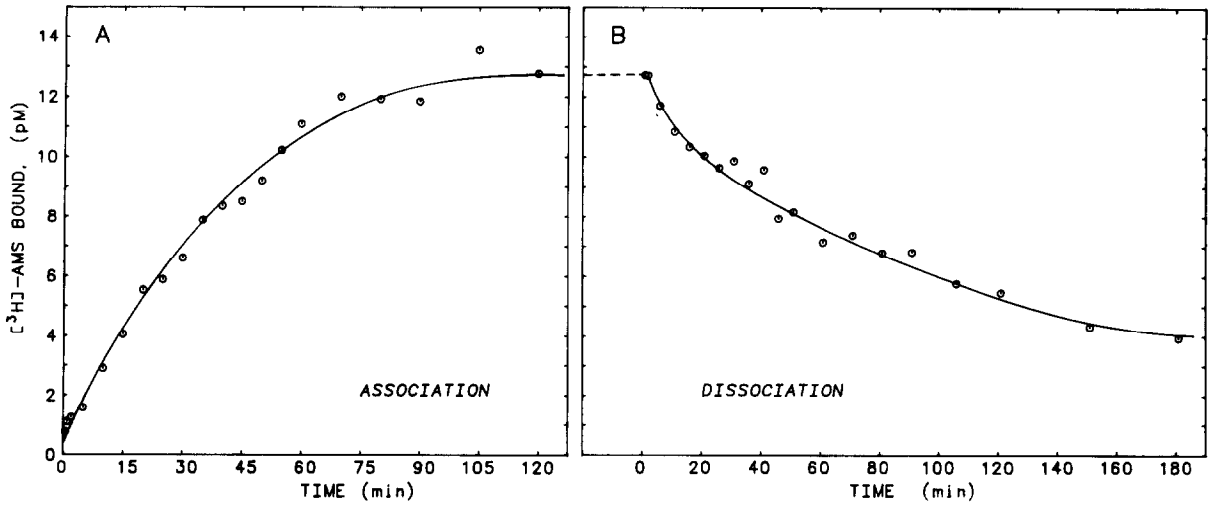

Fig.1. Kinetics of [^{3}H]AMS binding to striatal membranes. For association experiments (A), membranes were incubated in the dark with [^{3}H]AMS (70 pM) for various time periods and assayed for D_2 receptor activity. The association rate constant for [^{3}H]AMS was determined to be 4.167×10^{-4} $pM^{-1} \cdot min^{-1}$. (B) Dissociation of [^{3}H]AMS: following equilibrium, dissociation was initiated by the addition of 500 nM YM-09151-2, and the dissociation rate constant determined to be 0.006456 min^{-1}.

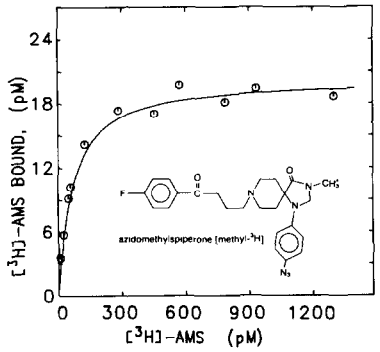

Fig.2. Saturation isotherm of [^{3}H]AMS binding to canine striatal membranes. Striatal homogenates were incubated in the dark with increasing concentrations of [^{3}H]AMS (10–1000 pM) for 120 min at 22°C and assayed for D_2 dopamine receptor activity. The data were analyzed by the non-linear least-squares computer program LIGAND. Each point is the mean obtained from triplicate determinations and is representative of three independent experiments. B_{max} and K_d values for [^{3}H]AMS binding are listed in the text. Inset: Structure of [^{3}H]AMS.

sites (table 1, where D_2^{high} and D_2^{low} represent agonist high- and low-affinity forms of the receptor). The competitive binding of [^{3}H]AMS to canine striatal membranes was virtually identical to that of the parent probe, [^{3}H]spiperone [12].

3.2. *Covalent photoincorporation of [^{3}H]AMS into D_2-receptors*

In membrane preparations [^{3}H]AMS labelled in a photo-dependent and specific manner a peptide of M_r 90000–94000, as assessed by liquid scintillation counting of gel slices following electrophoresis. However, the low amount of label incorporated into this peptide (100–300 cpm) precluded the fluorographic visualization of the receptor despite exposure times of up to six weeks. We chose to enrich [^{3}H]AMS-photolabelled membrane receptors by WGA-Sepharose affinity chromatography [16–18] (fig.4). Under these conditions [^{3}H]AMS incorporated covalently into a major protected band at M_r 92000. Moreover, one to two minor bands appear at M_r 70000–59000 that were protected by dopaminergic antagonists and the agonist (fig.4B) *N*-propylnorapomorphine (–NPA). The D_2 dopaminergic nature of these labelled peptides was also shown by the fact that

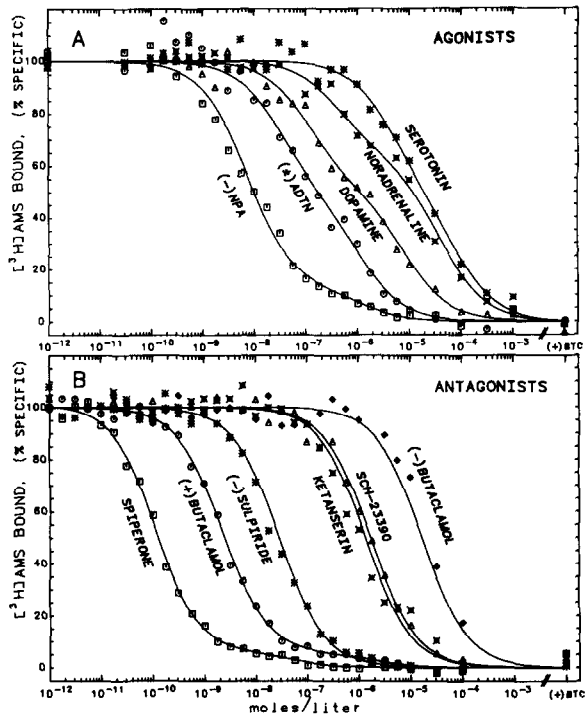

Fig.3. Ability of dopaminergic agonists (A) and antagonists (B) to compete for [^{3}H]AMS binding to striatal membranes. Increasing concentrations of dopaminergic agonists and antagonists were incubated in the dark with 70 pM [^{3}H]AMS and assayed for D_2 receptor activity. Data were analyzed for one and two site fits by the non-linear least-squares computer program LIGAND. The results shown here are the means of triplicate determinations and are representative of three independent experiments.

1 μM ketanserin, a serotonergic S_2 receptor antagonist only slightly attenuated [^{3}H]AMS photoincorporation. Neither SCH-23390 (1 μM), a dopamine D_1 receptor antagonist, nor the β-adrenergic receptor antagonist propranolol (10 μM), afforded any protection against [^{3}H]AMS photo-incorporation (not shown). Control experiments revealed that under non-photolysing conditions [^{3}H]AMS did not covalently incorporate into any peptide, and that radioactivity associated with the 92 kDa peptide could not be extracted by chloroform:methanol (3:1), indicating that it is a protein. Pre-exposure of [^{3}H]AMS to UV light did not affect its ability to bind to D_2 receptors in a reversible and saturable manner with high affinity (70 pM), satisfying most

Table 1

Agonist and antagonist dissociation constants for the D_2 dopamine receptor

Agonist	K_d (nM)		Proportion (%)		Antagonist	
	D_2^{high}	D_2^{low}	D_2^{high}	D_2^{low}		
(−)NPA	3	539	87	13	spiperone	0.050
(+)ADTN	16	532	52	48	(+)butaclamol	1.1
Dopamine	61	4047	49	51	(−)sulpiride	14.4
(+)NPA	82	1428	27	73	ketanserin	674
Noradrenaline	241	19840	38	62	SCH-23390	857
Serotonin	2292	37310	49	51	(−)butaclamol	8438

Canine striatal membranes were prepared and assayed for D_2 receptor activity as described in section 2. All data were analyzed by the computer program LIGAND for one and two site fits. Values represent the means of 3 independent determinations with a SE of <15%. NPA, 10,11-hydroxy-*N*-propylnorapomorphine; ADTN, 6,7-dihydroxy-2-aminotetralin

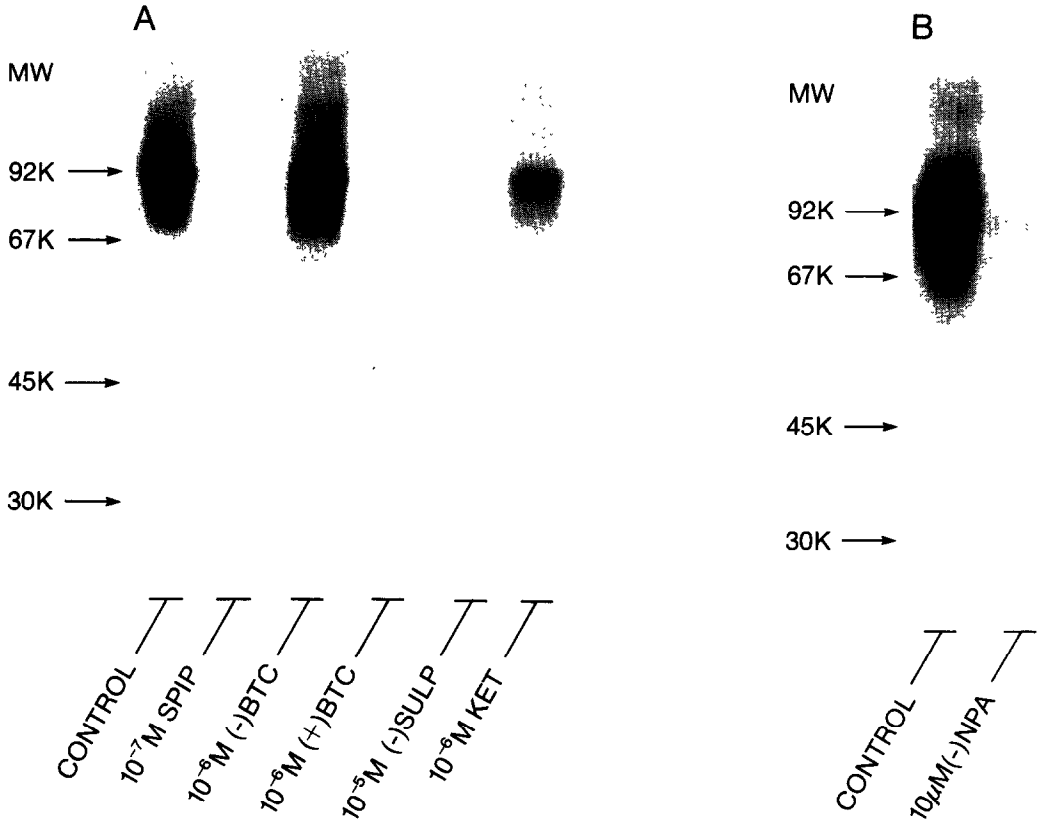

Fig.4. Photoaffinity labelling and pharmacological specificity of [^{3}H]AMS incorporation into canine striatal membranes. Striatal homogenates were incubated with [^{3}H]AMS (800–1000 pM) alone or with (A) antagonists or (B) *N*-propylnorapomorphine (−NPA), photolyzed, solubilized, applied to WGA-Sepharose and finally electrophoresed on 10% gels as described in section 2. The results shown are identical with two other experiments. Arrows represent the M_r of some known protein standards and shown × 1000 (K). SPIP, spiperone; BTC, butaclamol; SULP, sulpiride; KET, ketanserin.

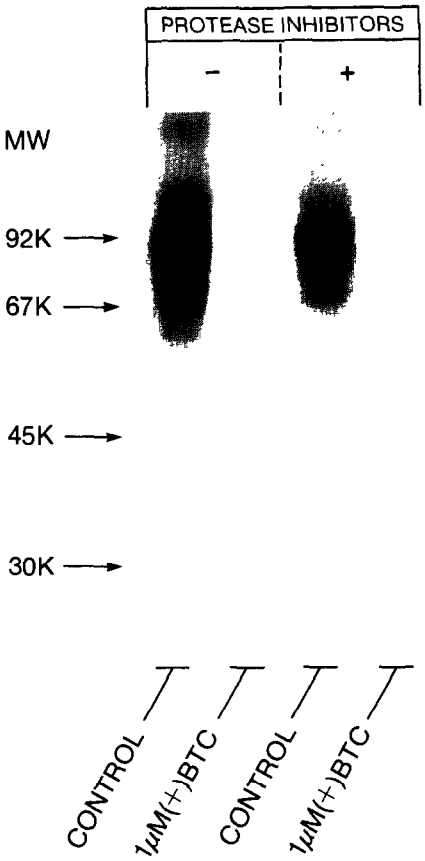

Fig.5. Effect of protease inhibitors on the photoaffinity labelling pattern of [^{3}H]AMS into canine striatal membranes. Striatal membranes were prepared either in the absence or presence of 10 mM EDTA, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 15 μg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride. Membranes were incubated with [^{3}H]AMS (1000 pM) alone and in the presence of 1 μM (+)-butaclamol, and processed for electrophoresis as described in section 2. Abbreviations used are as described in fig.4.

of the requirements of a photoaffinity label. The protein labelled by [^{3}H]AMS as the ligand binding subunit of the D_2 dopamine receptor (M_r 92000) corresponds to that recently reported [9,10,19].

Failure to inhibit endogenous proteinase activity during photoaffinity labelling may allow for the generation of peptide fragments which retain the pharmacological specificity of native receptors [20]. As seen in fig.5, photolabelling brain membrane receptors in the absence of protease inhibitors allows for the generation of multiple specifically labelled fragments with M_r values of 92000, 70000, 60000 and 55000. Photolabelling in the presence of multiple protease inhibitors, however, resulted in the labelling of a single major peptide of M_r 92000 while greatly attenuating the labelling of lower molecular-mass fragments. We conclude, therefore, that all specifically labelled peptides seen in fig.4A and B are probably the result of proteolytic degradation of the peptide at M_r 92000. The metalloprotease inhibitor, EDTA (10 mM), did not appear to prevent the appearance of multiple labelled peptides of M_r 70000–55000 (not shown).

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