A novel radioiodinated high affinity ligand for the D_2-dopamine receptor

Characterization of its binding in bovine anterior pituitary membranes

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Received 4 September 1984

A novel high affinity dopaminergic ligand, *N*-(*p*-aminophenethyl)spiroperidol, has been synthesized and radioiodinated to a specific radioactivity of 2175 Ci/mmol. Binding of this ligand to bovine anterior pituitary membranes is: (i) rapid (40–60 min to equilibrium at 25°C) and reversible ($t_{1/2}$ = 1 h at 25°C); (ii) saturable and of high affinity ($K_D \sim 20$ pM) and (iii) displays a typical D_2-dopaminergic specificity. The ligand, which identifies the same number of receptor sites as other tritiated antagonist ligands, can be used in different tissues and preparations to delineate the characteristics of the D_2 receptor. Thus, this high affinity, high specific radioactivity ligand (*N*-(*p*-amino-*m*-[^{125}I]iodophenethyl)spiroperidol) represents a tool which until now had not been available for the characterization of the D_2-dopamine receptor.

D_2-dopamine receptor *Radioiodinated ligand* *High affinity radioligand* *Anterior pituitary*

1. INTRODUCTION

Radioligands have been very powerful tools in studies aimed at elucidating the biochemical events by which hormones and neurotransmitters mediate their physiological effects as well as the molecular mechanisms by which responsiveness can be modulated. Thus, the ability to measure hormone and neurotransmitter receptors has revealed that receptors are points of dynamic regulation in target tissues [1]. However, the feasibility of such studies often depends on the availability of appropriate tools for the detection of low concentrations of these receptors in target tissues. The specific receptors which mediate the various physiological actions of dopamine in central and peripheral target tissues have been characterized extensively by direct ligand binding. These receptors have been classified into two distinct subtypes designated D_1- and D_2-dopamine receptors [2]. For the D_2-dopamine receptor, although the initial tritiated ligands were developed about a decade ago [3,4] there has been to data no high affinity, high specific radioactivity ligand available for receptor identification. The antagonist, [^{3}H]spiroperidol [5] and the agonist [^{3}H](−)*N*-propylnorapomorphine [6], two of the more widely used ligands, bind to the D_2-dopamine receptor with high affinity ($K_D \sim 50$–300 pM) [7]. However, their modest specific radioactivity (20–60 Ci/mmol) often limits their versatility as ligands.

We describe the properties of a new ligand, an analog of the antagonist spiroperidol, which can be radioiodinated to theoretical specific activity (2175 Ci/mmol) and which binds to the D_2-dopamine receptor with very high affinity ($K_D \sim 20$ pM). The properties of this ligand offer distinct advantages for the measurement of the D_2-dopamine receptor and its eventual biochemical characterization.

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2. MATERIALS AND METHODS

Materials used in these studies (drugs and radioligands) were from sources previously described [7]. Na^{125}I was obtained from New England Nuclear, Boston, MA, whereas chemicals were usually from Aldrich, Milwaukee, WI.

2.1. *Synthesis and radioiodination of the ligand*

The chemical synthesis and characterization of the newly developed compound *N*-(*p*-aminophenethyl)spiroperidol (NAPS) will be detailed elsewhere [12]. The radioiodination of NAPS was performed essentially as described previously [8] for the β-adrenergic antagonist *p*-aminobenzylcarazolol. The iodinated ligand was separated from the non-radioactive ligand by thin-layer chromatography in the solvent system methylene chloride:methanol:triethylamine (87.5:12.5:0.01) $R_{f(NAPS)}$:0.5; $R_{f([^{125}I]NAPS)}$:0.65. The [^{125}I]NAPS was eluted from the plate with ethyl acetate containing 1% triethylamine and stored at ~1 mCi/ml in ethanol, 0.01% phenol or isopropanol:water (50:50) for several weeks without chemical damage. Since carrier-free Na^{125}I was used for the radioiodination and [^{125}I]NAPS could be completely separated from unlabelled NAPS a specific radioactivity of 2175 Ci/mmol could be assumed for [^{125}I]NAPS.

2.2. *Anterior pituitary membranes*

Bovine anterior pituitary membranes were prepared essentially as in [7], resuspended in 25 mM Tris–HCl, pH 7.4 (25°C), 6 mM $MgCl_2$, 1 mM EDTA and stored frozen at −70°C until used.

2.3. *Radioligand and binding assays*

Assays were performed by incubating 100 μl of membranes containing 300–800 μg protein/ml (for [^{125}I]NAPS binding) with the appropriate ligand and competitors in a total volume of 1 ml of the resuspension buffer containing 0.1% ascorbate. After incubation for 1 h (or the indicated time) at 25°C bound ligand was separated from free ligand by filtration through GF/C glass fiber filters and washing the filters with 4 × 5 ml of 50 mM Tris–HCl, pH 7.5 (25°C), 0.1% BSA, 0.01% Triton X-100. [^{3}H]Spiroperidol binding was determined as previously described [7]. Bound ligand was determined by liquid scintillation (45% efficiency) or by counting in a gamma counter (75% efficiency). Bovine serum albumin and Triton X-100 were included in the wash buffer in order to reduce the level of nonspecific binding, contributed in large part by the interaction of the ligand with the glass fiber filter matrix.

Analysis of binding data was performed as in [7]. Protein determinations were performed by the method of [9].

3. RESULTS AND DISCUSSION

3.1. *Kinetics and optimal conditions for [^{125}I]NAPS binding*

At 25°C [^{125}I]NAPS binding to bovine anterior pituitary membranes reached equilibrium between 40 and 60 min of incubation (fig.1). Binding was completely reversible upon the addition of excess (+)-butaclamol (1 μM) with a half-time of ~60 min. The ratio of the dissociation to the association rate constants yielded an estimated dissociation constant (K_D) value for the binding of [^{125}I]NAPS to these membranes of 6–10 pM ($n = 2$). The binding of [^{125}I]NAPS to anterior pituitary membranes was found to be linear over at least a 20-fold range of protein in the assay (5–100 μg).

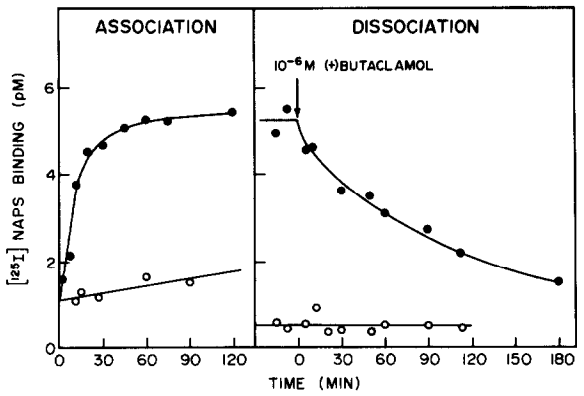

Fig.1. Kinetics of the binding of [^{125}I]NAPS to bovine anterior pituitary membranes. Membranes were incubated at 25°C as described in section 2 in the presence (○) and absence (●) of 1 μM (+)-butaclamol with 30–40 pM [^{125}I]NAPS. At the indicated times, samples were filtered through GF/C glass fiber filters to determine bound ligand. Dissociation was initiated by the addition of 1 μM (+)-butaclamol to incubation mixtures after an equilibration time of 90 min. The dissociation constant (K_D) of [^{125}I]NAPS from the ratio of the rate of dissociation and association was calculated according to [10]. Results are from 3 experiments.

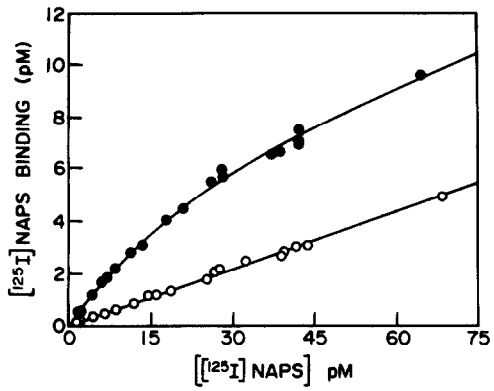

Fig.2. Saturation binding isotherms for [^{125}I]NAPS. Anterior pituitary membranes (62 μg) were incubated as described in section 2 with increasing concentrations of [^{125}I]NAPS in the presence (○) and absence (●) of 1 μM (+)-butaclamol. Incubations were carried out at 25°C for 3 h to insure equilibrium binding of the lower ligand concentrations. Total ligand concentrations were determined by sampling each incubation mixture at the end of the incubation period before separation of bound from free ligand. Results are from 3 experiments.

3.2. *Equilibrium binding of [^{125}I]NAPS*

Binding of [^{125}I]NAPS to bovine anterior pituitary membranes was saturable, as shown in fig.2. From the data shown, a K_D value of 17.1 pM (19.5 ± 2.3, $n = 3$) could be estimated, which is over an order of magnitude more potent than the reported K_D value for [^{3}H]spiroperidol in this tissue (0.3 nM) [11]. Specific binding ranged from 80–85% of total binding at low ligand concentrations to 50–60% at high ligand concentrations. In the experiment shown a B_{max} of 6.7 pM was determined for the number of sites occupied at saturation. This value corresponds to a concentration of binding sites of 52 fmol/mg and is in reasonable agreement with an estimate of 60 fmol/mg obtained using [^{3}H]spiroperidol as the ligand in the same membrane preparation.

3.3. *Specificity of the binding of [^{125}I]NAPS*

As shown in fig.3, the ability of a variety of agonists (A) and antagonists (B) to compete for the binding of [^{125}I]NAPS to bovine anterior pituitary was typical of a D_2-dopaminergic binding site. The typical order of potency NPA > ADTN ≥ APO > DA was observed for agonists. For antagonists, spiroperidol, unlabelled NAPS and (+)-butaclamol

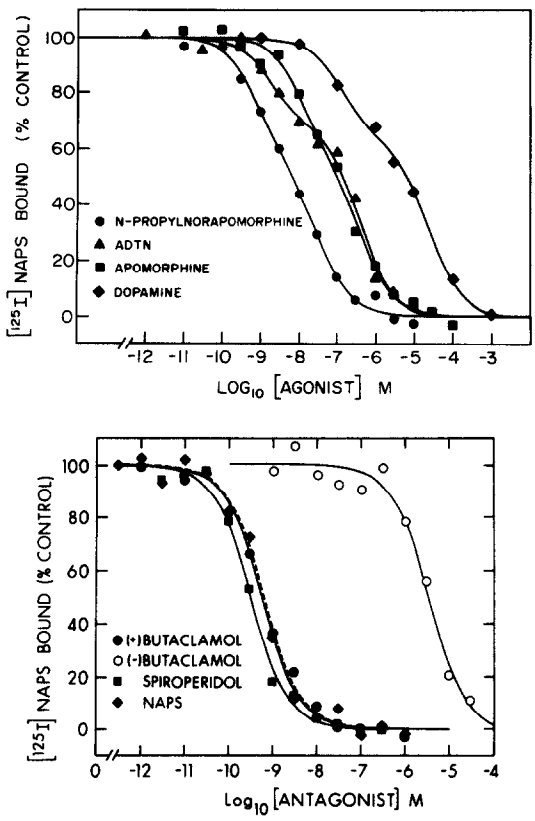

Fig.3. Competition curves of a series of dopaminergic agonists (A) and antagonists (B) for the binding of [^{125}I]NAPS to bovine anterior pituitary membranes. Membranes were incubated with ~40 pM [^{125}I]NAPS in the presence of increasing concentrations of the various agonists (A) and antagonists (B) shown. NPA, (−)-*N*-propylnorapomorphine; (±)-ADTN, (±)-2-amino-6,7-dihydroxytetrahydronaphthalene; APO, (−)-apomorphine; DA, dopamine. Binding is expressed in % of specific binding and 100% control corresponds to 4.8–6.5 pM [^{125}I]NAPS bound. Results are from 3–5 experiments.

were about equipotent whereas (−)-butaclamol was ~10 000 fold less potent than the (+) isomer of butaclamol. The estimated K_D values of these and other dopaminergic and non-dopaminergic drugs are shown in table 1. The calculated K_D values for these drugs are in good agreement with those obtained using [^{3}H]spiroperidol as the radioligand [7,11]. Note that for agonists the competition curves are shallow and model to two affinity sites, thus K_D values for the high and low agonist affinity states of the receptor have been compiled. As shown earlier using [^{3}H]spiroperidol [7,11], these biphasic

Table 1

Dissociation constants of dopaminergic and non-dopaminergic agents

Agents	Dissociation constants K_D (nM)	
	K_H	K_L
Agonists		
(−)-*N*-Propylnorapomorphine	0.43	15.8
(±)-ADTN	1.07	66.5
(−)-Apomorphine	13.4	338
Dopamine	67.2	9320
Antagonists		
(+)-Butaclamol	0.36	
(−)-Butaclamol	2620	
Spiroperidol	0.25	
NAPS	0.33	
(−)-Sulpiride	608	
Nondopaminergic antagonists		
Phentolamine	9400	
Mianserin	6200	
Cinanserin	7200	
Prazosin	5350	
Propranolol	>10000	

Dissociation constants (K_D) were estimated by quantitative analysis [7] from 8–12 points competition curves of the various agents shown for the binding of [^{125}I]NAPS to bovine anterior piruitary membranes. For agonists the curves were better fit to a two-affinity state model [7] and therefore both K_H and K_L are shown. The results represent the geometric average of 2–3 experiments performed in duplicate

agonist competition curves can be steepened and shifted to the right in the presence of guanine nucleotides. For example, in the presence of 100 μM Gpp(NH)p the competition curve of NPA for [^{125}I]NAPS binding displays a single affinity state, the K_D value of which corresponds to the value for the low affinity form of the receptor in the control curve (15–20 nM; cf., table 1) (not shown).

These findings are in excellent agreement with previous studies using [^{3}H]spiroperidol as the radioligand. Moreover, [^{125}I]NAPS has also been used in preparations such as rat striatal membranes and solubilized preparations of anterior pituitary membranes to monitor effectively D_2-dopamine receptors (not shown).

In summary, this newly developed ligand, [^{125}I]NAPS, possesses high affinity ($K_D \sim 20$ pM) for binding to the D_2-dopamine receptor. It binds to membrane preparations containing dopamine receptors with an appropriate D_2-dopaminergic specificity. In binding assays it yields a high ratio of specific to nonspecific binding. The ligand can be used in all preparations of D_2 receptors so far tested with higher affinity than [^{3}H]spiroperidol. However, the cardinal property of [^{125}I]NAPS is its high specific radioactivity (2175 Ci/mmol), which allows a 50–100-fold more sensitive detection of D_2 receptors than conventional low specific radioactivity [^{3}H]ligands. Therefore, this probe should be extremely useful in attempts at characterizing the receptor at the biochemical level.

The strategy employed in the development of this ligand was aimed at yielding a multifunctional probe for the D_2-dopamine receptor. The presence of an arylamine moiety on the molecule not only allows radioiodination of the ligand but this arylamine moiety can be easily converted to an azide [8] to yield a specific iodinated photoaffinity probe for the receptor. Moreover, the arylamine group can be easily derivatized for the purpose of affinity chromatography. Thus, these various applications should make this compound one of the most useful probes yet available for the characterization of the D_2-dopamine receptor.

ACKNOWLEDGEMENTS

This work was supported in part by grant from the National Institutes of Health NS19576. The authors would like to thank Dr Peter W. Jeffs, Department of Chemistry, Duke University for the availability of his laboratory facilities and Dr Sarah L. Heald for helpful discussions and suggestions. The technical assistance of Ms Beverly Hill and the secretarial assistance of Ms Lynn Tilley and Ms Donna Addison are greatly appreciated.

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