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Dopamine Receptor Binding on Intact Cells

Absence of a High-Affinity Agonist-Receptor Binding State

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SUMMARY

The ligand binding characteristics of D-2 dopaminergic receptors on dispersed bovine anterior pituitary cells were investigated. The binding of the radiolabeled butyrophenone antagonist, [^{3}H]spiroperidol, to intact cells was reversible, of high-affinity, and saturable, with an apparent dissociation constant (K_D) of 0.21 ± 0.05 nM and a maximal binding capacity of 9.0 ± 0.85 fmoles/10^6 cells. The stereospecificity and rank order of neuroleptic antagonists, catecholamines, and related drugs in competing for [^{3}H]spiroperidol binding were indicative of interactions with a dopamine receptor. There was no high-affinity, stereospecific, or saturable binding of the radiolabeled dopamine agonist, [^{3}H]*N*-*n*-propylnorapomorphine ([^{3}H]NPA), to these dispersed cells. In contrast, washed membranes prepared from these cells exhibited high-affinity [^{3}H]NPA binding, with a K_D of 0.25 ± 0.02 nM. Similarly, in membranes prepared from dispersed cells, agonist/[^{3}H]spiroperidol competition curves were heterogeneous, exhibiting high- and low-affinity displacement phases. In the presence of exogenous guanine nucleotides, these curves were shifted to the right and became uniphasic. Computer analysis indicated that agonist/[^{3}H]spiroperidol curves in membranes are best explained by assuming a two-state binding model, whereas in the presence of guanine nucleotides a one-state model is sufficient to fit the data. In intact cells, however, agonist/[^{3}H]spiroperidol curves were monophasic and exhibited affinities similar to the corresponding low-affinity agonist binding state seen in membranes. Addition of guanine nucleotides did not alter the agonist/[^{3}H]spiroperidol curves in intact cells. The data suggest that high-affinity dopamine agonist binding in pituitary membranes does not represent a distinct receptor subtype but rather a guanine nucleotide-sensitive, agonist-specific binding state of a single D-2 receptor. In this model, endogenous or exogenous guanine nucleotides in intact cells or membranes, respectively, mediate an interconversion from the high- to the low-affinity agonist binding state, thus effectively eliminating high-affinity agonist binding as measured directly with [^{3}H]NPA or indirectly with agonist/[^{3}H]spiroperidol competition experiments. Non-equilibrium binding experiments indicated that in the presence of guanine nucleotides the high-affinity agonist binding state is undetectable throughout the approach to equilibrium.

INTRODUCTION

Utilizing biochemical and pharmacological criteria, anterior pituitary dopamine receptors are classified as belonging to the D-2 category of dopamine receptor subtypes (1, 2). Agonist occupancy of these receptors results in a diminished release of prolactin from the anterior pituitary gland (reviewed in refs. 3 and 4). Dopamine agonists also inhibit basal (5–9) as well as vasoactive intestinal polypeptide-induced (10) adenylate cyclase activity in anterior pituitary preparations. Immunocytochemical data indicate that these dopamine receptors are located on the pituitary mammotroph cells that synthesize and secrete prolactin (11, 12). Subcellular fractionation studies additionally demonstrate that these receptors are localized to the plasma membrane (13). Using this homogeneous population of dopamine receptors, we have previously characterized the ligand binding properties of the D-2 receptor in washed plasma membrane preparations (14). Briefly stated, the binding of antagonists to the D-2 receptor is shown to exhibit mass-action characteristics consistent with a simple bimolecular re-

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action. In contrast, agonist binding displays heterogeneous properties indicative of two agonist-specific receptor binding states of high and low affinity. In the presence of guanine nucleotides, however, only the low-affinity receptor binding state is observed. We have suggested that a two-step, three-component, ternary complex model can explain these ligand binding properties of D-2 dopamine receptors in membrane preparations (14).

To date, all radioligand binding characterizations of dopamine receptor subtypes have been performed in tissue homogenates or in washed membrane preparations. In the central nervous system a multiplicity of binding sites for various dopaminergic [^{3}H]agonists and [^{3}H]antagonists has been identified and interpretated, in part, as identifying multiple dopamine receptor subtypes (2, 15). While there is no controversy concerning the pharmacological relevance of [^{3}H]antagonist binding to brain membranes (16), no concensus has yet been reached concerning the neuronal location, function, or pharmacological relevance of high-affinity [^{3}H]agonist binding. In contrast, we have conclusively demonstrated that [^{3}H]agonists label the high-affinity receptor state of the D-2 dopamine receptor in anterior pituitary membrane preparations (14). We now report that in intact, viable, bovine anterior pituitary cells there are no identifiable high-affinity dopaminergic agonist binding sites as determined by direct [^{3}H]agonist binding or by indirect agonist/[^{3}H]antagonist competition experiments. However, in membranes prepared from these cells, high-affinity agonist binding to the D-2 receptor is detectable in the absence of guanine nucleotides. Caution should thus be observed in classifying dopamine receptor subtypes solely on the basis of data from studies of radioligand binding in membrane preparations, as has been recently suggested (17).

EXPERIMENTAL PROCEDURES

Preparation of dispersed cells and membranes. Fresh steer pituitary glands were obtained from a local slaughterhouse. The glands were bisected, and the anterior lobes were removed and finely minced into pieces approximately 1 mm^3. The minced tissue was washed three times with ice-cold dispersion buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 0.2% glucose, gentamycin sulfate (50 μg/ml), pH 7.4 at 25° (hereafter referred to as HDB buffer). The tissue was then transferred to a Bellco spinner bottle and suspended to a final concentration of 100 mg/ml (wet weight) in 37° HDB buffer containing 0.4% collagenase (Worthington Type II), 0.4% bovine serum albumin (Sigma Fraction V), and DNAse II (10 μg/ml) (Sigma Type IV). After constant stirring for 2 hr at 37°, the dispersion solution was filtered through two layers of cheesecloth and centrifuged at 300 × *g* for 10 min. The resulting cell pellet was resuspended in standard Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, gentamycin sulfate (50 μg/ml), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4 at 25°. The cells were centrifuged as before and washed twice more in the Dulbecco's modified Eagle's medium. Before the final centrifugation, the cells were suspended in HDB buffer and cell counts were determined using a Coulter counter. Viability was determined using Trypan blue exclusion and was always greater than 90%. Primarily single cells as well as occasional clumps of between two and six cells were present. Routine yields varied between 2.1×10^7 and 6.4×10^7 cells/g of anterior pituitary tissue (wet weight). After the final centrifugation, the cells were resuspended either in ice-cold 50 mM Tris-HCl, pH 7.7 at 25° (for preparing membranes), or in 37° assay buffer containing 50 mM Tris-HCl (pH 7.7 at 25°), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.7 mM ascorbic acid (for direct use in the binding assay).

Radioligand binding assays. The radioligand binding assays were performed as previously described (14), using approximately 4.0×10^6 cells in a final volume of 1.0 ml. For membrane experiments, a known quantity of cells was homogenized with a Tekmar homogenizer and centrifuged at 50,000 × *g* for 10 min at 4°. The membrane pellet was resuspended in assay buffer to give a final membrane concentration representative of approximately 4.0×10^6 cells/ml. The cell or membrane suspensions were added to glass test tubes containing radioligands and other unlabeled drugs. The assay tubes were incubated for 15 min at 37° then terminated by rapid filtration over GF/C glass-fiber filters (Whatman) under vacuum. The filters were rinsed rapidly with 15 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.7 at 25°. Radioactivity trapped on the filters was counted by liquid scintillation spectroscopy at an efficiency of 45%. Nonspecific binding of all radioligands was determined in the presence of 1 μM (+)-butaclamol. Specific [^{3}H]spiroperidol binding at 0.2 nM, using intact cells, was 30–50% of the total binding, whereas with membranes it was 40–60% of the total binding. Specific [^{3}H]NPA binding in membranes was 30–40% of the total binding at 0.2 nM. As documented elsewhere (18), the inclusion of ascorbic acid was necessary to demonstrate receptor-specific agonist-receptor interactions. The viability of the cells was determined to remain unchanged during the radioligand binding assay.

Data analysis. The computer analysis employed was a weighted, nonlinear regression, curve-fitting program (19) using a generalized model for ligand-receptor binding systems (20). All computations were performed using a VAX 11 computer. The exact treatment of experimental data has been described previously in detail (14). Briefly stated, competition curves were analyzed according to models for the mass-action binding of the radioligand and competing drug to one receptor binding site or multiple receptor binding sites. Deviation of the observed points from the predicted values were weighted according to the reciprocal of the predicted variance. Testing for statistical difference between models was obtained by comparing their residual variances of fits to the data. Saturation curves were analyzed by Scatchard analysis. All experiments for which representative data are presented were replicated a minimum of three times, with the results varying by less than 20%.

Materials. [^{3}H]spiroperidol (25–35.9 Ci/mmole) and [^{3}H]NPA[3] (68.9–75 Ci/mmole) were obtained from New

[3] The abbreviations used are: NPA, (−)-*N*-*n*-propylnorapomorphine; ADTN, (±)-2-amino-6,7-dihydroxytetrahydronaphthalene; GppNHp, guanyl-5′-yl-imidodiphosphate.

England Nuclear Corporation (Boston, Mass.). Dopamine, norepinephrine, epinephrine, serotonin, and GppNHp were purchased from Sigma Chemical Company (St. Louis, Mo.). The following drugs were gifts from the respective pharmaceutical companies: apomorphine, Merck & Company (Rahway, N. J.); butaclamol, Ayerst Laboratories (Rouses Point, N. Y.); spiroperidol, Janssen Pharmaceuticals (Beerse, Belgium); fluphenazine, E. R. Squibb & Sons (New York, N. Y.); chlorpromazine, Smith Kline & French (Philadelphia, Pa.); promethazine, Wyeth Laboratories (Philadelphia, Pa.); NPA, Sterling-Winthrop (Rensselaer, N. Y.); (±)-ADTN, Burroughs-Wellcome (Research Triangle Park, N. C.).

RESULTS

Tissue dependence of [^{3}H]spiroperidol binding to intact cells. The specific binding of the butyrophenone dopamine antagonist, [^{3}H]spiroperidol, increased linearly with increasing cell concentration over the range of 0.5×10^6 to 5.0×10^6 cells/ml (Fig. 1). Cell concentrations higher than 5.0×10^6 cells/ml had a tendency to compromise the filtration rate during termination of the binding assay. Assays were routinely conducted using approximately 4.0×10^6 cells/ml.

Kinetics of [^{3}H]spiroperidol binding to intact cells. The specific binding of [^{3}H]spiroperidol was rapid at 37°, achieving equilibrium over a 15- to 20-min period (Fig. 2). Since the concentration of [^{3}H]spiroperidol utilized in the experiment shown in Fig. 2 (260 pM) was significantly larger than the receptor concentration in the assay (about 36 pM), the association reaction could be replotted as in the *inset* (Fig. 2), and a pseudo-first order association rate constant, k_{obs}, determined from the slope of the line. Pooled data from five experiments yielded an average k_{obs} of 0.21 ± 0.05 min^{-1}.

Specific [^{3}H]spiroperidol binding to anterior pituitary cells was reversible, as demonstrated in Fig. 3. Addition of excess (+)-butaclamol to the assay medium after equilibration resulted in the complete dissociation of [^{3}H]spiroperidol, with a $t_{1/2}$ of approximately 10 min. Addition of the agonist apomorphine to monitor [^{3}H]spiroperidol dissociation yielded identical results. The dissociation appeared to be a first-order process represented by a linear semilogarithmic plot of the data (Fig. 3). The dissociation rate constant, k_2, could be determined from the slope of the line. Pooled data from five experiments indicated a k_2 value of 0.07 ± 0.02 min^{-1}. The second-order association rate constant, k_1, could be calculated from the equation $k_1 = (k_{obs} - k_2)/([^3H]\text{-spiroperidol})$ and equaled 0.54 nM^{-1} min^{-1}. The ratio of the rate constants (k_2/k_1) provided a kinetic estimate of the equilibrium dissociation constant, K_D, for the [^{3}H]spiroperidol-receptor reaction. The kinetically estimated K_D was 0.13 nM.

Saturation experiments with [^{3}H]spiroperidol and [^{3}H]NPA using intact cells and membranes. Specific binding of [^{3}H]spiroperidol to intact anterior pituitary cells was of high affinity and saturable. Scatchard analysis (Fig. 4) demonstrated a homogeneous population of binding sites with an average K_D of 0.21 ± 0.05 nM (n = 3), which agreed well with the kinetically estimated dissociation constant. The average maximal binding capacity (B_{max}) for [^{3}H]spiroperidol was 9.0 ± 0.85 fmoles/10^6 cells (n = 3). Strikingly, there was no detectable high-affinity, stereospecific, or saturable binding of the radiolabeled agonist, [^{3}H]NPA, to the D-2 dopamine receptor on these dispersed cells. In contrast, washed membranes prepared from these cells exhibited high-affinity [^{3}H]NPA binding (Fig. 4). Scatchard analysis indicated a saturable population of homogeneous binding sites, with a K_D of 0.25 ± 0.02 nM (n = 3) and a B_{max} of 2.53 ± 0.05 fmoles/10^6 cells (n = 3). These sites exhibited a D-2 dopaminergic pharmacological specificty (see ref. 14 for a complete characterization of [^{3}H]NPA binding to anterior pituitary membranes) but exhibited only half the maximal binding capacity of the [^{3}H]spiroperidol-labeled receptors in these membranes (Fig. 4). At the concentrations of [^{3}H]NPA employed in the experiment shown in Fig. 4, [^{3}H]NPA labels only the high-affinity receptor state, as we documented previously using membranes prepared directly from whole anterior pituitary glands (14). Interestingly, in membranes prepared from the cells there was approximately a 50% loss in [^{3}H]spiroperidol binding (B_{max} = 4.78 ± 0.35 fmoles/10^6 cells; n = 3) but

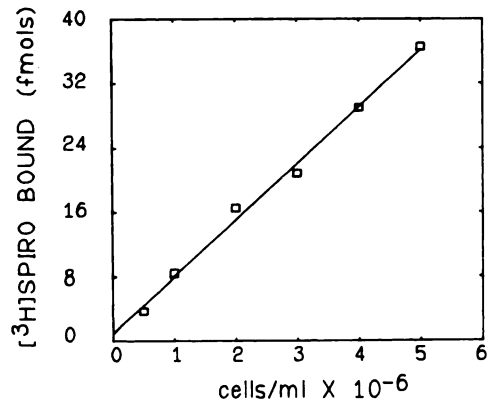

FIG. 1. *Tissue dependence of specific [^{3}H]spiroperidol binding*

[^{3}H]Spiroperidol (0.3 nM) was incubated with the indicated cell concentrations and specific binding was determined. The line drawn was determined from linear regression analysis (r = 0.99).

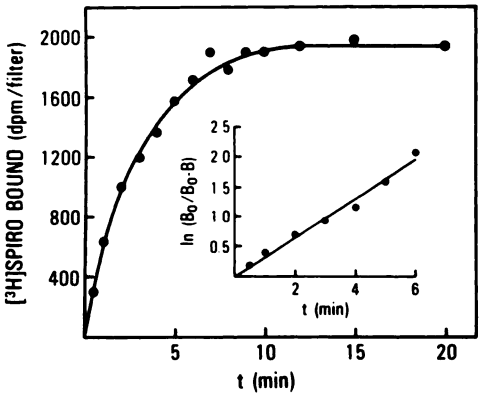

FIG. 2. *Time course of [^{3}H]spiroperidol binding to dispersed anterior pituitary cells*

[^{3}H]Spiroperidol (0.26 nM) was added to cells previously equilibrated at 37° and specific binding was sampled at the indicated time points. The *inset* shows a first-order kinetic plot of these data, where B represents the amount bound at time t and B_0 represents the amount bound at equilibrium.

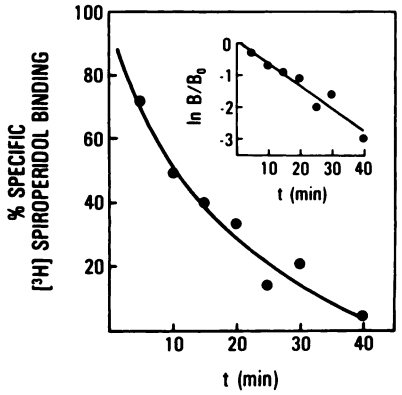

FIG. 3. *Dissociation of [³H]spiroperidol binding from dispersed anterior pituitary cells*

[³H]Spiroperidol was allowed to reach equilibrium and the amount of specific binding was determined. (+)-Butaclamol was then added without significant dilution of the samples to yield a final concentration of 2 μM, and the amount of [³H]spiroperidol remaining specifically bound was monitored. *Inset*, first-order rate plot of the data, where B represents the amount bound at time t and B_0 is the amount bound at $t = 0$. The first-order dissociation rate constant, k_2, was determined from the slope of the regression line and equaled 0.071 min^{-1}.

no change in affinity (K_D = 0.18 ± 0.04 nM; n = 3) from that seen in intact cells.

Pharmacological characterization of [³H]spiroperidol binding to intact anterior pituitary cells. The ability of various ligands to compete for [³H]spiroperidol binding to anterior pituitary cells was examined (Figs. 5 and 6; Table 1). Figure 5 shows representative antagonist/[³H]spiroperidol competition experiments. The competition curves for both spiroperidol and (+)-butaclamol

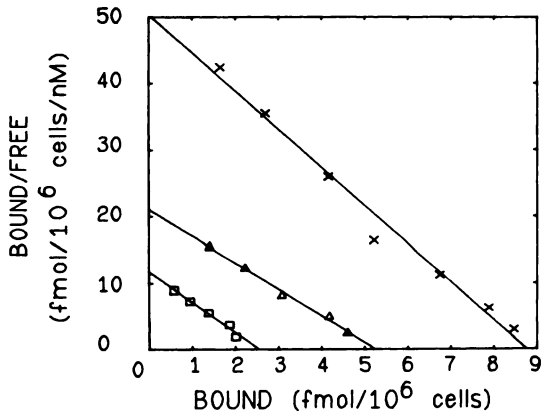

FIG. 4. *Scatchard plots of specific [³H]spiroperidol and [³H]NPA binding to intact cells and membranes*

Concentrations of [³H]spiroperidol were varied between 20 pM and 3 nM while [³H]NPA concentrations were varied between 30 pM and 2 nM. [³H]Spiroperidol binding to intact cells (×) was determined as described under Experimental Procedures. In the experiment shown, the apparent K_D was 0.17 nM and the B_{max} was 8.8 fmoles/10^6 cells. To perform membrane experiments, a known quantity of cells was homogenized and the membranes were collected as described under Experimental Procedures. The K_D and B_{max} values for the membrane [³H]spiroperidol curve (△) were 0.25 nM and 5.2 fmoles/10^6 cells, respectively. For the membrane [³H]NPA saturation curve (□), the K_D and B_{max} values were 0.22 nM and 2.5 fmoles/10^6 cells, respectively.

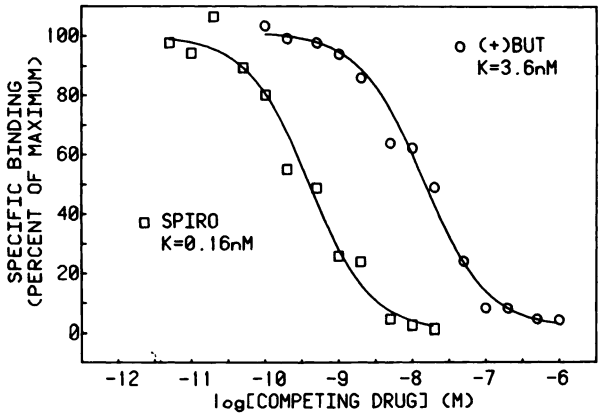

FIG. 5. *Computer-modeled curves for antagonist/[³H]spiroperidol competition experiments in dispersed cells*

The experimentally determined data points shown are from single representative experiments. The computer-drawn curves represent the best fit to the data, assuming a single homogeneous binding site in each experiment.

were uniphasic, with computer analysis indicating that the data are best explained by using a single-site binding model. Representative agonist/[³H]spiroperidol competition curves can be seen in Fig. 6. As with the antagonists, the agonist curves were monophasic and fitted best by using a one-site binding model. In Table 1 it can be seen that various ligands competed for [³H]spiroperidol binding on the dispersed cells with the stereospecificity and rank order of potency expected for a dopamine receptor. Dopamine was more than 1 order of magnitude more potent than either norepinephrine or serotonin. Fluphenazine was the most potent phenothiazine, followed by chlorpromazine and then promethazine. (+)-Butaclamol was more than 4 orders of magnitude more potent than its pharmacologically inactive isomer, (−)-butaclamol. The dopamine agonists (−)-NPA, apomorphine, (±)-ADTN, and dopamine also exhibited the rank order expected for a D-2 dopamine receptor (14).

Comparison of binding data between intact cell and membrane preparations. We have previously demon-

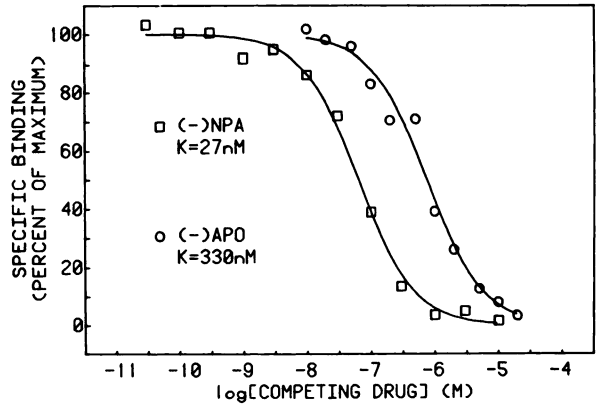

FIG. 6. *Computer-fitted curves for agonist/[³H]spiroperidol competition experiments in dispersed cells*

The experimentally determined data points are shown from single experiments, and the computer-drawn curves represent the best fit to the data. In each experiment, the data were modeled best according to a single binding site.

TABLE 1

Pharmacological characterization of [^{3}H]spiroperidol binding to intact anterior pituitary cells

Competition curves for various ligands were performed and analyzed as described in Figs. 5 and 6, using [^{3}H]spiroperidol concentrations between 0.17 and 0.39 nM. The data are presented as mean ± standard error; the number of independent replications is indicated by *N*.

Drug	Dissociation constant	*N*
	nM	
Spiroperidol	0.21 ± 0.05	2
(+)-Butaclamol	2.0 ± 0.81	3
(−)-Butaclamol	35,000 ± 7,000	4
Fluphenazine	2.9 ± 0.86	3
Chlorpromazine	15 ± 3.8	3
Promethazine	280 ± 56	3
(−)-NPA	43 ± 16	4
Apomorphine	380 ± 37	4
(±)-ADTN	3,200 ± 1,200	3
Dopamine	12,000 ± 1,700	4
Norepinephrine	170,000 ± 61,000	3
Serotonin	180,000 ± 30,000	3

strated that in membrane preparations the anterior pituitary D-2 dopamine receptor exhibits high- and low-affinity agonist-receptor binding states. The absence of detectable [^{3}H]NPA binding on dispersed anterior pituitary cells suggests that the high-affinity receptor state is not observable on intact cells. This phenomenon could be artifactually due to the collagenase dispersion procedure. However, the fact that [^{3}H]NPA binding to the high-affinity receptor state was observed after membranes were prepared from these cells argues against this hypothesis. To explore this further we performed agonist/[^{3}H]spiroperidol competition experiments using membranes derived from dispersed cells. As seen in Fig. 7, an apomorphine competition curve was biphasic, and computer analysis indicated that the data were best explained by assuming two agonist binding states of high and low affinity. These receptor states are designated R_H and R_L with the respective dissociation constants K_H and K_L. When the guanine nucleotide, GppNHp, was added to the membrane experiment, the apomorphine curve was shifted to the right and steepened, with the data then fitting best to a unitary binding site whose affinity was not significantly different from the low-affinity site (K_L) of the control curve (Fig. 7). These data are identical with those observed in membranes prepared directly from whole anterior pituitary glands without prior dispersion (14). The similarity between the membrane apomorphine curve in the presence of guanine nucleotides (Fig. 7) and the apomorphine curve observed in intact cells (Fig. 6) suggests that endogenous GTP regulates agonist binding in whole cells in a fashion identical with that of exogenously added guanine nucleotides in membrane preparations. Furthermore, the addition of guanine nucleotides did not alter agonist/[^{3}H]spiroperidol curves in whole-cell preparations (data not shown).

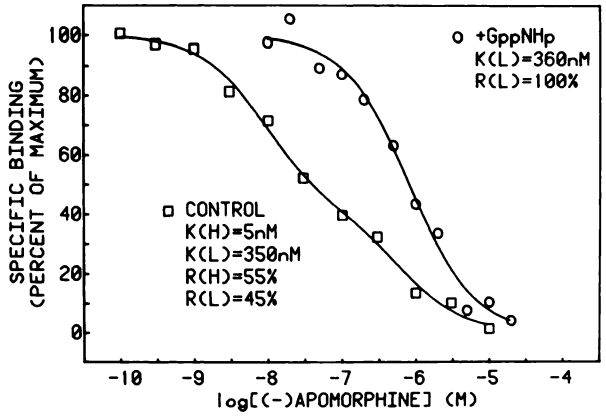

FIG. 7. *Computer-modeled curves for an apomorphine/[^{3}H] spiroperidol competition experiment in anterior pituitary membranes*

Membranes were prepared from dispersed cells as described under Experimental Procedures. Both competition experiments were performed with the same membrane preparation using 0.23 nM [^{3}H]spiroperidol. In the absence of guanine nucleotides the apomorphine curve was best fitted by assuming a two-site binding model. In the presence of 0.1 mM GppNHp, a one-site model was sufficient to fit the data. When the two curves were analyzed simultaneously and constrained to share a common K_L value, there was no significant ($p > 0.05$) worsening of the fit.

In Table 2 is shown a comparison of agonist and antagonist affinities for [^{3}H]spiroperidol binding in intact cell and membrane preparations. As suggested above, there was very good agreement between the agonist affinities determined in intact cells with agonist affinities exhibited for the low-affinity receptor state in membrane preparations. Since antagonists did not distinguish between the receptor states in membranes, their unitary membrane affinities correlated well with their corresponding affinities determined in cell preparations, as would be expected.

Non-equilibrium [^{3}H]spiroperidol binding. The results presented thus far indicate that the high-affinity, agonist-specific binding state of the D-2 receptor is not detectable with equilibrium binding assays in the presence of guanine nucleotides using either membranes or intact cells. If it is assumed that ternary complex formation is essential for agonist function, then there appear

TABLE 2

Comparison of dissociation constants derived from intact cell and membrane preparations

The cell dissociation constants are replicated from Table 1 for comparative purposes. Some of the membrane data were obtained from Sibley *et al.* (14). The membrane agonist dissociation constants are those for the low-affinity agonist binding state (K_L). Using Student's *t*-test, there was no significant ($p > 0.05$) difference between any pair of dissociation constants.

	Cells	Membranes (K_L)
	nM	*nM*
Agonists		
(−)-NPA	43 ± 16	26 ± 2.6[a]
Apomorphine	380 ± 37	350 ± 28[a]
(±)-ADTN	3,200 ± 1,200	1,850 ± 420[a]
Dopamine	12,000 ± 1,700	15,000 ± 1,900[a]
Antagonists		
Spiroperidol	0.21 ± 0.05	0.29 ± 0.05[a]
Fluphenazine	2.9 ± 0.86	2.7 ± 0.67[a]
Chlorpromazine	15 ± 3.8	22 ± 4.6
Promethazine	280 ± 56	310 ± 26
(+)-Butaclamol	2 ± 0.81	1.2 ± 0.06[a]
(−)-Butaclamol	35,000 ± 7,000	26,000 ± 3,600

[a] Obtained from Sibley *et al.* (14).

to be at least two possible explanations for this phenomenon. One possibility is that in the presence of guanine nucleotides, the high-affinity binding state is present initially but dissipates by the time equilibrium is reached in the radioligand binding assays. Another possibility is that the high-affinity receptor state undergoes continuous formation and dispersion with a short kinetic half-life in the presence of guanine nucleotides and never accumulates to a sufficient extent to be measurable in the time frame of a binding experiment. In order to distinguish between these two possibilities we performed [^{3}H]antagonist association experiments in the simultaneous presence of both agonist and guanine nucleotide, as suggested by Pittman and Molinoff (21). [^{3}H]-Spiroperidol associations were performed in the presence and absence of 30 nM apomorphine and 0.1 mM GppNHp (Fig. 8). This concentration of apomorphine would be expected to inhibit [^{3}H]spiroperidol binding by about 50% by initially forming the high-affinity receptor state; however, this concentration was not high enough to inhibit binding from the low-affinity state at equilibrium (Fig. 7). As seen in Fig. 8, there was no inhibition of [^{3}H]spiroperidol binding by apomorphine throughout the association reaction, suggesting that the high-affinity agonist binding state is undetectable in the presence of guanine nucleotides even under non-equilibrium conditions. Identical results were obtained when dispersed cells instead of membranes were used.

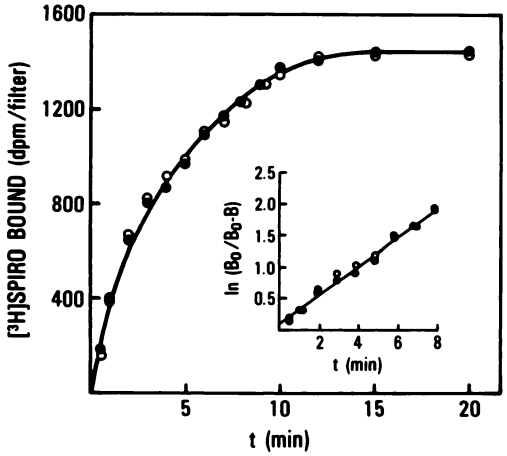

FIG. 8. [^{3}*H*]*Spiroperidol association to anterior pituitary membranes in the absence and presence of apomorphine and GppNHp*

Membranes were prepared as described under Experimental Procedures. Association experiments were run simultaneously as described in Fig. 2 in the absence (●) and presence (○) of 30 nM apomorphine + 0.1 mM GppNHp. As indicated in the *inset*, both curves exhibited an identical pseudo-first order association rate, k_{obs}, equal to 0.22 min^{-1}.

DISCUSSION

Ligand binding characterizations of dopamine receptors have heretofore been conducted exclusively with membrane preparations, primarily from brain. However, preliminary communications in which [^{3}H]spiroperidol was used have suggested the presence of dopaminergic receptors on intact lymphocytes (22, 23). We have been unable to obtain [^{3}H]spiroperidol binding on intact lymphocytes or lymphocyte membranes which demonstrates stereospecificity or other properties expected of a dopamine receptor.[4] Bloxham *et al.* (24) have obtained results similar to ours and concluded that [^{3}H]spiroperidol binding on lymphocytes is nondopaminergic in nature. In contrast, [^{3}H]spiroperidol binding on dispersed bovine anterior pituitary cells demonstrates properties expected for binding to a dopamine receptor. [^{3}H]Spiroperidol binds with high affinity to a homogeneous population of receptor sites in a reversible and saturable fashion. The intact cell binding also demonstrates an appropriate stereospecificity and rank order of potency for catecholamines, phenothiazines, and related drugs in competition experiments. In view of the similar properties such as kinetics, affinity, and pharmacological specificity of binding, it appears likely that the [^{3}H]spiroperidol binding sites on the intact cells are identical with the D-2 dopamine receptors previously characterized in membrane preparations (14).

There are, however, some dissimilar features in the ligand binding properties of dopamine receptors between intact-cell and membrane preparations. It is of interest that, when membranes are prepared from dispersed cells, there is about a 50% decrease in receptor binding capacity. This could result from the loss of receptor protein or receptor degradation during the homogenization process. Alternatively, there may be a random inside-out/right-side-out membrane vesicularization upon transfer from the hypotonic homogenization buffer to the isotonic assay buffer. These possibilities are currently under investigation. Hydrophilic agonists inhibit [^{3}H]spiroperidol binding on intact cells to the same extent as lipophilic antagonists (Figs. 5 and 6) and exhibit nonadditivity (data not shown), suggesting that [^{3}H]spiroperidol is not passively diffusing into the cells and binding to intracellular sites. A decreased receptor binding capacity in membranes as compared with intact cells has also been observed in the *beta*-adrenergic receptor/S49 lymphoma cell system (25).

Another difference in the ligand-receptor binding properties of intact cells and membranes is the lack of a detectable high-affinity receptor binding state on the cells. There was no observable receptor binding of [^{3}H]NPA nor was there high-affinity displacement of [^{3}H]spiroperidol by agonists. This phenomenon cannot be due to receptor or membrane damage during the dispersion procedure, since when washed membranes were prepared from these cells high-affinity agonist binding was demonstrable. [^{3}H]NPA binding to the low-affinity receptor state was difficult to demonstrate in cells or membranes because of the high levels of nonspecific binding at [^{3}H]NPA concentrations greater than 5 nM. The lack of high-affinity agonist binding on cells might be attributable to rapid destruction or uptake of agonists into the cells. However, incubation of cells with [^{3}H]NPA followed by centrifugation and sampling of the supernatant indicated that [^{3}H]NPA is not sequestered by the cells. Thin-layer chromatography of [^{3}H]NPA after exposure to cells also indicates a lack of agonist degradation. Since the affinities of agonists in displacing [^{3}H]spiroperidol binding to the cells correlates with their affinities in displacing [^{3}H]spiroperidol from the low-

[4] D. R. Sibley and I. Creese, unpublished data.

affinity receptor state in membranes, the most reasonable interpretation of the data is that in intact cells endogenous GTP regulates agonist binding in a fashion identical with exogenous guanine nucleotides in membrane preparations.

Our current and previous (14) data suggest the applicability of a two-step, three-component ternary complex model for the D-2 dopamine receptor. This model proposes that agonist binding to the receptor takes place in two steps which involve the interaction of the agonist and receptor with an additional membrane component to yield a high-affinity ternary complex:

$$L + R \rightleftharpoons LR \quad (1)$$

$$LR + X \rightleftharpoons LRX \quad (2)$$

Formation of the ternary complex results in the high-affinity agonist-receptor binding state. Guanine nucleotides are proposed to exert their effects by displacing the equilibrium of this reaction toward the low-affinity LR complex. It has been suggested previously that this model is applicable to *beta*-adrenergic (26, 27) and *alpha*$_2$-adrenergic (28) receptors. In the *beta*-adrenergic receptor system, the ternary complex is an intermediate which facilitates adenylate cyclase activation by GTP (26, 29). Conversely, in the D-2 dopaminergic receptor system, ternary complex formation and dissociation may be obligatory for inhibition of adenylate cyclase activity. This hypothesis is supported by the observation that D-2 dopamine receptor-mediated inhibition of adenylate cyclase requires GTP (7, 30). Thus, in this model, the affinity of dopamine agonists for inhibiting adenylate cyclase activity (K_i values) should correlate with their affinities for ternary complex formation (K_H values). Preliminary comparisons of these K_H (14) and K_i (5, 7, 10, 30) parameters suggest this to be the case; however, comparisons of agonist affinity parameters obtained under different assay conditions and between species should be performed with caution.

It was interesting to find that in the presence of guanine nucleotides (in membranes or intact cells) the high-affinity agonist-receptor binding state was unobservable even under initial, non-equilibrium binding conditions (Fig. 8). Assuming that ternary complex formation is necessary for agonist function, the data are consistent with the hypothesis that in the presence of GTP the ternary complex is a continuously formed but transient intermediate which never accumulates to an extent measurable by binding assays. This model would be similar to a collision coupling model proposed by Levitski (31) for adenylate cyclase activation except that in the D-2 receptor system the functional result is adenylate cyclase inhibition. In this case, the D-2 receptor ternary complex would perform a catalytic function in which the physiological result is adenylate cyclase inhibition. These results (Fig. 8) differ from previous findings with *beta*-adrenergic receptors on L6 cells (21). It was observed that *beta*-adrenergic agonists induced a "time lag" in the association of a radiolabeled antagonist to L6 cells. A fundamental difference between the L6 cell system and our system, however, is that with intact L6 cells agonist affinities are almost 100-fold lower than the low-affinity receptor state observed in L6 membranes. Thus, the observed L6 cell "time lag" may possibly be a manifestation of this latter phenomenon, which is not observed in the pituitary cell system.

Our present findings with dispersed anterior pituitary cells are also relevant with respect to certain recently proposed classification schemes for dopamine receptor subtypes (15, 17, 32). On the basis of radioligand binding studies in brain membrane preparations, Seeman (15, 17) has proposed that membrane binding sites with high (nanomolar) affinity for butyrophenone antagonists (e.g., spiroperidol) and low (micromolar) affinity for agonists be termed D-2 receptors. Conversely, binding sites with high (nanomolar) affinity for both butyrophenone antagonists and agonists are proposed as D-4 receptors. Schwartz and colleagues (32) have proposed an identical scheme except that the D-2 and D-4 designations are reversed. However, our data indicate that these proposed putative D-2 and D-4 receptors correspond exactly with the demonstrated high- and low-affinity agonist-receptor binding states of a single D-2 receptor and thus do not represent two discrete receptor subtypes, i.e., two separate protein molecules. The discrimination of dopamine receptor subtypes based solely on agonist binding affinities in brain membrane preparations (15, 17, 32, 33) could thus be premature and should be interpreted with extreme caution.

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