Dopaminergic Receptors in the Anterior Pituitary Gland

CORRELATION OF [3H]DIHYDROERGOCRYPTINE BINDING WITH THE DOPAMINERGIC CONTROL OF PROLACTIN RELEASE*

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MARC G. CARON,‡ MICHELLE BEAULIEU,§ VINCENT RAYMOND,§ BERNARD GAGNE, JACQUES DROUIN,§ ROBERT J. LEFKOWITZ,¶ AND FERNAND LABRIE∥

From the Medical Research Council Group in Molecular Endocrinology, Le Centre Hospitalier de l'Universite Laval, Quebec G1V 4G2 and Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The ergot alkaloid [3H]dihydroergocryptine, a potent dopaminergic agonist, has been used to study binding sites in bovine anterior pituitary membranes. One function of the anterior pituitary gland, prolactin secretion, as we show is under typical dopaminergic control and was measured in vitro in another series of experiments using rat anterior pituitary cells in primary culture in order to establish a correlation between binding and a biological function.

The dopaminergic specificity of ligand binding and the biological process was demonstrated by the fact that agonists competed for [3H]dihydroergocryptine binding and inhibited prolactin release with an identical order of potency: apomorphine > dopamine > epinephrine > norepinephrine > isoproterenol. Ergot alkaloids, which behave as agonists in this system, potentially inhibited prolactin release from pituitary cells to about the same extent as dopamine (80 to 90% of basal levels) and were potent competitors of [3H]dihydroergocryptine binding (Kᵦ 0.2 to 0.5 nM). Dopaminergic antagonists competed for [3H]dihydroergocryptine binding in bovine anterior pituitary membranes in parallel to their ability to reverse either dopamine or dihydroergocornine inhibition of prolactin release from rat anterior pituitary cells.

[3H]Dihydroergocryptine binding fulfilled another criterion of specific receptor sites in that binding to the anterior pituitary sites was saturable with an apparent dissociation constant (Kᵦ) of 2.2 ± 0.2 pmol/mg of protein.

The close correlation which exists between the properties of [3H]dihydroergocryptine binding to pituitary membrane receptors and characteristics of modulation of prolactin secretion by dopaminergic agents suggests that [3H]dihydroergocryptine binding sites are associated with mammotrophs and that these dopaminergic binding sites modulate prolactin secretion.

The diverse physiological effects of catecholamines appear to be mediated by three distinct types of receptors. These are known as α- and β-adrenergic and dopaminergic receptors. Each receptor type is characterized by a distinct order of binding affinities of agonist and antagonist agents (1, 2). In the case of the α- and β-adrenergic receptors, it has been possible to correlate binding data of specific radioactive ligands with a proximate effect of receptor occupancy, smooth muscle contraction (3, 4), or catecholamine-stimulated K⁺ efflux (5, 6) and adenylate cyclase activation (7-12), respectively.

Correlation of binding of specific ligands to the dopaminergic receptor with a physiological or biochemical response has been less successful. In the brain, where most dopaminergic binding studies have been performed (13-16), dopamine receptors appear to be coupled to adenylate cyclase (2, 17, 18). However, the potency of dopaminergic antagonists in inhibiting dopamine-sensitive adenylate cyclase activity did not always correlate well with the ability of all classes of compounds to compete with [3H]haloperidol or [3H]dopamine binding (19-25).

Prolactin secretion from the anterior pituitary gland appears to be under constant hypothalamic control (26). Accumulating evidence suggests that dopamine may be the main or even the only inhibitory substance involved (27-30). Dopaminergic agents have been shown to modulate prolactin secretion by a direct action at the anterior pituitary level in vivo and in vitro (31, 32) suggesting the presence of specific receptor sites for dopamine in the anterior pituitary.

Since adenohypophyseal cells in primary culture proved to be a very precise system for studying control of the secretion of many pituitary hormones (34-36), we have used this model in the present report in an attempt to correlate properties of
putative dopamine receptor binding sites with the specificity of action of a large series of dopaminergic agonists and antagonists on prolactin release.

Our original goal was to study control of prolactin secretion and binding of a specific dopaminergic ligand in the rat anterior pituitary system. In preliminary experiments, we have been able to demonstrate binding of [3H]dihydroergocryptine to rat anterior pituitary membrane or whole cells in culture which appears to possess features of dopaminergic specificity. However, due to the very small amount of membrane protein which can be derived from rat anterior pituitary, the relatively low concentration of receptors in these preparations and the modest specificity of the labeled ligand, it was felt that the bovine anterior pituitary would be a preferable tissue to carry out such detailed studies of binding to dopaminergic receptors.

We report that the characteristics of binding of the ligand [3H]dihydroergocryptine to bovine anterior pituitary membranes are typical of binding to a dopamine receptor. In addition, the binding data are shown to be in excellent agreement with the characteristics of the prolactin response to dopaminergic agents in rat anterior pituitary cells in primary culture. Thus, the ability of dopaminergic agonists and antagonists to interact with [3H]dihydroergocryptine binding sites in pituitary membranes correlates well with the relative potency of these agents for the dopaminergic response.
In experiments in which fractionation of the membrane preparation was performed, the top layer of the 30,000 \times g pellet collected after the first wash was suspended in buffer and adjusted to a density of 1.18 with sucrose before centrifugation in a five-layer discontinuous sucrose gradient of densities 1.14, 1.16, 1.18, 1.20, and 1.22 (42). Gradients were centrifuged at 40,000 \times g for 2.5 h in a Beckman L-55 (SW 27 rotor). Each layer was collected, diluted with buffer and pelleted at 30,000 \times g for 20 min. After resuspension in incubation buffer (25 mM Tris/HCl, 2 mM MgCl₂, pH 7.4 at 25°) fractions were ready to be assayed.

**Binding Assays** - [3H]Dihydroergocryptine binding to anterior pituitary membranes was assessed by a rapid filtration technique (43). [3H]Dihydroergocryptine was generally incubated with 0.7 to 2 mg of pituitary membrane protein for 60 min at 25° in a medium containing 25 mM Tris/HCl, 2 mM MgCl₂, pH 7.4 at 25°, in a total volume of 500 \mu l. At the end of the incubation, samples were diluted to 2 to 3 ml of cold washing buffer (25 mM Tris/HCl, 2 mM MgCl₂, pH 7.4 at 4°C) and rapidly filtered through Whatman (GF/C) glass fiber filters under reduced pressure. Filters were quickly washed with aliquots (3 \times 5 ml) of washing buffer. The filtration and washing procedure took less than 10 s. This procedure provides a good estimation of equilibrium binding since, as shown under "Results," dissociation was much slower than the filtration time. Filters were immediately placed into scintillation vials, stored overnight to become translucent, and then radioactivity counted with 10 ml of Aquasol (New England Nuclear) with an efficiency of 42 to 45%.

In all experiments, the amount of [3H]dihydroergocryptine non-specifically bound or trapped in membranes and filters was determined by performing parallel incubations in the presence of 10 mM (+)-butaclamol, a potent dopaminergic antagonist. Concentrations of (+)-butaclamol higher than 10 mM did not displace appreciably greater amounts of [3H]dihydroergocryptine. (+)-Butaclamol was used to determine non-specific binding since it was felt that its high affinity for the dopaminergic receptor, its specificity, and its stability under the assay conditions were all desirable. "Specific" binding determined as total binding minus "nonspecific" binding usually ranged from 60 to 70% of total binding at the concentrations of [3H]dihydroergocryptine routinely used (7 to 9 nM). In all experiments, the nonspecific binding value (except where stated) has been subtracted from total binding and results are presented as specific binding. In all experiments, determinations were done in triplicate and results presented are usually the average or representative of several experiments (n = 2 to 5). In figures, brackets represent standard error of the mean and where no bracket is shown, standard error of the mean was smaller than the symbol used.

[3H]Dihydroergocryptine-binding activity in bovine anterior pituitary membranes was stable to freeze-thawing and could be stored for long periods (1 to 2 months) at -90°C without any loss of activity.

**RESULTS**

**Specificity of Catecholaminergic Modulation of Prolactin Release from Rat Anterior Pituitary Cells in Primary Culture**

**Agonists** - Fig. 1A shows the inhibitory effect of increasing concentrations of various catecholamines and analogs on prolactin release during a 2-h incubation of rat anterior pituitary cells in primary culture. As measured by the concentration giving a 50% inhibition of hormone release by an agent the following order of potency was obtained: dopamine (35 nM) > epinephrine (420 nM) ≥ norepinephrine (540 nM) while the \(\alpha\)- and \(\beta\)-agonists phenylephrine and isoproterenol were without effect up to 10 \mu M. Apomorphine, the prototype of dopaminergic agonists (44), was approximately 10 times more potent than dopamine as an inhibitor of prolactin release with an \(ED_{50}\) value of 3 nM (Table I). At high concentrations, apomorphine and the catecholamines led to a maximal 85 to 90% inhibition of basal prolactin release. Serotonin, a neurotransmitter potentially involved in the control of prolactin secretion \textit{in vivo} (46, 47) was a very weak inhibitor of prolactin release in cells in culture (Table I). The inhibition of prolactin release by catecholamines showed the expected stereoselectivity with (+)-isomers being about 8-fold more potent than (-)-isomers (Table I). Ergot alkaloids which are thought to act mainly through a dopaminergic mechanism on prolactin secretion \textit{in vivo} (48) accordingly showed potent dopaminergic agonist activities in this \textit{in vitro} system. It can be seen in Fig. 1B that dihydroergocornine and dihydroergocryptine inhibited prolac-
Apparent dissociation constants \( (K_p) \) of dopaminergic agonists and antagonists and other compounds for their effects on prolactin release in anterior pituitary cells in primary culture and their ability to compete for \(^{3}H\)-dihydroergocryptine binding to bovine anterior pituitary membranes. \( K_p \) values for agonistic activity were taken directly as the concentration of an agent \( (43) \) giving 50% \( (E_{50}) \) of maximal inhibition of prolactin release by that agent. For antagonistic activity, compounds were tested in the presence of 3 nM dihydroergocornine or 50 nM dopamine and apparent dissociation constant \( (K_p) \) values were calculated according to the equation \( K_p = IC_{50}/(1 + S/K) \) (45). In this equation, \( S \) is the concentration of dopaminergic agonist, \( K \) the \( ED_{50} \) value of dopamine or dihydroergocornine for inhibition of prolactin release, and \( IC_{50} \) is the concentration of the agent giving 50% reversal of the inhibitory action of dopamine or dihydroergocornine. For the effects of agonists and antagonists on \(^{3}H\)-dihydroergocryptine binding, \( K_p \) values were calculated according to the relation \( K_p = IC_{50}/(1 + S/K) \) (46). In this equation, \( S \) represents the concentration of labeled ligand present in the assay mixture. \( K_p \) represents the apparent dissociation constant \( (K_p) \) of \(^{3}H\)-dihydroergocryptine for the binding sites as estimated by equilibrium or kinetic analysis (2-2 nM was used). \( IC_{50} \) is the concentration of an agent giving 50% competition of specific binding. Relative potencies for agonists and antagonists were calculated in reference to dopamine: \( K_p \) of dopamine \( / K_p \) of agent) x 100. Thus arbitrarily dopamine was chosen as having a relative potency of 100.

### Table 1

**Apparent dissociation constants of dopaminergic agents in anterior pituitary**

<table>
<thead>
<tr>
<th>Agents</th>
<th>Prolactin release from rat anterior pituitary cells in culture</th>
<th>(^{3}H)DHEC binding to bovine anterior pituitary membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_p ) ( nM )</td>
<td>Relative potency</td>
</tr>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apomorphine</td>
<td>2</td>
<td>1,170</td>
</tr>
<tr>
<td>Dopamine</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>(+)-Epinephrine</td>
<td>420</td>
<td>8.33</td>
</tr>
<tr>
<td>(-)-Epinephrine</td>
<td>3,100</td>
<td>1.13</td>
</tr>
<tr>
<td>(-)-Norepinephrine</td>
<td>540</td>
<td>6.48</td>
</tr>
<tr>
<td>(+)-Norepinephrine</td>
<td>4,200</td>
<td>0.83</td>
</tr>
<tr>
<td>(-)-Isoproterenol</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Clonidine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phenotamine</td>
<td>&gt;10,000</td>
<td>&lt;0.35</td>
</tr>
<tr>
<td>Propranolol</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dihydroergocryptine</td>
<td>0.2</td>
<td>17,500</td>
</tr>
<tr>
<td>Dihydroergocornine</td>
<td>0.4</td>
<td>8,790</td>
</tr>
<tr>
<td>Ergocryptine</td>
<td>0.2</td>
<td>17,500</td>
</tr>
<tr>
<td>2-Bromo-( \alpha )-ergocryptine</td>
<td>2.9</td>
<td>1,210</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)-Butaclamol</td>
<td>0.3</td>
<td>11,700</td>
</tr>
<tr>
<td>(-)-Butaclamol</td>
<td>&gt;1,000</td>
<td>&lt;3.5</td>
</tr>
<tr>
<td>( \alpha )-Flupenthixol</td>
<td>2.4</td>
<td>1,460</td>
</tr>
<tr>
<td>( \beta )-Flupenthixol</td>
<td>330</td>
<td>10.6</td>
</tr>
<tr>
<td>cis-Thiothixene</td>
<td>4.6</td>
<td>2.99</td>
</tr>
<tr>
<td>trans-Thiothixene</td>
<td>44</td>
<td>79.5</td>
</tr>
<tr>
<td>Haloperidol*</td>
<td>0.7</td>
<td>5,000</td>
</tr>
<tr>
<td>Pimozide*</td>
<td>0.3</td>
<td>11,700</td>
</tr>
<tr>
<td>Fluphenazine</td>
<td>0.6</td>
<td>5,830</td>
</tr>
<tr>
<td>Trifluromazine</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>Perphenazine</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>28</td>
<td>125</td>
</tr>
<tr>
<td>Serotonin</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>Cyproheptadine*</td>
<td>14</td>
<td>250</td>
</tr>
<tr>
<td>Methysergide*</td>
<td>190</td>
<td>18.4</td>
</tr>
</tbody>
</table>

* Indicates that when tested these antagonists were found to also have agonist activity as judged by their ability to inhibit prolactin release. The following \( K_p \) values were obtained: haloperidol \( (>10,000 \text{ nM}) \), pimozide \( (27 \text{ nM}) \), cyproheptadine \( (500 \text{ nM}) \), and methysergide \( (>10,000 \text{ nM}) \). \( K_p \) values for antagonistic activity of these agents were calculated from the concentrations giving 50% reversal of inhibition of prolactin release by an agent. At these concentrations pimozide, cyproheptadine, and methysergide had significant agonist activity and did not reverse the dopaminergic inhibition of prolactin completely. Note that not all antagonists were tested for agonist activity. —— indicates effect of agent was too weak to calculate a valid \( K_p \) value. n.t. = not tested. Several catecholamine analogs, precursors, and metabolites were tested for their ability to compete for binding. Ephedrine, \( \alpha \)-dopa, \( \alpha \)-methyl-dopa, and \( \alpha \)-dihydroxy-mandelic acid did not interact significantly with the binding sites below 100 \( \mu \text{M} \). Normetanephrine inhibited binding by 50% at 100 \( \mu \text{M} \). Various other agents having a presumed influence on dopaminergic systems in vivo have been screened for possible interactions with the receptor sites. None of the following drugs tested had any effect at 10 to 100 \( \mu \text{M} \): morphine, \( \beta \)-endorphin, thyrotropin-releasing hormone, triiodothyronine, \( \alpha \)-melanocyte-stimulating hormone, 17\( \beta \)-estradiol, 5a-dihydrotestosterone, substance P, neurotensin, somatostatin, and \( \gamma \)-aminobutyric acid.

Precursors, and metabolites were tested for their ability to compete for binding. Ephedrine, \( \alpha \)-dopa, \( \alpha \)-methyl-dopa, and \( \alpha \)-dihydroxy-mandelic acid did not interact significantly with the binding sites below 100 \( \mu \text{M} \). Normetanephrine inhibited binding by 50% at 100 \( \mu \text{M} \). Various other agents having a presumed influence on dopaminergic systems in vivo have been screened for possible interactions with the receptor sites. None of the following drugs tested had any effect at 10 to 100 \( \mu \text{M} \): morphine, \( \beta \)-endorphin, thyrotropin-releasing hormone, triiodothyronine, \( \alpha \)-melanocyte-stimulating hormone, 17\( \beta \)-estradiol, 5a-dihydrotestosterone, substance P, neurotensin, somatostatin, and \( \gamma \)-aminobutyric acid.

**Antagonists** To further study the specificity of this process we took advantage of the precision of the in vitro system to provide a more refined test of the interaction between dopamine and dihydroergocornine. A compound used as an inhibitor of prolactin secretion in hyperprolactinemia in humans (49, 50) was also tested. It was somewhat less potent with an ED\(_{50}\) value of 2.9 nM (Table 1). As mentioned, \(^{3}H\)-dihydroergocryptine was fully biologically active since it was equipotent with unlabeled dihydroergocryptine in inhibiting prolactin release.
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*O* H (+) BIJTACLAMOL A
w (-1 BUTACLAMOL

H a-FLUPENTHIXOL B - CIS-THIOTHIXENE c

T m-a p- FLUPENTHIXOL o-a Tram-THIOTHIXENE

ANTAGONISTS CONC LOG (M)

FIG. 2. Ability of various dopaminergic antagonists and their enantiomers to reverse the inhibition of prolactin release induced by 3 nM dihydroergocornine in rat anterior pituitary cells in primary culture. ○, basal prolactin secretion; ●, prolactin secretion in the presence of 3 nM dihydroergocornine. Increasing concentrations of examine the ability of various dopaminergic antagonists to reverse the inhibition of prolactin release induced by dihydroergocornine (3 nM) or dopamine (50 nM). As previously observed (Fig. 1B), 3 nM dihydroergocornine decreased basal prolactin release by more than 75% (Fig. 2, A, B, ○). This inhibitory action of 3 nM dihydroergocornine could be progressively and completely reversed by the addition of increasing concentrations of the potent neuroleptic (+)-butaclamol (K, = 0.3 nM) whereas its pharmacologically inactive enantiomer, (-)-butaclamol, had only slight activity at 10 μM (Fig. 2A). In agreement with their relative dopaminergic blocking activities in pharmacological tests cis-thiothixene and α-flupenthixol were approximately 10- and 100-fold more potent than trans-thiothixene and β-flupenthixol in reversing the dihydroergocornine-induced inhibition of prolactin release (Fig. 2, B and C). Comparable results were obtained when 50 nM dopamine was used to inhibit prolactin release (data not shown).

Several other compounds were tested for their effects on prolactin release. Thus, phenothiazines such as fluphenazine and chlorpromazine were able to reverse the inhibition of prolactin release caused by 3 nM dihydroergocornine (Table I). This is consistent with their known pharmacological activity (51-55).

Mixed Agonist-Antagonists—When assayed in the presence of 3 nM dihydroergocornine the classical dopaminergic antagonists pimozide and haloperidol led to a reversal of the inhibitory effect of the ergot alkaloid at relatively low concentrations (K, = 0.3 nM and 0.7 nM, Table I). However, at concentrations above 10 nM and 1 μM both pimozide and haloperidol caused a decrease of prolactin release characteristic of dopaminergic agonist activity. The dopaminergic agonist activity of these compounds was confirmed when the neuroleptics were tested alone, a 45% inhibition of prolactin release being measured at 10 μM haloperidol whereas pimozide inhibited prolactin release with potency similar (ED50 = 25 nM) to that of dopamine itself (35 nM) (Table I). In view of reported actions of serotoninergic agents in the control of prolactin secretion in vivo (46, 47), it was of interest to study the effect of such agents directly at the pituitary level on prolactin release. Cyproheptadine and methysergide, both putative serotoninergic antagonists, were found to be quite potent dopaminergic antagonists (Table I) but to also show weak partial agonist properties above 100 nM (Table I).

The order of potency of catecholaminergic agonists in inhibiting prolactin release and the specificity of the interaction of antagonists with this process clearly suggest that prolactin release is affected directly at the pituitary level via a dopaminergic process.

[3H]Dihydroergocryptine Binding to Bovine Anterior Pituitary Membranes

In order to correlate the biological activity of the various dopaminergic agents with their interactions with putative dopaminergic receptors we develop a direct ligand-binding technique using the potent dopaminergic agonist [3H]dihydroergocryptine. Binding to bovine anterior pituitary membranes was found to fulfill the expected criteria of specific binding namely kinetic, saturability, and specificity.

Kinetics of Binding—Binding of [3H]dihydroergocryptine to pituitary sites was time- and concentration-dependent. At concentrations of 8 to 10 nM, [3H]dihydroergocryptine binding reached equilibrium between 40 to 60 min of incubation at 25° (Fig. 3), whereas when lower ligand concentrations were used (0.2 to 0.5 nM), equilibrium was obtained after 2 to 3 h. Data presented in Fig. 3 have been replotted in the inset and a second order association rate constant k1 = 7.2 ± 1.1 × 106 M⁻¹ min⁻¹ was calculated. Similar k1 values were obtained at low ligand concentrations.

Fig. 4 shows that after the addition of 10 μM (+)-butaclamol to an incubation mixture at equilibrium specifically bound [3H]dihydroergocryptine completely dissociated after 12 h with...
Kz of 4.4 ? 1.0

ligand dissociation from binding sites.

indicating that apparent dissociation is a true reflection of a half-time of about 3 h. A first order dissociation rate constant to pituitary membranes was stable for at least 12 h at 25°C. Binding sites which is in good agreement with that derived from equilibrium binding data (Fig. 5).

In set, estimation of second order rate constant for [3H]DHEC binding to pituitary membrane preparations. R is the concentration of unoccupied binding sites at given time; κ1, the free receptor concentration at time zero, estimated by equilibrium saturation experiments; DHEC, the concentration of free [3H]DHEC, which was determined by subtracting bound [3H]DHEC concentration (DHECb). The linearity of this plot is consistent with a second order reaction (54). The slope provides an estimate of the association rate constant (k2).

Specificity and Affinity of Interaction of Dopaminergic and Other Agents with [3H]Dihydroergocryptine Binding Sites

Agonists—As shown in Fig. 6, agonists competed for [3H]dihydroergocryptine binding with the following order of potency: apomorphine > dopamine > epinephrine > norepinephrine >> isoproterenol = clonidine. This relative order of potency is typical of a dopaminergic process (2) and closely resembles the potency of these agonists in inhibiting prolactin secretion from rat anterior pituitary cells in culture. Ergot alkaloids which act as potent dopaminergic agonists on prolactin secretion (Fig. 1B, Table I, 57-59) were also potent competitors of [3H]dihydroergocryptine binding. These ergot compounds competed for binding of [3H]dihydroergocryptine to about the same extent as agonists such as apomorphine and dopamine (Fig. 6) and antagonists such as (+)-butaclamol (Fig. 7A), thus, suggesting that these compounds interacted with the same sites.

Antagonists—[3H]Dihydroergocryptine binding sites in bovine anterior pituitary displayed marked stereoselectivity toward various neuroleptics and their isomers. Thus, (+)-butaclamol, α-flupentixol, and cis-thiothixene which possess potent pharmacological dopamine-blocking activity (51-53, 60) were 2 to 5 orders of magnitude more effective than their
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Fig. 5. A, binding of [*H]DHEC to bovine anterior pituitary membranes as a function of increasing concentrations of ligand. Membrane preparations were incubated with increasing concentrations of [*H]DHEC (0.35 to 30 nM) for 180 min. A longer incubation period was chosen to ensure that binding at low ligand concentrations had reached equilibrium. Specific [*H]DHEC binding was measured as described under "Experimental Procedures" with the exception that samples containing greater than 10 nM [*H]DHEC were rapidly washed with an additional 20 to 40 ml of washing buffer to remove excess unbound ligand. Specific binding (O-O) was calculated from the difference between total and nonspecific (10 nM (+)-butaclamol) binding. Saturation binding was 0.32 ± 0.02 pmol/mg (n = five experiments). B, Scatchard plot of [*H]DHEC binding. Results from the experiment shown in A have been plotted here. An apparent dissociation constant \( K_d = 1.6 \text{nM} \) was obtained in this experiment. A mean apparent \( K_d \) value of 2.2 ± 0.6 nM was obtained from five such experiments. Results shown in A and B are means ± standard error of triplicate determinations from a representative experiment. Data where no bracket is shown indicate that the standard error of the mean was smaller than the symbol used.

Fig. 6. Competition of various agonists for [*H]DHEC binding to bovine anterior pituitary membranes. [*H]DHEC (8.2 nM) binding was determined as described under "Experimental Procedures" except that membranes were pretreated at 25°C for 10 min with 0.1% ascorbic acid and 10 \( \mu \)M pargyline. One hundred per cent or control specific binding was 0.185 ± 0.009 pmol/mg (n = 3). Results shown are means ± standard error of the means of three experiments determined in triplicate.

respectively pharmacologically less active enantiomers in competing for binding (Fig. 7A).

Various other compounds which possess dopamine antagonist activity in vivo were tested for their ability to compete for [*H]dihydroergocryptine binding. Fig. 7B shows that haloperidol reduced specific [*H]dihydroergocryptine binding with high affinity to about the same extent as (+)-butaclamol. Pimozide and chlorpromazine as well as other phenothiazines (Table I) also competed for binding with relatively high affinity whereas clozapine inhibited binding with somewhat lower potency. The \( \alpha \)-adrenergic antagonist phentolamine was a relatively weak competitor of [*H]dihydroergocryptine binding in pituitary membranes. Propranolol, a selective \( \beta \)-adrenergic antagonist, did not compete for binding below 100 \( \mu \)M. Other Agents—Serotonergic agents have been found to stimulate prolactin secretion in vivo (47), we have examined
The present study indicates that in anterior pituitary membranes, where α-adrenergic receptors could not be detected under the conditions used, \[^{3}H\]dihydroergocryptine appears to selectively and exclusively label dopaminergic sites. In the anterior pituitary system, haloperidol and (+)-butaclamol have been found to be, respectively, 300 and 2500 times more potent in competing for \[^{3}H\]dihydroergocryptine binding than the α-adrenergic antagonist phenolamine whereas in α-adrenergic systems, the reverse situation is observed. Similarly, whereas apomorphine and dopamine are more potent than epinephrine and norepinephrine in competing for binding in this system, epinephrine and norepinephrine were the more potent agonists in α-adrenergic systems (4). As summarized in Table I, the order of potency of a large variety of drugs in competing for \[^{3}H\]dihydroergocryptine binding in adenohypophysal membranes closely parallels their ability to elicit inhibition or reversal of inhibition of prolactin secretion in whole anterior pituitary cells in culture.

As shown by the studies with the rat anterior pituitary cells in culture the pharmacology of the control of prolactin release was found to be typical of a dopaminergic process. This was demonstrated: 1) by the order of potency of agonists in inhibiting prolactin release (apomorphine > dopamine > epinephrine > norepinephrine >> isoproterenol = phenylephrine), 2) by the high affinity of agonists such as haloperidol, pimozide, and various phenothiazines for this process, and 3) by the high degree of stereoselectivity (Table I) and the order of potency of agonists such as the neuroleptics butaclamol, flupenthixol, and thiouoxetine. Many previous investigations performed in vitro using intact pituitaries (29, 64-68) and in vivo (28, 69-71) have clearly shown a direct inhibitory effect of dopamine on prolactin release at the anterior pituitary level. The present experiments performed in cells in culture extend those observations and demonstrate the specificity of the dopaminergic action. In preliminary experiments, we have found that the pharmacology of prolactin control appears to be the same in the presence of thyrotropin-releasing hormone, yet thyrotropin-releasing hormone does not evoke a major change in prolactin release in these cells because prolactin levels are already very high.

The high degree of precision of the cultured cell system used has permitted the somewhat unexpected findings that several compounds so far classified only as dopaminergic antagonists, (e.g. haloperidol and pimozide) (44, 72), do in fact have mixed agonist-antagonist properties. The inhibitory effect (agonist activity) of high concentrations of haloperidol is in agreement with previous in vitro findings (67, 73) but may represent nonspecific effects. The presumed serotoninergic specific antagonists methysergide and cyproheptadine were also found to modulate prolactin release both as agonists and antagonists in addition to their ability to interact with \[^{3}H\]dihydroergocryptine binding sites in pituitary membranes. Therefore, interaction of these compounds with the dopaminergic receptors in anterior pituitary could offer an explanation for the in vivo observations of a stimulation of plasma prolactin levels after administration of these compounds (46, 47). These data show that the control of prolactin release in anterior pituitary cells in primary culture, beside its own intrinsic interest, offers a precise model for studying dopaminergic agonistic and antagonistic activities of compounds.

As mentioned above, \[^{3}H\]dihydroergocryptine binding to bovine anterior pituitary membranes displayed the specificity expected of a dopaminergic process and the relative potencies of more than 30 agents in competing for binding was identical.

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### Table II

**Distribution of \[^{3}H\]dihydroergocryptine-binding activity in adenohypophysal subcellular fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[^{3}H]Dihydroergocryptine binding</th>
<th>fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>74 ± 14</td>
<td></td>
</tr>
<tr>
<td>Interface 1.14-1.16</td>
<td>59 ± 2</td>
<td></td>
</tr>
<tr>
<td>Interface 1.16-1.18</td>
<td>355 ± 11</td>
<td></td>
</tr>
<tr>
<td>Interface 1.18-1.20</td>
<td>108 ± 3</td>
<td></td>
</tr>
<tr>
<td>Interface 1.20-1.22</td>
<td>38 ± 1</td>
<td></td>
</tr>
<tr>
<td>Pellet (secretory granules)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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the ability of serotonin and two serotoninergic antagonists, methysergide and cyproheptadine, to compete for binding. Serotonin was a very weak competitor whereas cyproheptadine and methysergide competed for binding with Kᵢ values of 0.23 and 11 μM, respectively (Table I). The interaction of these compounds with \[^{3}H\]dihydroergocryptine was comparable to their ability to inhibit or antagonize the dopaminergic inhibition of prolactin secretion and to compete for \[^{3}H\]haloperidol binding sites in brain (19).

Several other catecholamine analogs, precursors, and metabolites were tested for competition of binding and found to have little or no activity (listed in Table I).

### Subcellular Distribution of \[^{3}H\]Dihydroergocryptine Binding

Binding of \[^{3}H\]dihydroergocryptine to bovine pituitary membranes was studied in various fractions prepared by stepwise sucrose density gradient (42) centrifugation. The binding activity present at interfaces of density 1.14 to 1.16 and 1.16 to 1.18 of the stepwise sucrose density gradient constituted about 80% of the total activity present in all fractions. The specific activity of binding in these two fractions was increased by 5 to 8-fold over starting material (Table II). The distribution of \[^{3}H\]dihydroergocryptine binding in these fractions was found to closely parallel the distribution of adenylate cyclase, 5'-nucleotidase, and magnesium and sodium-potassium ion-activated ATPase activities obtained by Poirier et al. (42) using a similar procedure suggesting the likely association of the binding sites with the plasma membrane fraction of bovine pituitary cells.

### DISCUSSION

Ergot alkaloids are known to interact with a variety of drug receptors including α-adrenergic, serotoninergic, and dopaminergic receptors. \[^{3}H\]Dihydroergocryptine has proved a useful ligand for studying α-adrenergic receptor binding sites in membranes derived from uterine smooth muscle (3, 4), parotid acinar cells (5, 6), and human platelets (61). In membranes from brain, binding of the ligand is more complex appearing to label more than one type of receptor sites (62) but under certain conditions α-adrenergic receptors can be exclusively labeled with \[^{3}H\]dihydroergocryptine (63).
with their ability to modulate prolactin release from cultured cells. However, dissociation constant ($K_d$) values for all agents calculated from their effects on prolactin release were somewhat lower than those calculated for their interaction with the $[^3H]dihydroergocryptine binding sites (2- to 20-fold). These differences could possibly be due to differences in the two species used in these studies. More likely, however, these differences might be due to the use of high concentrations of protein in our binding assays. Indeed, because of the relatively low concentration of receptor in these membranes and the modest specific radioactivity of our tracer $[^3H]dihydroergocryptine, high concentrations of membrane protein (1.0 to 2.5 mg/ml) had to be used to obtain reasonable levels of specific binding (3000 to 6000 cpm/assay). Recent work of Brown et al. (22) on the binding of $[^3H]dihydrobenzylpindol to turkey erythrocyte membranes has shown that estimated $K_d$ values were directly dependent on the concentrations of receptor protein used in the assays. Using protein concentrations from 60 to 300 $\mu$g/ml, they showed variations of estimated $K_d$ values by 8 to 10-fold. Since binding assays were performed at room temperature while prolactin release was measured at 37°C, it is also possible that this difference of assay temperature has played a role.

Previous studies of dopaminergic receptors in membranes derived from brain have utilized either the agonist $[^3H]haloperidol (14-19) or the antagonists $[^3H]dopamine (14-16)$ and $[^3H]apomorphine (74). As noted above, in no previous study, has it been possible to observe a correlation of binding to putative dopaminergic receptors with a specific dopaminergic biological response as it has been done in the present investigation. Marked differences have been observed in the relative affinities of dopaminergic agonists and antagonists for $[^3H]haloperidol versus $[^3H]dopamine binding sites in brain membranes (19). Dopaminergic antagonists are relatively more potent in competing for $[^3H]haloperidol whereas agonists are more potent toward $[^3H]dopamine binding. It has therefore been proposed that the two ligands label, respectively, "agonist" and "antagonist" states of the dopamine receptors (19).

Data presented in this study are of particular interest with regard to this model. Dihydroergocryptine, as shown here, behaves as a full agonist, causing the same maximum inhibition of prolactin secretion as dopamine. Therefore, according to the two-state model, agonist should compete with much higher potencies than antagonists for $[^3H]dihydroergocryptine binding sites. However, antagonists were found to have very high potency in competing for $[^3H]dihydroergocryptine binding. In fact, binding affinities of both agonists and antagonists for $[^3H]dihydroergocryptine binding sites resemble more closely those obtained for competition with $[^3H]haloperidol than $[^3H]dopamine in brain studies (19). These observations suggest that, at least in the pituitary, the two-state (agonist versus antagonist) model of dopamine receptors might not fit the experimentally obtained data.

Several reports have previously presented supportive evidence that specific dopamine receptors might be present in pituitary (10-19). However, the studies reported here represent the first direct study and characterization of these binding sites in anterior pituitary and they represent the first study in which binding of a dopaminergic ligand has been correlated with a dopaminergic physiological process. Moreover, the close correlation observed between the properties of $[^3H]dihydroergocryptine binding sites with those of the dopaminergic inhibition of prolactin release strongly suggest that these sites correspond to the physiologically relevant dopamine receptors which regulate prolactin secretion. The present data indicate that the anterior pituitary gland, beside its own intrinsic interest, should represent a useful model for detailed study of the mechanisms of dopaminergic action. In fact, changes of $[^3H]dihydroergocryptine binding to the dopaminergic receptor can be correlated with an easily accessible and highly precise parameter of biological activity, prolactin release in cells in culture. Such a model of dopamine action has not been previously available and should be useful for a better understanding of the mechanisms controlling dopamine receptor-mediated actions.

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M G Caron, M Beaulieu, V Raymond, B Gagné, J Drouin, R J Lefkowitz and F 
Labrie


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