Bromocriptine (2-bromo-α-ergocryptine) inhibited prolactin release when added to primary cultures of dispersed pituitary glands from male rats. The total amount of prolactin (intracellular plus extracellular) was reduced to less than 20% of control values after 6 days. The amount of intracellular prolactin was increased in treated cultures and the total amount of hormone was the same in control and treated cultures for the first 8 h that prolactin release was inhibited. After 4 days with bromocriptine, treated cultures did not contain more intracellular prolactin than control cultures. The rate of accumulation of total prolactin from 3 to 4 days after bromocriptine was added varied from 6-fold less than controls to no accumulation at all. The apparent rate of synthesis at this time, measured by the incorporation of [3H]leucine for 1 or 2 h, was only 1.4- to 2.8-fold less in bromocriptine-treated cultures. After longer labeling periods, when the rate of accumulation of [3H]prolactin may increasingly reflect degradation as well as synthesis, the difference in the rate of accumulation became 6- to 7-fold less than control. Stability of [3H]prolactin was measured by incubating the cells for 2 h with [3H]leucine and then adding 3 μg/ml of cycloheximide and 100-fold excess leucine. [3H]Prolactin was stable for 9 h in control cultures, but in bromocriptine-treated cultures there was a 40% decrease by 9 h. Therefore, at least some of the decrease in prolactin accumulation caused by bromocriptine results from degradation of the hormone.

Secretion of prolactin from the anterior pituitary gland is primarily under negative control by the hypothalamus (1) and dopamine appears to be the hypothalamic inhibitory factor (2). The evidence is that dopaminergic agonists inhibit prolactin secretion in intact animals (3, 4), and direct inhibition of prolactin secretion has been demonstrated by adding these compounds to pituitary halves in organ culture or to primary cultures of dispersed pituitary glands (5, 6). In addition, the inhibitory activity found in hypothalamic extracts has properties of catecholamines (7).

Ergot alkaloids block prolactin secretion (5, 6). These compounds appear to act at the dopamine receptor in the pituitary gland since the inhibition of prolactin release by ergot alkaloids is prevented by dopaminergic antagonists (5, 6). Furthermore, the binding of ergot alkaloids to pituitary membranes can be blocked by dopaminergic agonists and antagonists (6).

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Ergot alkaloids inhibit prolactin release within 1 h (8–10) and hormone accumulates within the gland (8, 11–13). These drugs do more than just affect release since the total amount of prolactin is reduced in some experiments (11). We have used primary cultures of rat pituitary cells to characterize the effects of bromocriptine, a semisynthetic ergot derivative, on prolactin accumulation and to investigate its mechanism of action.

MATERIALS AND METHODS

Chemicals—Trypsin type III from bovine pancreas, soybean trypsin inhibitor, type I-S, and bovine serum albumin, Fraction V, were from Sigma Chemical Co. 1[4,5,3H]Leucine was from Amersham/Searle or New England Nuclear and had a specific activity of 5 Ci/ mmol. Prolactin used as standard for complement fixation and electrophoresis was NIAMDD rat prolactin RP-1 from the National Institute of Arthritis, Metabolism, and Digestive Diseases. Bromocriptine (2-bromo-α-ergocryptine) was a gift from Sandoz, Inc.

Preparation of Dispersed Cells in Culture—The method for preparing the cells is a modification of previously published procedures (14, 15). Male Sprague Dawley rats weighing between 200 to 250 g were used. All glassware was siliconized; all glassware, solutions, and implements were sterile. The rats were decapitated and the anterior pituitary glands were removed and placed in Krebs-Ringer bicarbonate solution supplemented with 14 mM glucose and 1% bovine serum albumin. All the glands were collected, they were cut with razor blades into pieces about 1 min across and rinsed twice with Krebs-Ringer bicarbonate solution with glucose and serum albumin but without calcium or magnesium. The pituitary pieces were incubated with 5 ml of 1 mg/ml of trypsin in Krebs-Ringer bicarbonate solution for several min. They were dissociated in Krebs-Ringer bicarbonate solution with glucose and serum albumin but without calcium or magnesium. These compounds did not affect prolactin production compared to appropriate control solutions were added in 0.05-ml volumes to each plate. These compounds did not affect prolactin production compared to untreated controls. When intracellular and extracellular prolactin were measured separately, the medium was collected and 1.5 ml of fresh medium was added to the plates. The cells were scraped from the plates into fresh medium and sonicated. When only total prolactin

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was measured, the cells were scraped directly into the medium which had been incubated with the cells. Cells were disrupted by sonication for 10 s on a Bransonic 1510 at half-maximal power. This resulted in a 2-fold or greater yield of intracellular prolactin than that obtained when cells were disrupted by repeated freezing and thawing and in the same yield as when cells were disrupted by homogenizing in 1 mM EDTA at pH 10.5.

Nonradioactive prolactin was assayed by the immunological technique of complement fixation (16). Incorporation of \[^{3}H\]leucine into prolactin was measured by immunoprecipitation and electrophoresis as previously described (17). Incorporation of \[^{3}H\]leucine into total protein was determined by precipitation with 10% trichloroacetic acid (18).

RESULTS

Characterization of Monolayer Cultures and the Response to Bromocriptine—The incorporation of \[^{3}H\]leucine into prolactin by cells which had been in culture for 3 days is shown in Fig. 1. Labeled hormone accumulated in the cell and was not released into the medium for over 1 h after addition of \[^{3}H\]leucine. \[^{3}H\]Prolactin released into the medium was only 5% of the total amount of labeled prolactin at 2 h. The time to process prolactin before secretion and the amount of intracellular hormone is consistent with the storage of the hormone in secretory granules, and electron micrographs of the cells after 1 week of culture show some cells with many granules. Therefore, the cells still appear to secrete prolactin normally, although they have been in culture for days.

The data in Fig. 2 show the total amount of prolactin (intracellular plus extracellular) that was present in the cultures of pituitary cells after six days of treatment with the indicated concentrations of bromocriptine. The drug caused a 6-fold decrease in prolactin at concentrations of 40 nM or greater; the half-maximal effect occurred at 3 nM.

Bromocriptine initially inhibited prolactin release but not total accumulation of the hormone. Control cultures released prolactin throughout the 8 h as shown in Fig. 3A, but there was no further secretion above the amount present at 30 min in cultures treated with bromocriptine. The hormone that was not released accumulated in the cells, so that the total amount of prolactin in control and treated cultures was the same at the end of 8 h (Fig. 3B).

At longer times, total prolactin accumulation was inhibited. Data in Fig. 4A show that the release of prolactin was almost completely suppressed during a 4-day incubation with bromocriptine. The intracellular hormone in the treated cultures was not enough to account for the decrease in prolactin in the medium; at 4 days, the intracellular amounts were the same in both control and treated cultures (Fig. 4B). From 3 to 4 days, there was no increase in the total amount of prolactin in the bromocriptine-treated cultures. In other experiments, we have found the rate of accumulation of total prolactin in treated cultures from 3 to 4 days varied from 6-fold less than controls to no accumulation at all.

The inhibition of the rate of prolactin accumulation by bromocriptine was not caused entirely by toxicity to the prolactin-producing cells since the effects were largely reversible. Table I shows the results of an experiment in which bromocriptine was incubated with the cells for 4 days, which reduced the concentration of both extracellular and intracellular prolactin. Then bromocriptine was removed and samples collected for the next two 24-h periods. The inhibition began to reverse in the first 24 h, and the cultures that had been treated with bromocriptine had almost as much intracellular and extracellular prolactin at the end of 48 h as control cultures. In other experiments (not shown) in which cells were incubated with the drug for 3 days instead of 4 days, the amount of prolactin released in the medium was the same for control and treated cultures 24 to 48 h after the bromocriptine was removed.
bromocriptine for various lengths of time. Medium containing 82 nm bromocriptine or control solutions was added at 0 time, and samples were collected at the indicated intervals. Each point represents the average of duplicate plates and the bars give the range. A, accumulation of prolactin in the medium: O——O, control; □—□, bromocriptine. B, intracellular prolactin: □—□, control; ■—■, bromocriptine. Total prolactin (sum of extracellular plus intracellular): △—△, control; ▲—▲, bromocriptine.

**Fig. 3.** Accumulation of prolactin during treatment with bromocriptine for various lengths of time. Medium containing 82 nm bromocriptine or control solutions was added at 0 time, and samples were collected at the indicated intervals. Each point represents the average of duplicate plates and the bars give the range. A, accumulation of prolactin in the medium: O——O, control; □—□, bromocriptine. B, intracellular prolactin: □—□, control; ■—■, bromocriptine. Total prolactin (sum of extracellular plus intracellular): △—△, control; ▲—▲, bromocriptine.

**Fig. 4.** Accumulation of prolactin during treatment with bromocriptine for various numbers of days. The procedure and symbols are the same as described in Fig. 3, except cells were treated with 410 nm bromocriptine. There were fewer cells/plate than in the experiments shown in Figs. 2 or 3, but similar results were obtained in other experiments at higher densities.

The Effect of Bromocriptine on [3H]Leucine Incorporation into Prolactin—The synthesis of prolactin after 3 days of incubation with bromocriptine was determined by measuring the incorporation of [3H]leucine into prolactin. Two such experiments are shown in Figs. 5 and 6. The rate of incorporation within the first 2 h was similar in control and bromocriptine-treated cultures. The rate of incorporation of the control cultures was 1.5 times the rate in the treated cultures from 1 to 2 h after the addition of [3H]leucine in the experiment shown in Fig. 5. In other experiments the values have ranged from 1.4- to 2.8-fold higher in controls for the first 1 or 2 h. Both incorporation of [3H]leucine into prolactin and the rate of accumulation of the total hormone were measured in 0-4 days, with bromocriptine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Treated</th>
<th>Control/treated</th>
</tr>
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<tbody>
<tr>
<td>Intracellular</td>
<td>6.65 ± 0.40</td>
<td>4.00 ± 0.51</td>
<td>1.7</td>
</tr>
<tr>
<td>Extracellular</td>
<td>21.2 ± 2.08</td>
<td>1.67 ± 0.17</td>
<td>12.7</td>
</tr>
<tr>
<td>Total</td>
<td>27.8 ± 2.20</td>
<td>5.67 ± 0.67</td>
<td>4.9</td>
</tr>
</tbody>
</table>
| 0-24 hours, after removing bromocriptine
| Intracellular              | 6.62 ± 0.35      | 4.22 ± 0.93     | 1.6             |
| Extracellular              | 6.53 ± 0.30      | 2.97 ± 0.23     | 2.2             |
| Total                      | 13.1 ± 0.50      | 7.19 ± 1.02     | 1.8             |
| 24-48 hours, after removing bromocriptine
| Intracellular              | 7.34 ± 0.32      | 6.19 ± 0.75     | 1.2             |
| Extracellular              | 10.4 ± 0.55      | 7.13 ± 0.82     | 1.5             |
| Total                      | 17.7 ± 0.80      | 13.3 ± 0.07     | 1.3             |

Reversibility of inhibition of prolactin secretion by bromocriptine

Cells were incubated with 410 nm bromocriptine or control solution for 4 days. At this time one set of plates was collected for hormone measurements. The rest of the plates were rinsed twice with 1.5 ml of fresh medium, and then more medium without the drug was added. A second set of plates was collected 24 h after the drug, bromocriptine; medium was replaced on the rest and these were collected 48 h after the bromocriptine was removed. Each point is the mean ± standard deviation of triplicate plates. Total prolactin is the sum of intracellular and extracellular.
Bromocriptine Causes Prolactin Degradation

Fig. 5. Effect of bromocriptine on [3H]leucine accumulation into prolactin and protein. Cultures were treated with 410 nM bromocriptine for 3 days and then 0.1 mCi of [3H]leucine was added to each plate without changing the medium at 0 time on the graph. Medium and cells were collected together at indicated intervals. Each point is the mean of triplicate plates and the bars give the ranges. A, accumulation of [3H]prolactin; ○—○, control; ●—●, bromocriptine. B, accumulation of [3H]leucine into protein: □—□, control; ■—■, bromocriptine.

Fig. 6. Effect of bromocriptine on [3H]leucine accumulation into prolactin and protein. The procedures and symbols are the same as described in Fig. 5. At the start and end of the labeling period, samples were collected to show the rate of total prolactin accumulation was greatly reduced in the presence of bromocriptine. The difference in rates of accumulation of labeled prolactin between control and treated cultures at times after 2 h can be explained by degradation of prolactin in the bromocriptine-treated cultures. We have tested this by measuring the stability of intracellular [3H]prolactin. Cells were incubated with [3H]leucine for 2 h and then fresh medium was added containing 10 mM leucine and 3 μg/ml of cycloheximide. The amount of cycloheximide reduced [3H]leucine incorporation into total protein to 5% of control levels. The leucine was 100-fold excess over the amount of [3H]leucine initially present to prevent further incorporation. Both the leucine and cycloheximide were necessary to prevent control cultures from continuing to incorporate label into prolactin. The amount of intracellular and extracellular prolactin remaining in the cultures was measured. There was no loss of labeled hormone from control cultures, but 30% of the labeled hormone had disappeared by 2 h and 40% by 9 h in bromocriptine-treated cultures (Fig. 7A). Cycloheximide did not interfere with the release process since control cultures still released [3H]prolactin, although protein synthesis was inhibited. In addition, bromo-
Bromocriptine Causes Prolactin Degradation

Fig. 7. Effects of bromocriptine on prolactin and protein. A, effect of bromocriptine on stability and release of prolactin. Cells were treated with 41 nm bromocriptine for 3 days and then 0.1 mCi of [3H]leucine was added to each plate. Two hours after the addition of [3H]leucine (0 time on the graph), the medium was removed and fresh medium was added containing 3 μg/ml of cycloheximide plus 10 nm leucine. Cells and medium were collected separately at the indicated intervals. The data are expressed as a per cent of the amount of total prolactin (extracellular plus intracellular) present at 0 time. Bromocriptine prevented the release of the [3H]prolactin, indicating cycloheximide did not interfere with the inhibition of prolactin release. We found the variability in the amount of [3H]leucine present in total protein increased in the presence of cycloheximide, but bromocriptine-treated cultures had not lost any detectable amount of labeled protein at the end of 9 h, compared to the 40% drop in [3H]prolactin. However, these cultures contain other cell types besides prolactin-producing cells and, therefore, specific effects on general protein synthesis or degradation in prolactin-producing cells may not be detected.

The disappearance of 40% of the labeled prolactin by 9 h is a relatively small effect which could not account for the large decrease in accumulation seen after 3 days of treatment. There are two reasons why the experiment may underestimate the amount of degradation. The first is that some labeled prolactin may be packaged into secretory granules and become stable for extended periods of time before degradation. The second reason is that cycloheximide is known to interfere with the degradation rate of some proteins (20) and may interfere with the degradation of prolactin.

The magnitude of the apparent decrease in the rate of synthesis, measured after 1- to 2-h labeling periods, varied from 1.4- to 2.8-fold. If this represents a true decrease in synthesis and if, in addition, 40% of what is synthesized is degraded, the rate of prolactin accumulation will be reduced 2- to 5-fold. This reduction is not enough to account for the times when there is no accumulation in the presence of bromocriptine since accumulation of prolactin over 24 h would be detectable even if the rate were reduced 5-fold. This indicates the rate of synthesis or the amount of degradation is being underestimated by these measurements.

DISCUSSION

We conclude prolactin is degraded after the cells are incubated with bromocriptine because: 1) labeled hormone is lost in drug-treated cultures and 2) the rate of prolactin synthesis measured by labeling periods of 1 or 2 h does not reflect the large decrease in the rate of prolactin accumulation, although the difference becomes greater at longer labeling times. The change in incorporation into prolactin after 1 or 2 h of labeling may reflect a decrease in synthesis but other factors may influence this rate as well. The decrease may occur because effects of degradation as well as synthesis are included at these times. Alternatively, prolactin cells may have reduced leucine uptake or different amino acid pools in the presence of bromocriptine that are not detected because other cell types are also present. There are several papers in the literature which suggest prolactin synthesis is reduced by bromocriptine (5, 12, 21). These results were obtained by incubating [3H]leucine with hemipituitary glands for 5 or 6 h. Such long labeling periods may include effects of both synthesis and degradation in addition to possible effects on amino acid uptake and pool size. Therefore, it is not yet possible to tell if prolactin synthesis is affected as well as degradation.

Prolactin is synthesized in the rough endoplasmic reticulum (22) and processed through the Golgi complex into secretory granules which begin to mature at 1 h (23). Degradation of the hormone may occur before or after prolactin is packaged into mature secretory granules. There is morphological evidence that degradation of prolactin-containing granules can be induced in lactating rats by removing suckling pups. Prolactin release is prevented and lysosomes are seen containing what appears to be the contents of secretory granules (24). In the experiments presented here the amount of intracellular prolactin was the same as or less than control cultures after 3 or 4 days of treatment. Therefore, if degradation occurs after prolactin is packaged, the cells must have some way of detecting an excess of granules. If degradation occurs after prolactin is packaged, granules containing newly synthesized prolactin must be degraded since we have measured newly synthesized prolactin in this paper; however, the contents of older granules may be degraded as well.

Alternatively, degradation may occur before the hormone is packaged into secretory granules. The synthesis of new granules may become rate-limiting so that all the newly synthesized prolactin cannot be stored and, therefore, this extra hormone is degraded. Since there is no evidence for degradation of prolactin in normal cells, the synthesis of
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prolactin would have to be coordinated with that of the secretory granules under noninhibited conditions. A second way in which prolactin could be destroyed before storage in granules is through the activation of a protease induced by bromocriptine. In this case, granules would be formed only when newly synthesized prolactin accumulates and the protease would prevent this accumulation.

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P S Dannies and M S Rudnick


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