Biochemical Pharmacology, Vol. 31, No. 18, pp. 2845–2849, 1982.
Printed in Great Britain.

0006-2952/82/182845-05 $03.00/0

COMMENTARY

PROLACTIN: MULTIPLE INTRACELLULAR PROCESSING ROUTES PLUS SEVERAL POTENTIAL MECHANISMS FOR REGULATION

PRISCILLA S. DANNIES
Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

Prolactin production is regulated by many factors at several different stages in the processing of prolactin, including synthesis, storage, release and intracellular degradation of prolactin. Many laboratories have investigated the mechanisms by which prolactin is produced and the production is controlled. Often prolactin release has been implicitly assumed to be a homogeneous process, regulated in a simple fashion, but work from several laboratories has shown that there is heterogeneity in the processing of prolactin. At this time, it is not clear whether all these reports are examining the same type of heterogeneity. This commentary is a summary of work showing that prolactin processing is not a homogeneous event and a discussion of the implications that this fact has for studies on mechanisms of prolactin release.

Prolactin is synthesized on the membranes of the rough endoplasmic reticulum, as other secreted proteins are (for review, see Ref. 1). The hormone is made as a larger precursor which contains a hydrophobic leader sequence that inserts the nascent polypeptide chain into the membrane. The precursor form is rapidly cleaved into prolactin in the cisterna of the endoplasmic reticulum, so it is not usually detectable within the cell. Farquhar and coworkers [2] have performed autoradiographic studies of tritiated leucine incorporated into prolactin to show the course of this hormone through the cell after synthesis. This technique is possible because mammotrophs appear to synthesize primarily prolactin and, therefore, background incorporation into other proteins is low. After a 5-min labeling period, incorporated leucine was located primarily with the rough endoplasmic reticulum. Fifteen minutes after the 5-min labeling period, radioactivity was located over the Golgi zone. Radioactivity was present in substantial amounts in granules after 1 hr, and the amount associated with the granules increased for the next 2 hr. Small granules and irregularly shaped granules had more radioactivity at early times; 2 or 3 hr after the labeling period, larger rounded granules were labeled, indicating these are the more mature of the granules.

All newly synthesized prolactin does not follow the same route out of the cell. Swearingen [3] showed that pituitary glands which had incorporated [^{3}H]leucine subsequently secreted prolactin with a higher specific activity than the prolactin which remained in the glands. This work has been extended recently using monolayer cells in culture, thereby avoiding problems of equal access to the medium by all cells. Walker and Farquhar [4] incubated cultured cells for 4 hr in [^{14}C]proline to label the slowly turning over pool of prolactin, followed 1 hr later by a 1-hr incubation period with [^{3}H]proline to label the prolactin that was rapidly released from the cell. Prolactin released basally had a higher ^{3}H/^{14}C ratio than that contained in the cells, a result consistent with that of Swearingen. These results indicate that the gland has a store of prolactin with a slow turnover rate. Prolactin released in response to TRH (thyrotropin-releasing hormone) had a lower ^{3}H/^{14}C ratio than did prolactin released from unstimulated cells, indicating that prolactin released by TRH contained more of the slowly turning over pool than prolactin released basally. Stachura* has seen similar results using GH cells; he has evidence for a more slowly turning over component which could be released by high potassium concentrations or by dibutyryl cyclic AMP.

These biochemical studies demonstrate the existence of two components of prolactin release, one in which prolactin is released rapidly after synthesis, and a second in which prolactin is released more slowly. It is not possible to determine from these studies the proportion of newly synthesized prolactin that enters each pool, but the second is stimulated more than the first by agents which stimulate prolactin release. These studies do not rule out the possibility that more than two pools may exist; pools which turn over very slowly would be difficult to label adequately. Finally, these studies do not distinguish whether the two detectable pools exist within the same cell. Walker and Farquhar have found evidence for heterogeneity of leucine incorporation into prolactin-producing cells [4]; they counted the autoradiographic grains over the cells and found that cells did not incorporate leucine at the same rates. Cells could be divided into three groups 30 min after a 5-min labeling period: lightly labeled, heavily labeled and an intermediately labeled group. Three hours after labeling, only lightly and intermediately labeled cells were detected. There are several possible explanations for this heterogeneity. First, the heavily labeled cells may synthesize more prolactin than the other cells

* M. E. Stachura, unpublished results.

BP 31:18 – A

and then rapidly release most of it, while the other two groups retain most of what they synthesize. Second, all cells may rapidly release prolactin but only the most actively synthesizing cells store the hormone. In this model, all three groups of cells lose label, so the heavy and intermediate groups become more lightly labeled. Third, the cells may all synthesize and release prolactin at the same rates or in the same way but may differ in the transport of leucine or the size of the intracellular leucine pools so that the specific activity of the intracellular leucine varies from cell to cell.

There is morphological evidence for heterogeneity of prolactin-producing cells. Prolactin cells in rats have the largest secretory granules of all the cells in the pituitary gland, ranging from 500 to 900 nm in diameter, but immunocytochemical stains have shown that cells exist in the normal pituitary gland which stain for prolactin but lack the largest granules [5, 6]. The large mature secretory granules cannot be the only component of the slowly turning over pool of prolactin, since GH cells have this pool but only small granules have been seen in these cells. Immunocytochemical studies have shown that GH cells are heterogeneous with respect to the amount of prolactin they store [7]. Therefore, it is possible that two pools exist in separate cells; one group of cells may process prolactin more slowly. A definitive way of demonstrating that the cells do not each contain both pools would be the physical separation of the cell types. Experiments separating pituitary cells by unit gravity sedimentation have shown differences in the amount of prolactin released into the medium [8]. At present, the morphology and the responses to releasing agents of the different fractions have not been reported.

If the release of prolactin from the cell is prevented, for example by dopaminergic agonists, prolactin not released from the cell is degraded [9, 10]. Prolactin degradation also occurs in some conditions in cells and glands where release has not been inhibited by drugs [10, 11]. Maurer [10] demonstrated that cycloheximide prevented degradation of prolactin if added with the dopaminergic agonist bromocriptine, but that cycloheximide did not prevent the effect if cells were pretreated with bromocriptine. This experiment suggests that protein synthesis may be necessary for the induction of prolactin degradation and, therefore, that degradation is an inducible function, not just a passive process which occurs because release is blocked. Degradation of prolactin has been studied morphologically by Smith and Farquhar [12] who showed that, when release is suppressed, granules can be degraded by lysosomes. They used suckling rats, which synthesize large amounts of prolactin that is not released if the rats are deprived of their young. Secretory granules accumulated in the mammotrophs for 12 hr; at later times, lysosomes were seen that contained the secretory granules inner cores. Whether chronic treatment with bromocriptine leads to the degradation of prolactin at the same stage in processing as this acute inhibition is not yet known. It is also not known whether the two pools of prolactin that have been distinguished are equally susceptible to degradation, or whether degradation is limited to one pool.

After prolactin is synthesized, it is possible for the hormone to leave the cell rapidly or to be stored in one or more slowly turning over pools. It is also possible for stored hormone to be released from the cell or to be degraded intracellularly. All of these processes can be regulated. Estrogen and TRH increase prolactin synthesis; hydrocortisone and bromocriptine decrease prolactin synthesis [13]. Release can be directly stimulated by TRH and VIP (vasoactive intestinal peptide); release can be inhibited by dopaminergic agonists, GABA (γ-aminobutyric acid,), and muscarinic agonists [1]. Bromocriptine, the dopaminergic agonist, induces degradation as previously discussed. Storage of a slowly turning over pool in GH cells, which normally store very little, can be increased 20-fold by insulin and estrogen, an effect which is independent of effects on prolactin synthesis.* An understanding of how prolactin release is controlled will involve all these steps.

For the last 15 years, efforts to understand these mechanisms have involved investigations of two factors, calcium and cyclic AMP. At times, too much emphasis has been placed on cyclic AMP without supporting evidence, so that review articles and textbooks cite TRH as a hormone whose receptor is coupled to adenylate cyclase, whereas a direct stimulation of adenylate cyclase in a cell-free system by TRH has not been demonstrated. At other times, attempts have been made to totally exclude cyclic AMP, based on experiments which showed that agents that increase cyclic AMP did not always cause an increase in prolactin release. Both of these views are oversimplifications for two reasons. The first reason is that the action of factors influencing prolactin production is complex and may involve more than one mechanism. The second reason is that heterogeneity in the pools of prolactin has not been considered. Both of these reasons are discussed below.

The two factors which have been studied most extensively, TRH and dopamine, both appear to have more than one action. There are several lines of evidence indicating that TRH increases the concentration of intracellular calcium. First, pituitary cells have action potentials which contain a calcium component, thus providing a mechanism for calcium entry into the cells [14, 15]. The frequency of the action potentials can be increased by TRH within seconds after its addition to either normal pituitary cells in culture or GH cells. Second, stimulation of prolactin release by TRH requires the presence of calcium and can be blocked by cobalt, a calcium channel blocker [16]. Third, Tan and Tashjian [17] have shown that TRH can release extracellular membrane-bound calcium and cause an influx of calcium into the cell. Since other factors which cause calcium to enter the cell, such as depolarization by high potassium concentrations or the use of a calcium ionophore, cause prolactin release, a logical possibility is that TRH causes prolactin release by increasing intracellular calcium concentrations. The mechanisms by which TRH acts, however, are probably

* D. R. Kiino and P. S. Dannies, unpublished results.

more complex than just increasing calcium concentrations. In the first place, calcium can affect cyclic AMP metabolism. A review by Rasmussen and Goodman [18] has already pointed out the many possible interactions between calcium and cyclic AMP. TRH has been shown to cause an increase in cyclic AMP in intact cells under some conditions [19, 20], even though a direct stimulation of adenylate cyclase has not been demonstrated. Calcium-dependent activation of adenylate cyclase through calmodulin has been found in other cell types. If such a mechanism exists in GH cells, it would explain the rise in cyclic AMP in intact cells sometimes seen with TRH. One other interaction between calcium and cyclic AMP in prolactin production is known, which is that calcium enhances release stimulated by derivatives of cyclic AMP [21]. Calcium and cyclic AMP may have additional interactions in the process of causing prolactin release.

The second reason why the action of TRH may be more complex than just causing cytosolic calcium concentrations to increase is that studies with TRH analogs suggest that there is more than one mechanism of TRH action. The structural requirements for increasing prolactin release are different from those for increasing prolactin synthesis in GH cells or for increasing thyrotropin release from normal cells [22]. Therefore, the hormone receptor interactions that mediate each of these processes cannot be identical. Use of analogs with increased specificity for release and synthesis will be important in relating effects of TRH on mediators, such as calcium, to the effects of TRH on release and synthesis.

The actions of dopamine and dopaminergic agonists like bromocriptine on prolactin production seem at least as complicated as those of TRH. Dopamine inhibits action potentials in pituitary cells [23], indicating that dopamine may block calcium entry. In addition, dopamine inhibits rises in cyclic AMP in intact cells induced by VIP or TRH [20, 24] and has been shown to directly inhibit adenylate cyclase in pituitary tumor membrane preparations [25]. The prolactin producing cells, therefore, seem to contain dopamine receptors that can inhibit the activation of adenylate cyclase, like some alpha-adrenergic receptors [26], in addition to blocking calcium entry. We have shown that bromocriptine blocks prolactin release stimulated by the calcium ionophore, A23187, which allows calcium to enter, and release caused by 8-bromocyclic AMP, which should increase cyclic AMP levels bypassing activation of cyclase [21, 27]. Because two steps, inhibition of calcium entry and inhibition of activation of adenylate cyclase, appear to be involved in dopamine action, adding back either calcium or cyclic AMP alone may not be sufficient to cause release. We tested to see if the dopaminergic inhibition could be overcome by using both calcium ionophore and 8-bromocyclic AMP. Calcium ionophore and 8-bromocyclic AMP together enhanced prolactin release more than either agent alone, but bromocriptine still blocked release caused by the combined use of the two agents as well as when they were added separately (S. W. Tam and P. S. Dannies, unpublished results). This experiment suggests that dopamine may block release at a step or steps in addition to blocking calcium entry and adenylate cyclase activation.

These brief summaries of what is known about the mechanisms of TRH and dopamine indicate it is an oversimplification to talk about *the* mechanism of action of these compounds; they each appear to have more than one. Other factors whose mechanisms now appear straightforward may also turn out to be more complicated. The second reason why approaches to the mechanisms of action regulating prolactin release have been oversimplified is that for the most part the heterogeneity of prolactin processing has not been considered. One important series of papers that have considered hormone pools in terms of mechanisms of release is that of Stachura. Most of his published work is on growth hormone. He measured changes in the rate of release of prelabeled growth hormone using perfused anterior pituitary glands. He found that high potassium concentrations trigger a rapid release of growth hormone from the perfused glands. The rate of release returns to basal levels quickly under these conditions in the continued presence of high potassium. Dibutyryl cyclic AMP also triggers a rapid release and, in addition, causes a second phase of increased release sustained over the next hour [28]. When release is blocked by somatostatin, dibutyryl cyclic AMP causes a build-up of growth hormone into the rapidly releasable pool, even though release into the medium is blocked. High potassium levels in the presence of somatostatin do not cause the build-up of the rapidly releasable pool [28, 29]. Stachura suggested that there are two components to stimulated release, a rapidly releasable pool, which can be released by either calcium or cyclic AMP, and a more slowly releasable pool, which can be released by cyclic AMP. If release is inhibited, cyclic AMP can still transfer growth hormone to the rapidly releasable pool, so that release from this pool is increased when the inhibition is removed. In the published work growth hormone release was measured but he has found similar results with prolactin release from pituitary glands of female rats (M. E. Stachura, unpublished results).

At this point we are talking about so many pools of prolactin that the situation may be confusing. There is the newly synthesized, rapidly released prolactin which does not appear to be stored. The labeling studies discussed earlier show that this pool is affected by agents which release prolactin to a lesser extent than the stored prolactin. The prolactin in this more slowly turning over pool appears to have two components, one which can be released rapidly by high potassium concentrations and dibutyryl cyclic AMP, and one which can be transferred to the rapidly releasable form by dibutyryl cyclic AMP. Consideration of these pools can explain some of the interactions among the various compounds which affect prolactin production. For example, Fagin and Neill [30] have done a study of the effect of dopamine on TRH-induced prolactin release using perfused pituitary glands. They found that, when the cells were exposed to dopamine, prolactin release was decreased to about 35% of untreated glands. When dopamine infusion was stopped, prolactin release rose to control levels within 12 min, and when it was

added back, prolactin release was inhibited to the same degree as before. When TRH infusion was begun 18 min after dopamine infusion was reinitiated, TRH stimulated prolactin release more than when TRH was administered to glands which had been continuously subjected to dopaminergic inhibition. Since one of the actions of TRH is to increase entry of calcium, as high concentrations of potassium do, TRH might therefore be expected to act as potassium did in the experiments of Stachura, and to stimulate release of the quickly releasable pool. One of the actions of dopamine is to inhibit adenylate cyclase, and dibutyryl cyclic AMP can cause transfer of hormone to the quickly releasable pool. Removal of dopaminergic inhibition may therefore allow increased levels of cyclic AMP and a build-up of this pool at the same time that release into the medium increases, so that after inhibition is reapplied, more prolactin is available to be released when the cells are subsequently stimulated by TRH. This interpretation may be oversimplified since it involves only one action of TRH and dopamine. One prediction from this interpretation is that treating glands with cyclic AMP analogs in the presence of dopamine should enhance the amount of release caused by a later infusion of TRH.

Grosvenor and colleagues in a series of papers have also defined and investigated more than one pool of prolactin involved in prolactin release. Prolactin release in the intact animal appears to go through two stages; the stages are first, "depletion" or transformation in the gland, and second, release from the gland. There are two kinds of evidence that the first stage exists. The first evidence is that the amount of prolactin in the pituitary gland decreases within minutes after lactating rats begin to suckle and the amount of prolactin that appears in the serum within that time does not reflect the apparent decrease in the gland [31, 32]. The method by which glands are prepared for the prolactin assay is probably important to see this effect. When the depletion was observed, the glands were homogenized at neutral pH; this procedure may not solubilize all the prolactin. The apparent depletion may reflect a change in the recovery of prolactin from the cell. This interpretation is supported by the results of Nicoll *et al.* [33] who did not see reproducible depletion when glands were homogenized at high pH. If a change in recovery is responsible for the depletion, it will, of course, be of interest to find out what is causing this change. The term transformation, which was used recently by Grosvenor and coworkers to describe the effect, seems more appropriate than depletion.

The second kind of evidence for a process besides release is based on the change in the response of the pituitary gland before and after a brief period of suckling. Grosvenor and coworkers used lactating rats that had been deprived of their young for hours and had, therefore, built up large stores of prolactin. Compounds were tested using rats either in this state, or after they had been allowed to suckle their young for just 10 min. Prolactin release rose during this 10-min period but dropped to basal release when suckling was discontinued. Using this system, Grosvenor and Mena [34] have found that TRH stimulates prolactin release only slightly in rats that have not suckled, but there is a larger elevation in release from rats which have first suckled and then received TRH after prolactin release returned to basal levels. They also found that TRH does not cause the apparent depletion of the gland the way suckling does. They suggested that TRH can release prolactin only after the prolactin stores are transformed by suckling and that brief suckling periods transform some of the prolactin in the gland so it can later be released. They found that dopamine and bromocriptine inhibit the suckling-induced increase if given to rats before suckling. When bromocriptine is given to rats after a brief suckling period, it does not inhibit release induced by further suckling [35]. They therefore suggest that dopamine can inhibit the transformation step but not the subsequent stimulation of release. It is tempting to consider the transformation of prolactin to be the equivalent of a shift in the slowly releasable pool of prolactin to the rapidly releasable pool. Dopamine, which blocks activation of adenylate cyclase, would therefore be expected to block the shift, or transformation, and TRH, if its effects were primarily mediated through calcium, should only be able to release prolactin after its transformation into the quickly releasable pool. This suggestion is not completely satisfying since electrophysiological evidence indicates that dopamine can prevent calcium entry into the cell; if this is true, dopamine should be able to block release of the quickly releasable pool as well as transformation. Other complicating factors may therefore have to be sorted out, but certain predictions can be tested. Agents which raise cyclic AMP levels or which mimic the action of cyclic AMP should be able to transform prolactin; cyclic AMP analogs given to rats deprived of their young should increase release by TRH administered subsequently. Increases in cyclic AMP concentrations caused by VIP can be blocked by dopamine [24] so this peptide should be able to cause transformation in the absence of dopamine but not in its presence. A series of experiments of this type could determine the relationship of the rapidly releasable pool seen in isolated glands to the transformed pool seen in intact rats.

At this time it is not known what the factor that causes transformation is. Suckling, mammary nerve stimulation, or exposure to pups without suckling can cause transformation [36, 37] but the factor or factors that mediate this response are not known. TRH did not cause transformation, and they did not find any such activity in extracts of stalk median eminence, although these extracts could stimulate release after transformation as TRH does [34]. Normal pituitary cells in culture do not release a large portion of their intracellular prolactin no matter what stimulus we use; there may be conditions or factors which can convert prolactin to a releasable form. Transformation may be caused by a substance not yet identified, or it may be caused by an unknown combination of factors already shown to affect prolactin production. The timing or sequence in which these factors are added may be important.

It is also not known at this time what the morphological correlates of the various biochemically distinguished pools are. One simple explanation may

be that prolactin that is quickly releasable is in granules near the membrane, and that transformation involves moving more granules closer to the membrane; obviously there are more complicated possibilities.

Investigations of the mechanisms of regulation of prolactin production have been performed by many different laboratories and there are many conflicting reports in the literature about factors which influence prolactin release and the mechanisms by which they do so [1]. The regulation of hormone synthesis and release is complex and the two factors studied in the most detail, TRH and dopamine, appear to have more than one mechanism. It is possible that different mechanisms dominate under different circumstances. In addition, the process by which prolactin is released from the cell is not homogeneous. Newly synthesized prolactin may be rapidly released or stored in a more slowly turning over pool. Release of the slower pool can be stimulated, and there are at least two components. If transformation does not correspond to the slowly releasable pool, there may be even more than two. The amount of stored prolactin can be varied, and the rates of the various components of prolactin storage may vary as well. As we learn more about conditions under which various pools are released, we may be able to understand why differences among various laboratories have occurred.

Acknowledgements—I wish to thank Dr. Max Stachura for allowing me to see unpublished data, and Dr. Diane Kiino for helpful discussions. This work was supported by USPHS Grant HD-11487 and Research Career Development Award HD-00272.

REFERENCES

1. P. S. Dannies and S. W. Tam, in *Cellular Regulation of Secretion and Release* (Ed. P. M. Conn). Academic Press, New York (in press).
2. M. G. Farquhar, J. J. Reid and L. W. Daniell, *Endocrinology* **102**, 296 (1978).
3. K. C. Swearingen, *Endocrinology* **89**, 1380 (1971).
4. A. M. Walker and M. G. Farquhar, *Endocrinology* **107**, 1095 (1980).
5. B. L. Baker, *J. Histochem. Cytochem.* **18**, 1 (1970).
6. C. Tougard, R. Picart and A. Tixier-Vidal, *Am. J. Anat.* **158**, 471 (1980).
7. R. F. Hoyt and A. H. Tashjian, Jr., *Anat. Rec.* **197**, 163 (1980).
8. J. Snyder, W. Wilfinger and W. C. Hymer, *Endocrinology* **98**, 25 (1976).
9. P. S. Dannies and M. S. Rudnick, *J. biol. Chem.* **255**, 2776 (1980).
10. R. A. Maurer, *Biochemistry* **19**, 3573 (1980).
11. R. Shenai and M. Wallis, *Biochem. J.* **182**, 735 (1979).
12. R. E. Smith and M. G. Farquhar, *J. Cell Biol.* **31**, 319 (1966).
13. R. A. Maurer, in *Cellular Regulation of Secretion and Release* (Ed. P. M. Conn). Academic Press, New York (in press).
14. Y. Kidokoro, *Nature, Lond.* **258**, 741 (1975).
15. P. S. Taraskevich and W. W. Douglas, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4064 (1977).
16. A. H. Tashjian, Jr., N. J. Barowsky and D. K. Jensen, *Biochem. biophys. Res. Commun.* **81**, 796 (1978).
17. K. N. Tan and A. H. Tashjian, Jr., *J. biol. Chem.* **256**, 8994 (1981).
18. H. Rasmussen and D. B. P. Goodman, *Physiol. Rev.* **57**, 421 (1977).
19. P. S. Dannies, K. M. Gautvik and A. H. Tashjian, Jr., *Endocrinology* **98**, 1147 (1976).
20. G. D. Barnes, B. L. Brown, T. G. Gard, D. Atkinson and R. P. Ekins, *Molec. cell. Endocrin.* **12**, 273 (1978).
21. S. W. Tam and P. S. Dannies, *Endocrinology* **109**, 403 (1981).
22. P. S. Dannies and A. H. Tashjian, Jr., *Nature, Lond.* **261**, 707 (1976).
23. P. S. Taraskevich and W. W. Douglas, *Nature, Lond.* **276**, 832 (1978).
24. P. Onali, J. P. Schwartz and E. Costa, *Proc. natn. Acad. Sci. U.S.A.* **78**, 6531 (1981).
25. P. DeCamilli, D. Macconi and A. Spada, *Nature, Lond.* **278**, 252 (1979).
26. J. N. Fain and J. A. Garcia-Sainz, *Life Sci.* **26**, 1183 (1980).
27. S. W. Tam and P. S. Dannies, *J. biol. Chem.* **255**, 6595 (1980).
28. M. E. Stachura, *Endocrinology* **101**, 1044 (1977).
29. M. E. Stachura, *Endocrinology* **108**, 1027 (1981).
30. K. D. Fagin and J. D. Neill, *Endocrinology* **109**, 1835 (1981).
31. C. S. Nicoll, *Gen. comp. Endocr. Suppl.* **3**, 86 (1972).
32. C. E. Grosvenor, F. Mena and N. S. Whitworth, *Endocrinology* **104**, 372 (1979).
33. C. S. Nicoll, F. Mena, C. W. Nichols, Jr., S. H. Green, M. Tai and S. M. Russell, *Acta endocr. Copenh.* **83**, 512 (1976).
34. C. E. Grosvenor and F. Mena, *Endocrinology* **107**, 863 (1980).
35. C. E. Grosvenor, F. Mena and N. S. Whitworth, *Endocrinology* **106**, 481 (1980).
36. C. E. Grosvenor, N. S. Whitworth and F. Mena, *Endocrinology* **108**, 820 (1981).
37. F. Mena, P. Pacheco and C. E. Grosvenor, *Endocrinology* **106**, 458 (1980).