SECRETION AND DEGRADATION OF PARATHORMONE AS A FUNCTION OF INTRACELLULAR MATURATION OF HORMONE POOLS

Modulation by Calcium and Dibutyryl Cyclic AMP

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ABSTRACT

The biosynthesis, processing, and secretion of parathormone and the effect of calcium on these processes were measured in dispersed porcine parathyroid cells incubated with [^{35}S]methionine. Proparathormone was detected at 10 min, the earliest time measured, and was rapidly and apparently quantitatively converted to parathormone. The half-life of the prohormone pool was 15 min. Secretion of parathormone was detected by 20 min. In pulse-chase experiments there was a period between 20 and 40 min during which the wave of newly-synthesized parathormone was secreted. After 40 min, little additional radioactive hormone was secreted, but dibutyryl cyclic AMP, an agent that can mobilize stored parathormone, when added to the incubation mixtures enhanced radioactive parathormone secretion but only after 60 min, although it increased net hormone secretion as determined by radioimmunoassay to the same extent at all times studied. When the ionized calcium concentration of the medium was lowered, more radioactive hormone was secreted at all times but the effect was greatest on that hormone that was synthesized <60 min previously; however, net hormone secretion in contrast to radioactive hormone was enhanced equally at all intervals. These data could mean that the refractoriness to secretion of parathormone 40–60 min of age was related to maturation of its secretory container preparatory to storage. Low calcium (0.5 mM) stimulated hormone secretion up to fivefold compared to high calcium (3.0 mM) but did not affect synthesis of parathormone or proparathormone or conversion of the latter to hormone. During processing at least 70% of the intracellular parathormone was lost, presumably through proteolysis and this degradation was greater at high calcium. These data have been interpreted in light of the concept that two secretable pools of parathormone exist within the parathyroid.

KEY WORDS parathormone · calcium · secretory protein-I · cyclic AMP · protein secretion

Parathormone is initially generated as preproparathormone on the endoplasmic reticulum (19, 21), converted during its synthesis at that site to proparathormone which upon transfer to the Golgi is converted to parathormone 9–11, 17, 27). The hormone is then believed to be packaged into secretory granules and either secreted or stored (11, 29). This pathway would be generally equivalent to the segregated processing route described by Palade and his colleagues (25, 36). Among the stimuli for secretion are hypocalcemia (13, 37, 43–45), hypomagnesemia (7, 35, 44), and a variety of other agents including most prominently, β-adrenergic agonists (2, 5, 6, 15, 52) and dibutyryl cyclic AMP[1] (1, 32, 52). In addition to affecting secretion, calcium enhances the degradation of hormonal protein, a process that has been postulated to regulate the amount available for secretion (8, 18).

During a study of the incorporation of radioactive amino acids by bovine parathyroid slices, MacGregor et al. (30) noted that the specific radioactivity of secreted parathormone was always many-fold greater than that remaining in the tissue. These data were interpreted to mean that a secretory "bypass" existed through which newly synthesized parathormone was released by the gland preferentially over stored hormone. At that time it was not clear if this "bypass" resulted from a non-equilibrium of new secretion vesicles with older stored granules or if instead "new" and "old" hormone represented different, and individually controllable, intracellular pools. Recently, we (32) confirmed the work of MacGregor et al. (30) using dispersed porcine parathyroid cells. We showed that the new and old pools of hormone were indeed different: hypocalcemia increased secretion of both the newer and older hormone to the same extent whereas the β-adrenergic agonist, isoproterenol, and dibutyryl cyclic AMP brought about release of the older hormone exclusively. Since the intracellular concentration of cyclic AMP was inversely related to the extracellular calcium concentration (3, 12, 32), it seemed likely that the effect of calcium on the secretion of new hormone was a primary one, and the effect on the secretion of older hormone was modulated through cyclic AMP (32).

These results showed that the intracellular processing and secretion of parathormone is under more complex control than hitherto suspected. This has led us to evaluate the kinetics of hormone biosynthesis and secretion in the dispersed parathyroid cells influenced by calcium and dibutyryl cyclic AMP. The present data have allowed us to more precisely define certain degradative and secretory phases in the intracellular processing of parathormone.

MATERIALS AND METHODS

Preparation and Incubation of Cells

Porcine parathyroid cells were prepared by the collagenase-DNase procedure of Brown et al. (4) with slight modification (31). In all experiments the cells were incubated at a density of 4×10^5 cells/ml in Krebs-Ringer supplemented buffer (31) at 37°C containing 1 mM magnesium sulfate and either 0.5 mM or 3.0 mM calcium chloride. In continuous label experiments, cells were incubated with 2.5 μCi/ml [^{35}S]methionine. In pulse-chase experiments, the cells were incubated with 50 μCi/ml [^{35}S]methionine for 10 min followed by the addition of nonradioactive methionine to a final concentration of 250 μM. This amount of unlabeled methionine halted further incorporation of labeled methionine into total trichloroacetic acid precipitable protein within 1 min.

In pulse-chase experiments, the cells were incubated at 0.5 mM or 3.0 mM calcium, pulsed with radioactive methionine, and chased with unlabeled amino acid as described. At various times portions of the pulse-chase cell mixture were transferred to secondary incubation vessels and incubation was continued for an additional 20 min in the presence or absence of 1 mM dibutyryl cyclic AMP for the 0.5 mM calcium medium or 2.5 mM EGTA (ethylene bis(oxyethylene nitrilo)-tetraacetic acid) for the 3.0 mM calcium medium. These experiments were designed to assess the amount of newly synthesized radioactive parathormone available for secretion in the tissue storage pool of hormone and also available for secretion in response to acute hypocalcemia. Cells were separated from the medium at the appropriate times during the experiments by centrifugation. Medium and cell pellets were stored frozen until analysis.

Analysis of Medium and Cells

The radioactivity of proparathormone and parathormone within the cells and of parathormone in the incubation medium was determined from gel electrophoresis profiles as described previously (31, 32). A particular advantage to the use of radioactive methionine as the sole marker amino acid in these studies is that porcine parathormone contains but a single methionine residue at position 8 (40). Therefore, carboxy-terminal fragments of the hormone that are believed to be derived by cleavage of the hormone in the central region of the molecule and are secreted by the parathyroid cells (34) would not contribute radioactivity to the hormone peaks separated from cell and medium in the present studies. Furthermore, large amino-terminal fragments

[1] *Abbreviations used in this paper:* cyclic AMP, adenosine 3′:5′-cyclic monophosphoric acid; dibutyryl cyclic AMP, N^6,O^2 dibutyryl adenosine 3′:5′-cyclic monophosphoric acid; EGTA, ethylene bis(oxyethylene nitrilo) tetraacetic acid.

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that could contain [^{35}S]methionine appear to be degraded within the cell since they are not found in the elution profiles (34). Assay of radioactivity was performed by liquid scintillation spectrometry.

Radioimmunoassay of parathormone was performed as described earlier (22, 31) using goat antisera (G-1811) that recognizes the carboxy-terminal portion of parathormone. [^{35}S]Methionine (SJ.204) was obtained from Amersham Corporation (Arlington Heights, Ill.). Other sources of reagents and minor procedures have been previously described (31–33).

RESULTS

The incorporation of [^{35}S]methionine into cellular parathormone and proparathormone and secreted parathormone (proparathormone was never detected in the medium) at low (0.5 mM) and high (3.0 mM) calcium in continuous labeling experiments is portrayed in Fig. 1. Radioactive prohormone rapidly accumulated and reached a steady state level within 10 min. This process was unaffected by calcium (Fig. 1 *A* and *B*). Calcium did, however, alter the kinetics of accumulation and secretion of the hormone. At low calcium, radioactive parathormone was detected in the cell by 20 min (Fig. 1 *A*). It increased in amount but at a progressively lesser rate and plateaued by 60 min. This decrease in rate of accumulation within the cell could be accounted for by its secretion into the medium since the total radioactive hormone (cell + medium, Fig. 1 *C*) increased linearly throughout the 90 min period of incubation. In contrast, at 3.0 mM calcium, radioactive hormone accumulated at a greater rate in the cell than at 0.5 mM calcium and plateaued at a higher level whereas its secretion was only one-fifth that at low calcium (compare Fig. 1 *B* to 1 *A*). By 60 min there was less total radioactive parathormone in the system (cell + medium) at 3.0 mM calcium than at 0.5 mM calcium and this difference increased with time (Fig. 1 *C*). Although not measured in the experiment illustrated, in this type of study the radioimmunoassayable parathormone in the medium invariably increased linearly with time and the rate of secretion was three to fivefold at 0.5 mM calcium than at 3.0 mM.

The experiment portrayed in Fig. 1 was performed three times with similar results. The total radioactive parathormone (cell + medium) at the 90 min experimental point was 4.7 ± 0.2 (mean ± SEM) × 10^4 dpm for 0.5 mM calcium and 2.9 ± 0.1 (mean ± SEM) × 10^4 dpm for 3.0 mM calcium. The difference in radioactive parathormone between low and high calcium could not be attributed to degradation of parathormone in the medium, since as shown previously (31) there was little if any degradation of exogenous parathormone that was added to the medium and cell mixture at either calcium concentration.

The synthesis, processing, and secretion of parathormone was next examined by the pulse-chase technique (Fig. 2). The dispersed cells were incubated for 10 min with radioactive [^{35}S]methionine and then chased for 80 min. Radioactive proparathormone was greatest at the end of the pulse period and decreased progressively during the chase period. Calcium did not affect its rates of synthesis or decay (compare Fig. 2 *A* and *B*). The

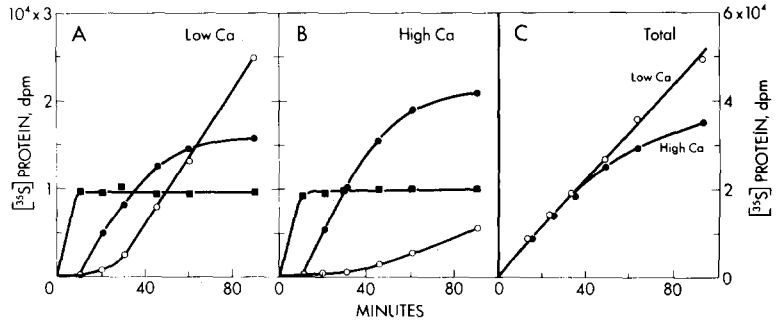

FIGURE 1 Continuous labeling study of the biosynthesis of radioactive proparathormone and parathormone and the secretion of the latter by porcine parathyroid cells. The cells were incubated in medium containing 25 μCi/ml [^{35}S]methionine at either 0.5 (*A*) or 3.0 mM Ca (*B*). Samples of the incubation mixture were taken at the indicated times and the cells and medium were analyzed for hormonal protein as indicated under Materials and Methods. Total parathormone and proparathormone in the cells and medium at 0.5 and 3.0 mM Ca are portrayed in *C*. For *A* and *B*: ■——■, proparathormone; ●——●, cellular parathormone; ○——○, secreted parathormone. For *C*; ●——●, 3.0 mM Ca; ○——○, 0.5 mM Ca.

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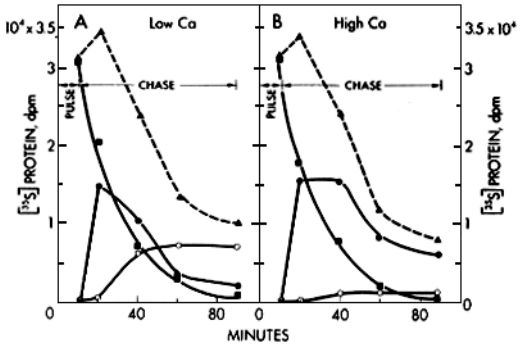

FIGURE 2 Pulse-chase study of the biosynthesis of radioactive proparathormone and parathormone and the secretion of the latter by porcine parathyroid cells. The cells were incubated for 10 min (pulse) with 50 μCi/ml of [^{35}S]methionine at 0.5 (*A*) and 3.0 mM Ca (*B*). Nonradioactive methionine was added (0.25 mM final concentration) to stop radioactive incorporation and incubation was continued as shown (chase). Samples of cell and medium were analyzed as indicated. ▲---▲, the sum of radioactive parathormone + proparathormone in cell and medium; ■—■ proparathormone; ●—●, cellular parathormone; ○—○, secreted parathormone.

loss of radioactive prohormone occurred at an apparent first-order rate with a half time of 15 min. At both concentrations of calcium, the decrease in radioactive prohormone between 10 and 20 min was matched by an appearance within the cell of an equivalent amount of radioactive parathormone (Fig. 2*A* and *B*). Therefore, up to the 20 min time point there was no difference in the parameters examined between high and low calcium. Between 20 and 40 min, substantially more parathormone was secreted at low than at high calcium, and there was correspondingly less in the cell such that the sums of secreted and cell hormone were similar at either calcium concentration. There was little additional secretion of hormone after 40 min at high or low calcium but the amount of cellular hormone continued to decrease at a rate somewhat greater at 3.0 mM calcium than at 0.5 mM calcium. In the study shown in Fig. 2 together with two additional experiments the percentage of proparathormone that survived as parathormone at 3.0 mM calcium was 19.2 ± 1.2 vs. 26.8 ± 2.8 at 0.5 mM calcium. Therefore, >70% of the total hormonal protein (parathormone and proparathormone) disappeared from the system and the loss was greater at 3.0 mM calcium due to the greater disappearance of parathormone at this concentration of calcium (compare Fig. 2*A* and *B*).

The lack of appreciable secretion of radioactive parathormone after 40 min was at first surprising since there was still a substantial amount of radioactive hormone within the cell (Fig. 2*A* and *B*). Since we showed earlier (32) that dibutyryl cyclic AMP elicited secretion from the older pool of hormone, whereas hypocalcemia mobilized hormone from both the older and newer pools, we repeated the pulse-chase experiment with the additional variable of adding either dibutyryl cyclic AMP or creating hypocalcemia by adding EGTA to the incubation medium at various times during the chase. Fig. 3 shows that dibutyryl cyclic AMP did not enhance the secretion of radioactive hormone until after 60 min. At the last period examined (120–140 min) the secretagogue raised hormone secretion ~80% over the untreated control, compared to ~60% in the 90–110 min interval and only 20% in the 60–80 min interval. That this additional radioactive hormone that was mobilized by dibutyryl cyclic AMP came from that cellular hormone that otherwise would have been retained is evident from the data of Table I. During the 60–80 min interval and thereafter there was less radioactive parathormone in those cells treated with dibutyryl cyclic AMP than in the control. The magnitude of decrease in cellular hormone was greater at the later intervals and in

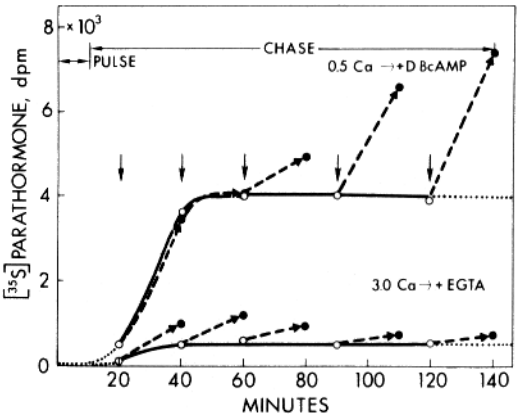

FIGURE 3 The stimulation of secretion of radioactive parathormone by dibutyryl cyclic AMP (DBcAMP) or low calcium. Cells were incubated under pulse-chase conditions described in the legend of Fig. 2. At the indicated times portions of the reaction mixture were incubated for an additional 20 min in the presence or absence of 1 mM DBcAMP (0.5 mM Ca) or 2.5 mM EGTA (3.0 mM Ca). The medium was assayed for both radioactive and immunoactive parathormone (see text).

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TABLE I

Effect of Dibutyryl cAMP on the Cell Content of Newly-Synthesized Parathormone

Interval	[^{35}S]Parathormone		
	Control	+DBcAMP	Change
min	*dpm*	*dpm*	*%*
20–40	15,500	14,600	−6
40–60	7,240	7,500	4
60–80	6,450*	5,750	−11
90–110	6,100*	4,850	−20
120–140	5,900*	3,300	−44

* Values were obtained for the end of the time interval by graphic extrapolation of values for intracellular parathormone in the experiment described in Fig. 3 obtained at 60, 90, and 120 min time points.

amount was generally equivalent to that secreted to the medium. These data contrasted sharply with those of net hormone secretion determined by immunoassay. At every time point dibutyryl cyclic AMP elicited the secretion of about the same amount of additional hormone above the basal level of secretion: the calculated cumulative levels of hormone secreted by the control cells at 40, 60, 80, 110, and 140 min were, respectively 4.2, 6.0, 8.1, 11.2, and 14.8 ng/10^5 cells; with dibutyryl cyclic AMP, the hormone secreted at the corresponding times was 13.8, 14.5, 18.0, 21.7, and 24.7 ng. The average increment and standard deviation in hormone secretion produced by dibutyryl cyclic AMP was 9.7 ± 0.7 ng/10^5 cells.

Fig. 3 also shows that switching the cells to low calcium increased radioactive hormone secretion at all times but was most effective at the earliest periods: stimulation of ~100% at 20–60 min vs. 25% at 90–140 min intervals. In contrast to the variable effect of radioactive hormone, hypocalcemia produced identical net increases in secretion of parathormone (3.9 ± 0.2 ng/20 min), at each interval examined. In the experiments with EGTA, in contrast to those with dibutyryl cyclic AMP, we found little difference in cellular radioactive hormone between control cells and those treated with EGTA. This is in keeping with the smaller effects of the treatment on secretion—particularly at the latter periods. These results with dibutyryl cyclic AMP and induced hypocalcemia showed that much of the cellular radioactive hormone was available for secretion, at either high or low calcium conditions, but required the appropriate secretagogue applied at the appropriate time to elicit its secretion.

DISCUSSION

The temporal relationships for the synthesis and secretion of parathormone established by this and previous studies in our laboratory (11, 29, 30) together with the recent work on preproparathormone (21) are portrayed in Fig. 4. Newly synthesized proparathormone is detected at the earliest measured time (11) and presumably is derived from preproparathormone within the cisternal space of the endoplasmic reticulum (21). During the next 10–20 min, the prohormone is transported to the Golgi zone where it is converted to parathormone (9–11, 27). These processes are unaf-

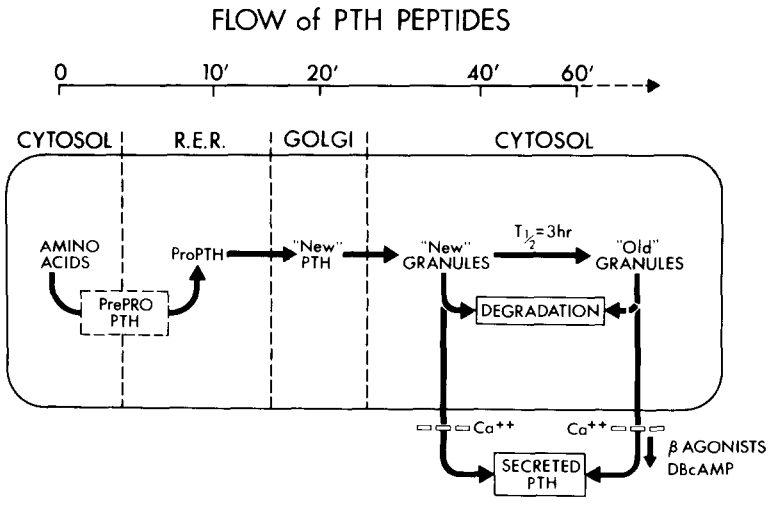

FIGURE 4 Postulated scheme for the biosynthesis, processing, and secretion of parathormone (PTH).

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fected by calcium and the prohormone that disappears is accounted for by formation of parathormone (Fig. 2). After 20 min, the newly formed parathormone, now in secretory granules, is subject to secretion, degradation or storage. In this study secretion was inhibited as much as 80% by high calcium concentration (Figs. 1 and 2). Degradation has two components: one that is unaffected by calcium, the other that is calcium sensitive. The former component makes itself evident in the pulse chase studies during between 20 to ~60 min (Fig. 2, parathormone + proparathormone). Overall, this component encompasses the disappearance of ~70% of the hormonal protein. The calcium sensitive component encompasses the disappearance in high calcium of an additional 10% of the total hormonal protein or ~30% of the surviving parathormone found in the low calcium situation (Figs. 1 and 2, 60–90 min intervals). The loss of hormonal protein is most likely the result of proteolysis (23) since in separate studies of fragments released by parathyroid cells, up to one half of the "missing" hormone could be accounted for by a major carboxyl terminal fragment released to the medium (34). The remainder of the hormone likely is degraded to smaller undetected fragments plus free amino acids.

Since the pulse-chase data revealed that the prohormone was converted stoichiometrically to the hormone (Fig. 2*A* and *B*, 10–20 min interval), parathormone must be the molecular species that specifically is degraded during cellular processing, an interpretation in accord with that of Habener et al. (18). This conclusion pinpoints the nature of this degradative event more closely than we were able to do in our previous studies with rat parathyroids in vitro (8). In our earlier study the pulse period was 1 h and the sampling times were 1–2 h, intervals that precluded us from distinguishing whether the degraded hormonal species was the hormone, prohormone or both. In the present study the short pulse and sampling intervals make it evident that the calcium sensitive component of degradation occurs well after the hormone is believed to have been discharged from the Golgi.

Whether the action of calcium on degradation is direct or indirect is not clear. Calcium could have acted in a primary fashion, for example by enhancing fusion of lysosomal vesicles with secretory granules (29, 47) or by activating a degradative enzyme (16). It is equally possible that its action was secondary to its effect on secretion. That is, since there was more hormone within the cell at the higher calcium level (Fig. 2*A* and *B*), more of it could have been degraded by the calcium-independent component of degradation. In any event, as a consequence of substantial degradation and secretion only a small amount of new hormone remains for transfer to storage—an observation in confirmation of the results previously reported by MacGregor et al. (30) with bovine parathyroid slices.

Our results should be viewed in light of the recent demonstration that two distinct pools of parathormone available for secretion exist in the parathyroid (32). One of these pools contains newly synthesized hormone, the other mature (storage) hormone. Accordingly, between 20 and 40 min after synthesis, hormone would be secreted directly from the newly synthesized pool (Fig. 2). The hormone that is mobilized after 60 min by dibutyryl cyclic AMP would be from the storage pool, and that contained in the cell between 40 and 60 min that is not secreted under the influence of hypocalcemia or dibutyryl cyclic AMP (Fig. 3, Table I) would be in a transition pool.

These data are in accord with a morphological picture of packets of hormone within a single cell moving through the cytoplasm after release from the Golgi and undergoing sequential chemical modifications that determine their secretory and degradatory fates. At least two other morphological models are equally plausible. A single parathyroid cell might manufacture two different types of secretory vesicles that require different processing times before secretion and whose chemistry or location within the cell is such that they respond differently to various secretagogues. Alternatively different cell types might exist within the parathyroid cell populations and these cells might have different modes of processing and secretion—an interpretation of Roth and associates (39, 41) for the parathyroid and by Walker and Farquhar for the pituitary mammotroph (51). Each of these situations would be analogous to having in one gland the two different pathways for intracellular transport of secretory proteins described for regulated vs. nonregulated cells (50), or to the slow and fast secretory mechanisms, respectively, described for the primary pituitary mammotrope and the cultured GH3 pituitary cell (14).

Regardless of which anatomical description proves correct, it is clear that heterogeneous pools of parathormone exist within the parathyroid gland. The concept that heterogeneous secretory protein pools exist in a secretory tissue is not novel.

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Evidence for such pools has been described in the placenta for placental lactogen (49), in the adenohypophysis for prolactin (48) and for gonadotropin (24), in the parotid for salivary enzyme (42) and in the pancreas for amylase (46). Indeed, our results are analogous to those of Slaby and Bryan (46) who found that myo-inositol specifically elicited secretion of pancreatic amylase that was newly packaged, in contrast to the effect of carbamylcholine that mobilized both newly synthesized and stored enzyme. They concluded that a period of 1 h was required for the flow of the most recently formed granules to enter a storage pool, a time similar to that which could exist in the parathyroid.

Since parathormone is membrane-associated in the gland (11, 20, 30) and since the hormone that is secreted at any time appears to have the same chemical structure (similar migration of gels [31], tryptic peptides and amino acid sequence [unpublished data]) as the major storage form, it is possible that the differences between the hormone pools are related to the chemical or physical nature of the membranes containing the hormone, or by undetermined material that may be associated with the hormone within the membranes. We note that the secretion of secretory protein-I (parathyroid secretory protein [26, 31, 32]) bears a close relationship to that of parathormone. Its secretion is strikingly inhibited by calcium and magnesium (even more so than that of parathormone); like parathormone, dibutyryl cyclic AMP and isoproterenol elicit secretion of its previously synthesized ("older") form (32); and it has been shown to be associated with secretory granules that contain parathormone (38). Moreover, we have recently shown that the protein undergoes glycosylation (33) and sulfation (31) in an apparently sequential fashion. It is attractive to speculate that secretory protein-I may be the substance that plays a specific role in determining the secretory state and differential secretion of parathormone. In any event, the growing awareness of the existence of functional heterogeneous pools of parathormone provide a new dimension for the study of the function of this gland.

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