PARATHYROID HORMONE BIOSYNTHESIS

Correlation of Conversion of Biosynthetic Precursors with

Intracellular Protein Migration as Determined by Electron Microscope

Autoradiography

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ABSTRACT

The formation of parathyroid hormone (PTH) in the parathyroid gland occurs via two successive proteolytic cleavages from larger biosynthetic precursors. The initial product coded for by PTH mRNA is pre-proparathyroid hormone (Pre-ProPTH), a polypeptide of 115 amino acids. Within 1 min of synthesis, the polypeptide, proparathyroid hormone (ProPTH), is formed as a result of the proteolytic removal of the NH_2-terminal 25 amino acids from Pre-ProPTH. After a delay of 15-20 min, the NH_2-terminal six-amino acid sequence of ProPTH is removed to give PTH of 84 amino acids. To investigate the subcellular sites in the parathyroid cell where the biosynthetic precursors undergo specific proteolytic cleavages, we examined, by electron microscope autoradiography, the spatiotemporal migration of autoradiographic grains and, by electrophoresis, the kinetics of the disappearance of labeled Pre-ProPTH and the conversion of labeled ProPTH to PTH in bovine parathyroid gland slices incubated with [^{3}H]leucine for 5 min (pulse incubation) followed by incubations with unlabeled leucine for periods up to 85 min (chase incubations). By 5 min, 85% of the autoradiographic grains were confined to the rough endoplasmic reticulum (RER). Autoradiographic grains increased rapidly in number in the Golgi region after 15 min of incubation; from 15 to 30 min they migrated within secretory vesicles still in the Golgi region and then migrated to mature secretory granules outside the Golgi area. Electrophoretic analyses showed that Pre-ProPTH disappeared rapidly (by 5 min) and that conversion of ProPTH to PTH was first detectable at 15 min and was completed by 30 min. At later times of incubation (30-90 min), autoradiographic grains within the secretion granules migrated to the periphery of the cell and to the plasma membrane, in correlation with the release of PTH first detected by 30 min. We conclude that proteolytic conversion of Pre-ProPTH to ProPTH takes place in the RER and that subsequent conversion of ProPTH to PTH occurs in the Golgi complex.

KEY WORDS intracellular protein migration ·
prohormone conversion · electron microscope
autoradiography

Parathyroid hormone (PTH)[1] is a polypeptide of
84 amino acids that is synthesized and secreted by
the parathyroid glands (16). The hormone acts on
the target organs, bone and kidney, to promote
the flow of calcium into the extracellular fluid
(43). The secretion of PTH, in turn, is regulated
by the concentrations of calcium in blood and
extracellular fluid, thus maintaining calcium ho-
meostasis (16, 43). In our studies of the factors
controlling the synthesis and release of PTH, we
identified a biosynthetic pathway for the hormone
that involves its formation via two sequential
proteolytic cleavages from a larger biosynthetic
precursor (8, 14, 18). The initial product synthe-
sized in parathyroid gland slices in vitro (18) or by
translation of parathyroid gland mRNA in cell-
free systems (12, 13, 27, 28, 30) is pre-propara-
thyroid hormone (Pre-ProPTH) of 115 amino
acids (8, 14, 27). This initial prehormone, either
during growth of the nascent chain or within 1 min
of completion of synthesis, is converted to an
intermediate precursor, proparathyroid hormone
(ProPTH) of 90 amino acids (22, 25), by removal
of the amino-terminal sequence of 25 amino acids.
After a delay of ~15 min, ProPTH undergoes a
second proteolytic cleavage, resulting in the re-
moval of the amino-terminal sequence of six
amino acids, with the resultant formation of PTH,
the major hormonal product stored in and se-
creted from the parathyroid gland (7, 8, 14, 16,
18). On the basis principally of the times during
the biosynthetic processes at which these cleavages
occur, we (8, 14, 18) and others (5, 21) have
proposed that the sequential proteolytic process-
ing of Pre-ProPTH to ProPTH and of ProPTH to
PTH takes place in the rough endoplasmic reticu-
lum (RER) and in the Golgi complex, respec-
tively, of the parathyroid cell. Further evidence
implicating the Golgi complex as the site of con-
version of ProPTH to PTH is that pharmacologic
agents, such as amine compounds (6) and the
ionophore X537A (20), produce both marked

cytologic disruption of the Golgi complex and
inhibition of the conversion of ProPTH to PTH.

To examine in further detail the subcellular
locus where proteolytic conversion of ProPTH to
PTH occurs, we have pulse-labeled bovine para-
thyroid gland slices in vitro with [3H]leucine and
have correlated the sequential translocation of
autoradiographic grains at the electron microscope
level with the conversion of radiolabeled ProPTH
to PTH as determined by electrophoretic analyses
of extracts prepared from the gland slices. Our
results indicate that conversion of ProPTH to
PTH coincides in time with the arrival of the pulse
of autoradiographic grains at the Golgi complex.
These findings further implicate the Golgi com-
plex as the subcellular site where ProPTH is
converted to PTH. Furthermore, at 5 min of
incubation of the gland slices with [3H]leucine, the
earliest time examined in these studies, autoradi-
ographic grains were predominantly located in the
RER. Inasmuch as Pre-ProPTH is converted to
ProPTH within 1 min of its synthesis (18), we can
conclude that the formation of ProPTH from Pre-
ProPTH occurs in the RER.

MATERIALS AND METHODS

Incubations of Parathyroid Gland Slices with Labeled Amino Acids

Bovine parathyroid glands obtained at the time of
slaughter were immediately placed in ice-cold Earle's
Balanced Salt Solution (EBSS) for transport to the
laboratory. Slices (0.5–1.0 mm) were made of the
glands, and ~100 mg of slices were preincubated at
37°C for 45 min in 1.0 ml of Eagle's Minimum Essential
Medium obtained without leucine, glutamine, calcium,
or magnesium (Grand Island Biological Co., Grand
Island, N. Y.) and supplemented with 0.8 mM $MgCl_2$,
0.5 mM $CaCl_2$, and 2 mM glutamine. After the 45-min
preincubation, the slices were "pulse-labeled" by addi-
tion of 0.4 ml of a solution consisting of 2 ml of L-[3,4,5-
3H]leucine (110 Ci/mmol, 1 mCi/ml) (New England
Nuclear, Boston, Mass.), 0.2 ml of 10× concentrated
EBSS, and 0.05 ml of 7.5% sodium bicarbonate. After
5 min of incubation with the solution containing
[3H]leucine, 5 vol of ice-cold EBSS was added to one of
the incubation vessels (5-min pulse incubation), and
0.05 ml of 0.1 M leucine was added to the remaining
vessels (5-min pulse and chase incubation). Cold EBSS
was then added to individual vessels at 10, 15, 25, 40,
55, and 85 min of incubation to inhibit further incorpo-
ration of radioactive leucine into the slices, and the slices
were immediately separated from the media and placed

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[1] Abbreviations used in this paper: EBSS, Earle's Bal-
anced Salt Solution; Pre-ProPTH, pre-proparathyroid
hormone; ProPTH, proparathyroid hormone; PTH,
parathyroid hormone; PSP, parathyroid secretory pro-
tein; RER, rough endoplasmic reticulum; SDS, sodium
dodecyl sulfate.

on ice. The cold tissue slices were divided into two approximately equal aliquots, and one aliquot was frozen on Dry-Ice for extraction and electrophoretic analyses of radioactive proteins, and the other aliquot was finely minced in a solution of 4% glutaraldehyde, 0.5 mM $CaCl_2$, 0.15 M NaCl, and 0.1 M sodium hydrogen phosphate (pH 7.4) in preparation for electron microscope autoradiography.

In other experiments, slices of parathyroid glands were incubated in Eagle's Minimum Essential Medium as above except without methionine, and pulse-labeling was carried out with [^{35}S]methionine (600–900 Ci/mmol, Amersham Radiochemicals, Arlington Heights, Ill.) at a level of 1 mCi/ml of medium. Chase incubations were done by addition of 0.05 ml of 0.1 M methionine. Tissues were extracted for electrophoretic analyses as described below.

Extraction and Electrophoretic Analyses of Radioactive Proteins in Parathyroid Gland Slices and in Incubation Media

The frozen parathyroid gland slices were ground to a powder with mortar and pestle and were extracted with 2 ml of 8 M urea-0.2 N HCl. The extracts were twice precipitated with cold 10% TCA (after solubilization in 0.2 N NaOH). The final TCA precipitates were suspended in water and lyophilized, and the dried powders were extracted with 1.0 ml of 8 M urea-0.15 N acetic acid, and the radioactive proteins in the urea-acetic acid extracts were analyzed by electrophoresis either on discontinuous 10% polyacrylamide gels containing 8 M urea and 0.1 N potassium acetate (urea-acetate gels) as described previously (15, 29) or on 10–20% polyacrylamide gradient slab gels containing sodium dodecyl sulfate (SDS) (33). The radioactive proteins in the media removed from the tissue slices were precipitated with 10% TCA, as described above, and the acid-insoluble precipitates were extracted with 8 M urea-0.15 N acetic acid. The radioactive proteins were analyzed by electrophoresis on cylindrical urea-acetate gels.

Amounts of radioactive proteins in cylindrical gels were assessed by scintillation counting of slices prepared from the gels (15, 29), and amounts in slab gels by densitometric (Zeiss PMQ II) analyses of autoradiographs prepared from the dried gel slabs using Kodak SB-5 film (33).

Subcellular Fractionation and Limited Proteolysis

Parathyroid gland slices (200 mg) that had been pulse-labeled with [^{35}S]methionine for 5 min and then followed by chase incubations of up to 55 min were homogenized at 2°C in a buffer consisting of 0.25 M sucrose, 50 mM Tris (pH 7.5), 50 mM KCl, and 5 mM $MgCl_2$ (STKM

buffer) by 10 strokes with a Teflon-glass homogenizer driven at 1,200 rpm. The homogenate was centrifuged at 1000 g for 10 min. The supernates (postnuclear) were centrifuged at 105,000 g for 60 min (Beckman type 65 rotor, 40,000 rpm) and the pellets were resuspended by gentle homogenization in 0.5 ml of STKM. For each pulse-chase time, 0.1-ml aliquots of these high-speed particulate suspensions were treated as follows: (a) addition of 10 μl of STKM (control), (b) addition of 10 μl of a solution of pancreatic chymotrypsin and trypsin (1.0 mg/ml) in STKM, (c) addition of 10 μl of water and 10 μl of a 10% solution of Triton X-100, (d) addition of detergent as in (c) plus enzyme solution as in (b). After a 30-min incubation at 2°C, 0.5 ml of cold 10% TCA was added, and the acid-insoluble precipitates were collected by centrifugation, dried by washing with acetone-ethylether (1:1 vol/vol), and dissolved in 0.1 ml of 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue. 0.025-ml aliquots of the samples were analyzed by electrophoresis on 10–20% polyacrylamide slab gels (33).

Processing of Tissue for Electron Microscope Autoradiography

For each time point of the pulse-chase experiments, three blocks were selected on the basis of the most satisfactory tissue preservation. Pale-gold sections (corresponding to ~1,000 Å on the thickness scale of Salpeter and Bachmann [48]) were cut from each selected block with an LKB Ultratom III (LKB Instruments, Inc., Rockville, Md.) equipped with a diamond knife. The sections were covered with an Ilford L4 emulsion according to the loop method of Caro (3, 4) and exposed in the dark at 4°C in the presence of Silicagel for 4–6 wk. Three types of developer were used. Development of the exposed emulsion was done with Phenidon (34).

From each block, 10 negatives (30 for each time point) were taken at a fixed magnification (× 7,233) controlled by a calibration grid. The negatives were further enlarged by photographic printing to × 21,700.

To estimate the probability with which the radiation source lies within one given tissue component, we applied to the developed autoradiographs the "95% probability" circle of Whur et al. (56), associated with a morphometric analysis according to Stäubli et al. (51) and Weibel (54, 55). This procedure is needed because a β-particle will not necessarily hit the silver crystal that lies immediately over the source and because a single developed silver grain often overlaps several structures (2, 47, 49, 56). The evaluation of developed silver grains and the morphometric study were performed on the same photographic print. In practice, the morphometric data were recorded first, then the circle method was applied (see below).

Morphometry

The volume density of the different cytoplasmic compartments was determined by the point counting method (55), using a multipurpose test screen with 168 points and 84 test lines.

The following cell compartments were considered: the nucleus, the RER (including the nuclear envelope), the Golgi apparatus (including the cytoplasmic matrix immediately surrounding the saccules and vesicles), mitochondria, secretory granules, lysosomes, plasma membrane, cytoplasm (defined as the cytoplasmic matrix containing none of the membrane compartments delimited above).

Autoradiography

A circle with a radius of 4.9 mm (corresponding to 2,250 Å at the 21,700 magnification of the prints) with five equidistant points on its perimeter was placed around each developed grain (i.e., the grain was considered as the circle's center). The probability that one of the cellular compartments defined above was responsible for the developed silver grain was then estimated as follows (see Fig. 3): Because three points of the circle were over RER cisternae, and two points were over mitochondria, the probability that each of these compartments was the source of radiation that generated the particular silver grain was rated 3 in 5 and 2 in 5, respectively (Fig. 3).

All the autoradiographic data were based on the analysis of 1,500–2,000 grains for each time point; the background was ~2.5 grains per 1,000 μm^2. Two modes of calculation were used (51, 54):

(a) The radiation label density indicating the relative labeling intensity of the different compartments, disregarding their contribution to the cell volume:

$$R_{VL} = Gi/Pc\ (5.\frac{\sqrt{3}}{d^2}.T),$$

where R_{VL} = the radiation label density, Gi = the number of points of the 95% probability circle falling on a compartment i, Pc = the number of points falling on the entire parathyroid cell, d = the point spacing of the lattice, T = section thickness; R_{VL} represents the number of autoradiographic grains in compartments (i) per μm^3 of parathyroid cell.

(b) The specific radiation label density indicating the grain counts per unit volume of a given compartment:

$$R_{VL^+} = Gi/Pi\ (5.\frac{\sqrt{3}}{d^2}.T),$$

where R_{VL^+} = the specific radiation label density, Pi = number of points of the morphometrical-test lattice falling on the compartment $i-d$ and T were expressed in μm; R_{VL^+} represents the number of grains in compartment i per μm^3 of compartment i.

RESULTS

Precursor Conversions Determined by Electrophoretic Analyses of Tissue Extracts

Fig. 1 shows the electrophoretic patterns of radioactive proteins in extracts of parathyroid gland slices that had been incubated with [^{3}H]leucine for 5 min (Fig. 1A) and also the radioactive proteins secreted into the medium during the course of a 5-min pulse incubation with [^{3}H]leucine followed by an 85-min chase incubation with unlabeled leucine (Fig. 1B). The predominant radiolabeled proteins synthesized by the gland slices are ProPTH and parathyroid secretory protein (PSP), a protein of a mol wt of 150,000, consisting of two subunits each of mol wt of ~70,000 (31). The cellular or biologic functions of PSP are unknown, but PSP and PTH are the only two distinct proteins released into the medium by the parathyroid gland slices (Fig. 1). In these and previous studies (10, 17, 31), we have
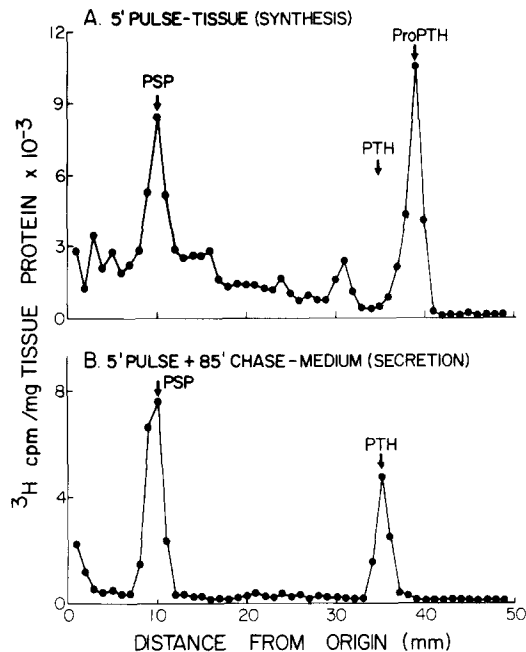

FIGURE 1 Electrophoretic patterns of radiolabeled proteins in extracts of (A) parathyroid gland slices incubated in [^{3}H]leucine for 5 min and (B) incubation media after a 5-min pulse with [^{3}H]leucine followed by an 85-min chase with unlabeled leucine. Extracts were analyzed by electrophoresis on 10% urea-acetate polyacrylamide gels. Arrows mark the migratory positions of PSP, ProPTH, and PTH.
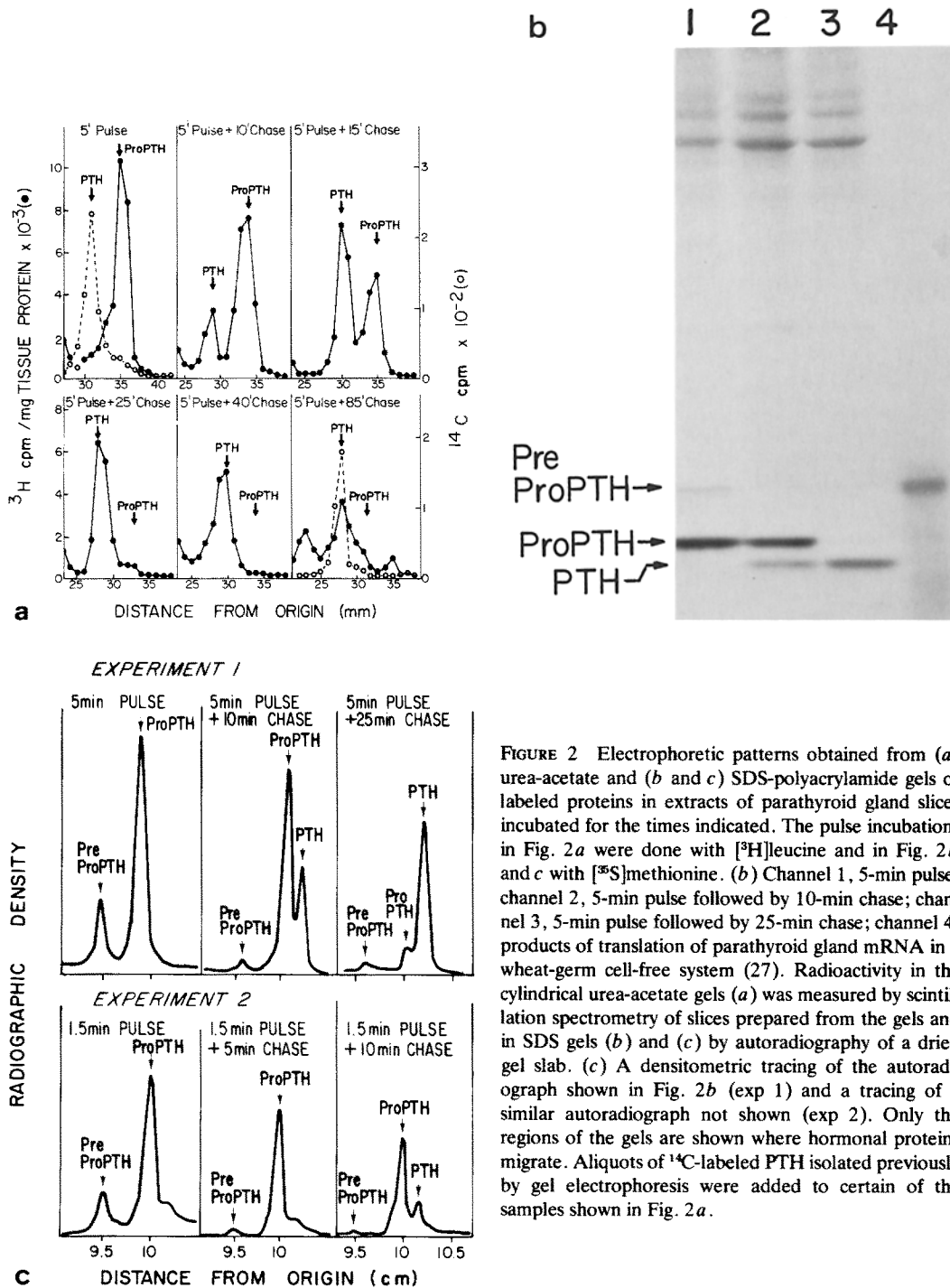
FIGURE 2   Electrophoretic patterns obtained from (a) urea-acetate and (b and c) SDS-polyacrylamide gels of labeled proteins in extracts of parathyroid gland slices incubated for the times indicated. The pulse incubations in Fig. 2a were done with [^{3}H]leucine and in Fig. 2b and c with [^{35}S]methionine. (b) Channel 1, 5-min pulse; channel 2, 5-min pulse followed by 10-min chase; channel 3, 5-min pulse followed by 25-min chase; channel 4, products of translation of parathyroid gland mRNA in a wheat-germ cell-free system (27). Radioactivity in the cylindrical urea-acetate gels (a) was measured by scintillation spectrometry of slices prepared from the gels and in SDS gels (b) and (c) by autoradiography of a dried gel slab. (c) A densitometric tracing of the autoradiograph shown in Fig. 2b (exp 1) and a tracing of a similar autoradiograph not shown (exp 2). Only the regions of the gels are shown where hormonal proteins migrate. Aliquots of ^{14}C-labeled PTH isolated previously by gel electrophoresis were added to certain of the samples shown in Fig. 2a.

not identified the presence of ProPTH or Pre-ProPTH in the media after incubation of parathyroid gland slices in vitro. On the basis of assessments of the amounts of radiolabeled PSP, ProPTH, and PTH in comparison with total acid-insoluble radioactive protein synthesized and secreted by the bovine parathyroid gland slices, we have estimated that as much as 40% of total cellular synthesis is directed toward the production of these specific parathyroid proteins and that up to as much as 80% of total protein secreted consists of PSP and PTH (10, 17, 31).

The time-dependent conversion of radiolabeled ProPTH to PTH and the rapid disappearance of Pre-ProPTH in the parathyroid gland slices during the pulse-chase experiments was determined by polyacrylamide gel electrophoresis, and the results are shown in Fig. 2.

At 5 min of incubation, Pre-ProPTH and ProPTH comprise all of the parathyroid hormonal protein (Fig. 2). After 10 min of chase incubation, labeled Pre-ProPTH has diminished markedly in amount (Fig. 2b and c). The rapid disappearance of Pre-ProPTH is further demonstrated by a shorter pulse-chase interval of 1.5 min of pulse followed by a 5-min chase incubation (Fig. 2c). The formation of PTH first appears by 15 min of incubation (Fig. 2a, 5-min pulse plus 10-min
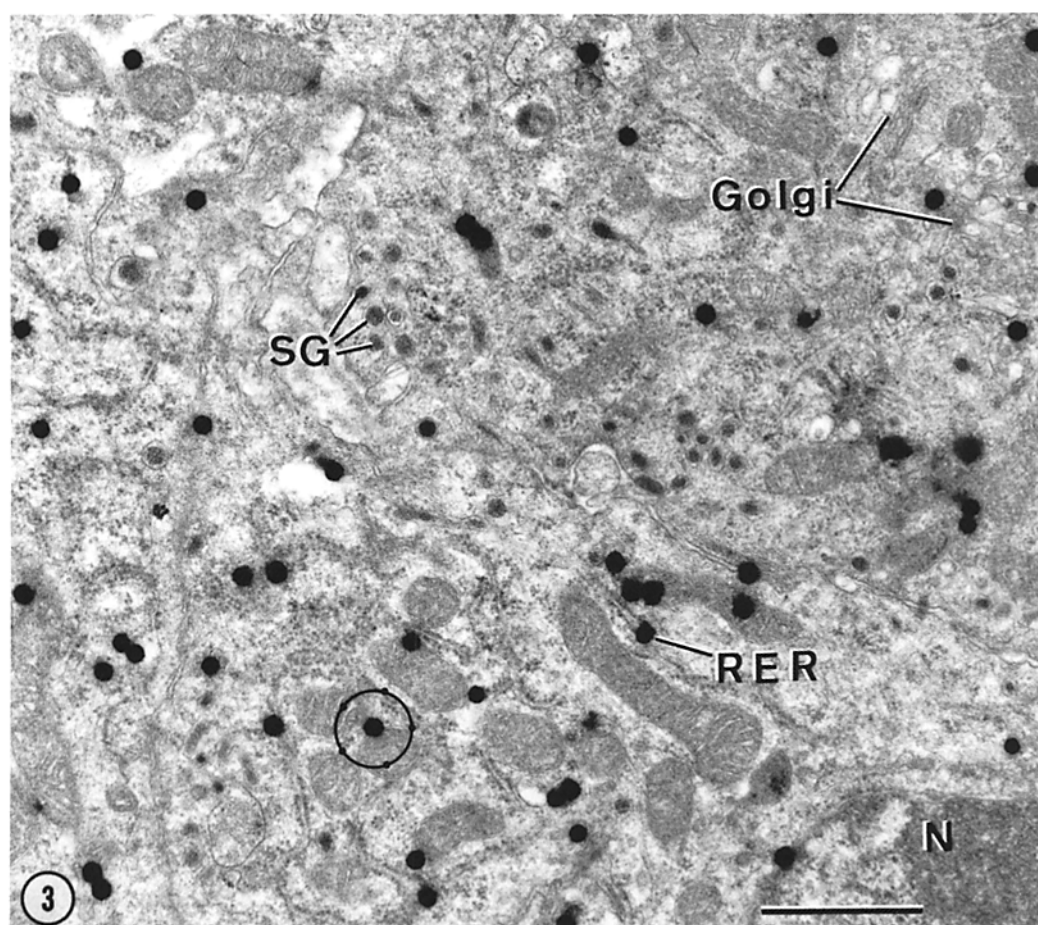

FIGURE 3 Autoradiograph of parathyroid cells at the end of the 5-min, [³H]leucine pulse. The three main intracellular membrane compartments of the parathyroid cell: rough endoplasmic reticulum (RER), Golgi apparatus, and secretory granules (SG) are identified. At this time point, many autoradiographic grains (black dots) relate to RER cisternae. Around one developed grain, the probability circle, used for quantitative evaluation of the labeling, has been drawn. On this circle, the five equidistant points are represented: three of five points fall on RER profiles whereas two of five are over mitochondrial profiles. N, nucleus. Bar, 1 µm. × 21,700.

chase). At longer times of incubation, there is a progressive fall in the amounts of radiolabeled ProPTH and an increase in radiolabeled PTH. By 30 min of incubation, essentially all ProPTH has been converted to PTH (Fig. 2).

Autoradiography of Pulse-Labeled Tissues

Typical images of autoradiographs of parathyroid cells in incubated slices pulse-labeled with leucine under the same conditions used for the biochemical studies are shown in Figs. 3–8. An unexpected difficulty was encountered with autoradiography of parathyroid cells. This was mainly due to the fact that the cytoplasm of these cells

appears less precisely compartmentalized (e.g., respective areas occupied by the RER cisternae, the Golgi apparatus, and secretory granules) than was the cytoplasm of previously studied endocrine polypeptide-secreting cells such as the B-cell of the islet of Langerhans (41) or the somatotrophs of the anterior pituitary gland (23). The relating of autoradiographic grains to precise compartments thus required a sensitive quantitative evaluation combining morphometry (51, 54, 55) and the probability circle method (56). Fig. 3 is taken at the end of the 5-min pulse of leucine. It shows many autoradiographic grains related to RER cisternae. Figs. 4 and 5 illustrate the labeling 15
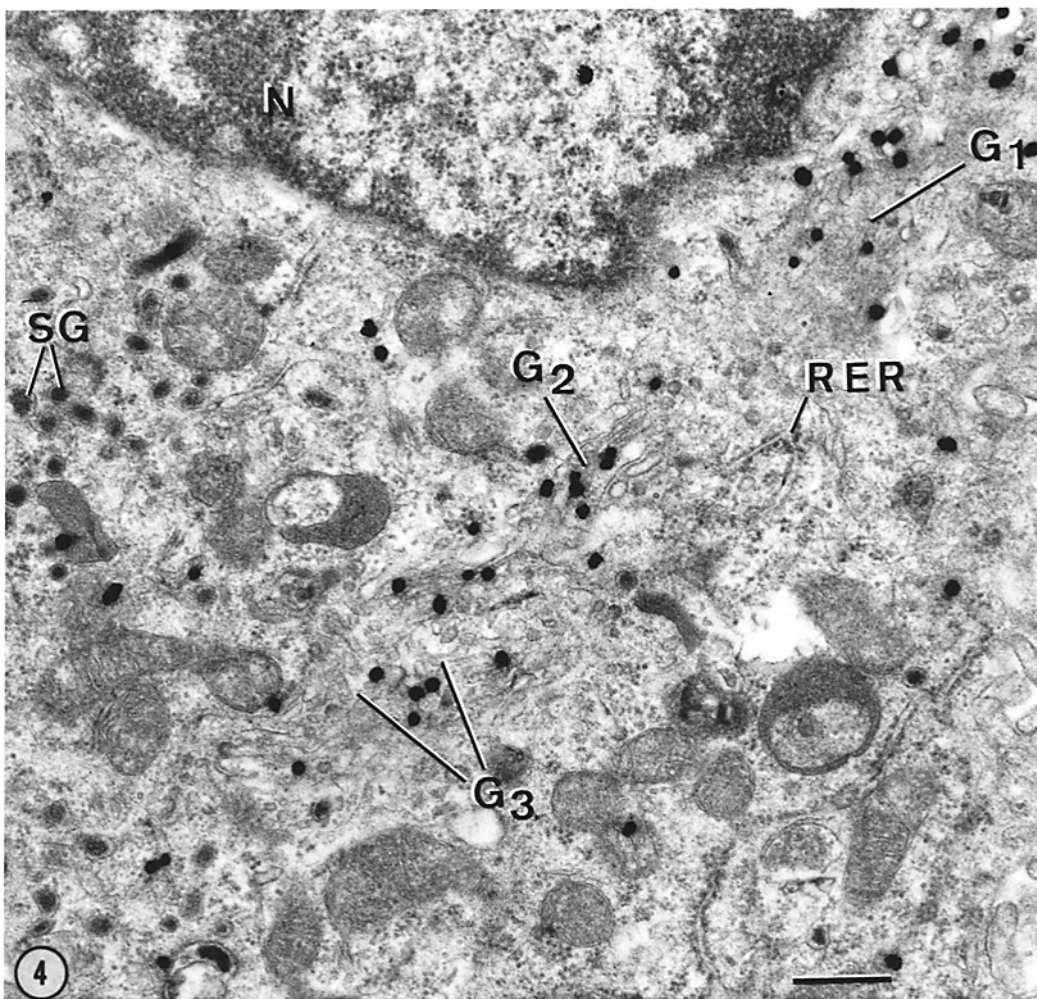

FIGURE 4 Parathyroid cell 10 min after the end of the pulse. At this stage, three Golgi regions (G_1, G_2, G_3) are preferentially labeled with developed autoradiographic grains. Rough endoplasmic reticulum cisternae (RER) and secretory granules (SG) are indicated. N, nucleus. Bar, 0.5 μm. $\times$ 26,000.
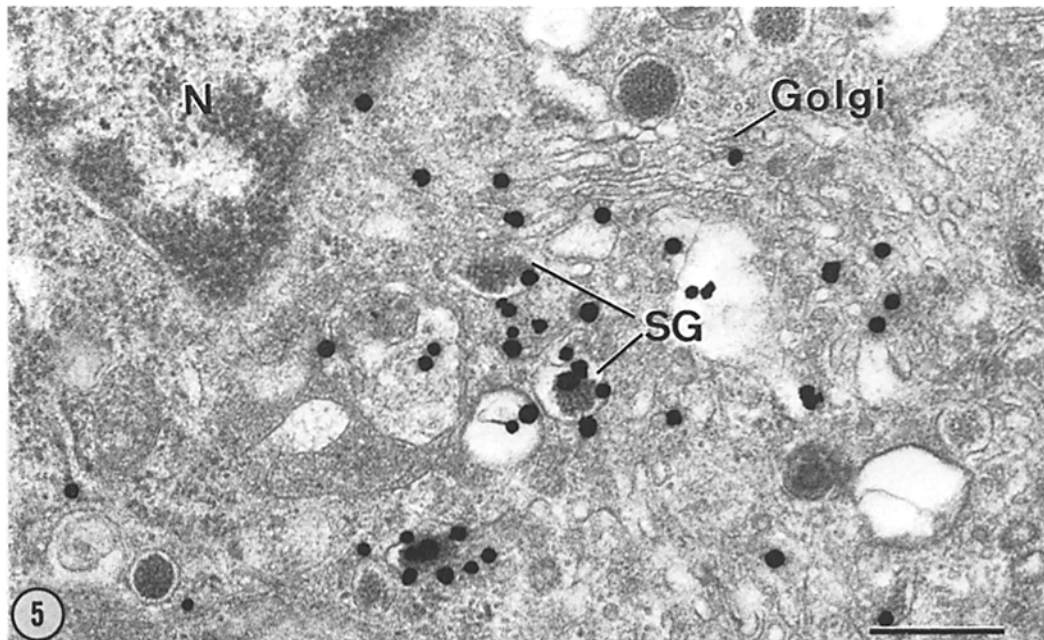
FIGURE 5 Detail of the Golgi region of a parathyroid cell 10 min after the end of the 5-min pulse. The Golgi cisternae are labeled with several developed grains, as well as secretory granules (SG) associated with Golgi cisternae and distinguished by their relatively large size and wide halo surrounding the granule core. Such granules are interpreted as "maturing" secretory vesicles. N, nucleus. Bar, 0.5 μm. × 32,000.

min after the beginning of the pulse. At this time point, Golgi apparatuses show a preferential labeling, as do large granules in the Golgi areas. These granules typically display a wide halo surrounding the core, and were interpreted as the counterparts of condensing granules in the parathyroid cells. At 30 min after pulse, all main intracellular membrane compartments (i.e., RER, Golgi, secretory granules) show labeling (Fig. 6), whereas 60 min after pulse there is evidence for preferential labeling of secretory granules, many of them located at the cell periphery (Fig. 7). At 90 min after the beginning of the pulse, a relatively diffuse, low-intensity labeling of the various cell compartments is observed (Fig. 8). A graphic representation of the results obtained by the quantitative evaluation of such qualitative images is shown in Fig. 9a and b for the main compartments involved in synthesis, packaging, storing, and release of the labeled protein (i.e., RER, Golgi, secretory granules, and plasma membrane). Moreover, a comparison between the pattern of gel electrophoresis of the labeled hormonal polypeptide and the composite profile of radioactivity in these intracellular compartments is represented

in Fig. 10. The complete numerical data obtained for all compartments studied are given in Table I.

Evidence that PSP and PTH Share the Same Transport Pathway

Inasmuch as PSP is synthesized and secreted along with PTH, it is clear that a substantial fraction of the autoradiographic grains represents PSP and not PTH, ProPTH, or Pre-ProPTH. Therefore, to correlate validly the conversion of hormonal precursors with the translocation of autoradiographic grains in these studies, it was necessary to obtain evidence that PSP and the hormone are transported and secreted in parallel. To obtain evidence in this regard, analyses were made of the subcellular distributions and the temporal secretion of the two proteins.

It was shown previously that PSP, PTH, and the hormonal precursors are associated with the particulate fractions of gland homogenates (9, 19, 35). Slices of parathyroid glands were pulse- and pulse-chase-labeled with [^{35}S]methionine for times the same as those used for the studies with [^{3}H]leucine. The tissues taken at each interval of
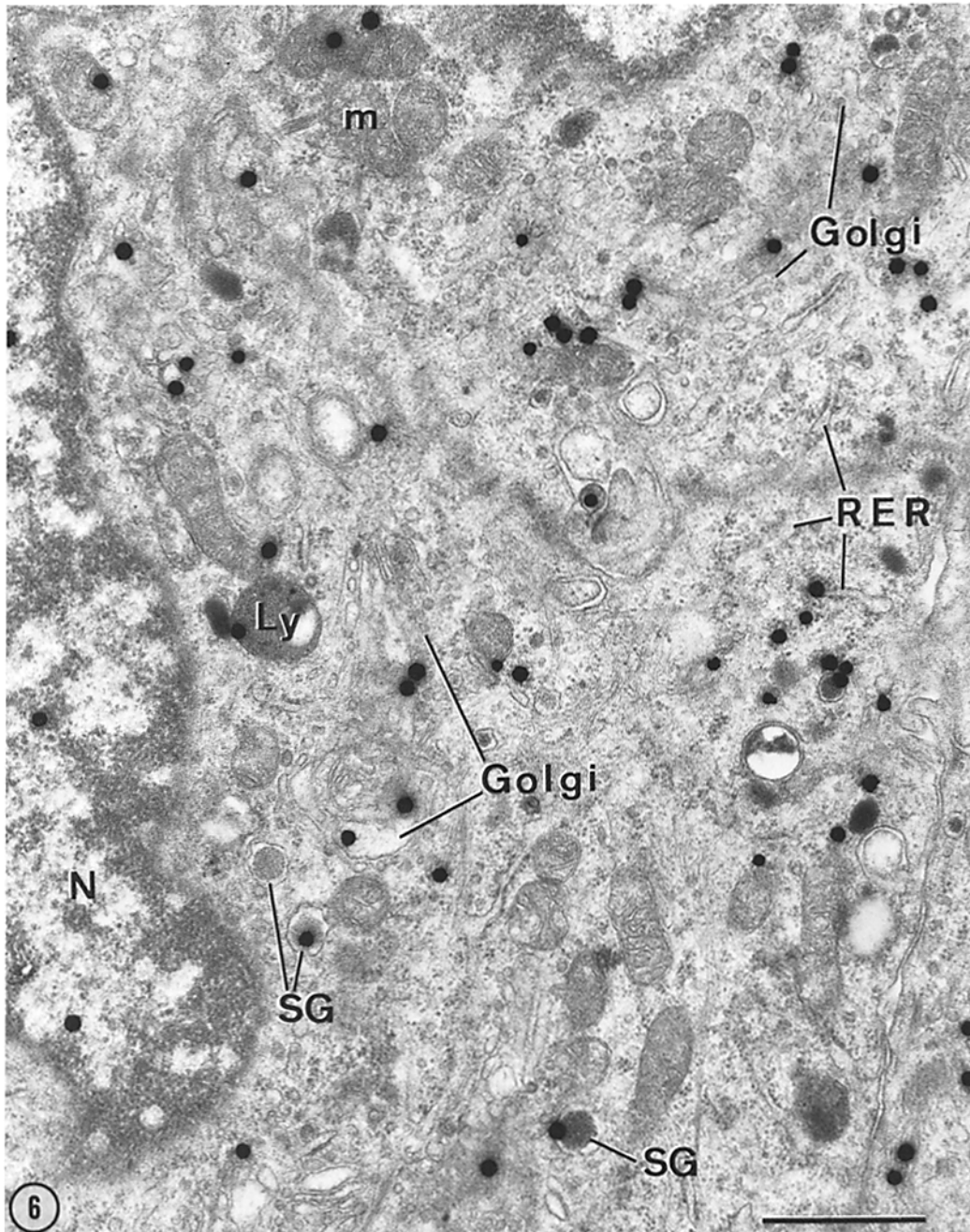
FIGURE 6   Parathyroid cells 25 min after the end of the 5-min pulse. At this time point, Golgi cisternae still contain substantial labeling; in addition, several labeled secretory granules appear outside Golgi areas. RER, rough endoplasmic reticulum; N, nucleus; Ly, lysosomes; SG, secretory granules; m, mitochondria. Bar, 1 μm. $\times$ 23,000.
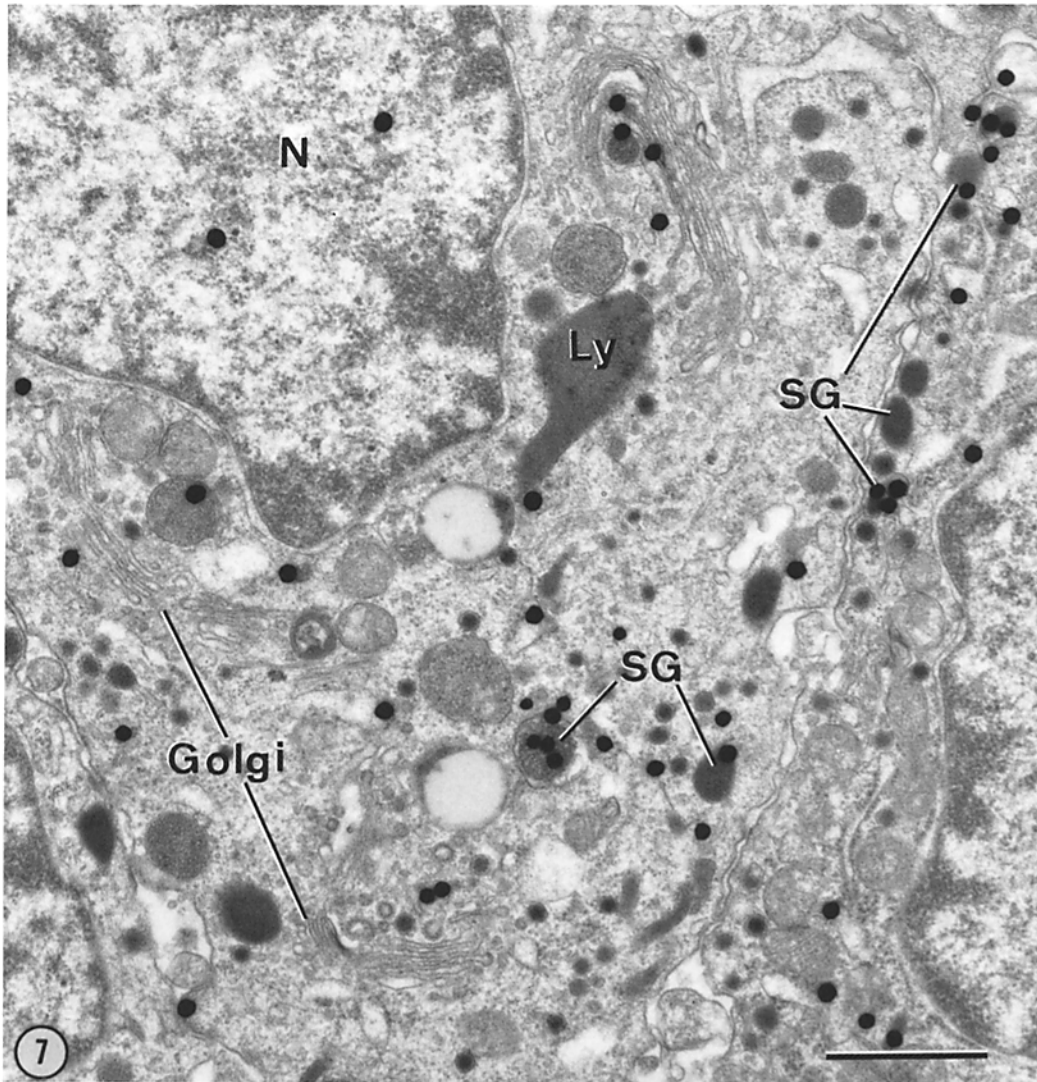
FIGURE 7   Parathyroid cell 55 min after the end of the 5-min pulse. This time point is characterized by the presence of numerous labeled secretory granules (*SG*), several of them marginated at the cell periphery. A moderate degree of Golgi labeling is still present. *N*, nucleus. Bar, 1 μm. × 21,000.

time were separated from the media and homogenized, and high-speed particulate fractions were prepared and subjected to limited proteolysis as described under Materials and Methods. Fig. 11 shows the densitometric tracings prepared from an autoradiograph of the labeled proteins after their separation on an SDS-polyacrylamide slab gel. In agreement with our previous report (19), we again found that, in the absence of detergent, PSP, ProPTH, and PTH are protected against proteolysis, whereas Pre-ProPTH is susceptible.

This situation is believed to exist as a result of the sequestration of the secretory proteins within membrane-limited vesicles (1, 46). In the presence of detergent (Triton X-100), however, these proteins are rendered susceptible to proteolysis but only up to 15 min after their synthesis (5-min pulse plus 10-min chase). Thereafter, both PSP and PTH become progressively resistant to proteolysis, and by 60 min (5-min pulse plus 55-min chase) they are nearly equally resistant to enzymic digestion in the presence and absence of deter-
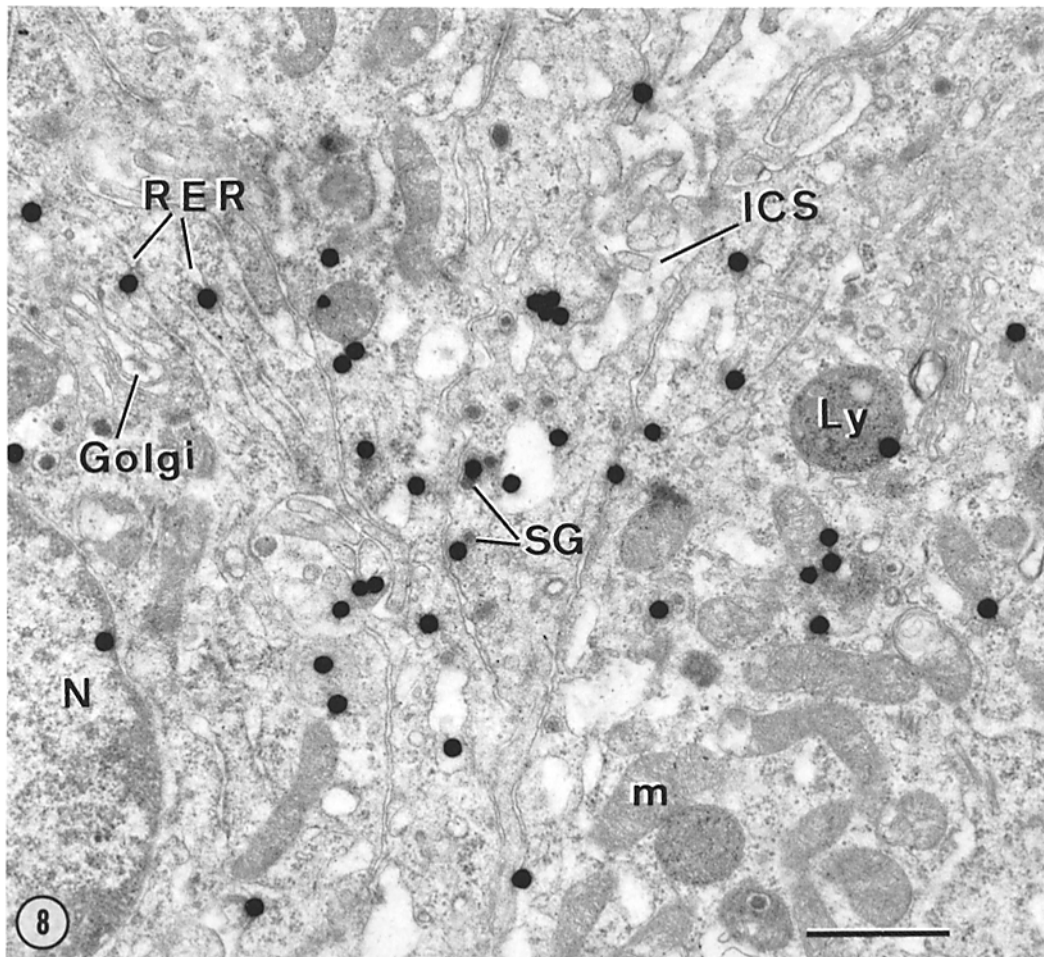
FIGURE 8 Parathyroid cells 85 min after pulse, showing a rather diffuse labeling punctuating most cell compartments: rough endoplasmic reticulum (*RER*), Golgi apparatus, secretory granules (*SG*), lysosomes (*Ly*), and mitochondria (*m*). *ICS*, intercellular space. Bar, 1 μm. × 19,000.

gent. These findings are consistent with the transfer, at 15–30 min, of both PSP and PTH from RER and Golgi compartments, bound by detergent-labile membranes, to secretory granules, which previously were shown to resist lysis by detergents (35). Thus, concomitant transfer of both proteins from detergent-labile to detergent-resistant forms indicates that the two proteins are transported together.

Analyses by electrophoresis of amounts of the newly synthesized PSP and PTH released into the media during the pulse-chase experiments are shown in Fig. 12. Both proteins are released at the same time and are present in a relatively fixed ratio. These observations indicate that the two

proteins synthesized (labeled) during the 5-min pulse were transported together from their site of synthesis to the plasma membrane and were released in parallel.

DISCUSSION

In these studies of pulse-labeled parathyroid gland slices in vitro, we have analyzed simultaneously both the kinetics of the cellular conversion of Pre-ProPTH to ProPTH and of ProPTH to PTH by electrophoresis and the migration of radiolabeled protein within specific subcellular organelles by electron microscope autoradiography. The results of the autoradiographic studies confirm those done previously by Nakagami et al. (38) using rat

comprises a synthesis of polypeptides on membrane-bound ribosomes in the RER and the segregation of newly synthesized polypeptides within the cisternal space of the RER followed by their transport within membrane-bounded vesicles to the Golgi region where secretory proteins are packed into membrane-bounded granules which are released into the extracellular fluid via the fusion of secretory granule membrane with the cell membrane.

Our studies using electrophoretic analyses of the kinetics of the conversion of ProPTH to PTH,
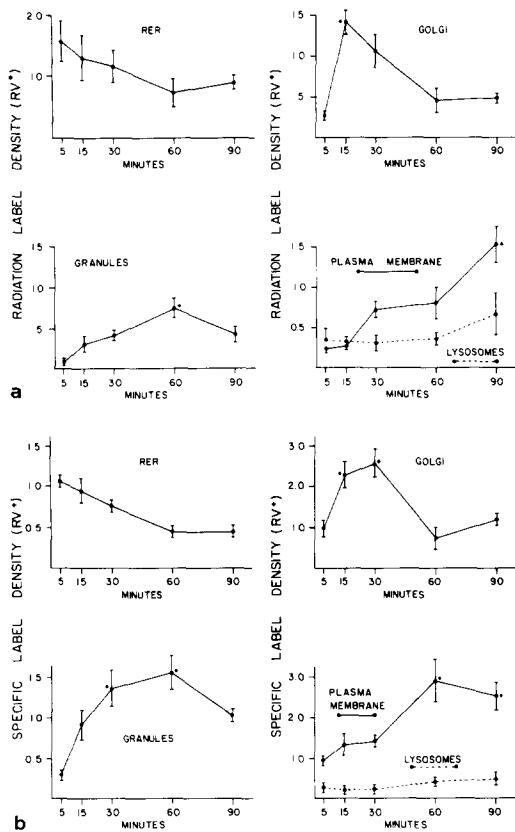

FIGURE 9 (a) Graphic representation of the evolution of radioactivity in five compartments of the parathyroid cell—rough endoplasmic reticulum (RER), Golgi apparatus, secretory granules, plasma membrane, and lysosomes—expressed as radiation-label density (RV·) in each compartment. This mode of expression gives the relative grain counts in compartments (i.e., without taking into account the volume of each compartment). In each group, the values identified with a star are significantly different from values without star ($P <$ 0.005). (b) Graphic representation of the evolution of radioactivity in five compartments of the parathyroid cell—rough endoplasmic reticulum (RER), Golgi apparatus, secretory granules, plasma membrane, and lysosomes—expressed as specific label density (RV⁺). This mode of expression gives the absolute number of developed grains in compartments (i.e., by taking into account the volume of each compartment). In each graph, the values identified with a star are significantly different from values without star ($P <$ 0.005).

parathyroid glands and support the concept that there exists in the parathyroid cell a transport pathway for PTH similar to that recently reviewed by Palade (42) for pancreatic exocrine and other polypeptide-secreting cells (45, 50). This pathway
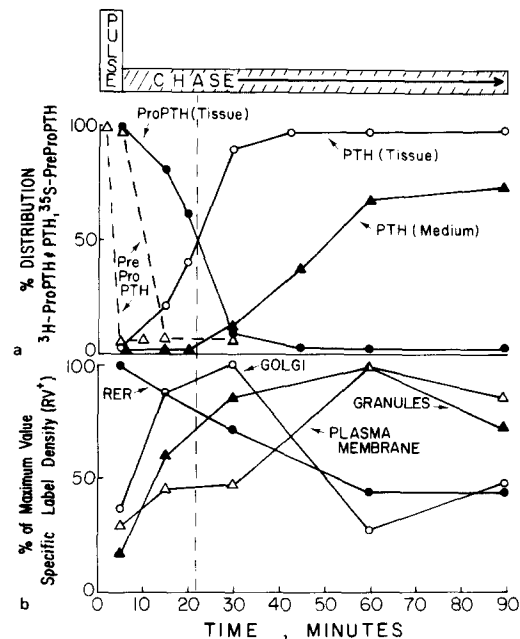
FIGURE 10 Summary of pulse-chase incubations with bovine parathyroid gland slices. Parathyroid gland slices were pulse-labeled with [³H]leucine for 5 min, after which incubations were continued in the presence of unlabeled leucine (chase incubations) for the times indicated. (a) Distributions of [³H]leucine-labeled ProPTH and PTH in parathyroid tissues and in incubation medium as determined by polyacrylamide-gel electrophoresis. Open and closed circles indicate percent distributions of PTH and ProPTH within tissues, where the sum of PTH and ProPTH at each time is taken as 100%. Open triangles indicate Pre-ProPTH in tissue where amount at end of pulse incubation is taken as 100%. Closed triangles indicate percent of PTH in the incubation medium, where PTH in medium plus PTH in tissue is taken as 100%. (b) Distribution of specific label density in the subcellular compartments of parathyroid cells analyzed by quantitative electron microscope autoradiography.

carried out on aliquots of the same tissues upon which autoradiographic studies were performed, lead us to conclude that the Golgi complex is the subcellular locus where the conversion of ProPTH occurs. Our present studies, as well as similar pulse-chase labeling studies reported previously by us (11, 18, 29, 32) and by other workers (5, 7), indicate that, after a delay of 15 min after the addition of a pulse-label to biosynthetically active parathyroid tissues, newly synthesized ProPTH undergoes a rapid and specific conversion to PTH with a half-time of conversion of ~8-10 min. The corresponding autoradiographs presented in this report show that newly synthesized proteins first appear in the Golgi region at the time when the conversion of ProPTH to PTH begins to take place (15-30 min) and that the maximum density of grains in the Golgi region occurs during the period of most rapid conversion of ProPTH to PTH. Moreover, radioactivity leaves the Golgi region and is transferred to the secretory granules only after the conversion of ProPTH to PTH is completed; no ProPTH was seen by electrophoresis at times later than 30 min, when autoradiographic grains had moved from the Golgi region
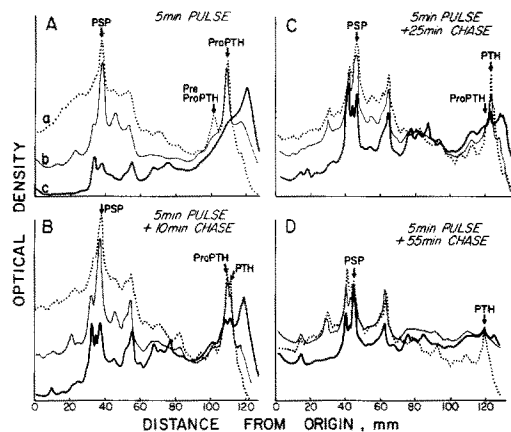

FIGURE 11 Densitometric tracings of autoradiographs prepared from a 10-20% gradient SDS in which ^{35}S-labeled parathyroid proteins were separated by electrophoresis. Proteins were extracted from 105,000-g particulate fractions prepared from pulse/chase-labeled gland slices after the particulates were subjected to limited proteolysis for 30 min with trypsin and chymotrypsin as indicated in Methods. For each of the four pulse-incubation times, tracings of film density are co-plotted that correspond to particulates incubated under three conditions: a, $\cdots$ = without enzymes or Triton X-100; b, — = with enzymes and without Triton X-100; and c, — = with enzymes and with Triton X-100.
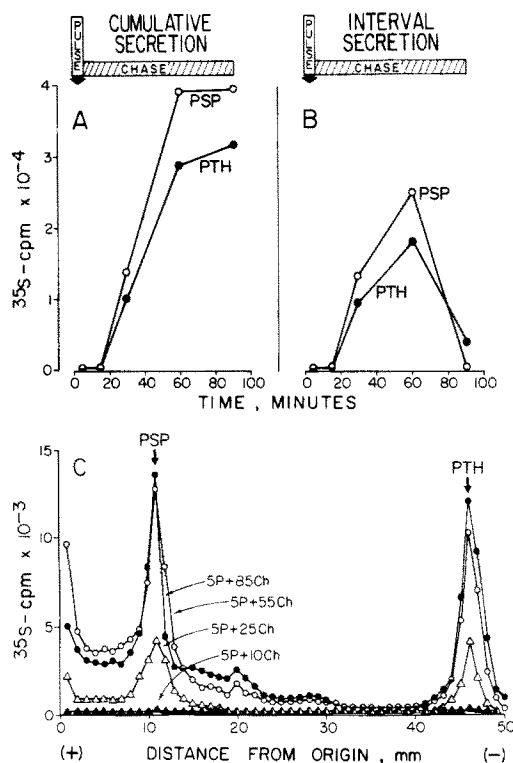

FIGURE 12 Analysis of labeled proteins released by parathyroid gland slices into the incubation media during a pulse/chase experiment. (C) Electrophoretic profiles (urea-acetate gels) of labeled proteins in the media. (A and B) Plots of cumulative and interval secretion of PSP and PTH determined from data shown in Fig. 12 C.

to the secretory granules.

Other lines of evidence obtained from previous reports also suggested that the Golgi complex may function in the conversion of ProPTH to PTH. Chu et al. (6) reported that incubation of parathyroid gland slices in the presence of amine compounds such as Tris buffer produces both a marked inhibition of the conversion of ProPTH to PTH and striking morphologic alterations in the Golgi complex of the parathyroid cells. Similar observations of both inhibition of prohormone conversion and morphologic disruption of the Golgi complex in response to incubation of parathyroid gland slices with the ionophore X537A have been made in our laboratory (20). Thus, these prior observations taken in conjunction with our present studies strongly implicate the Golgi complex as the intracellular site where the ProPTH is proteolytically converted to PTH.

Results similar to ours have been reported from

TABLE I

Pulse chase	RER	Golgi	Granules	Lysosomes
A. Distribution of specific label density (RV$^+$) in the main cellular compartments of the parathyroid cells				
5 min	1.057 ± 0.180	0.994 ± 0.200	0.264 ± 0.057	0.274 ± 0.117
5 + 10 min	0.950 ± 0.271	2.264* ± 0.331	0.934 ± 0.208	0.183 ± 0.077
5 + 25 min	0.767 ± 0.119	2.553* ± 0.312	1.349* ± 0.269	0.186 ± 0.087
5 + 55 min	0.472 ± 0.109	0.735 ± 0.186	1.537* ± 0.214	0.376 ± 0.069
5 + 85 min	0.486 ± 0.052	1.247 ± 0.102	1.147 ± 0.088	0.418 ± 0.127
B. Distribution of radiation label density (RV·) in the main cellular compartments of the parathyroid cells				
5 min	15.880 ± 3.203	2.613 ± 0.320	0.307 ± 0.043	0.371 ± 0.260
5 + 10 min	13.560 ± 3.996	14.210* ± 1.260	2.748 ± 0.693	0.277 ± 0.079
5 + 25 min	11.806 ± 1.802	10.540 ± 2.555	3.388 ± 0.339	0.275 ± 0.107
5 + 55 min	7.433 ± 2.148	4.243 ± 1.372	7.310* ± 1.051	0.356 ± 0.068
5 + 85 min	9.346 ± 0.277	4.926 ± 0.340	4.360 ± 0.900	0.657 ± 0.282

Values represent means ± SEM.

* Values differ significantly from values without asterisk ($P < 0.005$).

studies of the intracellular pathway of hormonal polypeptides in various cell types (23, 24, 36, 39, 40, 53). In B-cells of pancreatic islets, a strong correlation has been observed in the time that elapses between the appearance of radiolabeled proteins in the Golgi region of the islet cells and the conversion of proinsulin to insulin (41).[2] Moreover, Kemmler et al. (26) reported the partial isolation of specific proinsulin-converting activity from a subcellular fraction enriched in Golgi membranes. The enzymic activities responsible for the cellular conversion of both proinsulin and ProPTH to the hormonal products also are similar; combined trypsinlike and carboxypeptidase-B-like actions are involved (9, 26). Thus, it appears that pancreatic islet cells and parathyroid cells alike, and perhaps other secretory cells as well, have similar conversion and transport pathways and that the specific proteolytic activities involved in the conversion of the prohormones to the hormones reside in the Golgi complex.

In addition to our observations pointing to the Golgi complex as the intracellular site for the conversion of ProPTH to PTH, the present study also supports our proposal made previously (12,

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[2] Compared with the rate of formation of insulin from proinsulin (52), the formation of PTH from ProPTH (18) appears to occur considerably faster and to a greater extent. Conversion of proinsulin to insulin begins between 30 and 60 min after synthesis, and the process takes 1-2 h; secretory granules contain 5-10% proinsulin in addition to the insulin. The conversion of ProPTH to PTH takes place after 15 min and appears to be complete by 30 min; little residual radiolabeled ProPTH has been identified in the cell after a chase incubation (18), and no evidence that ProPTH is secreted from the cell has been found (10, 17, 20).

18, 28) that the RER is the site where Pre-ProPTH, the initial biosynthetic precursor of PTH, is converted to the intermediate precursor, ProPTH. Pre-ProPTH, first identified as a major product of the translation of parathyroid mRNA in cell-free systems (12, 27, 28, 30), was subsequently identified during short pulse-labeling studies of hormone biosynthesis in parathyroid gland slices (18). In the studies done previously (18), as well as in the present studies, ProPTH was observed as early as 1-2 min after the addition of the pulse label into the incubation medium. Our finding that, by 5 min of synthesis, most of the autoradiographic grains are situated over the RER can be taken as direct evidence that the proteolytic conversion of Pre-ProPTH to ProPTH must occur at, or close to, this subcellular compartment.

However, even using the shortest pulse-incubation times of 1-2 min, in which it is possible to demonstrate incorporation of [^{35}S]methionine into protein, amounts of newly synthesized ProPTH are greater than those of Pre-ProPTH, and, during chase incubations, it has not been possible to demonstrate quantitative transfer of label from Pre-ProPTH to ProPTH. Therefore, we are not able to distinguish between a biosynthetic pathway that involves synthesis of completed Pre-ProPTH followed by conversion to ProPTH and a pathway that involves cleavage of incompleted nascent chains to give nascent ProPTH chains that are subsequently completed. In the latter case, the Pre-ProPTH observed in the tissues may represent "heterotopic" synthesis, that is, a minor pathway involving chains that do not attach to endoplasmic reticulum, and, as a result, are completed without their cleavage and are then degraded in the cell matrix—a process that would account for their

TABLE I, continued

Plasma membrane	Cytoplasm	Nucleus	Mitochondria
0.866 ± 0.122	0.101* ± 0.005	0.110 ± 0.033	0.222 ± 0.058
1.323 ± 0.250	0.237 ± 0.064	0.184 ± 0.035	0.331 ± 0.073
1.401 ± 0.111	0.266 ± 0.049	0.200 ± 0.047	0.371 ± 0.045
2.876* ± 0.503	0.269 ± 0.051	0.284 ± 0.064	0.305 ± 0.070
2.530* ± 0.371	0.339 ± 0.021	0.364 ± 0.084	0.350 ± 0.049
0.232 ± 0.102	4.613* ± 0.434	2.132 ± 0.573	2.656 ± 0.679
0.244 ± 0.094	10.225 ± 3.015	3.743 ± 0.799	3.716 ± 0.474
0.712 ± 0.120	11.853 ± 2.146	3.750 ± 1.014	4.466 ± 0.663
0.764 ± 0.239	11.876 ± 2.368	4.856 ± 0.737	3.313 ± 0.783
1.503* ± 0.212	14.553 ± 0.827	5.623 ± 1.204	4.430 ± 0.733

disappearance during the chase incubations. The susceptibility of Pre-ProPTH but not of ProPTH (or PTH) to limited proteolysis (Fig. 11) is consistent with the situation in which Pre-ProPTH synthesized and released in the cell sol is adsorbed to the surface of the microsomes (105,000 g particulates), whereas ProPTH and PTH are sequestered within the cisternae of microsomal vesicles.

Some information, albeit limited, is available from these studies regarding events that occur late in the transport pathway. The initial appearance of substantial numbers of autoradiographic grains within mature secretory granules at the cell periphery was observed at the time when release into the medium of radioactive protein (^{3}H-labeled PTH and PSP) first takes place. By 90 min of incubation (5-min pulse plus 85-min chase), autoradiographic grains became rather diffusely distributed throughout the various cell compartments and there appeared to be an increase in grains within both the RER and lysosomes (Table I). Although speculative at this stage of the studies, these observations may reflect the intracellular turnover of radioactive proteins believed to occur in the parathyroid gland (10); the reappearance of grains in the RER may represent reutilization, in protein synthesis, of radioactive amino acids derived from the cellular metabolism of radiolabeled proteins.

Another outcome of these studies was the generation of additional data indicating that PSP accompanies PTH in the transport pathway. These findings are not in accord with the conclusions of Morrissey and Cohn (37) who suggested that the two proteins utilize different pathways. Nonetheless, our results are further supported by our

recent observations, using immunocytochemical techniques at the electron microscope level, that PSP and PTH reside in the same secretory granules (44).

The findings derived from these studies should prove useful in attempts to isolate and to characterize the enzymes involved in the proteolytic processing of the biosynthetic precursors of PTH. Specifically, the enzymic activity that converts Pre-ProPTH to ProPTH should be located in the endoplasmic reticulum, and the combined trypsin-like and carboxypeptidase-B-like activities should be within the Golgi complex. Future studies aimed toward identification of the specific enzymic activities in the parathyroid cell and of the metabolic fates of the peptides cleaved from the hormonal polypeptides during their cellular processing should provide a greater understanding of the role of biosynthetic precursors in the transport of proteins destined for export from the cell.

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