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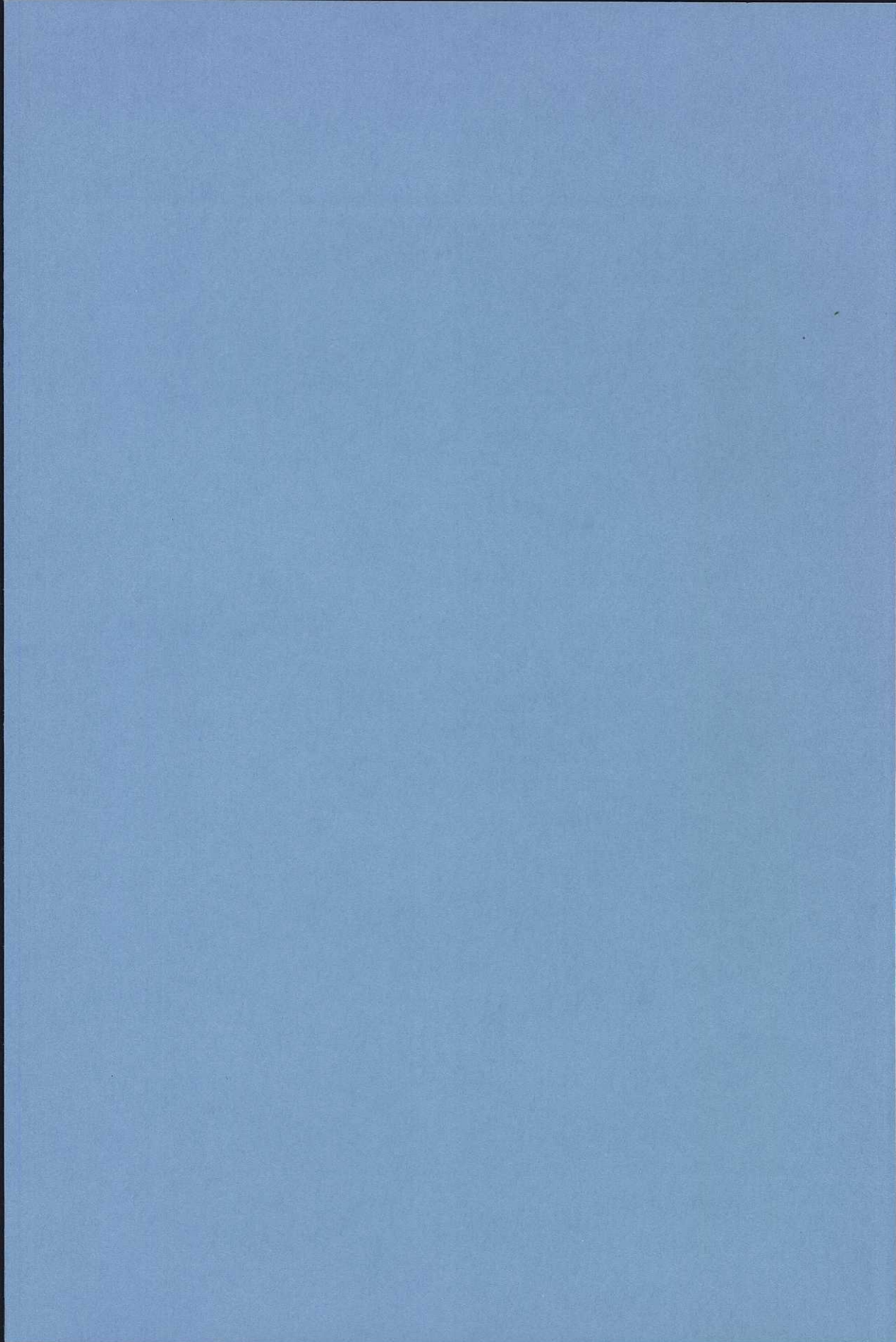
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Studies on Exocytosis and Endocytosis
in Thyroid Follicle Cells

By
GUNNAR ENGSTRÖM

GÖTEBORG 1979



Studies on Exocytosis and Endocytosis in Thyroid Follicle Cells

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ABSTRACT

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The thyroid follicle cell synthesizes thyroglobulin which is transferred through the apical part of the cell in vesicles. These vesicles (exocytotic vesicles) empty their content into the follicle lumen by exocytosis, a process that i.a. implies fusion between the membrane of the exocytotic vesicles and the apical plasma membrane. As the first step in the release of thyroid hormones, hormone-containing thyroglobulin is reabsorbed into the follicle cell by endocytosis. Endocytosis, which is rapidly stimulated by TSH, includes the formation of pseudopods, colloid droplets and micropinocytotic vesicles from the apical plasma membrane. In this thesis the effect of TSH on exocytosis and endocytosis in the follicle cells of the rat thyroid was studied during the first 30 minutes of stimulation by quantitative light and electron microscopy as well as biochemical methods.

A large dose of TSH (500 mU) induced exocytosis. This previously not known effect of TSH was manifested by a rapid depletion of exocytotic vesicles located in the apical part of the follicle cells and by a rapid transfer of newly synthesized proteins from the follicle cells to the follicle lumen. Electron microscopic stereology showed that TSH-induced exocytosis was part of an extensive redistribution of membrane. The first phase (0-5 min) of this redistribution was characterized by exocytosis resulting in addition of membrane to the apical plasma membrane. During the second phase (5-20 min) exocytosis continued but at the same time membrane from the apical plasma membrane was transferred to pseudopods. The third phase (20-30 min) was characterized by a transfer of membrane from pseudopods to colloid droplets. A quantitatively different, but qualitatively similar, redistribution of membrane was also induced at submaximal levels of TSH (5-100 mU). A log dose-response relation was demonstrated between TSH and exocytosis.

To further explore the relationship between exocytosis and endocytosis the pool of exocytotic vesicles was experimentally reduced by inhibition of protein synthesis (by long-term treatment with thyroxine or by cycloheximide). A parallel reduction in the membrane surface area of exocytotic vesicles and that of TSH-induced endocytotic structures was found. This indicates that the size of the membrane pool in the exocytotic vesicles, added to the apical surface at stimulation, determines the size of the endocytotic response. No evidence was obtained that the apical plasma membrane served as the primary source of membrane used in the formation of endocytotic structures.

Thus, exocytosis is part of the normal response of the follicle cell to stimulation. By exocytosis, which precedes endocytosis, the requirement of membrane for the formation of endocytotic structures is covered.

Key words: Exocytosis - endocytosis - thyroid gland - membranes - electron microscopy - stereology.

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The present thesis is based on the following papers:

1. Exocytosis of protein into the thyroid follicle lumen: An early effect of TSH.
Endocrinology 97, 337, 1975.
R. Ekholm, G. Engström, L.E. Ericson and A. Melander
2. Quantitative electron microscopic studies on exocytosis and endocytosis in the thyroid follicle cell.
Endocrinology 103, 883, 1978.
L.E. Ericson and G. Engström
3. Effect of graded doses of thyrotropin on exocytosis and early phase of endocytosis in the rat thyroid.
Submitted for publication.
G. Engström and L.E. Ericson
4. Effects of long-term thyroxine treatment on thyrotropin-induced exocytosis and endocytosis in the rat thyroid.
Endocrinology 1978. In press.
L.E. Ericson, G. Engström and R. Ekholm
5. Effect of cycloheximide on TSH-stimulated endocytosis in the rat thyroid.
Submitted for publication.
G. Engström, L.E. Ericson and R. Ekholm

The papers will be referred to in the text as paper 1, paper 2, etc.

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INTRODUCTION

The thyroid gland contains two endocrine systems, one producing the iodinated hormones thyroxine and triiodothyronine and the other secreting thyrocalcitonin. The present studies concern only the former system.

The structure of the thyroid is characterized by its organization in follicles. These cystlike units consist of a wall of a single layer of epithelial cells surrounding a closed cavity, the follicle lumen, which is filled with the colloid, a protein solution whose major component is thyroglobulin. This special structural organization reflects the fact that the thyroid, unlike all other endocrine glands, stores its hormones in an extracellular pool, namely the follicle lumen, where the hormones are bound to thyroglobulin.

The improved knowledge of the unique structure of the thyroid follicle achieved in recent years by electron microscopical studies has greatly promoted the understanding of the physiology of the follicle (18, 20). It is now possible to relate the processes of formation, storage and secretion of the iodinated hormones to the fine structure of the follicle.

Hormone formation. The basal step in thyroid hormone synthesis is the formation of thyroglobulin. This large glycoprotein, which has a molecular weight of about 670,000 daltons (9), is synthesized in the follicle cells. The peptide chains are formed on the attached ribosomes of the endoplasmic reticulum and transferred into the cisternae (3, 29). The size of the primary peptide has been much discussed; recent studies indicate that it may correspond to half the complete molecule (51). In any case, most of the secondary and tertiary structuring of the protein seems to take place in the cisternae of the endoplasmic reticulum and the protein backbone is probably completed when the molecule is transferred to the Golgi apparatus. Although there are no direct evidence for a transfer of

the protein from the endoplasmic reticulum to the Golgi, such a transfer can be deduced from observations on the incorporation of carbohydrates into the molecule. The carbohydrates are attached to the protein in a stepwise manner; only the monosaccharides closest to the protein backbone are incorporated in the cisternae of the endoplasmic reticulum (22, 57) whereas more peripheral sugars are linked to the molecule in the Golgi area (21, 22).

The last part of the intracellular migration of the newly synthesized thyroglobulin, from the Golgi area to the apical cell surface, takes place in smooth-surfaced vesicles (21, 22, 29). At the cell surface these vesicles empty their contents into the follicle lumen by exocytosis. This process involves a fusion between the membrane of the exocytotic vesicle and the plasma membrane and then a membrane fission, whereby the vesicle is opened up and its contents discharged into the lumen (32).

The thyroglobulin of the exocytotic vesicles is uniodinated (6, 12). Iodination of thyroglobulin and hormone formation seem to be confined to the follicle lumen and apparently occur at the apical plasma membrane of the follicle cells (12, 45). There are indications that peroxidase in the apical plasma membrane (46, 49) catalyzes all steps in hormone formation, including oxidation of iodide, iodine binding to tyrosyl residues in the thyroglobulin molecule and coupling of these residues to the hormones, thyroxine and triiodothyronine (48).

Hormone storage. The hormone-containing thyroglobulin is stored in the follicle lumen. Analysis of protein samples obtained by micropuncture of follicles has shown that almost all the protein consists of thyroglobulin and larger iodoproteins (43), consisting of aggregates of two or more thyroglobulin molecules (2, 44). Several observations clearly show that the follicle lumen is the only important store of thyroglobulin and, consequently, hormones in the thyroid (26, 47).

Hormone secretion. Since the hormones are bound to thyroglobulin in peptide linkage (37), release of the hormones necessitates that the thyroglobulin is hydrolyzed. This degradation is obviously not possible in the follicle lumen (43) but takes place in the follicle cells, which requires that thyroglobulin is reabsorbed by the cells (58). This occurs by endo-

cytosis. The best known type of endocytosis in the thyroid is macropinocytosis. Studies with transmission (10, 28, 38, 39, 40, 42, 45) and scanning (1, 25, 56) electron microscopy have revealed the structural details of this process. The first well defined step in macropinocytosis is the protrusion of pseudopods, formed by the apical plasma membrane of the follicle cells, into the follicle lumen. In its earliest stage the pseudopod has the appearance of a longitudinal fold. This fold curls, its margins fuse and a double-walled tube, open at the top, is formed. The tube is then closed and the pseudopod is transformed into a cystlike or more irregular structure, enclosing one or several portions of colloid. The pseudopod retracts and the enclosed portions of colloid, enveloped by the inner membrane of the pseudopod, are moved into the cell body where they appear as colloid droplets.

In addition to uptake of colloid by macropinocytosis there seems to exist a reabsorption by micropinocytosis. Thus, after injection of label into the follicle lumen in vivo the label can be detected in caveolae connected with the apical plasma membrane and in small vesicles in the apical cell region within a few minutes (38, 41). The recent observation on chronically stimulated thyroids that hormones are secreted in spite of the absence of signs of macropinocytosis in the follicle cells may also be taken as an indication of micropinocytotic activity (36). The relative importance of the two modes of colloid reabsorption is not known and may be different in different functional states. It seems possible that micropinocytosis plays an important role in hormone release under physiological conditions.

A prerequisite for the degradation of thyroglobulin in the colloid droplets and micropinocytotic vesicles is that hydrolytic enzymes gain access to these structures. The source of enzymes is the lysosomes of the follicle cells (58). Indications of interaction between micropinocytotic vesicles and lysosomes have not been reported, but signs of fusion between colloid droplets and lysosomes are often observed in the electron microscope (10, 40, 55) and mixing of the contents of colloid droplets and lysosomes has been described (55, 59). Furthermore, thyroid lysosomal enzymes have the capacity in vitro of degrading thyroglobulin and releasing thyroid hormones (11). It

is not known how far the hydrolysis of thyroglobulin goes under physiological conditions, nor is it known how the released hormones are brought through the cell to the basal cell surface in order to be released into the capillaries.

The most important regulator of thyroid activities is the pituitary thyrotropic hormone, TSH (7, 8). TSH stimulates a variety of the processes involved in hormone synthesis and secretion in the thyroid. The action of TSH is mediated by the adenylate-cyclic AMP system (7). The stimulatory effect of TSH on hormone secretion is very rapid and pseudopods can be observed within a few minutes after intravenous TSH administration (25, 29, 55).

The present problem. The present studies were performed in order to elucidate some aspects of endocytosis of thyroglobulin. From the above review it is obvious that endocytosis must involve redistribution of membrane from the apical cell surface to macropinocytotic and micropinocytotic structures. Estimates of the need of apical plasma membrane to cover the demand for membrane in colloid droplets and micropinocytotic vesicles indicate that this need is very great under steady state secretion of thyroid hormones (60). The very rapid stimulatory effect of TSH on endocytosis further means that there must exist in the follicle cell a mechanism by which an acutely increased need of membrane can be satisfied. This, in turn, indicates that the membrane requirements are supplied by preformed membrane. A possible source of this membrane could be the exocytotic vesicles. Although it has long been generally assumed that the newly synthesized thyroglobulin is transferred into the follicle lumen by exocytosis no clear idea of the exocytotic vesicle has existed until recently. The reason for this is that the apical region in the normal follicle cell contains a mixed population of exocytotic and endocytotic vesicular structures in which it has not been possible to identify the exocytotic elements. However, a recent study from this laboratory showed that elimination of the endogenous TSH secretion (by hypophysectomy or thyroxine treatment) results in a rapid and almost complete inhibition of endocytosis but leaves thyroglobulin synthesis and transport fairly unaffected (4); the vesicles remaining in the apical cell region under these conditions are almost ex-

clusively exocytotic vesicles. This functional condition has been utilized in the present studies in order to explore the possible role of exocytotic vesicle membrane as a source of endocytotic membrane and the possible functional interrelation between exocytosis and endocytosis in the thyroid follicle cells.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats (Anticimex, Stockholm, Sweden), weighing 200-300 g, were used. The animals were maintained on a standard pellet diet containing 3-5 mg iodine per kg (Astra-Ewos, Södertälje, Sweden) and tap water. In order to inhibit endogenous TSH secretion (4) the animals were injected sc with thyroxine (T_4 ; 20 or 50 μ g) 48 and 24 h before the experiments.

Light and electron microscopy: Under ether or pentobarbital anesthesia (60 mg/kg ip) the thyroid glands were fixed by perfusion via the heart with 2.5% or 3% glutaraldehyde in 0.05 or 0.075 M sodium cacodylate, pH 7.2. After excision, the thyroids were immersed in the same solution for 1-3 h. Transversely cut slices from the middle of the lobe were used in the subsequent preparation. The tissue was postfixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 or 2 h, dehydrated in ethanol and embedded in Epon. The specimens were sectioned on an LKB Ultratome fitted with glass or diamond knives. For light microscopy one μ m thick transverse sections of the whole thyroid lobe were cut. These sections were stained with PAS. Central parts of the lobes were cut for electron microscopy. Sections were picked up on uncoated copper grids, contrasted with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope. The magnification of the electron microscope was calibrated with a carbon grating replica with 2,160 lines/mm.

Light microscopic morphometry: To get a general idea about the rate of endocytosis the number of colloid droplets and/or pseudopods was counted in 25 consecutive follicles in each of four sections from each rat.

Electron microscopic morphometry: Cells to be analysed were randomly selected on the grids (10-12 cells/rat; 5 rats/group). The following selection principle was applied. In consecutive grid squares, the follicle cell located closest to the lower right corner and exhibiting an apical surface reaching the follicle lumen, a basal surface resting on the follicle basement membrane, and a nucleus with sharp contours and more than 3 μ m in diameter was photographed at 2 different magnifications. The analysis was performed on photographic prints

with final magnifications of x 9,600 and x 19,700, respectively. In prints with the lower magnification and comprising the whole follicle cell section, areas of the cytoplasm and nucleus were estimated by point-counting (13, 52, 53, 54). A transparent sheet with a printed multipurpose testline system was placed on top of the copy and the number of testpoints (represented by the ends of the testlines) located over the profiles was counted.

In addition, the apical and basal diameters as well as the cell height were measured. On these prints the apical and basal diameters were defined as the shortest distance between the points where the apical and basal plasma membranes, respectively, meet the lateral plasma membrane. The cell height was defined as the length of the shortest line connecting the apical and the basal plasma membranes and passing through the center of the nucleus.

Prints with the higher magnification and comprising the supranuclear region of the cell were used for estimation of surface areas of membranes in the apical part of the follicle cell. Membranes of exocytotic vesicles, apical plasma membrane, pseudopods, colloid droplets and micropinocytotic vesicles were included in the analysis. The surface areas were estimated by counting the number of intersections between the testline system (about 450 testlines/100 μm^2) and the contour of the structure measured (52). The values obtained were expressed as surface densities (μm^2 per μm^3 cell volume) or as number of intersections per follicle cell.

Electron microscopic autoradiography: The intracellular location of newly synthesized protein was studied with electron microscopic autoradiography after injection of 2.0 mCi of (4-5- ^3H)L-leucine. The thyroid tissue, fixed by perfusion 1.5 h after injection of radioleucine, was prepared for electron microscopy as described above. Stained sections, picked up on Formvar-coated grids, were covered with a layer of carbon by vacuum evaporation and the emulsion, Ilford L4, was then applied by means of a wire loop. After exposure for 8 weeks, development was performed in Kodak D-19B for 2 min and fixed in Kodak F-24 for 2 min.

Preparation of subcellular fractions: To evaluate the distribution of labeled proteins between thyroid compartments, subcellular fractionation technique was used. Rats were injected iv in the tail with 10 μCi of $(1\text{-}^{14}\text{C})\text{L-leucine}$ and killed with a head blow 1.5 and 6 h later. After excision, the thyroids were minced with scissors and gently homogenized in 0.44 M sucrose with a Potter-Elvehjem homogenizer equipped with a loose-fitting Teflon pestle. The homogenate was centrifuged at $700 \times g$ for 10 min. The pellet was rehomogenized and recentrifuged, and the resulting pellet was saved. The combined supernatants were centrifuged at $105,000 \times g$ for 1 h and the resulting pellet and the supernatant were saved. The radioactivity and protein content were determined in the different fractions.

Inhibition of protein synthesis: To evaluate the inhibitory effect of cycloheximide (CH) on thyroid protein synthesis the incorporation of $(1\text{-}^{14}\text{C})\text{L-leucine}$ in total thyroid protein was measured. CH (1-3 mg/kg bw), dissolved in saline, was injected iv at different time intervals before sacrifice, controls receiving saline only. The animals were killed by head blow under light ether anesthesia. The protein-bound radioactivity and protein content in pooled glands from 2-3 animals were then determined.

Thyroid hormone secretion: To evaluate the secretion of thyroid hormones the protein-bound ^{125}I (PB^{125}I) in blood plasma was measured. Rats were kept on a low-iodine diet (0.83 mg stable iodine/kg; Astra-Ewos, Södertälje, Sweden) and distilled water for 13 d before the experiments. The thyroglobulin in follicle lumens was pre-labeled by the iv injection of 30 μCi carrier free Na^{125}I (Radiochemical Centre, Amersham, England) 48 h before the experiments. T_4 (20 μg) was given immediately after injection of label and again 24 h before the experiments. Blood samples, drawn from the exposed femoral vein by heparinized syringes immediately before and 2 h after injection of TSH or saline, were centrifuged in a Beckman-Spinco Microfuge and 100 μl were collected from each tube. The radioactivity and protein content were measured after precipitation with TCA.

Protein determination: The protein content was determined according to Lowry et al. (27) with crystalline bovine serum albumin used as standard.

Measurement of protein-bound radioactivity: Protein in tissue homogenates and subcellular fractions, obtained from rats injected with (1-¹⁴C)L-leucine, was precipitated with 10% TCA, washed with TCA and ethanol and dissolved in NCA (Amersham-Searle Corporation, Ill., USA) or 1 N NaOH. The radioactivity was measured in a Packard Tri-Carb or Intertechnique SL 30 liquid scintillation spectrometer.

To measure protein-bound ¹²⁵I, plasma proteins were precipitated with ice-cold 10% TCA, washed twice in the same solution, and dissolved in 1 N NaOH. The radioactivity in the precipitate was determined in a Beckman Biogamma II Counter.

Statistics: All statistical assessments were performed with the Student's t test. Differences were considered significant when P<0.05.

RESULTS

The effect of TSH on exocytosis (paper 1)

Earlier studies with autoradiographic (21, 22, 29) and cell fractionation (3, 21) methods strongly indicate that newly synthesized thyroglobulin is transported through the apical zone of the follicle cells by smooth-surfaced vesicles. However, in the normal follicle cell thyroglobulin is transported not only towards the follicle lumen but also in the opposite direction, as a result of endocytosis (29, 38, 45, 55). This is reflected by the electron microscopic appearance of the apical cell zone in normal cells which shows a polymorphic population of vesicular structures (4). Some of these structures apparently are exocytotic and others endocytotic in nature.

In a previous study from this laboratory (4) it was demonstrated that elimination of the endogenous TSH secretion (by hypophysectomy or thyroxine treatment) creates within one day a functional condition in which endocytosis and thyroid hormone release are almost completely inhibited whereas thyroglobulin synthesis is only slightly decreased. In this functional state only one morphological type of apical vesicles remain out of the polymorphic population seen in the normal cell. These vesicles have a rounded shape, a diameter of 0.1-0.3 μm and a smooth surface membrane; they are characterized by a finely granular content of high density. By several observations on these vesicles, i.a. showing that they contain newly synthesized uniodinated thyroglobulin (6), it has been established that they are exocytotic vesicles.

The present study was performed in order to elucidate the effect of an acute elevation of the TSH level on the exocytotic vesicles. Rats, T_4 -treated for 2 d, were given 500 mU of TSH (Actyron, Ferring, Sweden) and the electron microscopical appearance of the follicle cells was studied at different times after the TSH administration. As was to be expected signs of endocytosis (pseudopods and colloid droplets) were observed but, more pertinent to the present problem, also a progressive decrease in the number of exocytotic vesicles. Counting of the number of vesicles showed a significant decrease after 5 min and at 20 min less than 10% of the original number remained.

The effect of TSH on the vesicle content was evaluated by quantitative electron microscopic autoradiography 1.5 hours after injection of ^3H -leucine. It was found that TSH, given 20 min before sacrifice, caused a drastic redistribution of labeled protein from the apical region of the follicle cells, containing the exocytotic vesicles, to the periphery of the follicle lumen. This transfer of radioactivity was concomitant with the disappearance of exocytotic vesicles.

The effect of TSH on redistribution of protein was also studied in subcellular thyroid fractions. A particle fraction, containing most of the cell organelles including exocytotic vesicles, was separated from a supernatant fraction, containing the luminal colloid. TSH, given 5-20 min before sacrifice to rats labeled with ^{14}C -leucine 1.5 h earlier, induced a redistribution of the label from the particle fraction to the supernatant fraction. This redistribution increased with time and appeared to be dose dependent when TSH was given in a dose range from 10 to 500 mU.

From the observations in this study it was concluded that TSH, administered to T_4 -treated rats, induces exocytosis by which newly synthesized protein is transferred from the follicle cells into the follicle lumen.

The effect of TSH on redistribution of membrane in the apical cell region (papers 2 and 3)

It is well known that TSH induces endocytosis in the thyroid follicle cells. After iv TSH administration, pseudopods appear within 5-10 min but the response rate seems to differ between follicles and between cells in the same follicle (25, 30, 56). At 15-30 min after a large dose of TSH all follicles have pseudopods. Colloid droplets first appear in the pseudopods and then in the body of the cell (28, 55, 59). The rate of increase and decrease of the number of colloid droplets is dependent on the dose of TSH (19, 58).

The formation of pseudopods and colloid droplets requires that a large amount of membrane can be mobilized in a short time (60). It seems unlikely that this is possible by de novo synthesis of membrane. A priori the most plausible source of preformed membrane available for endocytosis in the follicle

cell seems to be the microvilli of the apical plasma membrane. However, the observations in paper 1 show that TSH has a rapid stimulatory effect on exocytosis, in a later study found to be demonstrable within 3 min after TSH (15). Since exocytosis implies fusion of the exocytotic vesicle membrane with the apical plasma membrane, the exocytotic vesicles also appear to be a possible membrane source for endocytosis. To evaluate this possibility information is required about the amount of membrane present in structures involved in exocytosis and endocytosis in the follicle cell and about the effect of TSH on the distribution of membrane in these structures. These problems were studied by electron microscopical stereology in papers 2 and 3.

In a first series of experiments (paper 2) the membrane surface area of exocytotic vesicles, apical plasma membrane and endocytotic structures was measured in T_4 -treated controls and 5, 10, 20, and 30 min after administration of 500 mU of TSH. In the follicle cells in control rats, the membrane surface area of exocytotic vesicles was slightly larger than that of the apical plasma membrane. No endocytotic structures, i.e. pseudopods and colloid droplets, were observed. During the 30 min following TSH stimulation three phases in the action of TSH could be distinguished. A first phase (up to 5 min) was dominated by exocytosis; the membrane surface area of exocytotic vesicles decreased by about 40% and the apical plasma membrane showed a corresponding increase, manifested as an increase of both length and number of microvilli; pseudopods were rare. - The second phase (5-20 min) was characterized by a steep increase in the membrane surface area of pseudopods; colloid droplets and micropinocytotic vesicles also occurred but their membrane surface area was small; the surface area of exocytotic vesicles continued to decrease, but more slowly than during the first phase, and the apical plasma membrane returned to the control value. - A third phase (20-30 min) was characterized by a decrease in the membrane surface area of pseudopods and an equivalent increase in the membrane surface area of colloid droplets, while the total surface area of endocytotic structures was constant; the surface area of apical plasma membrane remained constant during the last 10 min whereas that of exocytotic vesicles slowly decreased.

The sum of the surface areas of all measured membranes did not differ significantly between the groups. It should also be pointed out that the membrane surface area of endocytotic structures at 20 and 30 min after TSH was large and approximately the same as that of the apical plasma membrane in unstimulated cells.

The observations in this study were interpreted to show that stimulation of the thyroid follicle cells with a large dose of TSH induces a transfer of membrane from exocytotic vesicles to the apical plasma membrane. Membrane is then transferred from the enlarged plasma membrane to pseudopods and, later, from pseudopods to colloid droplets.

The large dose of TSH (500 mU) used in the study of paper 2 induced an almost complete depletion of exocytotic vesicles within 20 min and probably also a maximal endocytotic response. As it was judged to be important to explore whether the pattern of membrane turnover observed at this high TSH dose holds true also at lower levels of TSH stimulation a complementary study was undertaken. In this (paper 3) the membrane redistribution was followed during 20 min after the administration of TSH in doses ranging from 5 to 100 mU.

Exocytosis and endocytosis were induced at all dose levels. Quantification of membrane surface areas after 5 and 50 mU of TSH showed that the rate of exocytosis was faster than that of endocytosis during the first 10 min of stimulation.

Exocytosis was signified by a decrease, progressive with time, in the number of exocytotic vesicles and in the membrane surface area of these vesicles. The rate of exocytosis was dependent on the dose of TSH; the number of vesicles remaining in the follicle cells at 20 min was linearly related to the logarithm of the dose. - At 5 and 10 min after 5 and 50 mU of TSH the surface area of the apical plasma membrane was increased; at 20 min this surface area did not differ from that of the controls. - The membrane surface area of endocytotic structures increased with increasing time of TSH stimulation. The surface area of endocytotic membrane was dose dependent as shown by the fact that it was significantly larger at 20 min after 20, 50, and 100 mU of TSH than after 5 mU.

The major part of membranes in endocytotic structures was present in pseudopods. The number of pseudopods increased progressively with time but was not influenced by the TSH dose. Since the membrane surface area of pseudopods was significantly larger at 100 mU of TSH than at 5 mU, this means that the pseudopods increased in size and/or morphological complexity with increasing TSH doses. The non-pseudopodal endocytotic membrane was present in colloid droplets (10% of total endocytotic membrane at 20 min and 100 mU) and micropinocytotic vesicles (about 30%).

In summary, this study showed that submaximal TSH doses, down to 5 mU, have an action on membrane redistribution in the follicle cell similar to that observed (in paper 2) after a large dose of TSH, implying that membrane is first transferred from exocytotic vesicles to the apical plasma membrane and then from the apical plasma membrane to endocytotic structures.

The results of the studies in paper 2 and 3 were interpreted to show that exocytosis is part of the normal response of the follicle cell to stimulation and that the membrane of the exocytotic vesicle population constitutes a pool of membrane that is used for the formation of endocytotic structures. This membrane turnover apparently occurs via the apical plasma membrane but the apical membrane itself does not seem to function as a membrane reserve in spite of its large - due to the presence of microvilli - surface area.

The effect of TSH on exocytosis and endocytosis after reduction of thyroid protein synthesis (papers 4 and 5)

The observations in papers 2 and 3 indicate that the availability of membrane in exocytotic vesicles may be a prerequisite for TSH-stimulated endocytosis. If so, the size of this membrane source could determine the size of the endocytotic response to a large dose of TSH. In order to test this idea experiments were undertaken in which the effect of a large dose of TSH was studied on membrane redistribution in follicle cells in which the pool of exocytotic vesicles had been reduced.

In the first study (paper 4) rats were treated with T_4 (20 μ g/day) for 2 days (controls) and 7, 14, and 21 days. Such prolonged T_4 treatment reduces the rate of protein synthesis

by suppression of TSH secretion (4, 33). Diminished synthesis of protein, including thyroglobulin, should reduce the need for thyroglobulin transport and thus the number of exocytotic vesicles was expected to decline.

Analyses by electron microscopic stereology showed a slow but progressive reduction in the size of the follicle cells, including a reduction of the surface area of the apical plasma membrane; the latter was reduced by 30% after 21 days. The exocytotic vesicles showed a faster progressive decrease and their membrane surface area was reduced by about 85% after 21 days of T_4 treatment. Administration of TSH (500 mU) 20 min before sacrifice induced both exocytosis and endocytosis in all groups. The exocytotic response to TSH was, in relative terms, similar in all groups: The membrane surface area of exocytotic vesicles remaining in the cells after TSH, expressed as the percentage of the membrane surface area of exocytotic vesicles in the corresponding non-TSH-stimulated groups, was almost the same at all times of T_4 treatment and less than 15%. This means, of course, that the absolute surface area of exocytotic vesicle membrane added to the apical plasma membrane by TSH-induced exocytosis was progressively reduced during T_4 treatment. The membrane surface area of the endocytotic structures showed a progressive reduction with increasing time of T_4 treatment. The sum of the membrane surface areas of endocytotic structures and remaining exocytotic vesicles in the TSH-stimulated groups was almost identical to the membrane surface area of the exocytotic vesicles in the corresponding non-TSH-stimulated groups. TSH did not change the total membrane surface area analysed.

In paper 5 we studied the effect of acute inhibition of protein synthesis on the TSH stimulation of exocytosis and endocytosis. For this purpose cycloheximide, known to be a potent inhibitor of thyroid protein synthesis (17, 35, 50), was used in a dose of 3 mg/kg bw. This dose, given to T_4 -treated rats, was shown to substantially reduce radioleucine incorporation into thyroid proteins without causing any apparent qualitative changes of the electron microscopical morphology of the follicle cells.

Morphometric analyses showed that the membrane surface area of exocytotic vesicles was reduced by about 20% at 30 min

after cycloheximide administration and by 50% at 3.5 hours as compared to saline-injected controls. Stimulation with 500 mU of TSH 30 min before sacrifice (30 min and 3.5 hours, respectively, after cycloheximide injection) induced exocytosis and endocytosis. The membrane surface area of endocytotic structures was smaller in the cycloheximide-treated animals than in the controls and this difference was of the same magnitude as the difference in membrane surface area of exocytotic vesicles between cycloheximide-treated animals and controls before TSH administration. The surface area of the apical plasma membrane did not differ between controls and cycloheximide-treated animals after TSH. The weakened endocytotic response to TSH agreed well with the observation that the increase in the plasma $PB^{125}I$ level 2 hours after 500 mU of TSH was reduced by 50% by administration of cycloheximide 3.5 hours before TSH.

In summary, the studies in papers 4 and 5 show that reduction of the rate of protein synthesis is accompanied by a reduction of the pool of exocytotic vesicles in the follicle cells. Irrespective of the method of protein synthesis inhibition (T_4 or cycloheximide), TSH induces exocytosis and this stimulatory effect of TSH is the same as in control animals when the disappearance of exocytotic vesicle membrane is calculated in per cent of the pool of exocytotic vesicle membrane available in the follicle cells before stimulation. The TSH-induced endocytosis, evaluated as the membrane surface area of endocytotic structures, is reduced by protein synthesis inhibition and this reduction corresponds to the decrease in membrane surface area of exocytotic vesicles. These observations strongly indicate that the size of the pool of exocytotic vesicle membrane in the follicle cells determines the size of the endocytotic response to a large dose of TSH.

SUMMARY AND CONCLUSIONS

The studies included in this thesis are based on the observation from this laboratory (4) that elimination of TSH secretion in the rat by treatment with thyroxine results in a functional condition of the thyroid follicle cells in which endocytosis and hormone release are almost completely inhibited whereas delivery of newly synthesized protein to the follicle lumen by exocytosis is little affected. In such functionally changed follicle cells endocytotic structures disappear while exocytotic vesicles, containing newly synthesized thyroglobulin (6), remain in the apical cell region.

The unequivocal identification of exocytotic vesicles in thyroxine-treated animals was a prerequisite for the detection that TSH induces exocytosis (paper 1). Morphologically this effect of TSH, previously not known, was demonstrated by a rapid depletion of exocytotic vesicles located in the apical part of the follicle cell; 20 min after a large dose of TSH (500 mU) less than 10% of the original number of vesicles remained. Functionally, as revealed by cell fractionation technique and by electron microscopic autoradiography, exocytosis was signified by a redistribution of newly synthesized protein from the apical region of the follicle cell to the follicle lumen.

Both exocytosis and endocytosis were early signs of TSH stimulation which indicated that a functional relationship may exist between these processes. To test this possibility a quantitative study on the distribution of membranes in the apical part of the follicle cells was performed using electron microscopic stereology (paper 2). This study of membrane surface areas showed that 500 mU of TSH induced a redistribution of membrane in the apical part of the follicle cell. The first phase of this redistribution (0-5 min) was characterized by exocytosis resulting in addition of membrane to the apical plasma membrane, which enlarged in size. During the second phase (5-20 min) exocytosis continued but, at the same time, membrane was transferred from the apical plasma membrane to pseudopods, resulting in a decrease of the plasma membrane towards control values. The third phase (20-30 min) was

characterized by a transfer of membrane from pseudopods to cytoplasmic colloid droplets. The amount of membrane involved in this redistribution was considerable; for instance, the surface area of membrane in endocytotic structures almost equalled that of the apical plasma membrane, which in turn constituted more than 50% of the surface area of the total plasma membrane of the follicle cell.

As shown in paper 3, exocytosis and redistribution of membrane did not only occur after a large dose of TSH, inducing a maximal endocytotic response, but also at submaximal levels of TSH stimulation. This study also demonstrated a log dose-response relation between TSH and exocytosis.

The studies in papers 2 and 3 suggested that addition of membrane to the apical cell surface by exocytosis might be a prerequisite for the TSH-induced formation of endocytotic structures; this was indicated by the facts that exocytosis preceded endocytosis and that the surface area of apical plasma membrane did not decrease during the formation of endocytotic structures whose membrane surface area was very large. The possibility of a quantitative relation between exocytosis and endocytosis was explored in papers 4 and 5. The pool of exocytotic vesicles in the apical cell region was experimentally reduced by inhibition of protein synthesis, either slowly by long-term (7-21 d) treatment with T_4 (paper 4) or acutely by administration of cycloheximide (paper 5). In both experimental situations a decrease in the number of exocytotic vesicles and a corresponding decrease in the endocytotic response to TSH was found. Electron microscopic stereology revealed a close relationship between the amount of membrane added to the apical surface and the amount of membrane appearing in endocytotic structures. These observations strongly indicate that the size of the membrane pool in the exocytotic vesicles determines the size of the endocytotic response. This conclusion is supported by a previous observation; when the store of exocytotic vesicles is depleted by a large dose of TSH, a second dose of TSH fails to induce endocytosis until the store of exocytotic vesicles is replenished (14). No evidence was obtained in any of the studies included in the present thesis that the apical plasma membrane itself served as the primary source of membrane used in the

formation of endocytotic structures.

Recent observations from this laboratory (5) indicate that TSH-stimulated exocytosis is also related to TSH-stimulated increase in iodination. Thus, not only endocytosis and hormone release but also iodination of thyroglobulin and formation of thyroid hormones appear to be influenced by the action of TSH on exocytosis.

The findings in the present thesis concerning the coupling of exocytosis and endocytosis are also of interest in a general cell biological perspective. In a number of different cell systems, including exocrine cells (23), endocrine cells (16, 31, 34), and nerve cells (24), in which exocytosis is a prominent phenomenon, stimulated exocytosis is accompanied by an interiorization of membrane by endocytosis. Thus, a relationship between exocytosis and endocytosis similar to that described in the thyroid follicle cell in the present thesis seems to be a common phenomenon.

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