

Thyrotropin Induces the Acidification of the Secretory Granules of Parafollicular Cells by Increasing the Chloride Conductance of the Granular Membrane

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Abstract. Secretory granules of sheep thyroid parafollicular cells contain serotonin, a serotonin-binding protein, and calcitonin. Parafollicular cells, isolated by affinity chromatography, were found to secrete serotonin when activated by thyrotropin (TSH) or elevated $[Ca^{2+}]_e$. TSH also induced a rise in $[Ca^{2+}]_i$. We studied the effect of these secretagogues on the pH difference (ΔpH) across the membranes of the secretory granules of isolated parafollicular cells. The trapping of the weak bases, acridine orange or 3-(2,4 dinitro anilino)-3'-amino-N-methyl dipropylamine (DAMP), within the granules was used to evaluate ΔpH . In contrast to lysosomes, which served as an internal control, the secretory granules of resting parafollicular cells displayed a limited and variable ability to trap either acridine orange or 3-(2,4 dinitro anilino)-3'-amino-N-methyldipropylamine; however, when parafollicular cells were stimulated with TSH or elevated $[Ca^{2+}]_e$, the granules acidified. Weak base trapping was also

used to evaluate the ATP-driven H^+ translocation into isolated parafollicular granules. The isolated parafollicular granules did not acidify in response to addition of ATP unless their transmembrane potential was collapsed by the K^+ ionophore, valinomycin. Secretory granules isolated from TSH-treated parafollicular cells had a high chloride conductance than did granules isolated similarly from untreated cells. Furthermore, ATP-driven H^+ translocation into parafollicular granules isolated from TSH-stimulated parafollicular cells occurred even in the absence of valinomycin. These results demonstrate that secretagogues can regulate the internal pH of the serotonin-storing secretory granules of parafollicular cells by opening a chloride channel associated with the granule membrane. This is the first demonstration that the pH of secretory vesicles may be modified by altering the conductance of a counterion for the H^+ translocating ATPase.

THE parafollicular cell of the mammalian thyroid gland is a neural crest derivative (Le Douarin et al., 1974; Polak et al., 1974) that remains capable of expressing neural characteristics when exposed to nerve growth factor (Barasch et al., 1987a). Parafollicular cells also produce the neurotransmitter, 5-hydroxytryptamine (5-HT)¹, which they costore in granules with the peptide hormone calcitonin (Pearse, 1966; Bussolati and Pearse, 1967; Falck and Owmann, 1968; Jaim-Etchevery and Zeiher, 1968; Gershon and Nunez, 1973; Nunez and Gershon, 1978a; Barasch et al., 1987b). The secretion of these hormones is stimulated by elevated $[Ca^{2+}]_e$ (Hirsch and Munson, 1969; Gershon et al., 1978). In addition to calcitonin and 5-HT, parafollicular cell granules contain a serotonin-binding protein (SBP) specific to 5-HT-storing cells of neuroectodermal origin (Barasch et al., 1987b). Although the function of SBP in the granules of

parafollicular cells or in the synaptic vesicles of serotonergic neurons has yet to be established, SBP binds 5-HT best at neutral or alkaline pH (Tamir et al., 1976). Since many secretory granules are known to maintain an acid pH interior (Johnson et al., 1982; Carty et al., 1980; Russell and Holz, 1981; Orci et al., 1986), we investigated the mechanisms that control the internal pH of the secretory granules of parafollicular cells.

The interior of lysosomes (Ohkuma et al., 1982) as well as that of secretory granules is acidified by a H^+ translocating ATPase that can generate a transmembrane H^+ gradient (ΔpH) and a membrane potential ($\Delta \psi$). The extent to which the H^+ translocating ATPase generates either gradient is dependent on the conductance of the organelle membrane to counterions, which permit charge compensation. In fact, the fractional contribution that ΔpH and $\Delta \psi$ make to the proton gradient can be experimentally manipulated by altering the concentration of permeant ions in suspensions of isolated vesicles (Johnson et al., 1979; Johnson and Scarpa, 1979; Glickman et al., 1983). Chromaffin granule (Pazoles et al.,

1. *Abbreviations used in this paper:* DAMP, 3-(2,4 dinitro anilino)-3'-amino-N-methyl dipropylamine; 5-HT, 5-hydroxytryptamine; SBP, serotonin-binding protein; TSH, thyrotropin.

1980; Johnson et al., 1979; Njus, 1983) and lysosomal (Schneider, 1981; Dell'Antone, 1979) membranes are sufficiently permeable to Cl^- that they rapidly acidify when treated with ATP in a Cl^- -containing buffer. When Cl^- is absent, however, these vesicles fail to generate a ΔpH and instead form a $\Delta\psi$.

In intact cells, a variation has been found in the internal pH of endocytic and secretory vesicles. This has been demonstrated for organelles of the endocytic pathway with fluorescein-labeled macromolecules (Yamashiro and Maxfield, 1984) and for organelles of the secretory pathway by weak base trapping (Anderson et al., 1984). The cause of the heterogeneity in intravesicular pH is unknown. Mechanisms accounting for variations in ΔpH may include differing conductances of the membranes of different vesicles to ions, including H^+ , or the presence of electrogenic ion translocating ATPases, in addition to the H^+ ATPase (Fuchs et al., 1988). The current study was done to test the hypothesis that the differences in the pH inside different granules result from a physiological regulation of the conductance of individual granules for counterions. In this paper, we demonstrate that granules of thyroid parafollicular cells can acidify in response to stimulation by secretagogues. Furthermore, secretagogues increase the granule membrane conductance to Cl^- , which would be expected to collapse the potential across the granular membrane, thereby allowing acidification of the granule interior. In this way, these cells can maximize the granule ΔpH by reducing the granule $\Delta\psi$; therefore, individual granules containing a H^+ translocating ATPase can vary in internal pH by varying the chloride conductance of their limiting membrane.

Materials and Methods

Isolation of Parafollicular Cells

Parafollicular cells were isolated by an affinity chromatographic method (Bernd et al., 1981) as modified by Barasch et al. (1987a,b). Essentially, sheep thyroid glands were dissociated with trypsin. The resultant suspension of thyroid cells (consisting of 85% red blood cells, 13.5% follicular cells, and 1.5% parafollicular cells) was treated with thyroid stimulating hormone (thyrotropin [TSH]; 5 mU/ml; Armour Pharmaceutical Co., Tarrytown, NY) and then loaded onto a column of Sepharose 6-MB beads (Pharmacia Fine Chemicals, Piscataway, NJ) at 37°C to which thyroglobulin (Sigma Chemical Co., St. Louis, MO) had been coupled. Follicular cells become phagocytic in the presence of TSH and "attempt" to phagocytize the beads; thus, they are retained on the column while parafollicular cells pass through. Parafollicular cells eluting from the columns were further purified by centrifugation (10 min at 800 g) on a 10% Ficoll (Pharmacia Fine Chemicals) cushion made in MEM. This step allowed separation of smaller particles such as red blood cells, bacteria, and yeast, from parafollicular cells. The purified parafollicular cells were washed and then plated onto plastic dishes and cultured for 2–3 d (Barasch et al., 1987a); 97% of the cells in the final suspension were parafollicular cells. In most experiments, parafollicular cells were resuspended in HEPES-buffered MEM (10 mM, pH 7.4) containing neither bicarbonate nor phosphate.

Electron Microscopic Immunocytochemical Demonstration of the Trapping of DAMP in Parafollicular Cell Granules

Aliquots of dissociated thyroid cells suspended in HEPES-buffered MEM, were incubated with 30 μM 3-(2,4 dinitro anilino)-3'-amino-N-methyl dipropylamine (DAMP), a weak base derived from dinitrophenol (Anderson et al., 1984; Anderson and Pathak, 1985) for 30 min at 37°C. The following substances were added to the suspending medium and their effects

on the intracellular distribution of DAMP were analyzed: (a) Ca^{+2} (5–20 mM); (b) TSH (30 mU/ml); (c) valinomycin (1.0–2.5 μM); (d) nigericin (1.5 μM). When the concentration of Ca^{+2} was raised above 2.5 mM, CaCl_2 replaced NaCl.

Cells were fixed at 37°C for 3 h with a mixture of 4% formaldehyde (from paraformaldehyde), 0.5% glutaraldehyde, 3% sucrose in 0.1 M KPO_4 buffer (pH 7.4). Fixed tissues were washed and excess aldehydes were quenched with 50 mM NH_4Cl in buffer for 15 min. The cells were again washed, dehydrated, and then embedded in a hydrophilic resin (type LR White; Ernest F. Fullam, Inc., Schenectady, NY). Thin sections of DAMP-treated cells were picked up on Formvar-coated nickel grids and treated with 10% horse serum in a solution containing 0.1 M Tris-HCl, 0.9% NaCl, and 0.125% Triton X-100 (TBS-X) for 30 min. The sections were then incubated overnight at 4°C with a mouse monoclonal antibody to dinitrophenol (50 $\mu\text{g}/\text{ml}$) that cross reacts with DAMP (clone HDP-1, No. 15, generously supplied by Dr. Anderson, University of Texas, Health Science Center, Dallas, TX) diluted in TBS-X with 4% horse serum. The sites where DAMP was concentrated were visualized with an anti-mouse secondary antibody coupled to colloidal gold (Janssen Pharmaceutica, Barse, Belgium). Sections were post fixed in 2.5% glutaraldehyde after immunocytochemical processing and examined in a JEOLCO JEM 1200 EX electron microscope.

Analysis of Acridine Orange Trapping in Parafollicular Cells

Chromatographically purified parafollicular cells were incubated with acridine orange (6 μM) for 5 min and were either examined by fluorescence microscopy or loaded into a fluorescence-activated cell sorter (FACS IV; Becton Dickinson & Co., Mountain View, CA). For fluorescence microscopy, cells were excited by light at 488 nm and emitted light was passed through a 520-nm-long band pass filter. For analyses with the FACS (Consort 400 Program) a single population of cells was selected by forward, narrow angle light scatter (a reflection of particle size), and was excited with light at 488 nm from an argon gas laser at 300–400 mwatts. The red fluorescence emission intensity (a function of the concentration of acridine orange) was collected through a 650 \pm 50-nm-wide band pass filter and plotted as a function of cell number. Parafollicular cells, suspended in HEPES-buffered MEM, were treated with TSH (5–30 mU/ml), CaCl_2 (5–15 mM), valinomycin (1.0–2.5 μM) or with nigericin (1.5 μM) for 0.5–30.0 min at 37°C, incubated with acridine orange (6 μM) for 5 min, and then introduced into the FACS. When the effects of brief exposures to these substances were studied, cells were incubated with acridine orange for 5 min before addition of the experimental substance and analyzed in the FACS immediately thereafter. To confirm the homogeneity of the selected cells and to obtain ultrastructural evidence that the analyzed cells remained viable in the FACS, the cells were sorted, collected in a fixative containing 4% glutaraldehyde in 0.1 M KPO_4 buffer, and then processed for examination by transmission electron microscopy. Cells were sorted at 1–2,000 cells/s and coincident fluorescent events were not allowed to exceed 15%.

Proton Transport Assay: Uptake of Acridine Orange by Isolated Parafollicular Cell Granules

ATP-dependent transport of H^+ into isolated parafollicular cell granules was evaluated by measuring granular accumulation of acridine orange in response to the addition of ATP. Acridine orange trapping in parafollicular cell granules was measured by dual wavelength absorbance spectroscopy (492–540 nm; Glickman et al., 1983; Gluck and Al-Awqati, 1984). Parafollicular cell granules were isolated on two sequential discontinuous metrizamide gradients as described by Barasch et al. (1987b), except with the addition of 1 mM dithiothreitol (DTT) to all solutions. Aliquots of the granular fractions of each gradient (0.2–0.4 mg protein) were diluted in 150 mM KCl, 6 mM MgCl_2 , 2 mM Tris, 2 mM morpholino ethane sulfonic acid, and 1 mM DTT, 1.5 ml (transport buffer) incubated with 6 μM acridine orange and placed in a stirred cuvette at room temperature. H^+ transport was initiated by adding Tris-ATP (0.33 mM, titrated to pH 7.0) after a steady baseline had been achieved, when the granules were equilibrated with acridine orange.

ATP-dependent transport of H^+ into parafollicular cell granules isolated from TSH-treated cells was evaluated by measuring the granular accumulation of acridine orange. Thyroid glands were dissociated with trypsin in MEM (see above) and aliquots of suspended cells were treated with TSH (30 mU/ml) for 30 min. Treated and unstimulated cells were diluted into 3 vol of 0.33 M sucrose (4°C) and granules were then rapidly isolated on

the first metrizamide gradient (see above). The trapping of acridine orange in granules treated with ATP was measured by fluorometry (490 excitation, 520 emission) in a stirred cuvette at room temperature, as described above.

5-HT Secretion by Isolated Parafollicular Cells

Plated, chromatographically purified parafollicular cells were treated with the monoamine oxidase inhibitor, nialamide (10 μ M), for 3 h. The cells (1.3×10^6 cells/ml) were then suspended in Hepes-buffered MEM without glutamine and aliquots were incubated at 37°C for 1–35 min with the secretagogues, TSH (30 mU/ml), or CaCl_2 (7.5 mM). The incubation was terminated by rapidly chilling the cells to 4°C and gentle centrifugation (800 g, 15 min). The supernatant was acidified to 0.1 N HCl and then analyzed by reverse phase HPLC (Reinhard et al., 1980; Barasch et al., 1987b) for 5-HT release. Secretagogue-stimulated release was reported as the difference in the 5-HT concentration in supernatants of control and treated cells.

Measurement of Intracellular Ca^{+2} in Isolated Parafollicular Cells

The intracellular concentration of free Ca^{+2} and the effect of TSH on this concentration was estimated from the fluorescence of the dye, Fura-2 (Grynkiewicz et al., 1985). Chromatographically purified parafollicular cells (Bernd et al., 1981) were loaded with Fura-2 by incubation at 37°C with 20 μ M Fura-2 AM (the permeant ester derivative of the dye; Molecular Probes, Inc., Junction City, OR) for 30 min. Fura-2 AM is hydrolyzed by cytoplasmic esterases to yield free Fura-2, which is trapped intracellularly. The Fura-2-loaded cells were washed, suspended in Hepes-buffered MEM solution, and loaded into a stirred cuvette. Cells were excited alternately with light at 380 and 340 nm and the intensity of the emitted light was measured at 505 nm. A calibration curve was constructed with 2.5 μ M Fura-2 acid in a solution containing 150 mM KCl, 1.0 mM MgCl_2 , 1.0 mM EGTA, and Ca^{+2} titrated with a calcium electrode in a range of 0.1 μ M to 100 mM. Highly purified bovine TSH (25 U/mg protein) was obtained from Dr. B. Erlanger at Columbia University (New York).

Measurement of Cytoplasmic pH in Isolated Parafollicular Cells

The pH of the cytosol of parafollicular cells was estimated from the fluorescence of the pH-sensitive dye, BCECF (Thomas et al., 1979). Chromatographically purified parafollicular cells were loaded with the permeant ester BCECF-AM (12.1 μ M; Molecular Probes, Inc., Junction City, OR) for 30 min at 37°C. BCECF-AM is hydrolyzed by cytosolic esterases to yield free BCECF, which is trapped in the cytosol. BCECF-loaded cells were washed, resuspended in Hepes-buffered MEM and loaded into a stirred cuvette (2×10^6 cells/ml). Cells were excited alternately with light at 460 and 490 nm and the intensity of emitted light was measured at 530 nm. A calibration curve was constructed by cleaving the BCECF ester (1.2 mM) with porcine liver esterase (Sigma Chemical Co.; 2 μ l/ml) for 2 h in a solution consisting of 140 mM KCl, 5 mM NaCl, 1 mM MgCl_2 , 10 mM Hepes, at pH 7.95.

Measurement of Chloride Efflux from Isolated Parafollicular Cell Granules

Parafollicular cell granules were isolated on metrizamide gradients (Barasch et al., 1987b) and were loaded with $^{36}\text{Cl}^-$ by freezing overnight in a buffer containing 130 mM KCl, 6 mM MgCl_2 , 10 mM imidazole (pH 7) with $^{36}\text{Cl}^-$ (16 mCi/g Cl^- ; New England Nuclear, Boston, MA). Granules were then thawed and diluted 63-fold into the same salt solution at 4°C, with 2.5 μ M valinomycin. Valinomycin was added to abolish a membrane potential. Aliquots were rapidly collected on Fiberglas GFC filters (Whatman International, Ltd., Maidstone, England) under a vacuum, and the filters washed with 2 ml of 250 mM sucrose (Landry et al., 1987). The effect of extragranular Cl^- on $^{36}\text{Cl}^-$ efflux was analyzed by diluting aliquots of granules 63-fold in Cl^- -free, gluconate salts (130 mM K gluconate, 6 mM Mg gluconate, 10 mM imidazole, pH 7) with 2.5 μ M valinomycin, and collecting granules on filters. In both sets of experiments, the amount of $^{36}\text{Cl}^-$ remaining associated with granules over time was expressed as a fraction of the retained radioactivity, 30 s after resuspension.

Results

ATP-dependent Transport of H^+ by Isolated Parafollicular Cell Granules

We assayed ATP-driven acidification of parafollicular cell granules by measuring the accumulation of the weak base acridine orange in purified granules. Using a previously characterized technique (Barasch et al., 1987b) with two sequential discontinuous metrizamide gradients, we isolated a granule fraction that is enriched 20-fold over the homogenate in the granule marker, 5-HT, but depleted in mitochondrial and lysosomal markers. In this fraction the granules, identified by electron microscopy, occupy $\sim 63\%$ of the particulate volume (Barasch et al., 1987b). When we added ATP (0.33 mM) to granules suspended in a KCl containing "transport buffer" there was no trapping of acridine orange. However, when we preincubated the granules with 2 μ M valinomycin, a K^+ -selective ionophore, the addition of ATP stimulated the initial rate of acridine orange trapping (from 0 to $8 \pm 2.5 \times 10^{-3}$ Δ absorbance/min per mg protein; Fig. 1). Proton-conducting ionophores such as 2 μ M nigericin (a H/K exchanger) or 5 μ M CCCP (a H^+ carrier) released the trapped acridine orange, implying that the uptake of acridine orange was due to the development of a transmembrane pH difference (Fig. 1). Furthermore, *N*-ethylmaleimide (10 μ M), which inhibits many nonmitochondrial ATPases, abolished the valinomycin/ATP-induced trapping of acridine orange.

Since the induction of a K^+ conductance in the granule membrane by valinomycin should collapse a membrane potential and thereby stimulate H^+ transport, these data suggest that H^+ transport, and thus acridine orange trapping, in isolated granules is limited by a membrane potential generated by electrogenic H^+ translocation. Such a transmem-

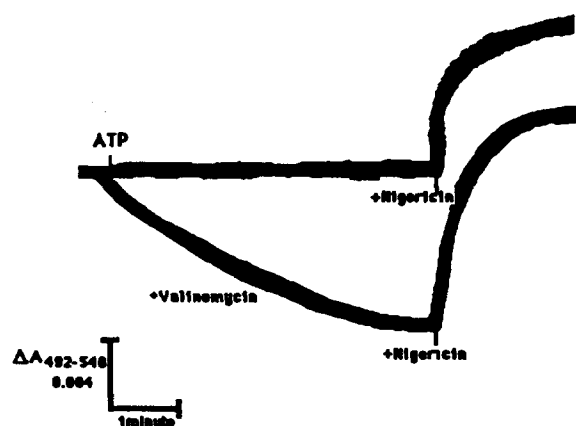


Figure 1. Uptake of acridine orange into purified parafollicular cell granules measured by dual wavelength spectroscopy. In the control trace little inward movement of acridine orange is initiated by the addition of ATP; however, subsequent addition of nigericin drives acridine orange out of the granules (upward deflection). In the superimposed tracing (+ valinomycin), the addition of ATP induces inward movement of acridine orange (downward deflection). The addition of nigericin causes rapid efflux of acridine orange from the granules.

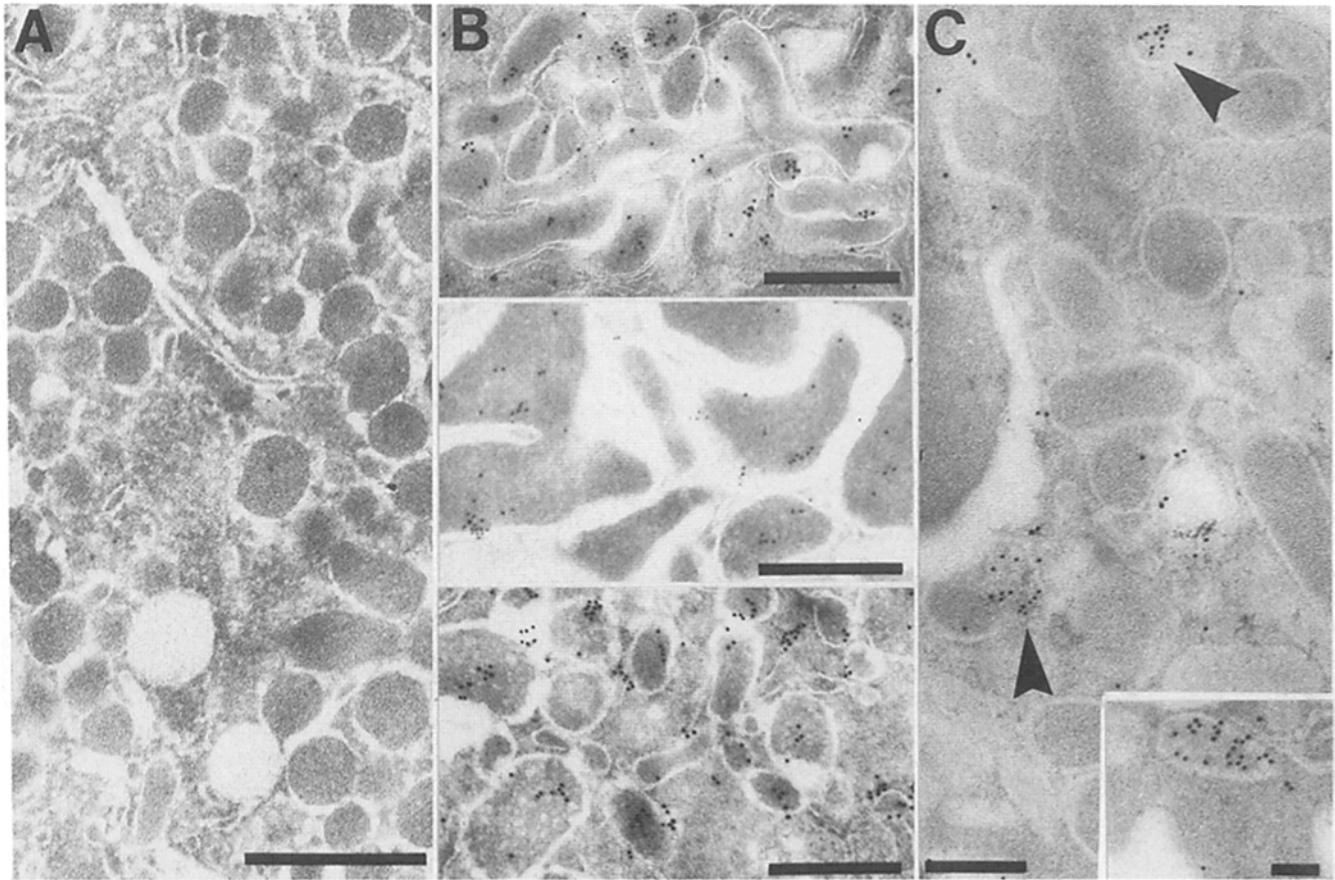


Figure 2. Electron microscopic immunocytochemical demonstration of subcellular sites of accumulation of DAMP in cells from the sheep thyroid. Thyroid cells were incubated with DAMP for 15 min (low exposure). (A) No accumulation of DAMP is seen in parafollicular cell granules. (B) DAMP accumulation can be detected in the lysosomes of three different follicular cells. (C) DAMP accumulation can be detected in a subset of the granules of a neutrophil (arrowheads). The labeling pattern is consistent with the trapping of DAMP in the less numerous primary (lysosomes) but not secondary (specific) granules. Bars: (A and B) 0.5 μm ; (C) 0.25 μm ; (inset) 0.1 μm .

brane potential difference could only arise if the granule membrane had little or no conductance to counterions such as K^+ or Cl^- .

Acidification of Parafollicular Cell Granules in Intact Cells

To examine whether granule acidification in intact parafollicular cells is limited in a manner analogous to that encountered in isolated granules, we compared the ΔpH of individual granules with that of follicular cell and neutrophil lysosomes by visualizing the subcellular distribution of the weak base DAMP with an electron microscopic immunogold technique (Anderson et al., 1984; Anderson and Pathak, 1985). When dissociated thyroid cells were incubated with DAMP for 15 min and fixed without pelleting, <1% of parafollicular cell granules showed concentration of the tracer (Fig. 2 A). In contrast, we found labeling of almost all lysosomes of follicular cells and of a subset of granules in neutrophils (Fig. 2, B and C). When we incubated thyroid cells for 30 min with higher concentrations of DAMP (by washing the cells free of albumin, to which DAMP binds) we found a variable concentration of DAMP immunoreactivity in gran-

ules (Fig. 3 A). Follicular cell lysosomes, however, were uniformly and more intensely labeled than parafollicular granules. These labeling patterns suggest that ΔpH varies from granule to granule in parafollicular cells and is generally less than that across lysosomal membranes; however, when we treated thyroid cells with valinomycin (10 μM) for 1 min before fixation of DAMP, we found an increased percentage of parafollicular cell granules labeled per cell (Fig. 4) and increased numbers of immunogold particles over granules (Fig. 3 B). Valinomycin treatment therefore maximized the ΔpH across the membranes of parafollicular cell granules in intact cells, just as it did in isolated granules. This observation suggests that in contrast to lysosomes, H^+ transport by parafollicular cell granules in situ is limited by a membrane potential. The addition of nigericin (7.5 μM) virtually eliminated all DAMP immunoreactivity from the granules (Fig. 3 C), indicating that accumulation of DAMP in granules reflects the ΔpH across the granular membrane. The effect of nigericin was apparent after brief exposure of cells to the ionophore (0.5 min).

To examine weak base trapping in a large population of cells, we incubated chromatographically purified parafollicular cells with acridine orange and examined them by

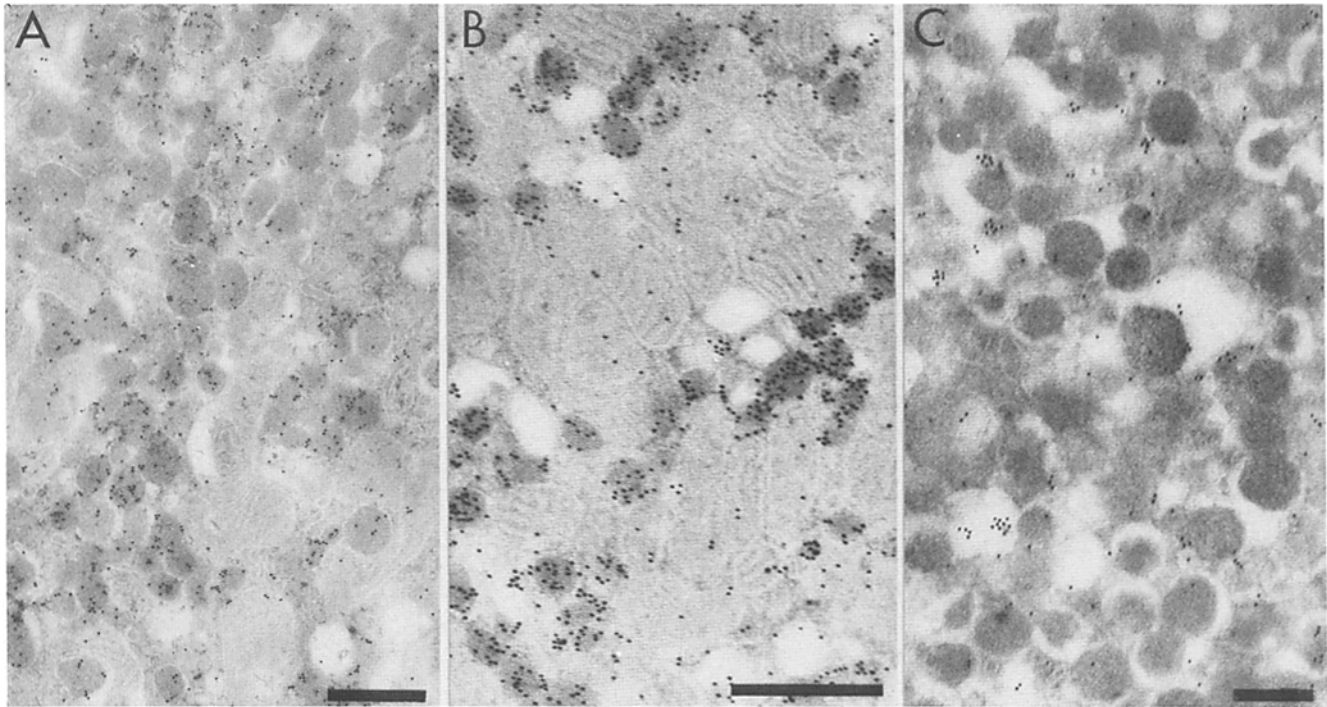


Figure 3. Electron microscopic immunocytochemical demonstration of subcellular sites of accumulation of DAMP in parafollicular cells from the sheep thyroid. Dissociated cells were incubated with DAMP under high exposure conditions in the absence (A) or presence of valinomycin (B) or nigericin (C). Valinomycin increases and nigericin abolishes labeling by DAMP of parafollicular cell granules. Bars: (A and B) 0.5 μm ; (C) 0.25 μm .

fluorescence microscopy (Allison and Young, 1964). We estimated the transmembrane H^+ gradient of intracellular granules by observing red shifts in the wavelengths of emitted light that parallel the intragranular concentration of the dye. The cells were heterogeneous in their ability to trap acridine orange; some cells showed a punctate orange-red fluorescence while others did not trap acridine orange at all

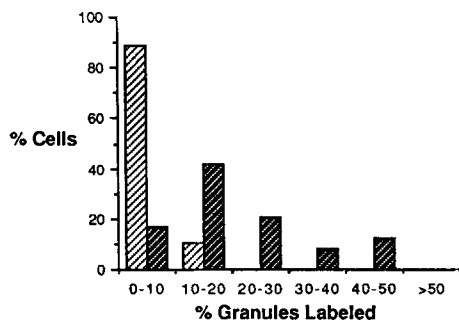


Figure 4. Histogram showing the effect of valinomycin on the accumulation of DAMP in parafollicular cell granules. Dissociated cells were incubated with DAMP (low exposure, as in Fig. 2) \pm valinomycin (1.0 μM). Granules were defined as labeled if two or more immunogold particles were found over them. The percentage of granules labeled per parafollicular cell was determined. Valinomycin increased the proportion of labeled granules per cell. ▨, control; ▩, valinomycin.

(Fig. 5). To evaluate this range in concentration of the dye quantitatively, we incubated purified parafollicular cells with acridine orange (6 μM , 5 min) and analyzed the intensity of red fluorescence of individual cells with a FACS. Cells were selected for analysis by size using forward angle light scatter. In one experiment these cells were collected by cell sorting and processed for electron microscopy. This population of cells was entirely composed of parafollicular cells ($\sim 97\%$; Fig. 6), indicating that the sorted cells were viable and that the intracellular distribution of acridine orange probably reflects its accumulation in granules. The cells varied over a 3.5-fold range in the intensity of red fluorescence of acridine orange with a large standard deviation equal to $84 \pm 8\%$ of the mean red fluorescence. When we added valinomycin (2.5 μM , 1 min), the mean intensity of red fluorescence increased to $122.4 \pm 6.8\%$ of control ($p < 0.01$; Fig. 7).

These results suggest that the membrane conductance of granules must be low in intact cells as it is in isolated granules thereby preventing the granules from maximally acidifying. The heterogeneity of the internal pH of granules may result from physiological regulation of the conductance of individual granules for counterions.

Secretagogue Induced 5-HT Secretion from Parafollicular Cells

We incubated chromatographically purified parafollicular cells with TSH or Ca^{+2} for varying periods of time. The concentration of 5-HT in the supernatant was analyzed to detect the release of endogenous 5-HT (Fig. 8). The maximum rate of 5-HT secretion induced by increased $[\text{Ca}^{+2}]_e$ ($5.2 \pm$

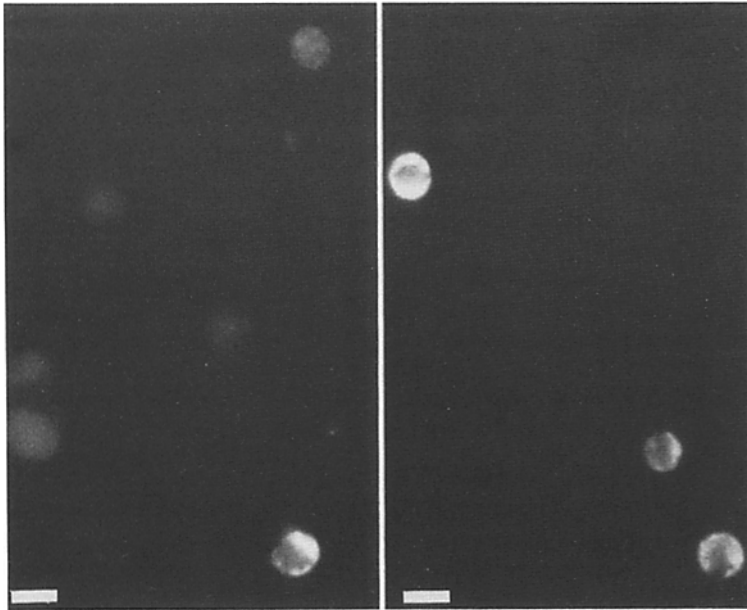


Figure 5. Chromatographically purified parafollicular cells were incubated with acridine orange (6.0 μ M) and examined by fluorescence microscopy. Cells were initially photographed in color and then rephotographed on panchromatic black and white film through a red filter. The brightness of the image reflects the intensity of red fluorescence. Note the heterogeneity between cells in accumulation of acridine orange. Bars, 10.0 μ m.

2.1 $\times 10^{-19}$ mol/cell per min) was faster than that induced by TSH ($0.4 \pm 0.2 \times 10^{-19}$ mol/cell per min; $p < 0.05$). Moreover, the effect of increased $[Ca^{+2}]_e$ peaked within 5 min while the amount of 5-HT released by TSH continued to increase for up to 20 min.

We investigated whether TSH affects the intracellular concentration of free Ca^{+2} ($[Ca^{+2}]_i$) in parafollicular cells by measuring the fluorescence of the calcium-sensitive probe, Fura-2. To validate the Fura-2 technique in parafollicular cells we added the calcium ionophore, ionomycin (2 μ M; extracellular $Ca^{+2} = 1.8$ mM) to cells loaded with Fura-2. Ionomycin increased the fluorescence signal of the cells identified in a fluorescence microscope (Fig. 9 A). Subsequent chelation of $[Ca^{+2}]_e$ with EGTA reduced the fluores-

cence ratio, suggesting that this method can measure changes in $[Ca^{+2}]_i$ in parafollicular cells. We found that the $[Ca^{+2}]_i$ was 176 ± 6 nM ($n = 13$) in isolated cells analyzed in a spectrofluorometer. The addition of a purified preparation (0.75 U/ml) or of a commercial preparation (30 mU/ml; Armour Pharmaceutical Co.) of TSH caused an immediate increase in $[Ca^{+2}]_i$ to 211 ± 7 nM (Fig. 9 B; $p < 0.001$; $n = 8$). The effect of TSH on Fura-2 fluorescence lasted 7 ± 1 min ($n = 8$).

Effect of Secretagogues on the Transmembrane Δ pH of Parafollicular Cell Granules

DAMP-loaded thyroid cells were treated with TSH (30 mU/ml for 30 min), elevated $[Ca^{+2}]_e$ (15 mM for 5 min) or $[K^+]_e$

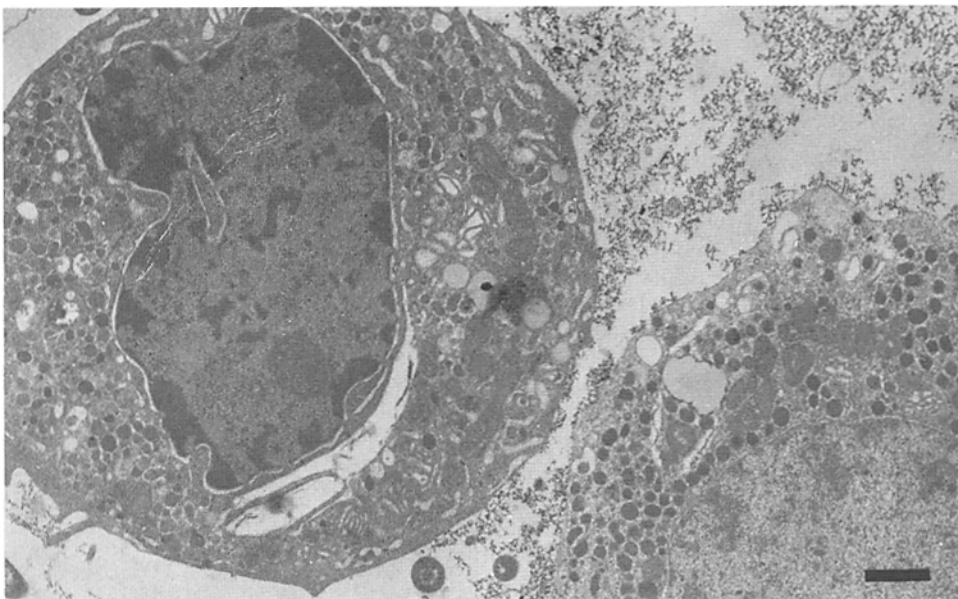


Figure 6. An electron micrograph of two typical parafollicular cells from the population of chromatographically purified cells incubated with acridine orange and selected by forward angle light scatter by the FACS. Bar, 1.0 μ m.

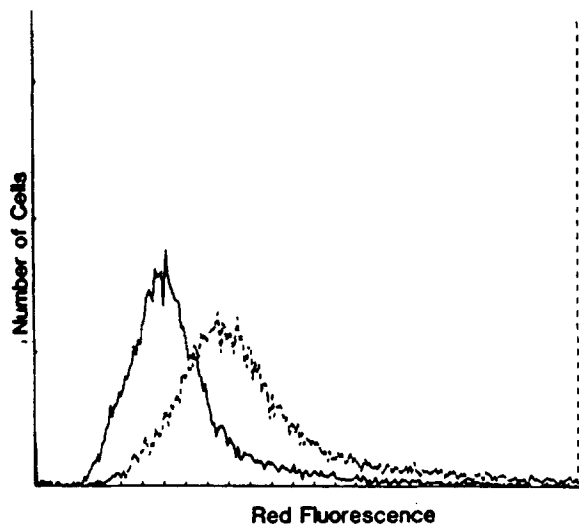


Figure 7. Distribution of the intensity of red acridine orange fluorescence of purified parafollicular cells in the absence (*solid lines*) and presence (*dashed lines*) of valinomycin. Fluorescence was analyzed by flow cytometry. (The accumulation [at the far right of each graph] of cells in the channel of brightest red fluorescence represents off-scale fluorescence intensity.) Incubation with valinomycin increased the average red intensity of the parafollicular cell population.

(56 mM for 5 min), and were examined by electron microscopic immunocytochemistry (as described above). With each of these treatments, the percentage of granules per cell labeled by DAMP increased (Fig. 10), as did the number of immunogold particles over granules (Fig. 11). In a similar manner, when we incubated isolated, acridine orange-loaded parafollicular cells with these same agents and analyzed the fluorescence with a FACS, we found an increased intensity of fluorescence emission. Elevation of the $[Ca^{+2}]_e$ (>5 mM; Fig. 12 A) was maximally effective when cells were incubated for ~ 1 min ($113 \pm 4\%$ of control; $p < 0.005$), and longer incubations failed to further change the intensity of

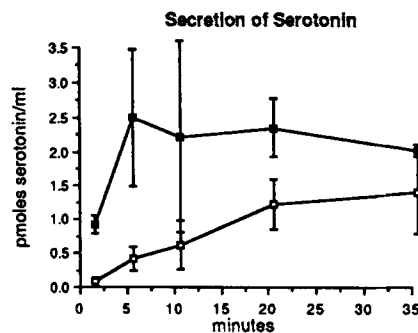


Figure 8. Secretion of 5-HT induced by Ca^{+2} or TSH. Chromatographically purified parafollicular cells were exposed to elevated extracellular Ca^{+2} (7.5 mM) or TSH (30 mU/ml) and the 5-HT concentration in the suspending medium was measured. Both agents stimulated the cells to release 5-HT, but Ca^{+2} did so more rapidly. ■, calcium; □, TSH.

the red fluorescence. TSH (30 mU/ml; Fig. 12 B) was maximally effective after 10 min of treatment ($134 \pm 10\%$ of control; $p < 0.025$), an effect that persisted with continued incubation with TSH to 35 min. Finally, elevation of $[K^+]_e$ (56 mM; 1 min) rapidly increased the red fluorescence (Fig. 12 C), approximately to the same extent as did TSH. The increased labeling of individual granules with DAMP or of populations of cells with acridine orange, therefore, demonstrates that secretagogues increase the transmembrane pH gradient of granules as does valinomycin.

An increase in the ΔpH across granular membranes must result from either acidification of the parafollicular cell granules or alkalinization of its cytoplasm. To ascertain whether secretagogues and valinomycin stimulate alkalinization of the cell cytosol, we loaded purified cells with the pH sensitive dye, BCECF, and measured the pH of the cytosol. The addition of NH_4Cl to BCECF-loaded cells increased the cell pH while nigericin caused a rapid acidification (probably by exchanging K^+ for H^+ at the plasma membrane) validating the use of BCECF as a probe of intracellular pH in parafollicular cells (Fig. 13 B). We found that the initial cytoplasmic pH

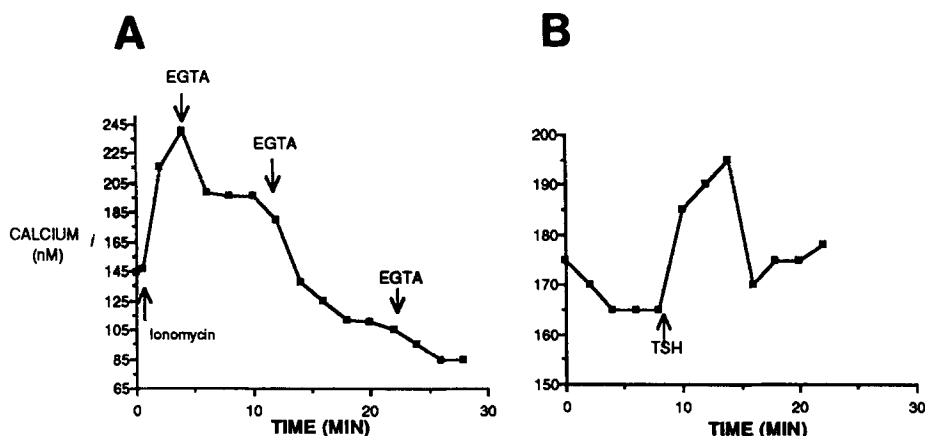


Figure 9. Intracellular-free Ca^{+2} levels ($[Ca^{+2}]_i$) estimated with Fura-2 fluorescence. (A) The $[Ca^{+2}]_i$ of a single cell is plotted as a function of time. Fluorescence was analyzed by fluorescence microscopy (cells were excited alternately at 350 and 380 nm, and emitted light was measured at 505 nm). Ca^{+2} (1.8 mM) is present in the suspending medium. The calcium ionophore, ionomycin, causes a rapid rise in $[Ca^{+2}]_i$ that is reversed when EGTA (1.0 mM) is added to the preparation. (B) Chromatographically purified parafollicular cells were loaded with Fura-2 and the fluorescence of the population was analyzed. Addition of TSH induced a rise in $[Ca^{+2}]_i$.

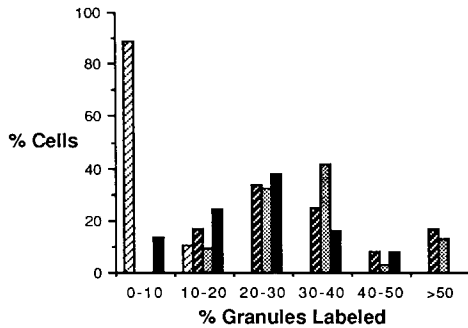


Figure 10. Histogram showing the effect of secretagogues on the accumulation of DAMP in parafollicular cell granules. Dissociated cells were incubated with DAMP (low exposure, as in Fig. 2) in the presence or absence of TSH (30 mU/ml) and elevated extracellular Ca^{+2} (10.0 mM) or K^{+} (56 mM). Granules were defined as labeled if two or more immunogold particles were found over them. The percentage of granules labeled per parafollicular cell was determined. Each agent increased the proportion of labeled granules per cell. ▨, control; ■, TSH; ▩, calcium; ■, KCl.

was 6.81 ± 0.26 ($n = 24$ experiments)². The addition of TSH caused an immediate acidification by 0.13 ± 0.02 pH units ($p < 0.001$; $n = 13$ experiments) that persisted with further incubation (Fig. 13, *A* and *B*). In contrast to TSH, neither the addition of Ca^{2+} ($n = 5$ experiments), nor valinomycin ($n = 5$ experiments) induced a change in the cytosolic pH. These results indicate that secretagogues, as well as valinomycin, stimulate the trapping of weak bases in granules by inducing granular acidification rather than by inducing cytoplasmic alkalization.

2. Calibration of BCECF was done by measuring the fluorescence of the dye in buffered solutions of different pH in a cuvette. Intracellular calibrations of fluorescein dyes show an alkaline shift in the pK_a of the dye by 0.3–0.4 pH U (van Adelsberg and Al-Awqati, 1986). The recorded intracellular pH of parafollicular cells therefore is a minimum value.

Effect of TSH on the Chloride Permeability of Parafollicular Cell Granule Membranes

Many intracellular vesicles such as Golgi and clathrin-coated vesicles, whose contents are acidified by a H^{+} translocating ATPase, have a chloride channel in parallel to the ATPase (Glickman et al., 1983). Removal of Cl^{-} (or closure of the Cl^{-} channel) reduces the pH gradient of these vesicles in vitro. Since valinomycin, an electrogenic ionophore, stimulated granule acidification, secretagogues might similarly change the internal pH of granules by altering the conductance of the granular membrane. We investigated the Cl^{-} conductance of granules purified from dissociated thyroid cells that had been treated with TSH, by loading the isolated granules with $^{36}\text{Cl}^{-}$ in a KCl-buffered solution (pH 7). We then measured tracer efflux from granules at 4°C upon dilution into 63 vol of the same buffer by collecting aliquots of granules on filters over time after resuspension. We found that granules isolated from cells treated with TSH showed a rapid efflux of $^{36}\text{Cl}^{-}$ (reaching background in 3 min), while the $^{36}\text{Cl}^{-}$ efflux from granules of untreated cells was very slow (Fig. 14). To test whether $^{36}\text{Cl}^{-}$ efflux was due to a $^{36}\text{Cl}^{-}/\text{Cl}^{-}$ exchanger, we resuspended $^{36}\text{Cl}^{-}$ -loaded granules in a buffered gluconate salt solution and collected aliquots over time. Again, we found that granules from TSH-stimulated cells showed $^{36}\text{Cl}^{-}$ efflux while control granules did not. This suggests that external Cl^{-} does not drive $^{36}\text{Cl}^{-}$ efflux and suggests that the tracer diffuses from the granules through a channel rather than by $\text{Cl}^{-}/\text{Cl}^{-}$ exchange. These results demonstrate that TSH induces the opening of a Cl^{-} channel, a modification that is stable during the period of granule isolation.

Granules Isolated from TSH-treated Cells Acidify Their Contents

To investigate whether the increased chloride conductance of granules from TSH-treated cells results in increased granule acidification, we measured ATP-dependent proton transport in granules from TSH-stimulated cells. Thyroid cells were

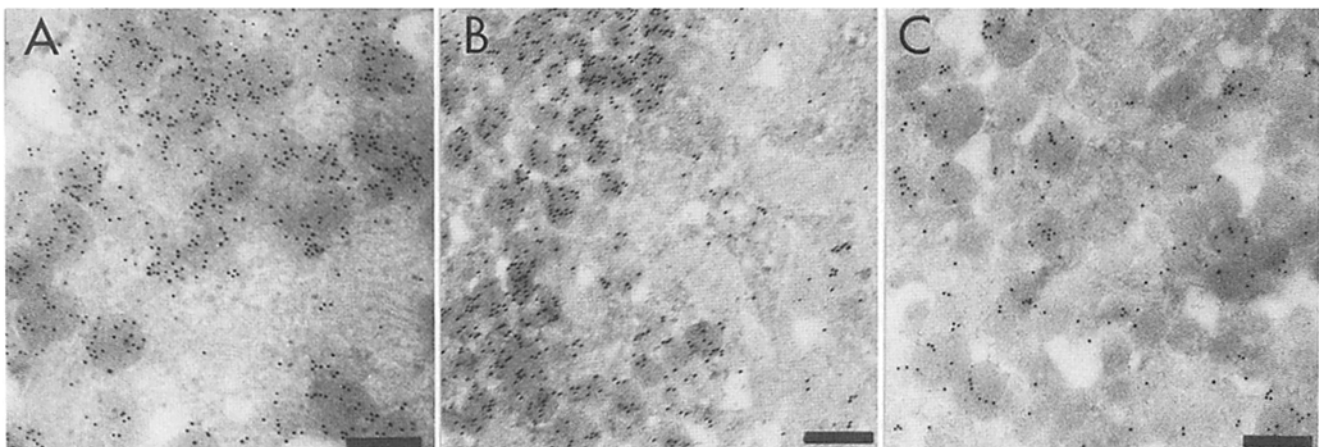


Figure 11. Electron microscopic immunocytochemical demonstration of subcellular sites of accumulation of DAMP in parafollicular cells from the sheep thyroid. Dissociated cells were incubated with DAMP (high exposure, as in Fig. 3) (*A*) in the presence of TSH (30 mU/ml) or (*B*) elevated concentrations of extracellular Ca^{+2} . Both of these secretagogues increase DAMP labeling of parafollicular cell granules over the level seen in control cells (*C*) incubated similarly (extracellular $\text{Ca}^{+2} = 1.8$ mM). Bars, 0.25 μm .

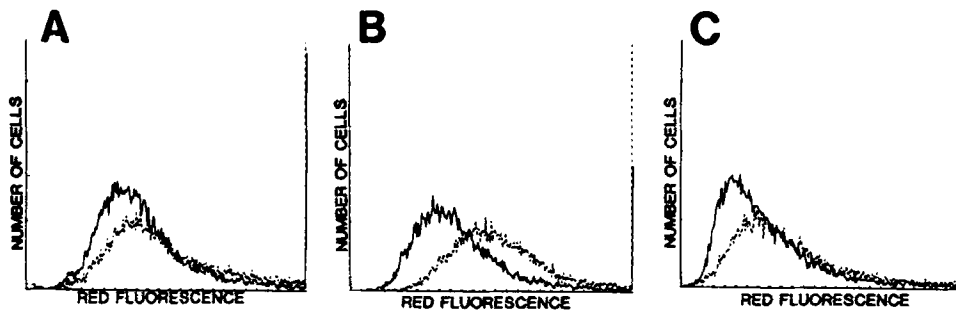


Figure 12. Graphs showing the distribution of the intensity of red acridine orange fluorescence of chromatographically purified parafollicular cells in the absence (solid lines in A, B, and C) and presence of secretagogues. Fluorescence of cells was analyzed by flow cytometry. (The accumulation of cells in the channel of brightest red fluorescence [at the far right of each graph] is due to the presence of cells the fluorescence intensity of which is off-scale.) Incubation with Ca^{+2} (10.0 mM; A, dashed line), with TSH (30 mU/ml; B, dashed line), or with an elevated concentration of K^{+} (56 mM; C, dashed line) increases the average red intensity of the parafollicular cell population.

dissociated and aliquots were stimulated with TSH for 30 min. Granules were then rapidly prepared on the first metrizamide gradient. We found that the addition of ATP to these granules resulted in a rapid uptake of acridine orange. Addition of valinomycin did not enhance the rate of uptake. Granules isolated from untreated cells of the same preparation, however, showed little trapping of acridine orange. Unlike the granules from TSH-stimulated cells, valinomycin increased the rate of H^{+} transport by the control granules at least threefold (Fig. 15; cf. Fig. 1). Since these results were obtained with a partially purified fraction, we can not exclude the possibility that other organelles, in addition to the granules, increased their proton pumping after TSH stimulation. The acidification of granules (and perhaps other organelles) from TSH-treated cells was due to the opening of a chloride channel since these granules failed to acidify in Cl^{-} -free, gluconate media (not shown). Granules from TSH-treated cells, unlike granules from unstimulated cells, can therefore maximally acidify their contents in the absence of an electrogenic ionophore. This result implies that the granule membrane conductance no longer limits H^{-} transport. TSH-induced Cl^{-} channel opening (as demonstrated above) would collapse a membrane potential and enhance granule acidification.

Discussion

In this paper we demonstrate that TSH has multiple effects on parafollicular cells, including the induction of secretion of 5-HT (see also Nunez and Gershon, 1983). This is the first

direct demonstration that TSH is a parafollicular cell secretagogue. 5-HT is taken up by follicular cells (Gershon and Nunez, 1976) where it stimulates follicular hormone secretion (Nunez and Gershon, 1978b; Melander and Sundler, 1972), while calcitonin has distant effects on osteoclasts. Thus, two cells of distinct embryological lineage both respond to TSH. It is possible that parafollicular cells regulate follicular cell activity through the release of 5-HT (Nunez and Gershon, 1978b). Whether follicular cell hormones influence parafollicular cell metabolism remains to be determined.

We found that parafollicular cell secretagogues (TSH and elevated $[\text{Ca}^{+2}]_i$) induce the acidification of 5-HT storage granules. Secretagogues have been found to induce acidification of other organelles. For example, glucose acidifies β cell granules of pancreatic islets (Pace and Sachs, 1982). Moreover, the intracellular canaliculi of parietal cells also acidify in response to histamine (DiBona et al., 1979; Berglindh et al., 1980) and microsomes from histamine-treated parietal cells have an increased membrane conductance for K^{-} and for Cl^{-} (Cuppoletti and Sachs, 1984). We demonstrated that TSH induces granule acidification by opening a Cl^{-} channel in the membranes of secretory granules. Opening of a Cl^{-} channel would allow charge compensation for electrogenic H^{-} pumping, thereby stimulating the H^{-} translocating ATPase and inducing granule acidification. Furthermore, we found that substitution of K^{+} gluconate for KCl reduced the rate of acidification of granules isolated from TSH-treated cells, suggesting that Cl^{-} , but not K^{-} , acts as the primary counterion.

TSH increases $[\text{Ca}^{+2}]_i$ of parafollicular cells; however,

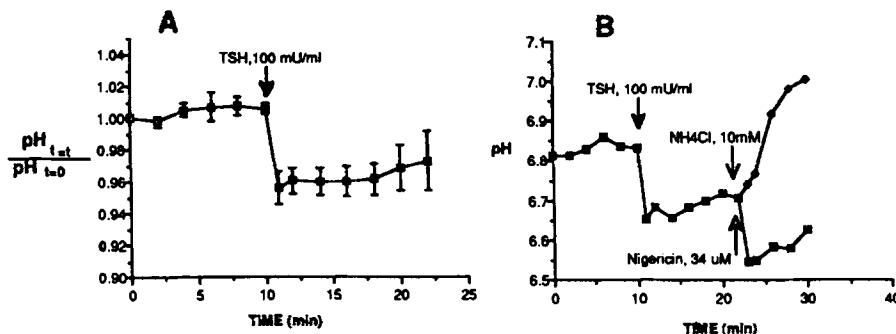


Figure 13. Intracellular pH estimated with BCECF fluorescence. (A) TSH induces cytoplasmic acidification in a population of chromatographically purified parafollicular cells. Cell pH is expressed as a fraction of the initial pH and plotted as a function of time. (B) Intracellular pH of a population of purified parafollicular cells. Addition of TSH induced a fall in cell pH. Addition of nigericin causes acidification, while ammonium chloride induces alkalization.

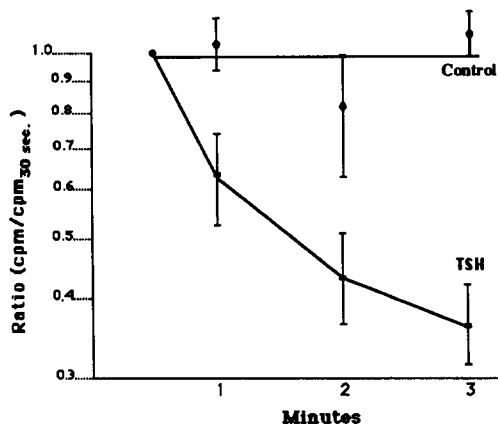


Figure 14. The effect of TSH on the granule membrane conductance for Cl^- , measured by $^{36}\text{Cl}^-$ efflux from purified granules. Dissociated thyroid cells were treated with TSH and granules isolated by density gradient centrifugation. Isolated granules were loaded with $^{36}\text{Cl}^-$, then diluted and rapidly collected on filters. Data are expressed as a fraction of the initial content of $^{36}\text{Cl}^-$. Granules isolated from TSH-treated cells show rapid efflux of the tracer, while control granules show little loss of the tracer over time.

since increased membrane conductance for chloride was present in the isolated granules hours after the cells were stimulated by TSH, modification of the channel was probably covalent rather than by direct Ca^{2+} activation. TSH is known to increase the cAMP concentration in follicular cells (Bastomsky and McKenzie, 1967) and thus a cAMP-dependent kinase may be involved in the opening of the chloride channel in granular membranes. In fact, Schoumacher et al. (1987) has demonstrated that epithelial chloride channels are opened by cAMP-dependent protein kinases. Alternatively, the increased $[\text{Ca}^{2+}]_i$ that follows stimulation by TSH, may trigger Ca^{2+} -activated kinases to induce the covalent modification. Although we have not examined the chloride conductance of granules isolated from KCl-depolarized

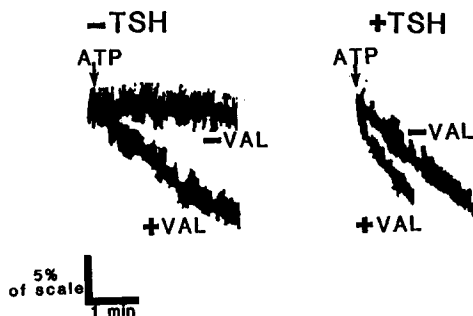


Figure 15. Acridine orange uptake into parafoollicular cell granules isolated from TSH-treated cells and from unstimulated thyroid cells measured by fluorimetry. In the control vesicles there is little ATP driven uptake of acridine orange; however, pretreatment with valinomycin stimulates the inward movement of acridine orange (downward deflection). In contrast, the addition of ATP to granules isolated from TSH-treated cells causes the rapid uptake of acridine orange, which is not further enhanced by treatment with valinomycin.

cells or from cells stimulated by elevated $[\text{Ca}^{2+}]_e$, a similar mechanism might also occur in these cells.

Variation in chloride conductance can also explain the heterogeneity in the internal pH of different organelles. Endosomes are less acidic than lysosomes (Murphy et al., 1984; Yamashiro et al., 1984) and differences in pH distinguish at least two types of endocytic vesicles involved in the recycling of receptors (Yamashiro et al., 1984; Yamashiro and Maxfield, 1984). Moreover, individual members of a single class of organelle can vary in pH, as occurs with maturing beta cell granules (Orci et al., 1986). Vesicles that generate a large ΔpH such as lysosomes (Schneider, 1981; Dell'Antone, 1979; Okuma et al., 1982) and chromaffin granules (Johnson et al., 1979; Njus, 1983) are highly permeable to Cl^- . In contrast, Golgi vesicles that maintain a small transmembrane pH gradient must conduct Cl^- poorly, since valinomycin increases proton pumping in these vesicles, even in the presence of 150 mM Cl^- (Glickman et al., 1983). This suggests that Golgi vesicles and lysosomes maintain their Cl^- conductances at different levels. Variation in conductance may be regulated by membrane-associated kinases and phosphatases that can produce stable modifications in Cl^- channels. Indeed, recent studies by Landry et al. (1987) demonstrated that the chloride conductance of vesicles enriched in Golgi membranes was reduced by treatment with ATP or ATP- γ -S, but not by nonhydrolysable ATP analogues. This suggests that these vesicles contain a membrane-associated kinase that can close the channel. Heterogeneity of organelar pH can not result from the regulation of the number or activity of the vesicular H^+ ATPase, since a single H^+ ATPase molecule with a turnover number of 50–100 ions/s would maximally acidify a granule (radius = 0.2 μm) within 1 min.

The role that the proton electrochemical gradient plays in the function of these granules remains to be determined. The electrical potential generated by the H^+ ATPase in the resting cell might act as a driving force for 5-HT accumulation in parafoollicular granules. It is not clear, at present, what the function of secretagogue-induced granule acidification is in the physiology of parafoollicular cells; however, it is possible that granule acidification may facilitate secretion of 5-HT by enhancing the dissociation of 5-HT from SBP before exocytosis. Upon fusion of granules with the plasma membrane and exposure of SBP to alkaline extracellular pH, rebinding of 5-HT would be prevented since SBP has a reduced binding capacity for 5-HT in the relatively high $[\text{Na}^+]_e$ and $[\text{Ca}^{2+}]_e$ (Tamir and Gershon, 1981).

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