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Biochemistry

Effects of thyrotropin on the thyroid cell membrane: Hyperpolarization induced by hormone–receptor interaction

(lipophilic ions/membrane vesicles/adenylate cyclase/toxins/interferon)

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ABSTRACT **Cultured thyroid cells accumulate the lipophilic cation triphenylmethylphosphonium, indicating that there is an electrical potential (interior negative) across the plasma membrane. Thyrotropin stimulates the uptake of the lipophilic cation 3-fold, and the proton conductor carbonylcyanide-*m*-chlorophenylhydrazone causes efflux of triphenylmethylphosphonium accumulated in the presence or absence of thyrotropin. The stimulatory effect of thyrotropin on triphenylmethylphosphonium accumulation is not mimicked by human chorionic gonadotropin, a glycoprotein hormone with a similar structure whose target organ is not the thyroid, and the effect is abolished if the thyrotropin-receptor activity of the cells is destroyed by treatment with trypsin. Analogous effects are observed with thyroid plasma membrane vesicles which are essentially devoid of mitochondrial and soluble enzyme activities. Triphenylmethylphosphonium uptake and stimulation by thyrotropin occurs when NaCl, KCl, or Tris·HCl concentration gradients are artifically imposed across the vesicle membrane ($[salt]_{out} > [salt]_{in}$). It seems likely, therefore, that triphenylmethylphosphonium uptake is driven by a chloride diffusion potential (interior negative) and that thyrotropin either increases the permeability of the membrane to anions or decreases its permeability to cations. Thyrotropin-stimulated triphenylmethylphosphonium uptake in the vesicle preparations reaches a quasi steady-state within 3 min; in contrast, thyrotropin-stimulated adenylate cyclase activity is negligible during this period of time, becomes measurable after about 4 min, and is optimal after 12–15 min. Thus, a primary mode of action of thyrotropin on the thyroid cell may be an alteration in the electrical potential across the plasma membrane. The relevance of this observation to the mechanism of action of other glycoprotein hormones, certain bacterial toxins, and interferon is discussed.**

Current views regarding the mechanism of action of thyrotropin (TSH) and other glycoprotein hormones invoke alterations in the concentration of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) as the second event in the transfer of information from the hormone to the appropriate cell. That is, the information carried by the hormone is translated purportedly into a "second message" by means of a change in intracellular cyclic AMP concentration (1–8). Recent evidence (9–19) also suggests, moreover, that the mechanism of action of TSH and other glycoprotein hormones is analogous in many respects to that of certain bacterial toxins (i.e., cholera and tetanus toxins) and the antiviral agent interferon. Generally, the schema proposed to explain the effects of these hormones, toxins, and interferon involves a multistep sequence of events that occur in the following order: (*i*) interaction of the effector with a specific ganglioside or ganglioside-like receptor in the cell membrane; (*ii*) a unique alteration in the conformation of the effector which allows one of its subunits to translocate within the membrane; (*iii*) an alteration in the state of the membrane which is reflected by changes in the exposure of certain glycolipids and glycoproteins and by positive receptor cooperativity; and (*iv*) an increase in adenylate cyclase activity leading to an increase in intracellular cyclic AMP concentration which represents the immediate stimulus for an alteration in cellular metabolism.

One implication of this type of scheme is that the increase in intracellular cyclic AMP may not necessarily represent a second message *per se* but rather an event that is sequentially or simultaneously coupled to a primary effect of these agents on the membrane. In other words, the common denominator of all of the effectors may be an alteration in the membrane that leads to generalized effects on cellular metabolism, one of which may be an increase in the concentration of intracellular cyclic AMP. Viewed in this manner, it is clear that one means of accomplishing this could be through alterations in electrochemical ion gradients across the cell membrane. In this regard, the following observations are notable: (*i*) the primary effect of tetanus toxin is an alteration in neuronal transmission (20); (*ii*) cholera toxin causes a dramatic loss of electrolytes and water through the intestinal epithelium (21); (*iii*) TSH increases the rate and extent of iodide uptake by thyroid cells (22); (*iv*) oubain, the classic inhibitor of Na^+,K^+-stimulated ATPase, blocks the antiviral action of interferon (23); and (*v*) electrochemical ion gradients play a primary role in active transport and metabolism in prokaryotes, eukaryotes, and many subcellular organelles (24).

In this report, we present preliminary evidence that is consistent with the postulate that a primary effect of TSH is to increase the electrical potential across the membrane of cultured thyroid cells and plasma membrane vesicles derived from these cells.

METHODS

Growth of Cells and Preparation of Membrane Vesicles. The thyroid cells were either primary cultures of normal bovine thyroid cells grown in the presence of TSH (22) or a single clone derived from a rat thyroid tumor (1-5G) developed in Fischer rats by Wollman (25). Preparation of the bovine thyroid cell cultures has been described (22); growth was in medium 199 supplemented with 15% (vol/vol) fetal calf serum, 0.02 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (pH 7.4), 0.02% (vol/vol) bicarbonate, penicillin (50 units/ml), streptomycin (0.05 mg/ml), and TSH (100 milliunits/ml). The

Abbreviations: TSH, thyrotropin; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NaCl/P_i, phosphate-buffered saline; ^{125}I-TSH, ^{125}I-labeled TSH; $TPMP^+$, triphenylmethylphosphonium.

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rat thyroid cells were derived from a single tumor that has been carried by subcutaneous implantation in male Fischer rats (25–27). The growth rate and metabolic properties of this tumor are similar to those reported for 1-8R rat thyroid tumor cells (25–27). Details of the cloning and passage process, as well as the growth characteristics of the rat tumor thyroid cells, will be the subject of a separate report. (F. S. Ambesi-Impiombato and H. G. Coon, unpublished). Cells were maintained in a modified F-12 medium (28) supplemented with 5% (vol/vol) calf serum and TSH at 50 milliunits/ml. Both types of cells were grown at 37° in a 95% air/5% CO_2 atmosphere.

Cell suspensions were obtained by gentle scraping of the culture flasks after a 10-min incubation with 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline ($NaCl/P_i$). Cells were recovered by centrifugation at 200 to 500 × *g*, washed twice in the same medium without EGTA or with an isotonic (0.25 M) sucrose solution containing 0.02 M Tris·HCl at pH 7.4, and suspended in one or the other of these media prior to use. Cells treated with trypsin to remove the glycoprotein component of the TSH receptor (22) were exposed to 2.5% (wt/vol) trypsin (Grand Island Biological Co.) for 20 min; tryptic digestion was terminated by addition of a 5-fold excess of soybean trypsin inhibitor. The cells were then washed twice with Ca^{2+}- and Mg^{2+}-free $NaCl/P_i$ and two additional times with either $NaCl/P_i$ or isotonic sucrose (0.25 M) containing 0.02 M Tris·HCl, pH 7.4.

Purified plasma membrane vesicle preparations from thyroid cells were obtained by using nitrogen cavitation as described by Lever (29, 30). The final vesicle preparation was suspended in 0.25 M sucrose containing 0.01 M Tris·HCl (pH 7.5) and stored at a concentration of 4 mg of protein per ml in liquid nitrogen. The vesicle preparations were evaluated for several marker enzymes. By comparison with whole cells or their homogenates, the vesicles retained 72% of the 5′-nucleotidase activity (plasma membrane marker), less than 3.5% of the succinic dehydrogenase and succinate-cytochrome *c* reductase activities (mitochondrial markers), and less than 0.5% of the *o*-nitrophenylacetate esterase and glucose-6-phosphatase activities (soluble enzyme markers). Assays for these enzymes were performed as described (31–35).

Binding of ^{125}I-Labeled TSH (^{125}I-TSH). Bovine TSH and ^{125}I-TSH were prepared as described (36–38). TSH was assayed at 25 international units (IU)/mg by a McKenzie bioassay (39). Binding of ^{125}I-TSH was routinely assayed by a filtration technique (9, 38, 40, 41) and, unless stated otherwise, binding assays contained 0.6% (wt/vol) bovine serum albumin and approximately 125,000 cpm of ^{125}I-TSH (2.5 nM) in addition to the cell suspension in a total volume of 100 μl. Ca^{2+}- and Mg^{2+}-free $NaCl/P_i$ (pH 7.4) equiosmolar to the growth medium was used for designated binding studies. Alternatively, binding assays were carried out in 0.25 M sucrose containing 0.02 M Tris·HCl (pH 7.4) as indicated. To ensure that ^{125}I-TSH binding was specific, control samples were incubated with 15 μM unlabeled TSH in addition to the other components of the reaction mixture.

Uptake of [^{3}H]Triphenylmethylphosphonium ($TPMP^+$). This was measured by filtration through Millipore Cellotate filters (0.5 μm) (42). Incubations were carried out at 20° in 100-μl reaction mixtures (total volume). For studies with intact cells, the reaction mixtures contained (final concentrations) 0.25 M sucrose, 0.02 M Tris·HCl (pH 7.4), and specified amounts of cell protein. Other additions were made as indicated, and the reactions were initiated by addition of 0.4 mM [^{3}H]$TPMP^+$ (120 Ci/mol). For studies with membrane vesicles, reactions were initiated by diluting vesicles suspended in 0.25 M sucrose containing 0.01 M Tris·HCl (pH 7.4) 1:10 into reaction vessels containing 0.05 M NaCl, KCl, or Tris·HCl (pH 7.4), 0.125 M sucrose, 0.4 mM [^{3}H]$TPMP^+$ (120 Ci/mol), and other additions as indicated. In both cases, uptake was terminated by addition of 5 ml of ice-cold 0.8 M NaCl containing 0.1 M Tris·HCl (pH 7.5), immediate filtration, and washing with an additional 5 ml of the same solution. Dilution, filtration, and washing were complete within 20 sec, and the filters were immediately removed from the suction apparatus and assayed for radioactivity by liquid scintillation spectrometry. Corrections for nonspecific adsorption were made by diluting the reaction mixtures with wash buffer before addition of [^{3}H]$TPMP^+$, followed by filtration and washing, and this value was subtracted from the experimental values presented. [^{3}H]$TPMP^+$ (bromide salt) was prepared by the Isotope Synthesis Group at Hoffman-La Roche, Inc., under the direction of Arnold Liebman.

Other Assays. Adenylate cyclase assays were performed by using the conditions of Rodbell and Krishna (43) as modified by Wolff and Jones (44). Cyclic AMP was measured by a binding protein assay (45). Protein was determined colorimetrically (46) on samples heated to 90° for 30 min in 0.1 M NaOH, and bovine serum albumin was used as a standard.

RESULTS

TSH Binding by Thyroid Cells. Thyroid cells suspended in a medium containing isotonic salts but no serum specifically bound small but significant amounts of ^{125}I-TSH (Fig. 1*A*). Binding was optimal at 37° (not shown), and inhibition of binding was not observed with albumin, human chorionic gonadotropin, insulin, glucagon, growth hormone, prolactin, or corticotropin (ACTH) at concentrations 10-fold higher than that of ^{125}I-TSH. Although not shown, at 20° and 1 nM ^{125}I-TSH, maximum binding was achieved after 10–15 min; at 100 nM, on the other hand, maximum binding was achieved in less than 30 sec.

The same cells suspended in a medium made isotonic with 0.25 M sucrose rather than salt bound significantly increased amounts of the same ^{125}I-TSH preparation with equal specificity (Fig. 1*B*). Moreover, under these conditions, as before, binding was maximal after 10–15 min at 1 nM ^{125}I-TSH and was effectively instantaneous at 100 nM. It is also noteworthy that inclusion of 0.05 M NaCl or KCl in this medium resulted in a 40–50% decrease in ^{125}I-TSH binding. Thus, salt inhibition of TSH binding to thyroid cells is analogous to that observed previously (40, 41) with membrane preparations derived from these cells. Finally, cells incubated with ^{125}I-TSH at 37° and pH 7.4 in isotonic sucrose were as viable as cells incubated in isotonic salt as judged by trypan blue exclusion or plating efficiency, and the same results were obtained in all binding studies independent of the source of the cells used [i.e., either bovine thyroid or rat tumor (1-5G)].

In previous studies (22), trypsin treatment of bovine thyroid cell suspensions was shown to cause the loss of TSH receptor activity when ^{125}I-TSH binding was measured with plasma membrane preparations from the trypsinized cells. Incubation of either bovine or rat tumor thyroid cells with trypsin resulted in complete loss of ^{125}I-TSH binding regardless of the medium in which the cells were suspended [i.e., either physiologic salt concentrations (Fig. 1*A*) or isotonic (0.25 M) sucrose (Fig. 1*B*)].

Influence of TSH on $TPMP^+$ Uptake. In the early 1970's, Skulachev and Liberman and their coworkers introduced the use of "lipophilic ions" to measure electrical potentials across biological membranes (see ref. 47 for a review), and these

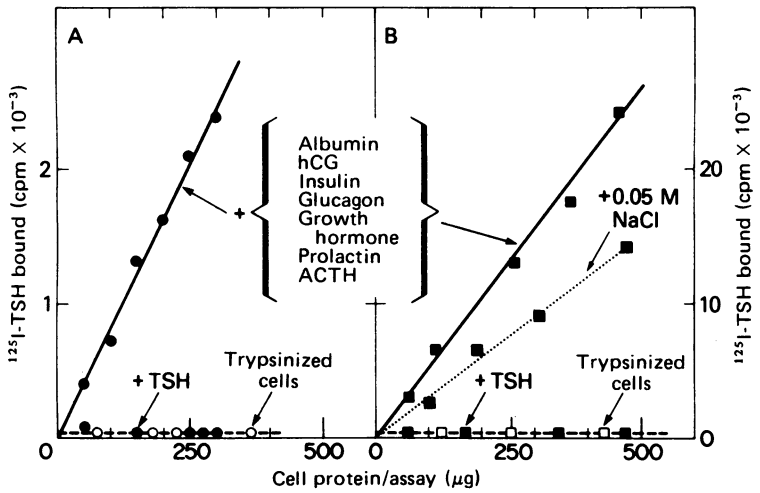

FIG. 1. ^{125}I-TSH binding to intact and trypsinized bovine thyroid (1-5G) cells. (*A*) Suspended in an isotonic medium containing physiological salt concentrations (Ca^{2+}- and Mg^{2+}-free NaCl/P_i, pH 7.4). (*B*) Suspended in an isotonic medium in which salts were replaced by 0.25 M sucrose containing 0.02 M Tris·HCl, pH 7.4. In both situations, incubations were carried out at 20° for 45 min at given protein concentrations. Where indicated, 10^{-5} M unlabeled TSH, albumin, human chorionic gonadotropin (hCG) (R. E. Canfield, Columbia University College of Physicians and Surgeons, NY), bovine insulin, bovine glucagon, porcine corticotropin (ACTH) (Calbiochem, San Diego, CA), bovine prolactin, or bovine growth hormone (highest purity preparations available from the NIH Endocrinology Study Section, Bethesda, MD) were also added to the reaction mixtures. Except in the case of albumin, which was always present at the inception of the experiment, binding reactions were initiated by the addition of cells. Although not shown, similar results were obtained with rat thyroid tumor cells.

compounds have now been utilized successfully in a number of systems (42, 48–50). The ions are constructed in such a fashion that they are sufficiently lipophilic to enter the hydrophobic core of the membrane, but they must also have the propensity for charge delocalization in order to equilibrate passively with the electrical potential across the membrane (51). As shown in Fig. 2*A*, thyroid cells rapidly took up the lipophilic cation $TPMP^+$, achieving a steady-state level of accumulation of about 0.5 nmol/mg of protein within 30 sec to 1 min. Strikingly, when TSH was added to the cells, $TPMP^+$ uptake increased, approaching a steady-state level of accumulation of about 1.5 nmol/mg of protein within 5–6 min. Moreover, when the proton conductor carbonylcyanide-*m*-chlorophenylhydrazone was added, the accumulated $TPMP^+$ was lost from the cells within 10–15 min. These observations are consistent with the suggestion that there is an electrical potential across the thyroid cell membrane (interior negative) and that this potential is increased in the presence of TSH. It is also clear that human chorionic gonadotropin elicited no effect on $TPMP^+$ uptake and that treatment of the cells with trypsin abolished the stimulatory effect of TSH (Fig. 2*B*). Thus, it is apparent that a specific receptor–hormone interaction is necessary for TSH-stimulated $TPMP^+$ uptake.

Analogous effects of TSH on $TPMP^+$ uptake also were observed with membrane vesicles prepared from thyroid cells (Fig. 2*C*). In this case, a small amount of $TPMP^+$ uptake was observed in the presence of an artifically induced NaCl gradient (vesicles prepared in 0.25 M sucrose containing 0.01 M Tris·HCl, pH 7.4, were diluted into a reaction mixture containing 0.05 M NaCl), and $TPMP^+$ uptake was enhanced when TSH was added under the same conditions. It is important to note that the same effects were observed with KCl and with Tris·HCl. Thus, it seems likely that the $TPMP^+$ uptake observed in these experiments is driven by a chloride diffusion potential. Moreover, under the experimental conditions used, $TPMP^+$ was slowly lost from the vesicles after a 5-min incubation; by 30 min, almost all of the accumulated radioactivity was lost. This effect is due presumably to the transient nature of the chloride concentration gradient. In any event, as demonstrated with the intact cells, human chorionic gonadotropin did not stimulate $TPMP^+$ uptake, and treatment of the vesicles with trypsin abolished the effect of TSH. Finally, it is also clear from the results presented in Fig. 2*D* that TSH caused an increase in adenylate cyclase activity in the vesicles; however, the effect of TSH in this respect was considerably delayed temporally relative to the effect of the hormone on $TPMP^+$ uptake: almost no increase in adenylate cyclase activity was observed during the first 4 min after addition of TSH whereas $TPMP^+$ accumulation reached a maximum over the same period. Subsequently, TSH-stimulated adenylate cyclase activity increased and became relatively constant at about 12–13 min.

DISCUSSION

The findings presented in this paper demonstrate that TSH causes an increase in the uptake of the lipophilic cation $TPMP^+$ when added to cultured thyroid cells or to membrane vesicles derived from these cells under appropriate conditions. It is also clear from the results that this effect depends on a specific interaction of TSH with its receptor at the cell surface. Because uptake of $TPMP^+$ and other lipophilic cations has been shown to reflect the presence of an electrical potential across the membrane (interior negative) (42, 47–51), it is tempting to speculate that one of the primary effects of TSH is to hyperpolarize the thyroid cell membrane. Experiments with membrane vesicles isolated from thyroid cells support this tentative conclusion. Because analogous effects of TSH are observed with respect to $TPMP^+$ uptake in this *in vitro* system, it is unlikely that the effect of the hormone is mediated by intracellular mitochondria. Moreover, it should be emphasized that results of experiments with thyroid slices utilizing a direct electrophysiological approach (52) are consistent with this interpretation. Because the driving force for $TPMP^+$ accumulation in the vesicles under the experimental conditions described appears to be a chloride diffusion potential, it is not unreasonable to suggest that TSH binding may cause an increase in the permeability of the membrane to chloride. However, it should be emphasized that we have made no attempt to investigate the ion specificity of this effect. Thus, it seems possible that TSH may induce a generalized increase in anion permeability or,

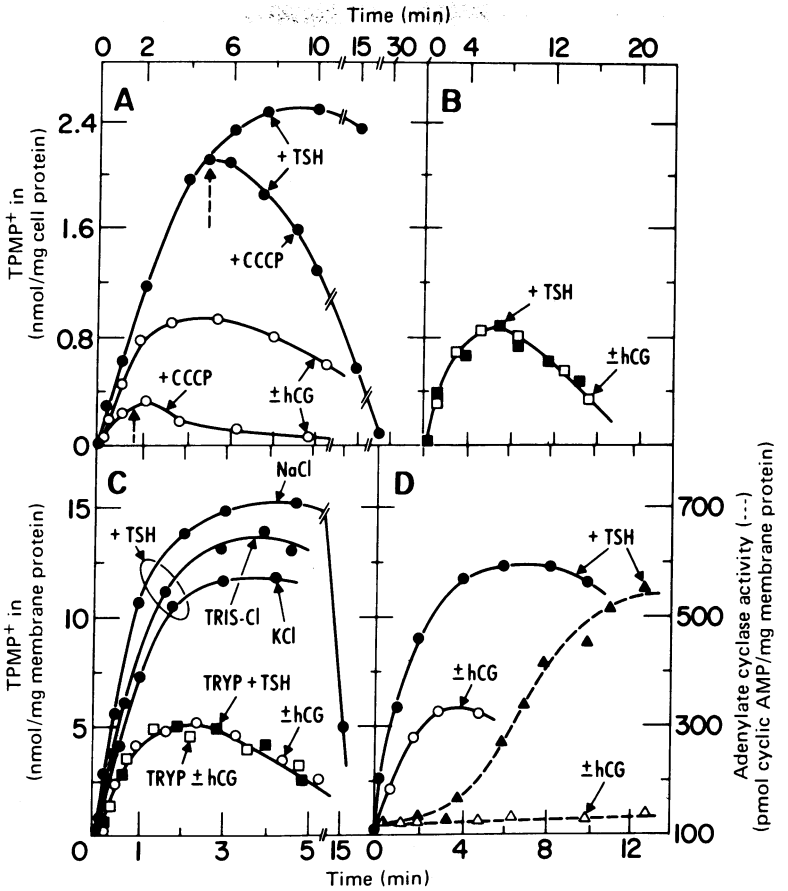

FIG. 2. TSH-stimulated $TPMP^+$ uptake by intact bovine thyroid cells (*A*), trypsinized bovine thyroid cells (*B*), plasma membrane vesicles prepared from bovine thyroid cells (*C*), and TSH-stimulated $TPMP^+$ uptake in bovine thyroid vesicles relative to TSH-stimulated adenylate cyclase activity in the same vesicles (*D*). In *A* and *B*, the experiments were carried out as described in *Methods* with a cell protein concentration of 500 μg/100 μl of reaction mixture. Where indicated, TSH and human chorionic gonadotropin (hCG) were added at final concentrations of 100 nM (i.e., about 7 milliunits/100 μl of assay volume) and μM, respectively, and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was used at a final concentration of 2.5 μM (dashed arrows). *C*, vesicles were diluted into reaction mixtures containing 0.05 M NaCl, KCl, or Tris·HCl (pH 7.4) as described in *Methods*, and the concentration of membrane protein was 115 μg/100 μl of assay. In the experiments shown in *D*, vesicles suspended in 0.25 M sucrose containing 0.01 M Tris·HCl (pH 7.4) were diluted 1:10 into medium containing 0.05 M NaCl, 0.125 M sucrose, 1 mM $MgCl_2$, 1 mM ATP, and 200 nM TSH; the final concentration of membrane protein was 115 μg/100 μl of assay. Incubations were performed in quadruplicate, and duplicate samples were evaluated for $TPMP^+$ uptake and for adenylate cyclase activity as described in *Methods*.

alternatively, a decrease in cation permeability. Finally, it is apparent from the results that the effect of TSH on $TPMP^+$ uptake precedes the effect of the hormone on adenylate cyclase activity, an observation that may have far-reaching implications with respect to the mechanism of action of other glycoprotein hormones, certain toxins, and interferon. Possibly, a primary mode of action of each of these effectors is to alter electrochemical ion gradients across the cell membrane. This concept not only is attractive with respect to the preliminary observations presented here but also would serve to explain certain other findings. Thus, human chorionic gonadotropin causes changes in adrenal cell ion transport that not only precede adenylate cyclase stimulation but also occur at effector concentrations that have minimal effects on cyclase activity (53). Similarly, cholera toxin and its B protein induce alterations in the permeability of liposomes reconstituted with "receptor" ganglioside in the absence of adenylate cyclase (54, 55). Whether it is in fact the alteration in the electrochemical gradient itself that triggers adenylate cyclase stimulation or whether this effect is coincident with an independent action of these effectors on the membrane is not yet clear.

With these ideas in mind, it is interesting that there is a small area of sequence homology in the A_1 protein of cholera toxin and the α subunit of TSH (10) and that this sequence is startlingly similar to that of the nonapeptide neurohypophyseal hormones oxytocin and vasopressin. Six of the nine residues match, either as identical residues (Cys, Pro), as analogously charged residues (Gln, Glx, or Glu; Arg, Asn, Asx, or Lys), or as similarly hydrophobic residues (Ile, Val, Phe, or Met) (15). These analogies are intriguing, particularly in view of the fact that the primary actions of these neurohypophyseal peptide hormones most certainly involve alterations in the transport properties of the receptor cells. The possibility exists, therefore, that this common sequence in the α subunits of the glycoprotein hormones is responsible for the membrane effects of these molecules. In this regard, recent studies have reported that a 1400 molecular weight fragment of the A_1 subunit of cholera toxin (i.e., a 10- or 11-residue sequence) may be sufficient to induce the changes in adenylate cyclase activity observed with the intact toxin (56).

One last point that deserves discussion concerns the binding of ^{125}I-TSH to cultured thyroid cells. The results obtained with cells suspended in isotonic salts are analogous to those presented by Lissitzky *et al.* (57). However, replacement of the salts with isotonic sucrose resulted in a major increase in TSH binding, and the properties of the binding observed under these conditions approximated those observed *in vitro* with plasma membrane preparations (40, 41). The reasons for this are not

clear at present. However, preliminary evidence (G. Lee and L. D. Kohn, unpublished data) suggests that the increased binding may be due to differences in the state of receptor aggregation in the membrane and that this state *in vivo* may differ from that in thyroid cells in culture.

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