Stimulation of Adenylate Cyclase Activity in Retro-orbital Tissue Membranes by Thyrotropin and an Exophthalmogenic Factor Derived from Thyrotropin

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Retro-orbital tissue membranes have been shown to have adenylate cyclase activity which can be stimulated by thyrotropin and by an exophthalmogenic factor derived from the thyrotropin molecule by partial pepsin digestion. This stimulable activity is maximal after 15 min and is optimal in the presence of 3 mM magnesium and 1.5 mM ATP. Calcium salts are exquisitely inhibitory to the hormonal stimulation; sodium, lithium, and ammonium salts are significantly less inhibitory. Thyrotropin and the exophthalmogenic factor induce similar maximal levels of stimulation but a 4- to 5-fold higher concentration of exophthalmogenic factor is required to achieve this level. Fluoride stimulates adenylate cyclase activity 2- to 3-fold higher than either thyrotropin or the exophthalmogenic factor; thyrotropin, luteinizing hormone, the β subunit of thyrotropin, and the α subunit of thyrotropin have relative activities for stimulation of cyclase activity of 100:2:2: <0.5. Several other polypeptide and glycoprotein hormones have no effect. The γ-globulin from patients with malignant exophthalmos has no significant effect on cyclase activity either alone or in the presence of maximal levels of thyrotropin or the exophthalmogenic factor; this γ-globulin does, however, stimulate cyclase activity at submaximal hormone levels. Trypsin not only destroys the hormone-stimulable adenylate cyclase activity on retro-orbital tissue plasma membranes, but also destroys it on the 15,000 to 30,000 molecular weight receptor fragment released from the membranes by the trypsic action.

It is the present belief that many polypeptide and glycopeptide hormones produce their cellular effects by activating adenylate cyclase and elevating intracellular cyclic adenosine 3':5'-monophosphate levels (1, 2). In this regard, recent years have witnessed numerous reports (3-14) which not only document the fact that thyrotropin (TSH) can stimulate adenylate cyclase activity and elevate cAMP1 levels in the thyroid but also show that this TSH-adenylate cyclase interaction occurs on thyroid plasma membranes. Additional reports have correlated TSH stimulation of adenylate cyclase activity with TSH stimulable thyroid functions such as glucose oxidation (15), iodide trapping and organification (16-18), colloid droplet formation (19), and hormone secretion (20). Our own studies have demonstrated that TSH binding to plasma membranes in vitro can be correlated with adenylate cyclase activation in these membranes in vitro (21-23) and that the reappearance of the TSH receptor after trypsinization of thyroid cells in culture correlates with the reappearance of TSH-stimulable adenylate cyclase activity (24).

In our studies on experimental exophthalmos (25-32), we have demonstrated that TSH or an exophthalmogenic factor derived from the TSH molecule by partial pepsin digestion can induce exophthalmos in guinea pigs and that this exophthalmos is associated with an increased synthesis of sulfated and nonsulfated glycosaminoglycans in guinea pig retro-orbital tissue. We have further demonstrated that plasma membranes from guinea pig retro-orbital tissue have a TSH receptor which is capable of binding either TSH or the exophthalmogenic derivative of the TSH molecule (EPF) produced by partial pepsin of TSH preparations. The demonstration of a TSH receptor in retro-orbital tissue membranes (28, 32), the finding that these same TSH or EPF preparations could induce experimental exophthalmos (27), and the above described linkage of the TSH receptor to thyroid function via adenylate cyclase and cAMP changes raised the question of cAMP mediation of the biochemical events leading to exophthalmos.

In the present report we show that TSH and EPF can stimulate adenylate cyclase activity in retro-orbital tissue plasma membranes; we characterize the optimal conditions of

1 The abbreviations used are: cAMP, cyclic adenosine 3':5'-monophosphate; TSH, thyroid-stimulating hormone or thyrotropin; EPF, the exophthalmos-producing factor derived from the TSH molecule by partial pepsin digestion; LH, luteinizing hormone.
this stimulation; and we correlate adenylate cyclase activation with TSH and EPF binding in these membranes.

**MATERIALS AND METHODS**

**Hormone and γ-Globulin Preparations**—TSH and the exophthalmogenic derivative of the TSH molecule (EPF) were obtained and purified as previously described (21-28, 31, 32). The properties of these preparations, their thyroid-stimulating activities (33), and their exophthalmogenic activities (34, 35) are summarized in our accompanying reports (23, 31, 32). Luteinizing hormone (LH), the subunits of TSH, γ-globulin preparations from patients with malignant exophthalmos, γ-globulin from patients with Graves' disease without exophthalmos, and γ-globulin from normal subjects were prepared as described (22, 29, 32, 36-38).

Membrane Preparations and Adenylate Cyclase Assays—Retro-orbital tissue membranes were prepared as previously reported (29) or as reported with the exception of a change in buffer throughout the procedure to 0.02 M Tris-chloride, pH 7.4, containing 1 mM MgCl₂ (ethylendiaminetetraacetic acid) and 1 mM dithiothreitol.

Adenylate cyclase was assayed by the method of Krishna et al. (39) as modified by Birnbaumer et al. (40). The incubation volume of 60 μl contained 1.5 mM [α-32P]ATP, 3 mM MgCl₂, 10 mM theophylline, 0.1% bovine serum albumin, 10 mM Tris-Cl, pH 7.6, 10 mM creatine phosphate, and 20 μg of crystalline rabbit muscle creatine kinase. The standard incubation mixture was at 37° for 15 min. The TSH or EPF was dissolved in buffer containing 1% albumin and was always freshly prepared. Fluoride was 10 mM unless otherwise specified. The reaction was initiated by the addition of membranes to prewarmed incubation mixtures. All points were the average of duplicate or replicate determinations.

The product was routinely checked by two-dimensional paper chromatography in isopropanol:concentrated NH₄OH:H₂O (7:2:1) and 1-butanol:acetic acid:acetic acid:65% NH₄OH:H₂O (7.5:3:3:2) using a cold cAMP standard; over 80% of the radioactivity chromatographed under the standard with all cases.

Other Assays and Materials—Protein was determined by the method of Lowry et al. (41) using crystalline bovine serum albumin as the standard. Membranes were dissolved in 0.1 N NaOH and heated to 90° for 30 min to insure their solubilization. Cyclic nucleotide phosphodiesterase was measured as described (39, 40); conditions closely approximating normal incubation conditions were used. Assays to evaluate hormonal binding to plasma membranes and solubilized receptors are detailed in our other reports (21-23, 32, 42).

[α-32P]ATP was obtained from the International Chemical and Nuclear Corporation; [3H]cAMP was from Schwarz/Mann Bioresearch Corporation or Amersham/Seearie Corporation. All other reagents were of the highest purity available from commercial sources.

**RESULTS**

**Properties of Adenylate Cyclase in Retro-orbital Tissues and Its Stimulation by Both TSH and EPF**—The adenylate cyclase activity of retro-orbital tissue plasma membranes was stimulated by both TSH and the exophthalmogenic derivative (EPF) of the TSH molecule obtained by partial peptic digestion of TSH preparations. Both the basal and the hormone-induced activities were time-dependent with maximal stimulation occurring at 15 min (Fig. 1).

Preincubation of the membranes at 37° resulted in a decrease in both TSH- and EPF-stimulable activity whereas preincubation at 0° had no such effect (Fig. 1). When the preincubation was performed in the absence of substrate, the 37° effect on adenylate cyclase activity was enhanced; preincubation in the absence of substrate but in the presence of TSH or EPF demonstrated partial protection (Fig. 1). The 37° preincubation induced decrease in cyclase activity could be partially reversed by returning the membranes to 0° for 1 to 2 hours. This sensitivity to preincubation temperatures was very similar to the temperature sensitivity of 125I-TSH, [3H]TSH, and [3H]EPF binding to retro-orbital tissue membranes (32).

Sensitivity of adenylate cyclase to preincubation temperatures has been reported in thyroid plasma membranes (12).

Activation of adenylate cyclase activity by either TSH or EPF was sensitive to both the concentration of magnesium ions and the concentration of the substrate, ATP (Fig. 2). A Mg₂⁺:ATP ratio of 2:1 seemed optimal for the TSH and EPF effect, as was reported for thyroid plasma membrane adenylate cyclase activation. The minimum concentrations of ATP and Mg²⁺ which appeared optimal within this ratio were 1.5 and 3.0 mM, respectively. As pointed out previously (23, 32), 3.0 mM Mg²⁺ will inhibit TSH and EPF binding over 90%; therefore, "effective" TSH or EPF concentrations must be at least 10-fold lower than TSH or EPF concentrations added to the incubation mixture.

In the presence of excess TSH or EPF (1 x 10⁻⁴ M), the optimum pH for EPF and TSHI activation was between 7.4 and 7.6 with half-maximal activation occurring at pH 6.0. Activation at 1 x 10⁻⁴ M TSH or 3 x 10⁻⁵ M EPF showed no difference between Tris-chloride, Tris-maleate, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffers; however, at 1 x 10⁻⁶ M and 1 x 10⁻⁷ M TSH or EPF concentrations, activation in Tris-chloride buffers was lower than in Tris-maleate or N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffers. The buffer effect on adenylate cyclase activity at less than maximal levels of TSH or EPF may well reflect the effect these buffers have on TSH and EPF binding to retro-orbital tissue plasma membranes (32). In vitro, therefore, TSH and EPF binding are only half-maximal at the pH optimum for adenylate cyclase activation; similarly, adenylate cyclase activity is only half-maximal at the pH optimum for binding (pH 6.0).

The activation by either EPF or TSH was sensitive to membrane protein concentration. Linear responses were obtained between 5 to 25 μg of membrane protein per assay; at protein concentrations higher than 25 μg per assay proportionality was lost. Significant inhibition occurred at levels of protein greater than 50 μg per assay. Although binding of TSH and EPF can be inhibited at high membrane protein concentrations, cyclase activity is significantly more sensitive, i.e., binding activity would just begin to be inhibited at 60 μg/60 μl.

![Fig. 1. A, time course of adenylate cyclase stimulation in retro-orbital tissue plasma membrane by TSH (●) or EPF (○). Basal activity (△) is presented for comparison. Standard conditions were used (see "Materials and Methods") with 20 μg membrane and either 1 x 10⁻⁴ M TSH or 5 x 10⁻⁵ M EPF. B, effect of preincubation at 0° and 37° on the TSH- and EPF-stimulable adenylate cyclase activity of retro-orbital tissue plasma membranes. Membranes were preincubated under the conditions noted, i.e., in buffer at 0°; in buffer plus TSH at 37°; in buffer plus substrate at 37°; or in buffer above at 37°. At the end of the preincubation period, assays of TSH- and EPF-stimulable adenylate cyclase activity were measured as described in A and under "Materials and Methods."](http://www.jbc.org/doi/10.1074/jbc.5623.6523)
assay volume whereas cyclase activity is over 50% decreased at this point. Similar cyclase sensitivities to membrane protein concentration have been described previously (10-14, 40).

Under the optimal conditions described, adenylate cyclase activation is sensitive to both the TSH and EPF concentrations (Fig. 3) with 4- to 5-fold higher concentration of EPF required to yield similar levels of cyclase activity. As predicted by the binding data (32) which demonstrated that TSH receptors are heterogeneous in regard to their affinities for the hormone, double reciprocal plots of the adenylate cyclase stimulation data (Fig. 3) yield complex curves (Fig. 3, inset). Using the criteria of Levitzki and Koshland (43), curves such as these indicate negative cooperativity among the TSH receptor sites relative to adenylate cyclase stimulation, a phenomenon already suggested in binding studies presented in an accompanying report (32). Independent of the existence of negative cooperativity, however, the adenylate cyclase data (Fig. 3, inset) can be grouped into two major classes of TSH-stimulable sites with $K_m$ values for TSH of approximately $2 \times 10^{-10} \, \text{M}$ and $2 \times 10^{-11} \, \text{M}$ for TSH if the TSH concentrations are accepted at face value. Consideration of these $K_m$ values should be made, however, in terms of effective TSH concentration, the concentration of TSH that actually can bind to the receptor at the concentrations of Mg$^{2+}$ in the buffer and at the pH optimal for the adenylate cyclase assay. As noted in an accompanying report (32) and in Fig. 3, this correction results in a decrease in $K_m$ values of approximately 10-fold and a resultant change in $K_m$ values to $0.2 \times 10^{-10} \, \text{M}$ and $0.2 \times 10^{-9} \, \text{M}$, respectively.

The adenylate cyclase data above were obtained using membranes prepared as was usual for our binding studies (32). Although initial basal and stimulable cyclase activities were the same in these membrane preparations as well as in membrane preparations analogously prepared in a modified buffer consisting of 0.02 M Tris-chloride, pH 7.4, containing 1 mM Mg$^{2+}$ ethylene glycol bis(s-b-aminoethyl ether)-N,N'-tetraacetic acid and 1 mM dithiothreitol, the stability of the two preparations was different. Thus adenylate cyclase activity was stable for 2 to 3 weeks at liquid N$_2$ temperatures in the first preparation and 4 to 6 weeks in the second preparation. The addition of 1 mM MgCl$_2$ to the first preparation partially reversed this problem.

Other Agents Altering Adenylate Cyclase Activity.—As with thyroid plasma membranes (12), fluoride-stimulated cyclase activity in retro-orbital tissue plasma membranes is about 2 to 3 times higher than optimal TSH or EPF stimulable cyclase activity. Half-maximal stimulation occurred at 4 mM F$^-$ under these assay conditions, maximal stimulation was between 8 and 15 mM, and inhibition occurred at greater than 15 mM F$^-$ concentrations.

Despite its significant effect on TSH binding to retro orbital tissue plasma membranes (32), luteinizing hormone had a minimal effect on adenylate cyclase activation. Thus its relative activity for binding was $\frac{1}{10}$ of that of TSH (32), but its cyclase stimulable activity was $\frac{1}{3}$ to $\frac{1}{5}$ of that of TSH. The subunit of TSH had $\frac{1}{3}$ to $\frac{1}{5}$ the stimulable-cyclase activity of TSH and the $\alpha$ subunit of TSH had less than $\frac{1}{100}$ the activity of TSH. These data are analogous to those previously reported using thyroid plasma membranes (22). In both that study and the present one, the luteinizing hormone and $\beta$-TSH effects could not be accounted for by TSH contamination as assayed in the McKenzie bioassay (33) whereas TSH contamination could not be excluded for the $\alpha$-TSH effect. Parathyroid hormone, glucagon, insulin, adrenocorticotropic hormone, prolactin, and human growth hormone either did not stimulate or inhibited adenylate cyclase activity.

TSH- and EPF-stimulable adenylate cyclase activities as well as fluoride-stimulable adenylate cyclase activity were significantly inhibited (75%) by calcium ions (0.5 mM). LiCl, NaCl, and NH$_4$Cl also inhibited TSH or EPF stimulable-cyclase activity, 50% inhibition being achieved at 50, 150, and 50 mM concentrations, respectively. Inhibition of the binding of TSH and EPF to retro-orbital tissue membranes by these agents has already been demonstrated (32); hence these effects are at least in part related to interference at the receptor level. N-Ethylmaleimide, p-chloromercuribenzoate, and sodium dodecyl sulfate at concentrations of $5 \times 10^{-5}$ M, $5 \times 10^{-4}$ M, and $5 \times 10^{-3}$ M, inhibited the stimulation of F$^-$, TSH; or EPF-stimulable adenylate cyclase activity to the same degree (75%).

With all activators or inhibitors, changes in cAMP levels could have resulted from inhibition or activation of cyclic nucleotide phosphodiesterases. Under the assay conditions we are indebted to Dr. Gerald D. Aurbach of the Metabolic Diseases Branch of the National Institute of Arthritis, Metabolism, and Digestive Diseases, for his help in obtaining samples of many of these hormones and for his encouragement and help in the early phases of this project.
described these activities were more than 90% inhibited and were not altered by the agents tested as inhibitors or activators above.

Effect of Trypsin—As demonstrated in an accompanying report, the TSH receptor, or a fragment thereof, is released from the plasma membrane of retro-orbital tissues by exposure to trypsin (32). In Fig. 4A we show that simultaneous with the loss in TSH receptor activity, there is a loss of TSH- or EPF-stimulable adenylate cyclase activity. Fluoride stimulable cyclase activity is also lost but there appears to be a difference in the sensitivity of this activity as a function of time. Whereas binding activity can be measured in the supernatant phase of the trypsinized membranes, no recovery of adenylate cyclase activity was measured (Fig. 4B).

Effect of γ-Globulin from Patients with Malignant Exophthalmos—When tested alone, neither the γ-globulin from patients with malignant exophthalmos, the γ-globulin from patients with Graves' disease and no exophthalmos, nor the γ-globulin from normal subjects stimulated adenylate cyclase activity (Table I). Similarly, at maximal levels of TSH and EPF stimulation, negligible or no effect by the γ-globulin was detected (Table I). At low levels of TSH and EPF stimulation, however, the γ-globulin from patients with malignant exophthalmos increased the TSH- and EPF-stimulable adenylate cyclase activity but not the fluoride-stimulable activity (Table I). The γ-globulin from patients with Graves' disease and no exophthalmos or from normal subjects had no effect at low levels of TSH and EPF stimulation (Table I). The γ-globulin from patients with Graves' disease but not the fluoride-stimulable adenylate cyclase activity but not the fluoride-stimulable activity (Table I). The γ-globulin from patients with Graves' disease but not the fluoride-stimulable adenylate cyclase activity but not the fluoride-stimulable activity (Table I).

**TABLE I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Adenylate cyclase activity* (nmol/mg protein/15 min)</th>
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</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td></td>
</tr>
<tr>
<td>2 x 10^{-4} M</td>
<td>0.98</td>
</tr>
<tr>
<td>1 x 10^{-5} M</td>
<td>1.9</td>
</tr>
<tr>
<td>EPF</td>
<td></td>
</tr>
<tr>
<td>1 x 10^{-4} M</td>
<td>1.0</td>
</tr>
<tr>
<td>5 x 10^{-5} M</td>
<td>2.1</td>
</tr>
<tr>
<td>γ-Globulin from patients with malignant exophthalmos</td>
<td></td>
</tr>
<tr>
<td>15 μg</td>
<td>0.41</td>
</tr>
<tr>
<td>15 μg + TSH 2 x 10^{-4} M</td>
<td>1.6</td>
</tr>
<tr>
<td>15 μg + TSH 1 x 10^{-5} M</td>
<td>2.0</td>
</tr>
<tr>
<td>15 μg + EPF 1 x 10^{-5} M</td>
<td>1.72</td>
</tr>
<tr>
<td>15 μg + EPF 5 x 10^{-5} M</td>
<td>2.0</td>
</tr>
<tr>
<td>γ-Globulin from patients with Graves' disease and having no exophthalmos</td>
<td></td>
</tr>
<tr>
<td>15 μg</td>
<td>0.36</td>
</tr>
<tr>
<td>15 μg + TSH 2 x 10^{-4} M</td>
<td>0.96</td>
</tr>
<tr>
<td>15 μg + TSH 1 x 10^{-5} M</td>
<td>2.0</td>
</tr>
<tr>
<td>15 μg + EPF 1 x 10^{-5} M</td>
<td>0.98</td>
</tr>
<tr>
<td>15 μg + EPF 5 x 10^{-5} M</td>
<td>2.1</td>
</tr>
<tr>
<td>γ-Globulin from normal subjects</td>
<td></td>
</tr>
<tr>
<td>15 μg</td>
<td>0.37</td>
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<tr>
<td>15 μg + TSH 2 x 10^{-4} M</td>
<td>0.98</td>
</tr>
<tr>
<td>15 μg + TSH 1 x 10^{-5} M</td>
<td>2.0</td>
</tr>
<tr>
<td>15 μg + EPF 1 x 10^{-5} M</td>
<td>1.0</td>
</tr>
<tr>
<td>15 μg + EPF 5 x 10^{-5} M</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Assays were identical with those described in Fig. 1A and under "Materials and Methods." The γ-globulin preparation used in the present experiments was the same preparation as was used to enhance binding of TSH and EPF to retro-orbital tissue plasma membranes in an accompanying report (32). This γ-globulin was LATS-positive (32). LATS-negative preparations were the same. LATS, long-acting thyroid stimulator.

**DISCUSSION**

The present report demonstrates that both TSH and EPF, the exophthalmogenic factor derived from partial pepsin digests of TSH preparations, stimulate adenylate cyclase activity in retro-orbital tissue plasma membranes. It further demonstrates that in several respects there is a close correlation of the stimulatory effect of TSH and EPF on cyclase activity with the binding of TSH and EPF to these membranes. Thus, for example, both TSH binding and TSH stimulation of adenylate cyclase exhibit similar dependencies on hormone concentration, i.e. the TSH receptors exhibit negative cooperativity and can be visually grouped to reflect two major groups of TSH receptor sites having high and low affinities. Moreover, when corrections are made for the TSH and EPF concentrations which are able to bind at the magnesium ion content, buffer composition, and pH of adenylate cyclase assays, the binding constants for the two major classes of TSH receptors as determined from adenylate cyclase activity in retro-orbital tissue plasma membranes compared to the effect of trypsin on TSH binding (---) to these membranes. Membranes were exposed to L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin at room temperature at a 20:1 ratio of membranes to trypsin and in 0.025 M Tris-maleate, pH 7.8. At the noted times, aliquots were removed and mixed with an amount of cold soy bean trypsin inhibitor (Calbiochem) which was 5-fold in excess of the trypsin concentration and the suspensions were chilled to 2-4°C. Aliquots from this suspension were assayed for TSH binding and TSH-stimulated adenylate cyclase activities and for their basal (A) adenylate cyclase activities. The binding assay has been described (32); the adenylate cyclase assays were the same as described in Fig. 4A and under "Materials and Methods." Fluoride was at 10 mM concentration. B, recovery of TSH binding activity (■) in the supernatant solutions from tryptic digests of retro-orbital tissue membranes in the absence of recoverable TSH- or EPF-stimulable adenylate cyclase activity. Aliquots of the tryptic digests in A were centrifuged in a Beckman microfuge and the supernatant solutions were assayed for TSH binding (■) for TSH-(O), EPF- (■), and fluoride-(A) stimulated adenylate cyclase activities, and for basal (A) adenylate cyclase activity. Assays were the same as in A with the exception of the binding assay; this used a solubilized receptor assay described in an accompanying report (42).
assays are similar to those measured in binding assays. Salts which inhibit binding inhibit TSH- and EPF-stimulable adenylate cyclase activities at similar concentrations. Trypsin which destroys the TSH binding activity on the membranes has the same destructive effect on TSH- and EPF-stimulable adenylate cyclase activity. Even the sensitivity of the membranes to 37° preincubation is similar whether binding or adenylate cyclase activation are assayed.

The correlations between TSH or EPF binding and TSH and EPF stimulation of adenylate cyclase activity in retro-orbital tissue plasma membranes are similar therefore to the correlations which exist between TSH binding and TSH stimulation of adenylate cyclase activity in thyroid plasma membranes. Given the additional correlation between adenylate cyclase activation and thyroid function, it is reasonable to presume that subsequent experiments in vivo will correlate TSH- or EPF-stimulable cyclase activity in retro-orbital tissue with the biochemical changes which result in exophthalmos. At the very least it is an avenue of research which must be pursued.

Three additional points are worth noting in the present data. First, the relative activities of TSH and EPF are similar in these studies measuring adenylate cyclase activation and in our accompanying studies measuring binding (32). In both cases 4- to 5-fold higher concentrations of EPF are necessary to achieve TSH activity levels. Second, the relative activity of TSH and LH for adenylate cyclase are low (50:1) whereas their relative activities in binding assays is much higher (10:1). This finding was also noted in our studies of TSH binding to thyroid plasma membranes (22) and suggests that LH is a structural analog of TSH which can act as a competitive inhibitor of TSH actions in vitro. Last, the γ-globulin effect on adenylate cyclase activity would appear to be compatible with its activity in binding studies. Thus γ-globulin from malignant exophthalmos patients does not itself bind to retro-orbital tissue plasma membranes nor stimulate adenylate cyclase activity in these membranes. In the presence of TSH or EPF, the γ-globulin does bind and does activate adenylate cyclase activities but not higher than cyclase activities determined with maximal levels of TSH or EPF. The suggestion from these two studies would thus be that the role of the γ-globulin is to increase the number and affinities of the TSH receptors and allow TSH or EPF to stimulate the adenylate cyclase response at lower hormonal concentrations.

Acknowledgments—We are indebted to Dr. J. E. Rall of the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland, and to Professor A. Nizet, Institut de Médecine, Université de Liège, Liège, Belgium. Their continual encouragement and support was our constant inspiration throughout this project.

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