Effect of thyroid phospholipids on the interaction of thyrotropin with thyroid membranes
(hormone receptors/gangliosides/phospholipase A/adenylate cyclase)

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ABSTRACT Various lipids extracted from bovine thyroid glands were tested for their ability to affect the binding of ¹²⁵I-labeled thyrotropin to bovine thyroid membranes. The most potent inhibitors were the acidic phospholipids in the order cardiolipin > phosphatidylethanolamine > phosphatidylcholine > phosphatidylglycerol > phosphatidylserine. Other phospholipids, neutral lipids, and neutral glycolipids were ineffective. As reported previously (Mullin, B. R., Pacuszkza, T., Lee, G., Kohn, L. D., Brady, R. O., & Fishman, P. H. (1975) Science 199, 77-79), thyroid gangliosides also inhibited thyrotropin binding but not as effectively as phospholipids. In addition, the mode of action of these two classes of acidic lipids was different. When thyroid membranes were preincubated with the phospholipids and then separated by centrifugation, their ability to bind thyrotropin was still diminished. In contrast, gangliosides appear to interact with the hormone and not with the membranes. The effect of phospholipids on thyroid membranes was further examined by incubating the membranes with phospholipase A. The treated membranes now bound more labeled hormone. These results suggest that certain acidic phospholipids, which are present in only small amounts in thyroid membranes, influence the state of the thyrotropin receptor and its ability to bind thyrotropin.

The initial event in thyrotropin (TSH)-mediated effects on thyroid cells is the binding of the hormone to specific receptors on the plasma membrane of the cells. Subsequently, a transmembrane signaling event occurs, which leads to the generation of a second messenger inside the cell. For TSH, this second messenger is cyclic AMP (1), and transmembrane signaling causes the activation of adenylate cyclase by mechanisms not yet understood. Recently, it has been suggested that gangliosides are involved in this process (see ref. 2). Thus, gangliosides (3) and, in particular, gangliosides from thyroid tissue (4) inhibit the binding of TSH to thyroid membranes. There are certain structural similarities between TSH and cholera toxin (3, 5, 6). The latter agent utilizes ganglioside G₃M₁ as its specific cell surface receptor (see ref. 2) and can activate adenylate cyclase in thyroid preparations (7, 8). Furthermore, thyroid tumor cells that are deficient in gangliosides more complex than G₃M₁ were unresponsive to TSH (9).

On the other hand, an important role for phospholipids has been implicated in hormone-responsive adenylate cyclase systems (10-15). Treatment of thyroid slices and membranes with phospholipases profoundly depressed the stimulation of adenylate cyclase by TSH (10-13) but resulted in enhanced binding of TSH to membranes (13-15). In order to explore further the relative roles of these two classes of acidic phospholipids on TSH action, we separated the various lipid classes from bovine thyroid glands and studied their ability to affect the binding of bovine TSH to bovine thyroid membranes.

MATERIALS AND METHODS

Materials. Highly purified bovine TSH was a generous gift from John Pierce (University of California at Los Angeles) and was iodinated by the chloramine-T procedure as described (16); the specific radioactivity of different preparations ranged from 38 to 71 μCi/μg. Sigma was the source of the unlabeled TSH used in the binding assays as well as purified phospholipids and phospholipase A₂ (bee venom, 1300 units/mg). Phospholipase C (Clostridium welchii, 6.6 units/mg) was obtained from Calbiochem.

Binding Assay. Plasma membranes from bovine thyroid glands were prepared as described by Wolff and Jones (17), stored frozen in liquid nitrogen, and washed prior to use by suspending them in 25 mM Tris acetate buffer (pH 6.0) and centrifuging at 50,000 × g for 10 min (4). The standard binding assay was similar to that described elsewhere (4) and contained 125,000 cpm of ¹²⁵I-labeled TSH (¹²⁵I-TSH), 5 μg of membrane protein, 0.5% bovine serum albumin, and 25 mM Tris acetate buffer (pH 6.0) in a total volume of 100 μl. Incubations were for 1 hr at 4°C, and bound radioligand was separated from free ¹²⁵I-TSH by filtration on cellulose acetate filters (4). Non-specific binding was determined by incubating the membranes in the presence of an excess of unlabeled TSH (1.5 μM). Specific binding of 5 μg of membranes was 20,000–25,000 cpm, a value that is on the upward slope of the binding curve when cpm bound is plotted against amount of membranes. Lipids to be tested in the binding assay were dried from chloroform/methanol (2:1, vol/vol) under a stream of N₂ and dispersed in 25 mM Tris acetate buffer (pH 6.0) by sonication for 10 min just prior to being assayed.

Extraction and Separation of Thyroid Lipids. Lipids were extracted from bovine thyroid glands and partitioned into upper and lower phases as described (4). The upper-phase lipids were chromatographed on a DEAE-Sephadex column (18) and eluted stepwise with methanol, 0.25 M ammonium acetate in methanol, and 0.5 M ammonium acetate in methanol. The fractions containing gangliosides were combined, taken to dryness, dissolved in water, dialyzed, and lyophilized. To separate gangliosides from other acidic lipids, we used a slight modification of the method of Yu and Ledeen (18). The lyophilized material was dissolved in chloroform/methanol (19:1, vol/vol) and applied to a Unisil column (200–325 mesh) that was eluted stepwise with increasing concentrations of methanol in chloroform as described in Table 1. Any remaining phospholipids were extracted with chloroform/methanol (7:3, vol/vol) and dried under a stream of N₂.

Abbreviations: G₃M₁, galactosyl-N-acetylgalactosaminyl-(N-acetyll neuraminyl)galactosylglucosylceramide; G₃G₃, N-acetylmuramylgalactosylglucosylceramide; CL, cardiolipin; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; SM, sphingomyelin; TSH, thyrotropin; ¹²⁵I-TSH, ¹²⁵I-labeled TSH.

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phospholipids were removed from the ganglioside fraction by saponification for 1 hr at 37°C in chloroform/0.6 M NaOH in methanol (2:1, vol/vol). The mixture was neutralized with 0.05 vol of 4 M acetic acid and desalted on Sephadex G-25 (19).

Lower-phase lipids (corresponding to 100 g of tissue) were fractionated on a Unisil column (10 g) according to Vance and Sweely (20). The column was eluted with 150 ml of chloroform (fraction A), 300 ml of acetone/methanol (9:1, vol/vol) (fraction B), and 150 ml of methanol (fraction C). Fraction B was further purified on a DEAE-Sephadex column (1 g) that was eluted with 100 ml of chloroform/methanol/water (30:60:8, vol/vol) (fraction B1) and 130 ml of chloroform/methanol/0.8 M sodium acetate (30:60:8, vol/vol) (fraction B2) (18). Fraction B2 was desalted on Sephadex G-25 (19) and further separated by preparative thin-layer chromatography. After exposure of the chromatogram to iodine vapor, individual zones were scraped from the plate and eluted with chloroform/methanol/water (65:25:4, vol/vol) and chloroform/methanol (2:1, vol/vol) (refer to Fig. 2 for the fractionation scheme).

Other Methods. Thin-layer chromatography was carried out on silica gel 60 on glass plates (E. Merck). Gangliosides (21), phospholipids (22, 23), and other lipids (24) were detected by conventional procedures. Phospholipids were further identified by two-dimensional thin-layer chromatography with chloroform/methanol/water (65:25:4, vol/vol) or chloroform/methanol/28% NH₄OH (65:35:5, vol/vol) in the first direction and chloroform/methanol/acetic acid/water (60:40:4:2, vol/vol) or chloroform/acetonelmethanol/acetic acid/water (50:20:10:10:5, vol/vol) in the second direction. Phospholipid phosphorus (25), ganglioside sialic acid (4), and membrane protein (26) were determined by established procedures. Membranes were treated with phospholipases as described by Pohl et al. (27) with minor modifications (12).

RESULTS

Analysis of Upper-Phase Lipids from Bovine Thyroid. As reported (4), the crude upper-phase lipids contained a complex mixture of gangliosides. In addition, there was a considerable amount of phospholipid (2 nmol/nmol of sialic acid). When the upper-phase lipids were chromatographed on Unisil, most of the gangliosides were recovered in fraction 3 and most of the phospholipids were in fraction 2 (Table 1). Analysis by thin-layer chromatography indicated that fraction 1 contained cardiolipin (CL) and phosphatidylglycerol (PtdGro), fraction 2 contained phosphatidylserine (PtdSer), and a trace of GM₃, and fraction 3 contained gangliosides with lesser amounts of PtdIns, PtdSer, and an unknown phospholipid (summarized in Table 1).

2 contained phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), and a trace of GM₃, and fraction 3 contained mainly gangliosides with lesser amounts of PtdIns, PtdSer, and an unknown phospholipid. The total lipid extract from bovine thyroid glands was partitioned into upper and lower phases, and the lower phase was fractionated as indicated. Each fraction was tested for its effect on 125I-TSH binding to bovine thyroid membranes. +, Inhibition of binding; −, no inhibition.

![Fig. 1. Inhibition of 125I-TSH binding to bovine thyroid membranes by thyroid ganglioside fraction: effect of purification from phospholipids.](image)

![Fig. 2. Procedure for separation of lower-phase lipids from bovine thyroid. The total lipid extract from bovine thyroid glands was partitioned into upper and lower phases, and the lower phase was fractionated as indicated. Each fraction was tested for its effect on 125I-TSH binding to bovine thyroid membranes. +, Inhibition of binding; −, no inhibition.](image)
Table 2. Inhibition of binding of $^{125}$I-TSH to bovine thyroid plasma membranes by acidic phospholipids

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Addition per assay, nmol</th>
<th>Inhibition of $^{125}$I-TSH binding, %</th>
<th>Bovine thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>4</td>
<td>67</td>
<td>70</td>
</tr>
<tr>
<td>PtdGro</td>
<td>10</td>
<td>74</td>
<td>78</td>
</tr>
<tr>
<td>PtdIns</td>
<td>10</td>
<td>64</td>
<td>58</td>
</tr>
<tr>
<td>PtdSer</td>
<td>10</td>
<td>26</td>
<td>ND*</td>
</tr>
</tbody>
</table>

Binding of $^{125}$I-TSH to bovine plasma membranes was determined in the presence and absence of the indicated phospholipids obtained from commercial sources or isolated from bovine thyroid glands. Each binding assay was done in triplicate and was corrected for binding in the absence of membranes.

* ND, not determined.

compared to fraction 3 on the basis of sialic acid content, it was found to be more inhibitory (Fig. 1). After saponification to destroy any remaining phospholipids, fraction 3 still inhibited the binding of TSH to membranes but was considerably less potent than the original upper phase; 50 nmol of lipid-bound sialic acid in fraction 3 after saponification caused 54% inhibition whereas 5 nmol in the original upper phase caused 57% inhibition. This latter value is similar to that reported previously (4).

Analysis of Lower-Phase Lipids from Bovine Thyroid. When the lower phase lipids were fractionated on silicic acid and DEAE-Sephadex columns, the inhibitory activity was recovered in fraction B2 which contained the acidic phospholipids (Fig. 2). These were separated by preparative thin-layer chromatography and identified by two-dimensional chromatography as CL, PtdGro PtdIns, and PtdSer. The first three of these were potent inhibitors of TSH binding and were as effective as highly purified phospholipids obtained commercially (Table 2). Thus, the inhibitory effects observed with our preparations from bovine thyroid appear to be due to these phospholipids. CL was the most effective inhibitor; 1.3 nmol (2.6 nmol of phosphorus) caused 50% inhibition (Fig. 3). This degree of inhibition was obtained with 4.6 nmol of PtdGro and 7.1 nmol of PtdIns; PtdSer was a weak inhibitor. The major phospholipids present in bovine thyroid were phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), and sphingomyelin (SM) (Table 3). These phospholipids as well as

Fig. 3. Effect of acidic phospholipids on binding of $^{125}$I-TSH to bovine thyroid plasma membranes. Binding was determined in the presence and absence of the indicated commercial phospholipid (added as nmol of phosphorus) in triplicate and was corrected for binding in the absence of membranes.

Table 3. Phospholipid composition of bovine thyroid glands

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>% of total phospholipid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiolipin</td>
<td>3</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>1</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>9</td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>8</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>3</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>14</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>27</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>26</td>
</tr>
</tbody>
</table>

Phospholipids were isolated from bovine thyroid glands. The phospholipid content was 8.76 µmol/g of fresh tissue. Our data are in good agreement with previous results for bovine thyroid (28) and porcine thyroid (29) except that our value for phosphatidylcholine was significantly lower.

* Total phospholipids include other minor unidentified components.

FIG. 4. Effect of preincubating bovine thyroid membranes with phospholipids on subsequent binding of $^{125}$I-TSH to membranes. Bovine thyroid membranes (1 mg of protein) were incubated for 8 min at 24°C in 1 ml of 25 mM Tris-HCl buffer (pH 7.6) containing no phospholipids or 300 µg of the indicated phospholipid. Then, 2 ml of the same buffer (ice cold) was added and the suspension was centrifuged at 30,000 x g for 20 min. The membranes were suspended in 25 mM Tris acetate buffer (pH 6.0) and assayed for $^{125}$I-TSH binding in triplicate. Binding was corrected for nonspecific binding as determined in the presence of excess unlabeled TSH.
Table 4. Effect of phospholipase A on binding of $^{125}$I-TSH to bovine thyroid membranes

<table>
<thead>
<tr>
<th>Phospholipase A, units/ml</th>
<th>$^{125}$I-TSH bound, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>122</td>
</tr>
<tr>
<td>25</td>
<td>138</td>
</tr>
<tr>
<td>100</td>
<td>124</td>
</tr>
</tbody>
</table>

Bovine thyroid membranes (2 mg of protein per ml) were incubated with the indicated concentration of phospholipase A in 1 ml of 25 mM Tris-HCl, pH 7.6/1 mM CaCl$_2$ for 5 min at 30°C. The reaction was stopped by adding 40 $\mu$l of 0.1 M EDTA. The membranes were washed and assayed for $^{125}$I-TSH binding in triplicate. Binding was corrected for nonspecific binding determined in the presence of excess unlabeled TSH. Prior to phospholipase treatment, the membranes were washed in the Tris-HCl buffer to remove dithiothreitol present in the membrane preparations.

assay, it appeared that the inhibitory effect of these phospholipids was due to their interaction with the membranes and not with the hormone.

We explored this effect further by incubating the membranes with phospholipases. In agreement with reports of others (13-15), treatment of the membranes with phospholipase A resulted in an increase in TSH binding (Table 4). When the membranes treated with phospholipase A were exposed to acidic phospholipids as described above, there was an apparent decrease in TSH binding (data not shown). In contrast, we did not observe a similar effect with phospholipase C. When we incubated the membranes with and without phospholipase C under identical conditions at 37°C, we observed a loss in TSH binding with both sets of membranes. Amir et al. (15) reported that incubation of bovine thyroid membranes at elevated temperatures caused a loss in TSH receptor sites. They also reported that phospholipase C treatment stimulated TSH binding (15), whereas Haye and Jacquemin (14) found that phospholipase C caused an increase in TSH binding to bovine thyroid membranes at low but not high concentrations of TSH.

DISCUSSION

Of the various lipid classes isolated from bovine thyroid gland, we found that only two classes, gangliosides and acidic phospholipids, affected the interaction of TSH with thyroid plasma membranes. The inhibition of TSH binding by gangliosides has been described (3, 4), but it is apparent from our present work that some of this inhibition was due to the presence of phospholipids in the ganglioside preparations. Gangliosides, however, do inhibit TSH binding because, even after extensive purification and saponification to destroy contaminating phospholipids, thyroid gangliosides are still inhibitory. In addition, TSH binds to liposomes containing gangliosides (16, 30). Finally, the inhibitory effects of gangliosides are distinct because gangliosides appear to interact with TSH (3), and, as we now show, phospholipids interact with the membrane.

1 After further purification and saponification, brain gangliosides were also found to be less inhibitory.
2 The nature of this interaction is still unclear. Oligosaccharides prepared from individual as well as mixed gangliosides did not inhibit the binding of TSH to bovine thyroid membranes even at concentrations as high as 3 mM (unpublished observations). A similar lack of interaction between these oligosaccharides and gonadotropin had been reported previously (16). In contrast, the oligosaccharide from GM$_1$ ganglioside, the cholera toxin receptor, binds to cholera toxin and blocks toxin binding to cells (31) and thyroid membranes (unpublished observations).

Our results indicate that certain phospholipids alter the membrane components responsible for the binding of TSH. Only the highly polar, negatively charged phospholipids (CL, PtdIns and PtdGro) were able to affect the interaction of the receptor with the hormone. Although CL was the most potent inhibitor, it is presumed to be a mitochondrion membrane component (32) and its presence in plasma membranes has not been described. PtdIns and PtdGro are plasma membrane components. A role for these phospholipids in influencing the ability of the TSH receptor to bind hormone is supported further by our experiments with phospholipase A. Treatment of thyroid membranes with this enzyme caused an increase in TSH binding and exposure of these treated membranes to the above phospholipids reduced TSH binding.

The effects of phospholipids on hormone-stimulated adenylyl cyclase systems has been best examined with a glucagon-sensitive system (27, 33-35). Levey and coworkers (33) observed that Lubrol PX-solubilized adenylyl cyclase lost its sensitivity to glucagon and addition of phospholipids to the solubilized enzyme restored the hormone response. The phospholipid effect appeared to be specific because PtdSer but not PtdIns was active. Rodbell and collaborators (27, 34) found that phospholipase A treatment of liver membranes abolished the response of adenylyl cyclase to glucagon and addition of membra phospholipids restored hormone sensitivity. There were corresponding effects on the binding of glucagon to the membranes. Further studies with phospholipases of different specificities indicated that the enzymes that hydrolyzed acidic phospholipids were the most effective (35).

The involvement of membrane phospholipids in the TSH-stimulated adenylyl cyclase system appears to be more complex. Treatment of thyroid membranes with phospholipase A or C caused a decrease in TSH-stimulated adenylyl cyclase activity (10-13) and subsequent addition of PtdCho or PtdSer partially restored hormone response (12). In addition, membranes treated with Lubrol PX lost their TSH response, and addition of PtdCho partially restored hormone sensitive adenylyl cyclase activity (12). However, our results as well as reports of others (13-15) indicate that phospholipase A treatment of thyroid membranes causes an increase in TSH binding to the membranes. In addition, we find that treatment of the membranes with certain acidic phospholipids results in decreased hormone binding. These apparently conflicting results suggest that membrane phospholipids may be involved at several different sites and different phospholipids may act at different sites. Thus, as in the glucagon-sensitive system, certain phospholipids (PtdCho and PtdSer) may play a role in the coupling or transducing site between the TSH receptor and adenylyl cyclase in the thyroid membrane. In addition, other acidic phospholipids may be involved at the receptor site.

We can only speculate on how these acidic phospholipids modulate the ability of the receptor to bind TSH. Current concepts of membrane structure envision membrane receptors as asymmetric with their hydrophobic regions buried in the fluid lipid bilayer. In addition, membrane phospholipids may be distributed asymmetrically both laterally and transversely in the bilayer. Thus, the receptors may exist in local domains enriched in certain phospholipids, and the composition of these phospholipids surrounding the hydrophobic region of the receptor as it traverses the membrane may be different in the two halves of the bilayer. Small changes in phospholipid composition could have profound effects on local fluidity and charge in such domains. An increase in negative charge could lead to a local increase in cations such as Ca$^{2+}$ which has been reported to inhibit TSH binding (13, 15) or to a repulsion of other negatively charged membrane components that are involved in hormone
binding. Changes in fluidity could lead to a vertical displacement of the receptor (37) or to lateral movement of the receptor in the plane of the membrane. Either type of movement could influence the ability of the receptor to bind hormone directly by masking the exposure of the receptor or indirectly by disrupting associations between the receptor and other membrane components required for hormone binding.

Finally, we suggest a potential physiological function for the effects we observe. It is well known that one of the actions of TSH on the thyroid is to stimulate phospholipid synthesis. TSH greatly enhances the incorporation of $^{32}$P into acidic phospholipids, especially PtdIns (14, 38). Hormone-induced changes in these phospholipids could in turn lead to a reduction in hormone binding. Such a model could be involved in the phenomenon of desensitization at the level of the membrane receptor.

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