Receptor for Thyrotropin-releasing Hormone in Plasma Membranes of Bovine Anterior Pituitary Gland

ROLE OF LIPIDS*

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SUMMARY

The receptor for thyrotropin-releasing hormone has been located in the plasma membranes of bovine anterior pituitary gland. Treatment of membranes with phospholipase A, phospholipase C, or digitonin or addition of lysophosphatidylcholine diminishes or completely destroys the ability of membranes to bind 3H-labeled thyrotropin-releasing hormone. Phospholipase D was without effect. These reductions in thyrotropin-releasing hormone binding capacity stem from a decrease in the affinity for thyrotropin-releasing hormone of the membrane-located receptor. The effects of phospholipase A were completely Ca²⁺-dependent. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine are the four major phospholipids of adenohipphysyal cell plasma membranes. When added in excess of 1 mg per ml to intact plasma membranes, sonicated suspensions of these four phospholipids present either singly or in combination result in an inhibition of thyrotropin-releasing hormone binding. If plasma membranes are used with a concentration of phospholipase A or C to give a reduction in thyrotropin-releasing hormone binding of at least 50%, partial recovery of binding activity is achieved on phospholipid addition. Inhibition of thyrotropin-releasing hormone binding by phospholipid addition to intact membranes is mediated by a decrease in the Vₘₐₓ of the reaction. These data show membrane phospholipids to be intimately involved in the receptor for thyrotropin-releasing hormone.

EXPERIMENTAL PROCEDURE

Materials

Thyrotropin-releasing hormone (L-[2,3-3H]proline-labeled, 40 Ci per mmole) was purchased from New England Nuclear. Phospholipase A (EC 3.1.1.4) from Vipera russelli, phospholipase C (EC 3.1.4.3) from Clostridium welshii, and phospholipase D (EC 3.1.4.4) from cabbage were obtained from Sigma. Phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine, lyso-phosphatidylcholine, bovine brain cerebrosides, sphingomyelins, and digitonin were from Sigma. Chloroform and methanol were redistilled before use.

Methods

Plasma Membranes—Plasma membranes were prepared from bovine anterior pituitary glands by a previously described modification of the method of Brecher and Seligman (14). The abbreviations used are: TRH, thyrotropin-releasing hormone; EGTA, ethylene glycol bis[β-aminoethyl ether]-N,N'-tetraacetic acid; cyclic AMP, cyclic adenosine 3':5'-monophosphate.
cation (5, 12) of the technique of Neville (13). The membranes used for these studies were the fractions collecting at the interfaces of buffered sucrose layers of densities 1.14 to 1.16 and 1.16 to 1.18. These fractions, as evidenced by electron microscopy and enzyme markers, consisted of pure plasma membranes.²

**[3H]TRH Binding Assay**—In the standard assay, an aliquot of plasma membranes containing 50 μg of membrane protein is incubated in 60 μl of Buffer A (50 mM Tris-HCl, pH 7.6; 7.5 mM KCl; 2 mM MgCl₂; 5 mM 2-mercaptoethanol) with 50 nM [3H]TRH for 40 min at 0°C. The reaction is stopped by the addition of 2.0 ml of ice-cold Buffer A and the mixture immediately is filtered through HAWP Millipore filters (4). After drying, radioactivity on the filter is measured in a toluene based scintillation fluid, using a Packard liquid scintillation spectrometer. Unless otherwise stated, the results of all binding assays are the mean of triplicate determinations.

**Extraction of Membrane Lipids**—Plasma membranes were resuspended in chloroform-methanol (2:1, v/v) containing 0.005% 2,6-di-tert-butyl-4-methylphenol (Aldrich Chemical Company) and extracted at 0°C overnight. After shaking with 0.2 volume of 0.9% NaCl (14), the two phases were separated by centrifugation and the upper phase was discarded. Insoluble material and water droplets were removed from the lower phase by passing through a Pasteur pipette containing 0.5 cm of Sephadex G-10, and washing with chloroform. Dispersal of lipids in buffers was achieved by sonication after solvent removal under N₂. Sonication was performed at 0°C until no further increase in clarification of the suspension occurred.

**Thin Layer Chromatography of Lipids**—Precoated Silica Gel H glass plates (Merck, Germany) were activated at 110°C for 30 min prior to use. Lipids dissolved in chloroform were spotted and the plate was developed with CHCl₃-MeOH-CH₃COOH-H₂O (50:25:8:4, v/v) (15). Spots were routinely detected by spraying with 50% H₂SO₄ and heating at 140°C. Identification was achieved by specific detection tests and co-chromatography of known standards.

**Phosphate Assay**—Areas of equal size corresponding to spots and bands were scraped from thin layer chromatography plates and, after digestion in 70% HClO₄ containing 10% HNO₃ at 180°C for 30 min, their phosphate content was measured by the method of Marnetti et al. (16). The phosphate content of lipids dissolved in CHCl₃ was measured by the technique of Ames (17).

**Phospholipase Digestion**—Plasma membranes were resuspended at a concentration of 1.0 mg of protein per ml in Buffer A containing 2 mM CaCl₂ and were incubated with the appropriate concentrations of phospholipase for 5 min at 37°C. Reaction was stopped by addition of ice-cold 10 mM EGTA. Membranes were resedimented at 30,000 × g for 20 min and the pellet was rinsed before resuspension in Buffer A.

**Protein Determination**—Protein was assayed by the method of Lowry et al. (18) using bovine serum albumin as standard.

## RESULTS

The specificity of [3H]TRH binding to plasma membranes of bovine anterior pituitary gland and the characteristics of the binding reaction have been previously described (4). Treatment of adenohypophyseal plasma membranes with phospholipase A at concentrations up to 10 milliunits per ml led to a rapid and marked loss of thyrotropin-releasing hormone binding activity (Fig. 1). Unlike the binding of glucacon to its receptor (10), very dilute concentrations of phospholipase A did not stimulate the binding of thyrotropin-releasing hormone. Binding of [3H]TRH to adenohypophyseal plasma membranes. Adenohypophyseal plasma membranes were centrifuged for 30 min at 30,000 × g, washed, and resuspended in Buffer A and the binding of [3H]TRH was measured as described under "Materials and Methods." Results are the mean of triplicate assays.

![Fig. 1. Effects of phospholipase A on the binding of [3H]TRH to adenohypophyseal plasma membranes. Adenohypophyseal plasma membranes containing 50 pg of membrane protein were incubated with increasing concentrations of phospholipase A for 5 min at 37°C.](http://www.jbc.org/)

² G. Poirier, A. de Lean, G. Pelletier, and F. Labrie, manuscript in preparation.

³ M. Berlinguet, F. Labrie, and N. Barden, unpublished observations.
The binding of [3H]TRH is similarly diminished following incubation of adenohypophyseal plasma membrane with phospholipase C (Fig. 2). The presence of 40 milliunits per ml of phospholipase C for 5 min at 37° inhibits completely the binding of thyrotropin-releasing hormone while such treatment releases only 9% of the total membrane phosphate into the supernatant. However, since not all damaged phospholipids are released from phospholipase-treated membranes (30, 31), the actual percentage hydrolysis of the membrane phospholipids is probably much higher than this visible 9%. In contrast to the actions of phospholipase A and phospholipase C, phospholipase D incubated at pH 7.6 had no effect on the ability of membranes to bind [3H]TRH. At pH 5.6, a value close to the pH optimum for this enzyme, and with a concentration of phospholipase D as high as 1 unit per ml, the binding of thyrotropin-releasing hormone was reduced by only 20%. Digitonin, a compound known to interact with membrane lipids and disrupt membrane structure (20), led to a marked inhibition of the binding of [3H]TRH to plasma membranes while ouabain, which inhibits the plasma membrane (Na+,K+)-dependent ATPase (21) and has been reported to prevent lipolytic hormone action at a stage at or before cyclic AMP formation (22) was without effect (Fig. 3). The effects of digitonin were the same whether the glycoside was added directly to the binding assay or membranes were pre-treated and the glycoside was subsequently removed.

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Effect of calcium ion, EGTA, and lima bean trypsin inhibitor on activity of phospholipase A and phospholipase C preparation.</td>
</tr>
<tr>
<td>Plasma membranes were incubated as described under &quot;Materials and Methods&quot; with additions as indicated. After washing with Buffer A and recentrifugation, binding of [3H]TRH is expressed as a percentage of the control and is the mean of triplicate assays. Phospholipase A or C was treated at 60° for 5 min prior to use (60°-heated phospholipase A or C).</td>
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<tr>
<th>Incubation conditions</th>
<th>Phospholipase A (10 milliunits/ml)</th>
<th>Phospholipase A (10 milliunits/ml)</th>
<th>Phospholipase C (20 milliunits/ml)</th>
<th>Phospholipase C (20 milliunits/ml)</th>
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<tr>
<td>Ca²⁺ (2 mM)</td>
<td>EGTA</td>
<td>Lima bean trypsin inhibitor (μg/ml)</td>
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<tr>
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<td>60°-heated phospholipase C (20 milliunits/ml)</td>
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<th>Table II</th>
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<tr>
<td>Effect of phospholipase A on phospholipid composition of adenohypophyseal plasma membranes.</td>
</tr>
<tr>
<td>Plasma membranes were incubated with increasing concentrations of phospholipase A and the lipids were extracted and chromatographed as described under &quot;Materials and Methods.&quot; Phospholipids were removed from equal areas of silica gel for assay of phosphorus content. Results are shown as micrograms of phosphorus recovered (μgP) and as percentage of the total phosphorus content (%). Recovery of phosphorus varied from 95 to 104%.</td>
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<th>Phospholipid</th>
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<th>%</th>
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<td>0.47</td>
<td>1.04</td>
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<td>13.61</td>
<td>1.55</td>
<td>16.79</td>
<td>1.15</td>
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FIG. 3. Effects of digitonin and ouabain on the binding of [3H]TRH to adenohypophyseal plasma membranes. The binding of [3H]TRH was measured as described under “Materials and Methods.” Ouabain, or digitonin, dissolved in 40% ethanol was added at the indicated concentrations. The final concentration of ethanol in all assays was 4%, a level which inhibited the binding of [3H]TRH by 35%. O--□, digitonin; ○-○, ouabain.

FIG. 4. Effects of phospholipase A and of phospholipase C on the affinity of adenohypophyseal plasma membranes for thyrotropin-releasing hormone. The binding of [3H]TRH to control membranes (□-□) and to membranes previously incubated with 5 milliunits per ml of phospholipase A (○-○) or 10 milliunits per ml of phospholipase C (●-●) was measured as a function of the thyrotropin-releasing hormone concentration as described under “Materials and Methods.”

Following treatment of plasma membranes with either phospholipase A or phospholipase C, the reduction in thyrotropin-releasing hormone binding stems from a decrease in the affinity for thyrotropin-releasing hormone of the membrane receptor (Fig. 4). The concentration of thyrotropin-releasing hormone required for half maximal binding is increased from the control value of $3.5 \pm 0.5 \times 10^{-8}$ M (mean ± S.E.M. of seven experimental determinations) to $2.5 \times 10^{-8}$ M and $5.0 \times 10^{-8}$ M following incubation with 5 milliunits per ml of phospholipase A and 10 milliunits per ml of phospholipase C, respectively. At these concentrations of phospholipases, no change of the maximum velocity of the binding reaction could be detected. Pre-treatment of plasma membranes with lysophosphatidylcholine, or direct addition of this agent in the binding assay, inhibited the binding of [3H]TRH at all concentrations tested between 5 and 200 µg per ml, the latter concentration giving an inhibition of 55%. As with phospholipase A and C, this inhibition was related to a lowering of affinity of the receptor for thyrotropin-releasing hormone (Fig. 4) and not to a reduction of the number of available binding sites.

Addition of phospholipids to membranes treated with either phospholipase A or phospholipase C only resulted in a partial recovery of binding activity (Fig. 6). In fact, unless the membranes were sufficiently damaged by phospholipases so as to reduce the binding of thyrotropin-releasing hormone by 50% or more, addition of phospholipids led to further inhibition of thyrotropin-releasing hormone binding. The final concentration of phospholipids used was important, a concentration of less than 1 mg per ml giving neither the inhibitory nor the stimulatory effects on thyrotropin-releasing hormone binding. The inhibition of thyrotropin-releasing hormone binding by addition to intact membranes of phospholipids in excess of 1 mg per ml resulted
from a decrease in the maximal velocity of the binding reaction (Fig. 7), while no change of the $K_D$ thyrotropin-releasing hormone receptor could be detected. Several methods were employed to regain thyrotropin-releasing hormone binding activity in phospholipase-treated membranes. These included addition of sonicated suspensions of standard phospholipids or phospholipids extracted from membranes. Membranes were preincubated with phospholipid suspensions at 0 or at 37° or mixed with phospholipid-saturated bovine serum albumin solutions. The maximal recovery of thyrotropin-releasing hormone binding activity achieved was 25%, and equal success was achieved with all individual phospholipids and with the phospholipid mixtures.

**DISCUSSION**

In current models of membrane structure (23, 24), phospholipids play a role both in the structural integrity of the membrane and in the interaction with protein components and possible modulation of their activity. Our results indicate that the activity of one component of bovine adenohypophysial plasma membranes, the receptor for thyrotropin-releasing hormone, is acutely dependent on membrane phospholipids. Digestion of membranes with extremely small quantities of phospholipase A, phospholipase C, or treatment with digitonin markedly diminishes the binding of thyrotropin-releasing hormone. These observations are in agreement with the necessity of membrane phospholipids for the binding of glucagon to its receptor (10). Glucagon appears to exert its effect on cellular metabolism via adenylate cyclase (25, 26) as most probably does thyrotropin-releasing hormone (5).

In contrast, the receptor for insulin, a hormone which does not stimulate adenylate cyclase, exhibits no phospholipid requirement for activity (27). In fact, digestion of liver or fat cell membranes with phospholipase increases the binding of insulin, an effect attributed to exposure of receptor sites (27). Although the binding of glucagon to its receptor is increased following membrane digestion with dilute concentrations of phospholipase (10), this has not been observed for the binding of thyrotropin-releasing hormone. This discrepancy may be attributed to the concentrations of phospholipases used. The binding of thyrotropin-releasing hormone is inhibited by concentrations of phospholipases far lower than those necessary for the inhibition of glucagon binding. It is possible that unmasking of additional binding sites for thyrotropin-releasing hormone is not observed because of simultaneous degradation of phospholipid components essential for activity.

Digestion of plasma membranes with phospholipase D did not produce the inhibition of thyrotropin-releasing hormone binding observed with the other phospholipases. This lack of effect with phospholipase D has been noted for the insulin receptor (27). Current models of membrane structure attach importance to interactions between hydrophobic groups of lipids and proteins in the interior of membranes for structural integrity while removal of exterior hydrophilic heads of phospholipids by phospholipase C or D has no effect on the membrane conformation, as evidenced by circular dichroism and electron spin resonance (23, 28). The fact that removal of only the base by phospholipase D does not greatly affect the binding of thyrotropin-releasing hormone while it is completely inhibited by removal of the complete polar head chain with phospholipase C suggests a requirement of the thyrotropin-releasing hormone receptor for exterior hydrophilic groups. Binding of thyrotropin-releasing hormone to its membrane receptor was inhibited most markedly following digestion with phospholipase A. This may be attributed first to the disruption of membrane conformation following $\beta$-ester bond cleavage by phospholipase A (29) and second to the fact that the end products of phospholipase A digestion, lyso-derivatives of phospholipids, have a well-known detergent effect and themselves inhibit the binding of thyrotropin-releasing hormone (Fig. 5).
The decrease in thyrotropin-releasing hormone binding following digestion with phospholipase A or C results from a decrease in the affinity of the receptor system and is not due to a loss of binding sites (Fig. 4). This could be the result of a secondary change in the conformation of the hormone binding site, or for a requirement of a specific phospholipid at a specific site or both. It is clear that the effects observed cannot easily be attributed to the preferential degradation of any one particular phospholipid. Although in terms of percentage change, phosphatidylserine is most rapidly hydrolyzed by phospholipase A (Table II), at the same time, larger amounts of phosphatidylcholine and phosphatidylcholanolamine are degraded. Furthermore, partial reversal of the effects of phospholipase digestion was achieved equally well with several individual phospholipids or phospholipid mixtures. The binding of thyrotropin-releasing hormone to intact plasma membranes was inhibited by phospholipid addition (Fig. 7). Similar results have been observed by POHL, S. L., KRANS, H. M. J., KOZYREFF, V., BIRNBAUMER, L., POHL, S. L., & RODBELL, M. (1971) J. Biol. Chem. 246, 1857–1860.

REFERENCES
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