Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 8-13

ROLE OF LIPIDS IN GONADOTROPIN RELEASING HORMONE AGONIST AND ANTAGONIST BINDING TO RAT PITUITARY

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Received January 13, 1982

Digestion of pituitary membranes with phospholipase A_2 and phospholipase C abolished, in a dose responsive manner, the specific binding of gonadotropin releasing hormone agonist and antagonist. These reductions in gonadotropin releasing hormone binding capacity stem from a decrease in the affinity of both agonist and antagonist. Digestion with phospholipase A_2 was calcium-dependent, whereas digestion with phospholipase C was calcium-independent. Treatment of the membranes with phospholipase D reduced the binding to a smaller degree but affected more significantly the binding of the agonist than that of the antagonist. These data show that membrane phospholipids are involved in the gonadotropin releasing hormone-receptor interaction.

INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide, which affects gonadotropin release by interacting with specific binding sites (receptors) on gonadotrope cells. The binding of GnRH or its stable analogs to pituitary membrane preparations or pituitary cells, has indicated the presence of a single class of high affinity binding sites for both agonists and antagonists (1-8).

Recent studies have shown that the binding of GnRH agonists and antagonists to rat pituitary membranes are affected differently by cations, lectins, and by pretreatment of membranes with proteolytic and glycosidic enzymes (9-10). Because membrane lipids have been demonstrated to be involved in hormone receptor interaction, e.g. thyrotropin-releasing hormone (11), insulin (12) and glucagon (13), we investigated the effects of various phospholipases on the binding of GnRH agonists and antagonists to rat pituitary membranes.

MATERIALS AND METHODS

Materials

Phospholipase A_2 (E.C. 3.1.1.4; 970 U/mg) from Naja Naja Venom, phospholipase C (E.C. 3.1.4.3; 24 U/mg) from C. perfringens and phospholipase D (E.C. 3.1.4.4: 26 U/mg) from cabbage were purchased from Sigma.

0006-291X/82/050008-06$01.00/0

Iodination and pituitary membrane preparations

[D-Ser(t-Bu)^{6}, des-Gly^{10}, ethylamide]-GnRH (Buserelin, GnRH agonist, provided by Dr. J. Sandow, Hoechst) and [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-GnRH (GnRH antagonist, kindly supplied by Drs. W. Vale and J. Rivier, Salk Institute) were iodinated by the lactoperoxidase method (9); specific activity of the labeled peptides was approximately 1.0 mCi/µg. Pituitary membranes were prepared from 25 to 28 day-old Wistar derived female rats as described previously (9). Briefly, the glands were homogenized gently with a Dounce homogenizer at 4°C in assay buffer (10 mM Tris.HCl, pH-7.4, 0.1% bovine serum albumin, BSA) containing 1 mM dithiothreitol and centrifuged for 10 min at 1000xg. The supernatant was then centrifuged for 20 min at 20,000xg. The pellet was resuspended in assay buffer, centrifuged at 20,000xg for 20 min and finally suspended in assay buffer.

Binding assay

The labeled peptides (40,000 cpm agonist and 20,000 cpm antagonist) were incubated with 0.1 to 0.2 mg protein of pituitary membranes in a total volume of 0.5 ml assay buffer for 90 min at 4°C (equilibrium conditions). The binding was measured by filtration under vacuum through Whatman GF/C filters. Specific binding represents the bound radioactivity which can be competed for by 10^{-6} unlabeled Buserelin or [D-pGlu^{1}-D-Phe^{2},D-Trp^{3,6}]-GnRH.

Phospholipase digestion

Pituitary membranes were incubated with appropriate concentrations of phospholipase at 37°C in assay buffer containign 2 mM $CaCl_2$. Reaction was stopped by addition of ice-cold 10 mM ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), centrifuged at 20,000xg for 20 min and the pellet washed before resuspension in assay buffer. The specific details are presented in legends to figures and tables.

RESULTS AND DISCUSSION

[^{125}I]-Buserelin (GnRH agonist) and [^{125}I][D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]GnRH (GnRH antagonist) exhibited high affinity binding to pituitary membranes (9,10). The characteristics of the binding have previously been described and indicated that GnRH agonists and antagonists bind differently to the same receptor (9-10).

The effects of phospholipase A_2 and phospholipase C on the binding of ^{125}I-labeled Buserelin and [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]GnRH are shown in Figs. 1 and 2. Pretreatment of the membranes with phospholipase A_2 and phospholipase C (0.1 to 5 U/ml) resulted in a rapid decrease of the specific binding of both ^{125}I-labeled agonist and antagonist. These results are similar to the effects of phospholipase A_2 and phospholipase C on the binding of thyrotropin-releasing hormone to pituitary membranes (11). However, unlike the binding of insulin (12) to its receptor very dilute concentrations of the phospholipases did not stimulate the binding of the GnRH analogs.

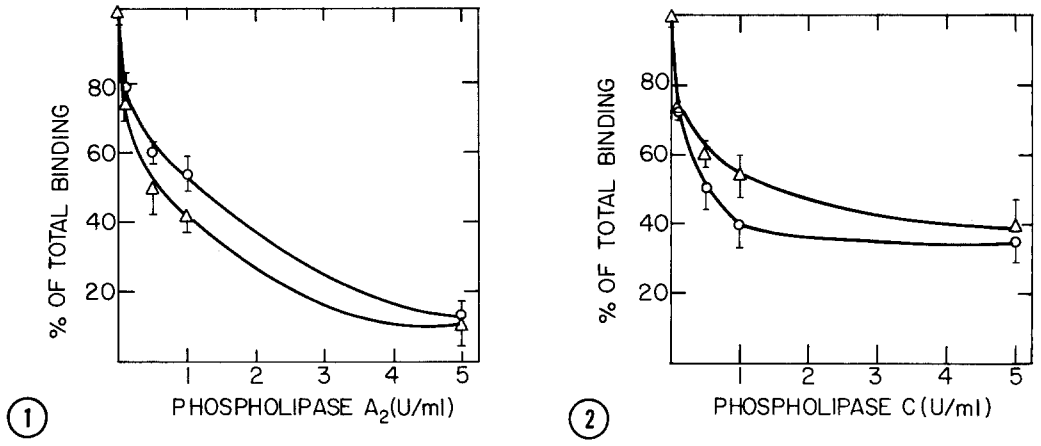

Fig. 1. Effects of phospholipase A_2 on the specific binding of ^{125}I-labeled Buserelin (o——o) and [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH (Δ–Δ) to pituitary membranes. Pituitary membranes in assay buffer plus 2 mM Ca^{+2} were incubated with increasing concentrations of phospholipase A_2 for 10 min at 37°C. After addition of ice-cold 10 mM EGTA, membranes were centrifuged for 20 min at 20,000xg, washed and resuspended in assay buffer. Specific binding was determined as described under Materials and Methods. The results are expressed as the mean ± S.E. of two separate experiments.

Fig. 2. Effects of digesting pituitary membranes with phospholipase C on the specific binding of ^{125}I-labeled Buserelin (o——o) and [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH (Δ–Δ). Pituitary membranes were incubated with increasing concentrations of phospholipase C for 10 min at 37°C. Experimental details for termination of reaction and determination of specific binding are as described in the legend to Fig. 1.

The effects of phospholipase A_2 and phospholipase C treatment on the competition of binding of [^{125}I]Buserelin by unlabeled Buserelin are shown in Fig. 3. Preincubation with phospholipase A_2 (1 U/ml, 10 min at 37°C) and phospholipase C (1 U/ml, 10 min at 37°C) resulted in a decrease of the specific binding of [^{125}I]-labeled Buserelin by about 50% and 66%, respectively. As shown in Table 1, this was accompanied by a decrease of the apparent IC_{50} values (the concentration of unlabeled ligand at which the specific binding of labeled ligand is displaced by 50%) from 0.5 nM (control membranes) to 5.0 nM (phospholipase A_2-treated membrane) and 4.0 nM (phospholipase C-treated membranes). Similarly, the apparent IC_{50} values (Table 1) of the antagonist were decreased from 0.06 mM (control membranes) to 1.0 nM (phospholipase A_2-treated membranes) and 0.6 nM (phospholipase C-treated membranes).

Since proteolytic enzymes were shown to affect the binding of GnRH agonists and antagonists (9), it was important to ascertain that the observed effects

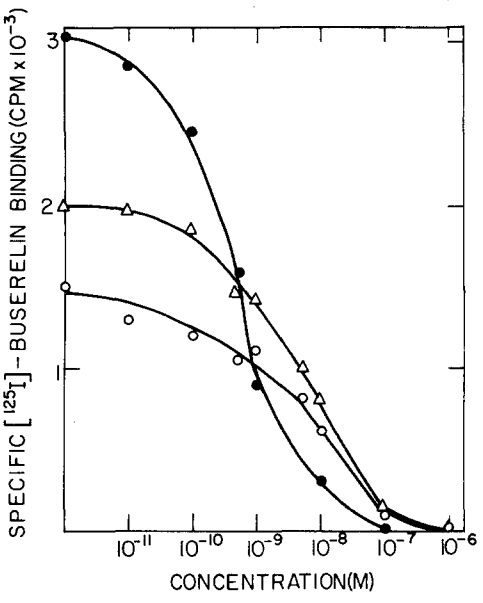

Fig. 3. Competition of binding of [^{125}I] Buserelin binding by unlabeled Buserelin to control pituitary membranes (●——●), pituitary membranes previously incubated with 1 U/ml of phospholipase A_2 (o——o) or 1 U/ml of phospholipase C (Δ—Δ). Membranes were incubated with phospholipases for 10 min at 37°C, as described in legends to Figs. 1 and 2. The radioactive Buserelin (40,000 cpm) was incubated with different concentrations of unlabeled Buserelin for 90 min at 4°C in a final volume of 0.5 ml containing pituitary membranes (0.1 to 0.2 mg of protein/ml) and the binding was measured as described in Materials and Methods. The S.E.M. (10% of the indicated means) were not added to the figure for the sake of clarity.

of phospholipase A_2 and phospholipase C were dependent upon hydrolysis of membrane lipids. The following lines of evidence have been obtained: (a) The effects of enzymes were examined in the presence of bovine serum albumin which prevents proteolytic activity. (b) Heating of phospholipase A_2 and phospholipase C at 60°C for 5 min had no effect on their ability to inhibit the binding

Table 1

IC_{50}[a] values of Buserelin and [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH in control and phospholipase treated pituitary membranes[b]

Membranes	Buserelin	IC_{50}[a](nM) [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH
No enzymic digestion	0.5 ± 0.1	0.06 ± 0.02
Phospholipase A_2	5.0 ± 0.8	1.0 ± 0.3
Phospholipase C	4.0 ± 0.5	0.6 ± 0.2

[a] The concentration of unlabeled ligand at which the specific binding of labeled ligand is displaced by 50%.

[b] For experimental details see legend to Fig. 3.

Table 2

Effect of calcium ion, temperature and EGTA on the activity of phospholipase A_2 and phospholipase C.

Incubation Condition Ca + 2 (2 mM)	EGTA (10 mM)	Enzyme	[^{125}I]-Buserelin specific binding (%)
+	-	-	100 ± 4
-	+	-	100.1 ± 5.8
+	-	Phospholipase A_2 (1 U/ml)	40.9 ± 2.9
-	+	Phospholipase A_2 (1 U/ml)	91.3 ± 7.6
+	-	60°C-heated phospholipase A_2 (1 U/ml)	56.9 ± 4.8
+	-	Phospholipase C (1 U/ml)	60.6 ± 3.8
-	+	Phospholipase C (1 U/ml)	62.8 ± 5.6
+	-	60°C-heated phospholipase C (1 U/ml)	70.3 ± 4.2

Pituitary membranes were incubated as described under Materials and Methods with additions as indicated. After washing by centrifugation in assay buffer and resuspension, specific binding was determined. Phospholipase A_2 or C was treated at 60°C for 5 min prior to use (60°-heated phospholipase A_2 or C).

of [^{125}I] Buserelin (Table 2). (c) In accordance with the cation requirements for phospholipase activity, digestion with phospholipase A_2 was calcium-dependent, whereas digestion with phospholipase C was calcium-independent (Table 2).

As shown in Table 3, digestion with phospholipase D at a longer incubation period (30 min at 37°C) slightly affected the binding of Buserelin and had a very small effect on antagonist binding (Table 3). The digestion with phospholipase D was done at pH 7.4 and not at pH 5.6, a value close to the pH

Table 3

Effect of digesting pituitary membrane with phospholipase D on specific binding of [^{125}I]Buserelin and [^{125}I][D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH

Condition[a]	Specific binding (%)[b] [^{125}I]Buserelin	[^{125}I][D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH
Control (4°C)	100 ± 5	100 ± 4
30 min at 37°C	59.3 ± 3.6	65.4 ± 2.5
Phospholipase D, 0.33 U/ml	49.6 ± 3.7	63.4 ± 4.8
1.67 U/ml	33.4 ± 4.2	49.8 ± 3.9

[a] Pituitary membranes were incubated in assay buffer containing 2 mM Ca+2 with increasing concentration of phospholipase D for 30 min at 37°C. After addition of ice-cold 10 mM EGTA, membranes were treated as described in legend to Fig. 1.

[b] Specific binding was measured as described under Materials and Methods.

optimum for this enzyme, since incubation of the membranes at pH 5.6 resulted in about 80% inhibition of the specific binding (9). In addition, incubation of the membranes (30 min at 37°C, without the enzyme), resulted in about 40% inhibition of the specific binding (9 and Table 3).

Phospholipids play an important role in the structural integrity of the membranes and in the interaction with protein components. Our previous studies have indicated that GnRH agonists and antagonists can interact with the same receptor (9). However, the binding of agonists and antagonists differs with respect to the effect of ions, lectins, proteolytic and glycosidic enzymes and sulfhydryl-blocking reagents (9-10). Those results (9-10) suggest that the GnRH receptor, apparent molecular weight of 60,000 daltons (14), is a glycoprotein which contains sialic acid residue. The present results indicate that digestion of pituitary membranes with phospholipases has similar effects on the specific binding of GnRH agonists and antagonists, which result from a decrease in their apparent affinities. In addition, the binding of GnRH agonists and antagonists is acutely dependent on both, the exterior hydrophilic head groups and the fatty acid on the β carbon of the phospholipid. These findings indicate additional binding properties of the pituitary GnRH receptor, and may have important implications towards the purification of the receptor.

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