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SEDIMENTATION BEHAVIOR OF SOLUBILIZED GONADOTROPIN RECEPTOR FROM PLASMA MEMBRANES OF BOVINE CORPUS LUTEUM

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Summary

The gonadotropin receptors associated with plasma membrane fractions were solubilized by detergents, including Triton X-100, Lubrol WX, Lubrol PX and sodium deoxycholate before and after equilibration with ^{125}I-labelled human chorionic gonadotropin. The binding activity remained in solution even after centrifugation at 300 000 $\times g$ for 3 h. The solubilized gonadotropin receptor or gonadotropin receptor complex was characterized by gel filtration and sucrose density gradient centrifugation. Sucrose density gradient centrifugation of solubilized gonadotropin-receptor complex in the presence of Triton X-100 had a sedimentation coefficient of 6.5 S whereas the solubilized uncomplexed receptor had a sedimentation coefficient of 5.1 S. In the absence of the detergent, solubilized hormone receptor complex from plasma membrane fractions I and II sedimented with an apparent sedimentation coefficient of 6.6 S and 7.4 S, respectively. Similarly, the free receptor also showed higher sedimentation profile with an apparent sedimentation coefficient of 6.7 S for fraction I and 7.2 S for fraction II. Treatment of plasma membranes with phospholipase A and C inhibited the binding of ^{125}I-labelled human chorionic gonadotropin in a dose dependent manner, whereas phospholipase D was without any effect. Doses of 1.4 mI.U. of phospholipase A or 0.6 mI.U. of phospholipase C were required to produce 50% inhibition of the binding activity. These phospholipases had no effect on the preformed ^{125}I-labelled human chorionic gonadotropin-receptor complex nor on the sedimentation profile of solubilized gonadotropin receptor complex.

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Introduction

The initial step in the action of luteinising hormone or human chorionic gonadotropin involve the binding of the hormone to its receptor on plasma membrane of the ovarian cell which results in the activation of adenylate cyclase [1,2] and increased steroidogenesis [3—6]. Recently, several reports have appeared which describe the properties of the membrane receptors from ovarian preparations [7—13].

We have reported previously on the isolation of plasma membranes from bovine corpus luteum possessing gonadotropin sensitive adenylate cyclase and ^{125}I-labelled gonadotropin binding activities [2]. In our continuing efforts to understand the mechanism of action of gonadotropin in the corpus luteum, we are investigating the properties of the isolated plasma membrane with respect to its various catalytic properties [2,14—16]. In this report we describe the sedimentation properties of the solubilized gonadotropin receptor from the plasma membranes of bovine corpus luteum.

Materials and Methods

Corpora lutea. Fresh bovine corpora lutea were obtained from a local slaughterhouse, placed in normal saline, transported to the laboratory in ice, and either processed immediately or frozen at $-80°C$ until further use.

Chemicals. Phospholipase A (Bee Venom) and phospholipase C (*Clostridium welchii*) were obtained from Sigma Chemical Co. ^{125}I-labelled human chorionic gonadotropin was generously supplied by Dr. A. Rees Midgley, Jr. of this University. All other chemicals used were conventional commercial products.

Preparation of plasma membrane fraction from bovine corpus luteum. Plasma membrane fractions (fractions I and II) were prepared as described by Gospodarowicz [8]. Purity of plasma membranes was checked by assaying various marker enzymes [8,14]. These included Na^+, K^+ and Mg^{2+} dependent ATPase, 5'-nucleotidase and glucose-6-phosphatase by the method of Solyom and Trams [17], NADH cytochrome C reductase by the procedure of Phillips and Langdon [18], succinic dehydrogenase by the method of Veeger et al. [19] and glucose-6-phosphate dehydrogenase according to Langdon [20].

Assay of ^{125}I-labelled human chorionic gonadotropin binding to plasma membranes. The assay for specific binding of ^{125}I-labelled gonadotropin to membranes was carried out as described earlier [2]. Membranes (100 μg protein) were incubated in 0.3 ml of buffer (10 mM Tris-HCl buffer of pH 7.2, 1 mM $CaCl_2$ containing 0.1% bovine serum albumin) with ^{125}I-labelled gonadotropin (70 000 cpm) at 37°C for 90 min. Following incubation, 1 ml of buffer was added to each tube and centrifuged at 10 000 × g for 10 min. The supernatants were discarded and sediments were again suspended in 1 ml of buffer and centrifuged as above. The supernatant from second was were again discarded and the tubes were then counted in a Packard automatic gamma spectrometer. In each case specific binding of labelled gonadotropin was determined by subtracting from the total counts radioactivity associated with membrane fraction in the presence of 1000-fold excess of unlabelled gonadotropin [2].

Assay of ^{125}*I-labelled gonadotropin binding to Triton X-100* solubilized receptor.* The ^{125}I-labelled gonadotropin binding to solubilized receptor was carried out employing a double precipitation of the gonadotropin-receptor complex with polyethylene glycol as described by Dufau et al. [21].

Solubilization of prelabelled plasma membranes. Aliquots of plasma membranes (100 µg protein) in Buffer A were incubated at 37°C for 90 min in the presence of 70 000 cpm of ^{125}I-labelled gonadotropin, washed three times with 2 ml of buffer to remove unbound hormone and the membranes were recovered by centrifugation at 20 000 × g for 15 min. The prelabelled membranes were then extracted separately with various concentrations of Triton X-100, Lubrol XW* and Lubrol PX* for 1 h at 4°C. After detergent treatment the suspensions were centrifuged at 20 000 × g for 20 min. The soluble fractions were then subjected to gel filtration on Sephadex G-200 columns and sucrose density gradient centrifugation. Centrifugation of the supernatant solution at 300 000 × g for 3 h was also carried out to determine if the radioacitivity associated with hormone receptor complex remained in the supernatant.

Solubilization of the free receptor. Plasma membranes were suspended in buffer containing 1% Triton X-100 and incubated at 4°C for 1 h. After dilution of the detergent to 0.1% with buffer, the suspension was centrifuged at 27 000 × g for 20 min to remove the unsolubilized membrane. The solubilized binding fraction was not sedimented on further centrifugation at 300 000 × g for 3 h.

Sucrose density gradient centrifugation. Sucrose density gradient centrifugation in 5 to 20% (W/V) sucrose with or without 0.1% Triton X-100 was performed as described by Martin and Ames [22] and Kuczenski [23]. Catalase (11.6 S), glyceraldehyde-3-phosphate dehydrogenase (7.7 S) and alcohol dehydrogenase (4.5 S) were used as internal markers [14].

Protein determinations. The protein content was determined by the method of Lowry et al. [24] using bovine serum albumin as standard.

Results and Discussion

Plasma membranes. The purity of plasma membranes were ascertained by assaying various plasma membrane marker enzymes that are specific for plasma membranes and other cellular organelles [17]. Relative to the 5'-nucleotidase the purification of plasma membrane fraction averaged 9—10 fold, a value similar to that reported by Gospodarowicz [8]. Contamination of the isolated plasma membranes by other cellular components was less than five percent as judged by assaying specific marker enzymes (see Materials and Methods).

Binding of ^{125}*I-labelled gonadotropin to bovine corpus luteum plasma membranes.* Plasma membrane fractions I and II isolated from bovine corpus luteum showed specific binding of ^{125}I-labelled human chorionic gonadotropin (Table I). Both fractions I and II showed comparable binding activities when assayed either separately or mixed together (Table I). In subsequent experiments, a mixture of fractions I and II were used for binding studies. Saturation of the binding of ^{125}I-labelled gonadotropin to plasma membrane was achieved at a concentration of approximately $5 \cdot 10^{-9}$ M to $1 \cdot 10^{-8}$ M ^{125}I-labelled gonado-

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* Triton X-100, octyl phenoxy polyethoxyethanol; Lubrol PX and Lubrol WX, polyoxyethylene alcohols.

TABLE I

^{125}I-LABELLED GONADOTROPIN BINDING TO PURIFIED PLASMA MEMBRANE FRACTION FROM BOVINE CORPUS LUTEUM

The incubation medium in a final volume of 0.3 ml contained Tris·HCl buffer, pH 7.2, 3 μmol; CaCl$_2$ 0.3 μmol, bovine serum albumin 0.1%, ^{125}I-labelled gonadotropin 1 ng (1.6 X 10^{-10} M), 100 μg plasma membrane protein and where required, 3 μg of unlabelled gonadotropin. After incubation at 37°C for 90 min the tubes were then processed for radioactivity as described under Materials and Methods.

Plasma membrane	^{125}I-labelled gonadotropin bound (dpm X 10^{-3})/mg membrane protein*
Fraction I	57.3 ± 2.8
Fraction II	68.2 ± 3.2
Fractions I and II	62.3 ± 5.1

* Each value represents the mean ± S.E. of three determinations.

tropin (Fig. 1). The binding activity increased with increasing concentrations of membrane protein, reaching saturation at about 250 μg in the presence of a fixed amount of ^{125}I-labelled gonadotropin (1.6 · 10^{-10} M). Specific binding of ^{125}I-labelled gonadotropin to plasma membranes was also dependent on incubation time and temperature. The general properties of the binding of ^{125}I-labelled gonadotropin to plasma membranes are comparable to those described earlier [7—13].

Solubilization of bovine corpus luteum plasma membranes pre-labelled with ^{125}*I-labelled gonadotropin by detergents.* Extraction of the preformed ^{125}I-labelled gonadotropin-receptor complex was attempted with Lubrol WX, Lubrol PX and Triton X-100. Triton X-100 was found to be a better solubilizing agent as compared to other detergents. Almost 50% of hormone receptor complex was solubilized by Triton X-100 (Fig. 2) at a concentration of 0.5%. Lubrol WX and Lubrol PX solubilized 30—40% of the prelabelled hormone receptor complex at the concentration range of 0.1 to 1%. Ionic detergents such a sodium deoxycholate also solubilized the hormone-receptor complex, but was not as effective as Triton X-100. Preliminary experiments using a solu-
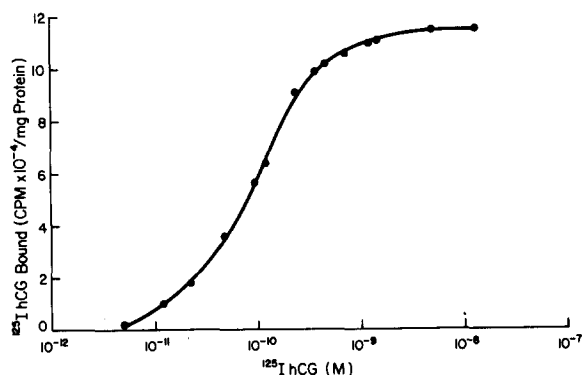

Fig. 1. Binding of ^{125}I-labelled gonadotropin to bovine corpus luteum plasma membranes as a function of the concentration of labelled hormone. Plasma membranes (100 μg of protein) were incubated for 90 min with increasing amounts of ^{125}I-labelled gonadotropin in Buffer A (10 mM Tris—HCl, pH 7.5, containing 1 mM CaCl$_2$ and 0.1% bovine serum albumin). Other details were similar to that described in the text.
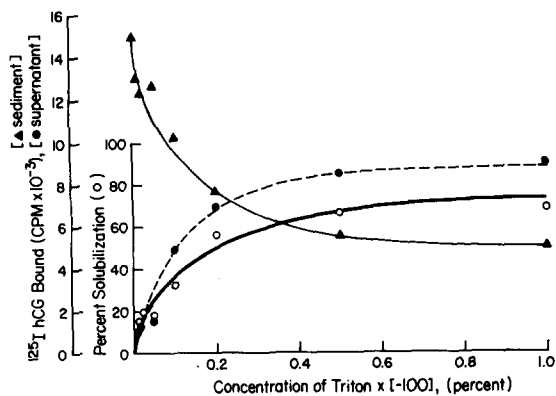
Fig. 2. Solubilization of prelabelled ^{125}I-labelled gonadotropin-receptor complex of bovine corpus luteum with increasing concentration of Triton X-100. Experimental details were similar to that described in the text.
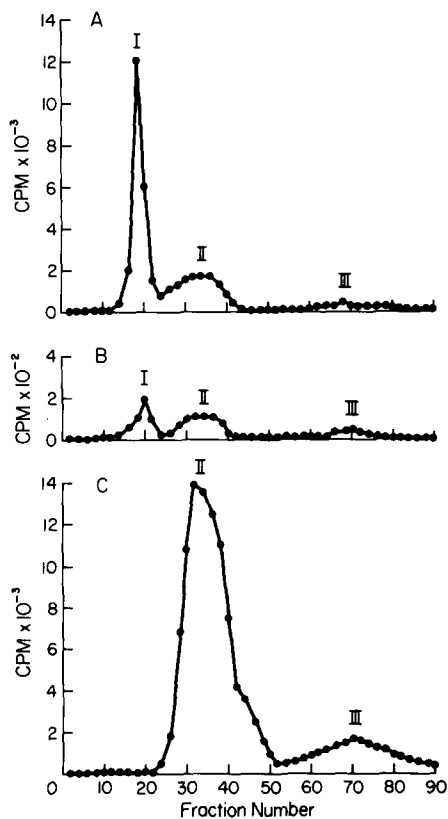

Fig. 3. Filtration of ^{125}I-labelled gonadotropin-receptor complex through sephadex G-200 column. After prelabelling of membranes with ^{125}I-labelled gonadotropin, the hormone receptor complex was solubilized by Triton X-100 (0.4%). The solubilized complex was filtered through Sephadex G-200 column (2.5 × 40 cm) equilibrated with 10 mM Tris—HCl pH 7.2 containing 1 mM $CaCl_2$, 0.1% bovine serum albumin and 0.1% Triton X-100. 3-ml fractions were collected and radioactivity was then determined in each fraction. (A) ^{125}I-labelled gonadotropin-receptor complex; (B) ^{125}I-labelled gonadotropin-receptor complex preincubated in the presence of excess unlabelled gonadotropin (1 · 10^{-7} M); (C) Free ^{125}I-labelled gonadotropin.

tion of high ionic strength failed to solubilize either the free receptor or hormone receptor complex. Only detergent treatment described above could solubilize both the free receptor as well as hormone receptor complex.

Gel filtration of Triton X-100 solubilized ^{125}I-labelled gonadotropin-receptor complex. When Triton X-100 solubilized plasma membrane extracts containing ^{125}I-labelled gonadotropin-receptor complex was chromatographed on Sephadex G 200 in the presence of 0.1% Triton X-100 (Fig. 3A), most of the radioactivity was eluted in the void volume (peak I) and the remainder was distributed between two small included peaks (II and III). On subsequent sucrose density gradient centrifugation, peak I was identified as ^{125}I-labelled gonadotropin-receptor complex (results are presented in the next section). Peak II was identified as free ^{125}I-labelled gonadotropin, which coincided with free ^{125}I-labelled gonadotropin as shown in Fig. 3C. The specificity of the interaction of ^{125}I-labelled gonadotropin with plasma membrane is shown in Fig. 3B. Saturation of the hormone binding sites with unlabelled gonadotropin ($1 \cdot 10^{-7}$ M) prior to treatment with ^{125}I-labelled gonadotropin prevented the association of the labelled hormone with binding proteins which were eluted from the column. In contrast, prior exposure of the membranes to bovine serum albumin
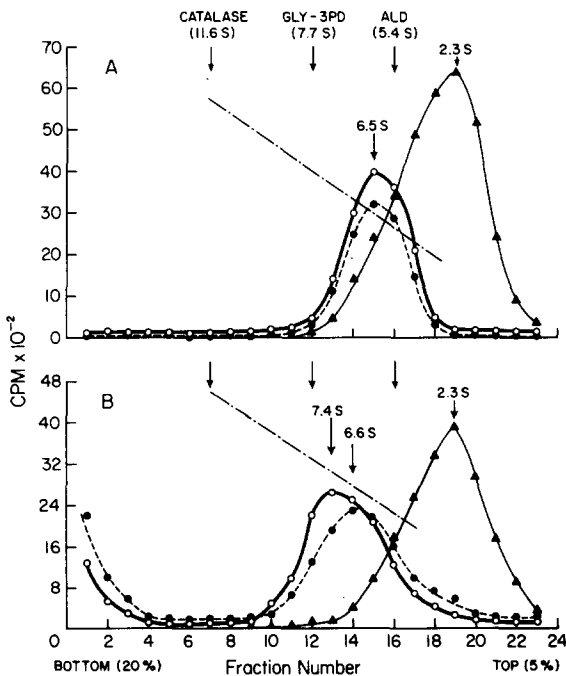

Fig. 4. Sucrose density gradient centrifugation of Triton X-100 solubilized ^{125}I-labelled gonadotropin-receptor complex. (A) Plasma membrane fractions were prelabelled with ^{125}I-labelled gonadotropin and then hormone receptor complex was extracted with 0.4% Triton X-100 in Buffer A (100 mM Tris—HCl pH 7.2 containing 1 mM $CaCl_2$ and 0.1% bovine serum albumin). An aliquot (200 μl) was then applied on 5—20% sucrose gradient (5 ml) made up in Buffer A containing 0.1% Triton X-100. The centrifugation was carried out at 38 000 rev./min in SW 50.1 rotor for 16 h. Five-drop (230 μl) fractions were collected and radioactivity was determined in each fraction. (B) Experimental details were the same as in A except gradient did not contain 0.1% Triton X-100. ●————●, fraction I plasma membrane; ○————○, fraction II plasma membrane; ▲————▲, Free ^{125}I-labelled gonadotropin.

showed no effect upon the subsequent binding and elution of ^{125}I-labelled gonadotropin-receptor complex.

Sucrose density gradient centrifugation of detergent solubilized ^{125}I-labelled gonadotropin-receptor complex. Aliquots of Triton X-100 solubilized ^{125}I-labelled gonadotropin-receptor complex from plasma membrane fractions I and II were also subjected to sucrose density gradient centrifugation in the presence of detergent. The mean sedimentation coefficient of hormone-receptor complex was found to be 6.5 S for both plasma membrane fractions (Fig. 4A). When sucrose density gradient centrifugations were carried out in the absence of Triton X-100, the hormone receptor complex transformed into an aggregate with rapidly sedimenting peaks. Under such experimental conditions the apparent sedimentation coefficients of hormone receptor complex was found to be 6.6 S and 7.4 S for plasma membrane fractions I and II, respectively (Fig. 4B).

Sucrose density gradient centrifugation of solubilized gonadotropin receptor. The sedimentation behavior of the detergent solubilized gonadotropin receptor from two plasma membrane fractions are presented in Fig. 5. Like ^{125}I-labelled gonadotropin-receptor complex, free receptors from both membrane fractions also exhibited identical sedimentation coefficients (5.1 S). Similarly, aggregation of free receptor was also observed when the preparations were centrifuged
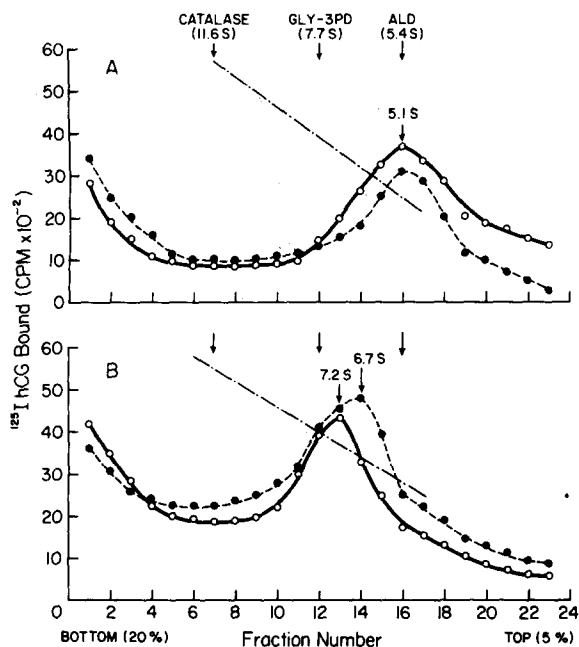

Fig. 5. Sucrose density gradient centrifugation of ^{125}I-labelled gonadotropin binding activity of detergent solubilized plasma membrane. (A) Plasma membranes were extracted with 0.4% Triton X-100 in Buffer A for 1 h at 4°C. The clear supernatant after centrifugation was then applied (200 μl) on 5–20% sucrose gradients (in Buffer A) containing 0.1% Triton X-100 and centrifugation was carried out at 39 000 rev./min for 16 h. Five drop fractions were collected and ^{125}I-labelled gonadotropin binding was carried out in each fraction by polyethylene glycol precipitation. (B) Experimental details were the same as in A except gradient did not contain Triton X-100. ●————●, fraction I plasma membrane; ○————○, fraction II plasma membrane; ▲————▲, Free ^{125}I-labelled gonadotropin.

in the absence of Triton X-100 (Fig. 5B). In the absence of the detergent, free receptor from plasma membrane fractions I and II sedimented as single peaks with sedimentation coefficients of 6.7 S and 7.2 S, respectively. These results are indicative of the possibility that one molecule of gonadotropin might bind to one molecule of the receptor. The increased sedimentation coefficient of free receptor or hormone receptor complex observed in the absence of Triton X-100 could be due to aggregation of proteins following the removal of detergent. At the same time, we cannot rule out the possibility that the removal of detergent may also result in the removal of phospholipids which may be required to maintain the structural integrity of solubilized receptor or the hormone receptor complex.

Modification of gonadotropin binding activity with phospholipases. Since bovine corpus luteum plasma membranes have been found to contain relatively higher proportions of phospholipid [8] the effect of phospholipases were tested on ^{125}I-labelled gonadotropin binding to membranes. Treatment of plasma membranes with phospholipase A or phospholipase C inhibited the binding of ^{125}I-labelled gonadotropin in a dose dependent manner while treatment with phospholipase D had no effect. The concentration of phospholipase A and C that resulted in 50% inhibition was between 1.4 mI.U. and 0.6 mI.U., respectively. The exposure of the labelled plasma membranes to phospholipases A and C prior to Triton X-100 treatment, did not relase sig-

TABLE II

SEDIMENTATION PROFILE OF GONADOTROPIN RECEPTOR AND ^{125}I-LABELLED GONADOTROP-INRECEPTOR COMPLEX FROM BOVINE CORPUS LUTEUM PLASMA MEMBRANES UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Condition	Sedimentation coefficient (S)	
	With Triton X-100 (0.1%)	Without Triton X-100
^{125}I-labelled gonadotropin-receptor complex fraction I	6.5	6.6
^{125}I-labelled gonadotropin-receptor complex fraction II	6.5	7.4
Free receptor fraction I	5.1	6.7
Free receptor fraction II	5.1	7.2
Free ^{125}I-labelled gonadotropin	2.3	2.3
^{125}I-labelled gonadotropin-receptor complex from fraction I membranes pretreated with phospholipase C	6.5	—
^{125}I-labelled gonadotropin-receptor complex from fraction I membranes treated with phospholipase C after ^{125}I-labelled gonadotropin binding	6.5	—
^{125}I-labelled gonadotropin-receptor complex from fraction I membranes pretreated with phospholipase A	6.5	—
^{125}I-labelled gonadotropin-receptor complex from fraction I membranes treated with phospholipase A after ^{125}I-labelled gonadotropin binding	6.5	—

nificant amounts of radioactivity into the medium nor did it affect the sedimentation profile on sucrose density gradient centrifugation. Similarly, pretreatment of plasma membranes with phospholipase A and C, followed by ^{125}I-labelled gonadotropin binding and subsequent solubilization by Triton X-100 showed no effect on the apparent sedimentation coefficient of ^{125}I-labelled gonadotropin-receptor complex when compared to untreated plasma membranes. Identical results were obtained with either plasma membrane fraction I or II. A summary of sedimentation profile of gonadotropin receptor under various experimental conditions is given in Table II.

Susceptibility of gonadotropin binding activity to phospholipases further suggests a role of plasma membrane phospholipids in the interaction between ^{125}I-labelled gonadotropin and receptor or receptor activity. The observed inhibitory effects of phospholipase A and C are in agreement with the results of Gospodarowicz [8], Lee and Ryan [25], but not with Rao and Saxena [9] and Haour and Saxena [10]. However, commercial preparations of phospholipase C showed a biphasic effect. Concentrations of phospholipase C up to 10 milliunits inhibited the binding of ^{125}I-labelled gonadotropin, and 1.0 unit and higher concentrations of phospholipase C was stimulatory. However, upon further purification of phospholipase C from calcium phosphate gel [26,27] the stimulatory activity was not observed. This suggested that the stimulatory effect was due to a contaminating enzyme activity associated with phospholipase C, probably neuraminidase activity [27]. The lack of effect on phospholipase A or C on sedimentation profile of solubilized gonadotropin-receptor complex suggests that phospholipids may constitute a small portion of ^{125}I-labelled gonadotropin-receptor complex, yet are important in the interaction of hormone with the receptor.

Acknowledgement

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