Eur. J. Biochem. *118*, 25–31 (1981)

Receptor-Mediated Gonadotropin Action in Ovary

Possible Regulatory Role of Cell-Surface Sialic Acid in Gonadotropin Interaction to Purified Bovine Corpus Luteum Plasma Membranes

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(Received January 27/April 28, 1981)

The role of cell-surface sialic acid in the interaction of ^{125}I-labeled choriogonadotropin with gonadotropin receptors from plasma membranes of bovine corpus luteum was investigated. Pretreatment of plasma membranes with neuraminidases purified from *Clostridium perfringens* or *Vibrio cholerae* enhanced the binding activity of ^{125}I-choriogonadotropin in a concentration-dependent manner. *C. perfringens* neuraminidase (100 mU/ml) and *V. cholerae* neuraminidase (1.0 I.U./ml) maximally stimulated gonadotropin-binding activity. The neuraminidase stimulation of binding was due to unmasking of gonadotropin-binding sites and not due to a change in affinity of the receptor for ^{125}I-choriogonadotropin. The activity of plasma-membrane 5′-nucleotidase, a glycoprotein enzyme, however, was not affected by neuraminidase treatment. The stimulatory effect of *C. perfringens* enzyme was abolished by co-incubation of plasma membrane with glycoproteins, fetuin and mucin. Furthermore, the stimulatory effect of neuraminidase was due to an intrinsic property of the enzyme and was accompanied by a parallel dose-dependent loss of plasma-membrane-associated sialic acid residues. Other neuraminidase preparations including that purified from *Arthrobacter ureafaciens* also enhanced ^{125}I-choriogonadotropin binding with concomitant hydrolysis of plasma-membrane sialic acid. By contrast, enzyme purified from influenza virus did not release plasma-membrane sialic acid and consequently failed to modulate receptor activity. Treatment of plasma membrane with neuraminidase and subsequent fractionation demonstrated that the enzyme hydrolyzed 50–60% sialic acid from glycoprotein and ganglioside components. Preincubation of plasma membrane with wheat-germ agglutinin partially blocked the hydrolysis of glycoprotein sialic acid as well as stimulation of ^{125}I-choriogonadotropin-binding activity. Incubation with other lectins, concanavalin A and peanut agglutinin, neither affected the hydrolysis of glycoprotein/ganglioside sialic acid by neuraminidase nor prevented the stimulatory action of enzyme on ^{125}I-choriogonadotropin-binding activity. These studies demonstrate a possible involvement of plasma-membrane-associated sialic acid (sialoglycoproteins) in the activity of gonadotropin receptors.

In the ovary, gonadotropin-receptor interaction and subsequent expression of biological response(s) represent a complex process [1–3]. Although in recent years considerable studies have been reported regarding the properties of ovarian gonadotropin receptors in isolated systems [2–13], relatively little is known about the function of receptors within the membrane environment. Major membrane constituents, phospholipids and carbohydrate-containing structures, not only are required for structural integrity of active molecules including receptors but could also exert a regulatory role. The cell-surface carbohydrates originating from glycoproteins, glycolipids and mucopolysaccharides have often been reported to play important roles in cell-surface-mediated events including adhesion and recognition phenomena [14–16]. Since cell-surface glycoproteins are localized mainly on the outer cell surface, the external orientation of carbohydrate groups makes them more suitable agents in the regulation of gonadotropin receptor interaction and/or function. However, at present little information is available regarding the role of cell-surface carbohydrates in gonadotropin-receptor-mediated events. Utilizing both the binding inhibition and fluorescence spectroscopy studies it has been demonstrated that glycoprotein hormones, thyrotropin, lutropin and choriogonadotropin-like cholera toxin, interact with gangliosides [17–21]. Each hormone seems to recognize a specific oligosaccharide sequence on the ganglioside molecule [17, 20, 21]. Further, the inhibition in hormone binding by ganglioside was closely correlated with the number and location of the sialic acid residues within the ganglioside structures. However, earlier studies reported from this laboratory suggest that gangliosides play no obligatory role in gonadotropin interaction to ovarian plasma membranes and/or gonadotropin-induced ovarian responses [22–24]. By employing an entirely different experimental approach, Pacuszka et al. [25] reached a similar conclusion that gangliosides or ganglioside-like structures are not directly involved in choriogonadotropin interaction to testicular plasma membranes. Besides these studies, few investigators have also reported modulation of gonadotropin binding to target plasma membranes of cells following incubation of membranes with relatively impure preparations of neuraminidase [4–7, 12]. However, the exact mechanism or the specificity of the enzyme and whether the modulation of binding activity represented an intrinsic property of the enzyme were not established.

Enzymes. Neuraminidase or mucopolysaccharide *N*-acetylneuraminyl hydrolase (EC 3.2.1.18); 5′-nucleotidase (EC 3.1.3.5); ATPase or ATP pyrophosphohydrolase (EC 3.6.1.8).

Previous studies from this laboratory suggest that phospholipids are required for structural integrity as well as for maximal activity of gonadotropin receptors associated with the plasma membrane bovine corpus luteum [26,27]. In continuation of our efforts to understand and gain additional insight into the role of cell-membrane constituents in gonadotropin-receptor interaction, we have now focused our attention on the role of cell-surface carbohydrates in these processes. In the present studies we have chosen sialic acid as a representative of cell-surface carbohydrates and attempted to modify the existing concentration of this sugar residue in glycoprotein and ganglioside fractions by neuraminidase treatment and to monitor accompanying changes in cell-surface sialic acid and in the properties of gonadotropin-receptor interaction. Our results demonstrate that enzymatic removal of cell-surface sialic acid led to the unmasking and stimulation of gonadotropin receptor activity in plasma membranes of bovine corpus luteum. Further, a relationship between gonadotropin-receptor interaction and glycoprotein-bound sialic acid is demonstrated. These studies thus demonstrate a potential regulatory role of sialoglycoprotein(s) in gonadotropin interaction with plasma-membrane-associated receptors.

MATERIALS AND METHODS

Bovine corpora lutea obtained from a slaughterhouse (Murco Slaughterhous, Plainwell, MI) were transported to the laboratory in ice-cold normal saline. They were kept frozen at $-80\,^{\circ}C$ until use. Concanavalin A (extracted from *Canavalia ensiformis*), purified neuraminidase from *Clostridium perfringens* (type V, 0.31 U/mg protein using *N*-acetylneuraminyl-lactose, 0.17 U/mg protein using bovine submaxillary mucin), chromatographically purified neuraminidase from *C. perfringens* (type VI, 5.4 U/mg protein using *N*-acetylneuraminyl-lactose; 2.3 units/mg protein using bovine submaxillary mucin) and neuraminidase purified by affinity chromatography from *C. perfringens* (type IX, 30–50 U/mg protein using *N*-acetylneuraminyl-lactose) were purchased from Sigma Chemical Company. Wheat-germ agglutinin (extracted from *Triticum vulgaric*), peanut agglutinin (extracted from *Arachis hypogea*), purified neuraminidase from *Arthrobacter ureafaciens* (50 U/mg protein using *N*-acetylneuraminyl-lactose), purified neuraminidase from *Influenza virus* (1.6 U/mg at 37 at pH 5.6 using *N*-acetylneuraminyl-lactose) and purified neuraminidase from *Vibrio cholerae* (500 U/ml using *N*-acetylneuraminyl-lactose) were purchased from Calbiochem-Behring Corp. Purified neuraminidase from *C. perfringens* (0.6 U/mg dry powder using bovine submaxillary mucin) and purified neuraminidase extracted from *C. perfringens* (0.5 U/mg using bovine submaxillary mucin) were supplied by Boehringer-Mannheim Corp. and United States Biochemical Corp., respectively. Other reagents used were of analytical grade. ^{125}I-choriogonadotropin was prepared according to Dufau et al. [28].

Preparation of Plasma Membranes of Bovine Corpus Luteum

Plasma membranes were prepared from bovine corpora lutea as previously described [26,27] according to the procedure of Gospodarowicz [4] and were kept frozen at $-20\,^{\circ}C$ until use. Protein was assayed according to the procedure of Lowry et al. [29] using bovine serum albumin as standard.

Neuraminidase Treatment of Plasma Membranes

The plasma membranes from bovine corpus luteum were centrifuged at $10000 \times g$ for 15 min and resuspended in Tris buffer (10 mM Tris/HCl pH 7.4; 1 mM $CaCl_2$) at a protein concentration of 1 mg/ml. Unless otherwise indicated the membranes were incubated with 100 mU/ml neuraminidase. Where required, 0.1 mg/ml mucin, fetuin or bovine serum albumin were also added. After incubation usually at 37 °C for 60 min, 5 ml Tris buffer was added and membranes were sedimented by centrifugation at $10000 \times g$ for 15 min. The membrane were washed three times with excess Tris buffer. The membranes treated with neuraminidase and buffer (control) were then assayed for binding of ^{125}I-choriogonadotropin, activity of 5′-nucleotidase and content of sialic acid as described below.

Gonadotropin Binding to Plasma Membranes of Bovine Corpus Luteum

The assay for specific binding of ^{125}I-choriogonadotropin to plasma membranes was carried out by a slight modification of the procedure described earlier from this laboratory [26,27]. Briefly, aliquots of membrane fractions containing known amounts of protein (≈ 100 μg) were usually incubated at 37 °C for 60 min in 0.3 ml Tris/albumin buffer (10 mM Tris/HCl pH 7.4; 1 mM $CaCl_2$, and 0.1% bovine serum albumin) with ^{125}I-choriogonadotropin (80000–120000 counts/min; $20-50$ counts $\times$ min^{-1} $\times$ pg choriogonadotropin^{-1}). Following incubation, 0.2 ml of 0.1 M sodium phosphate buffer pH 7.0, 0.5 ml of 0.2% bovine immunoglobulin (w/v) in phosphate buffer and 1 ml of 25% poly(ethyleneglycol) (Carbowax 6000, Union Carbide, w/v) in phosphate-buffered saline were added. After rapid mixing, the tubes were left on ice for 10 min, centrifuged at $1500 \times g$ for 20 min at 4 °C and the supernatants were aspirated. The precipitates were resuspended in 1 ml of phosphate buffer and following addition of 1 ml of 25% poly(ethyleneglycol), the tubes were centrifugaed and the supernatants aspirated. The tubes containing sedimented precipitate (bound hormone) were counted for radioactivity in an automatic γ-spectrometer (Searle Analytic, model 1195). The specific binding of ^{125}I-choriogonadotropin was determined by subtracting from total radioactivity (in counts/min) the radioactivity that remained associated with the membrane fraction in the presence of a 1000-fold excess of unlabeled choriogonadotropin.

Determination of Sialic Acid

Sialic acid was assayed by the colorimetric procedure of Aminoff [30]. Membranes (1 mg protein) pretreated with buffer or neuraminidase (as described above) were incubated with 1 ml of 0.05 M H_2SO_4 at 80 °C for 1 h. After incubation, the samples were cooled to 4 °C and centrifuged at $15000 \times g$ for 10 min. Aliquots (0.5 ml) of the clear supernatant were assayed for sialic acid [30]. Sialic acid hydrolyzed by neuraminidase was then calculated from the difference between buffer-treated and the enzyme-treated membranes [31].

Fractionation of Plasma Membrane Sialic Acid into Ganglioside and Glycoprotein Fractions

Unless otherwise stated neuraminidase-treated and buffer-treated membranes were fractionated into ganglioside and glycoprotein fractions according to the procedure of Tetta-

manti et al. [32]. Briefly, membranes (approximately 10 mg protein) in 0.5 ml of 10 mM potassium phosphate buffer pH 6.8 were extracted with 4 ml of tetrahydrofuran. The suspension was centrifuged at $5000 \times g$ for 10 min at 15 °C. The supernatants were saved. The sedimented pellet was extracted three times with 0.5 ml buffer and 2 ml of tetrahydrofuran. To the pooled fractions, 0.3 vol. of diethyl ether was added and the tubes were vortexed for 2 min, then centrifuged at $5000 \times g$ for 10 min at 15 °C. The aqueous phase was saved and organic phase after the addition of 0.1 vol. of glass-distilled water was vortexed and centrifuged as above. The pooled aqueous phase from each sample after initial concentration in vacuum was extensively dialyzed against distilled water for 3 days with at least three changes a day. The dialyzed fraction was lyophilized and referred to as 'ganglioside fraction'. The tetrahydrofuran-extracted plasma-membrane residues which contained glycoproteins and the extracted ganglioside fractions were hydrolyzed in 0.05 M H_2SO_4 for 1 h at 80 °C [31,33] and released sialic acid was quantified by the colorimetric procedure of Aminoff [30]. One-hour hydrolysis in 0.05 M H_2SO_4 was found sufficient to quantitatively release all the bound sialic acid from glycoprotein and ganglioside fractions. In some cases gangliosides from plasma membranes of bovine corpus luteum were also extracted according to the published procedures of Suzuki [34], Ledeen et al. [35] and Harth et al. [36]. The sedimented residue in each case was used to quantify glycoprotein sialic acid. Thin-layer chromatography of gangliosides was performed as described earlier [24].

Enzyme Assays

Neuraminidase was assayed according to Cassidy et al. [37] and 5'-nucleotidase was determined by a modification of the method of Solyom and Trams [38]. Incubation mixtures for 5'-nucleotidase in a final volume of 1 ml contained 75 mM Tris/HCl pH 9.0, 10 mM KCl, 5 mM $MgCl_2$, 5 mM 5'AMP, and 50–100 μg of enzyme protein. Incubations were carried out at 37 °C for 15 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v). After centrifugation, aliquots (0.5 ml) of clear supernatants were assayed for liberated phosphate by the method of Fiske and Subbarow [39]. ATPase activity was assayed according to Kimelberg and Papahadjopoulos [40].

RESULTS

Effect of Incubation pH on Neuraminidase-Stimulated Receptor Activity

Since neuraminidases exhibit optimum pH in acidic range [37], we tested the ability of the enzyme to stimulate the receptor at pH 5.0, 6.0, 7.0 and 7.5. As shown in Fig. 1, at all pH values tested, neuraminidase effectively stimulated ^{125}I-choriogonadotropin binding. Similarly, the extent of stimulation was more or less constant although the receptor activity itself was less stable around pH 5.0. In subsequent experiments, plasma membranes were pretreated with neuraminidase at pH 7.4.

Effect of Increasing Concentrations of Neuraminidase on Plasma-Membrane-Associated Sialic Acid and Gonadotropin-Binding Activity

Since the stimulatory effect of neuraminidase presumably results from the hydrolysis of plasma-membrane-bound sialic acid, we examined this possibility by measuring sialic acid release from membranes following neuraminidase treatment concomitant with ^{125}I-choriogonadotropin-binding measurements. Treatment of membranes with neuraminidase resulted in progressive loss of cell-surface sialic acid with concomitant increase in ^{125}I-choriogonadotropin-binding activity (Fig. 2). The maximum hydrolysis of membrane-bound sialic acid was observed to 100 mU/ml of enzyme. Interestingly, the same concentrations of enzyme also maximally enhanced the ^{125}I-choriogonadotropin-binding activity.

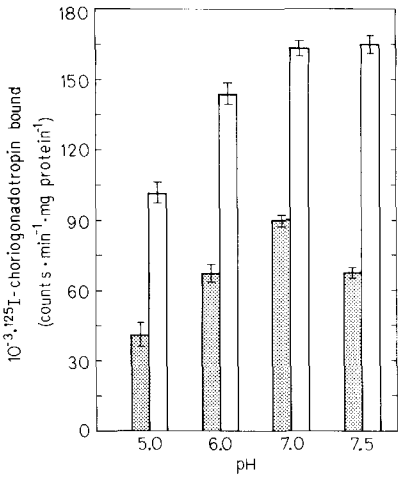

Fig. 1. *Effect of varying incubation pH on neuraminidase-stimulated ^{125}I-choriogonadotropin-binding activity.* Plasma membranes (≈ 1 mg protein) were pretreated with or without 100 mU *C. perfringens* neuraminidase (Sigma type V) at indicated pH for 60 min at 37 °C. After incubation, the membrane preparations were washed in Tris buffer and assayed for ^{125}I-choriogonadotropin binding. For pH 5.0 and 6.0 acetate buffer, pH 7.0 morpholinopropanesulfonic acid/NaOH and pH 7.5 Tris/HCl buffer were used. (▨) Buffer-treated membranes; (□) neuraminidase-treated membranes. The concentration of ^{125}I-choriogonadotropin used was 4.8 ng (120000 counts/min). Results are mean ± S.E.M.

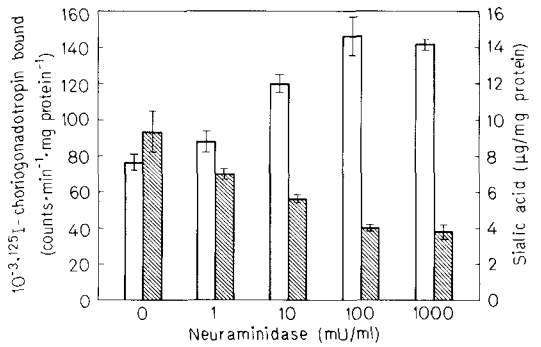

Fig. 2. *Effect of incubation of plasma membranes with increasing concentrations of neuraminidase on ^{125}I-choriogonadotropin binding and extent of hydrolysis of membrane-bound sialic acid.* Two sets of plasma membranes (1 mg protein) were incubated with indicated concentrations of neuraminidase. After enzymic treatment and washing of plasma membranes, one set of membranes were used to monitor ^{125}I-choriogonadotropin-binding activity (□). Samples from other set of membranes were extracted in 0.05 M H_2SO_4 and released sialic acid was assayed colorimetrically as described in Materials and Methods (▨). The concentration of ^{125}I-choriogonadotropin used was 3.8 mg (95000 counts/min). Results are mean ± S.E.M.

Table 1. *Effect of addition of mixed gangliosides, various glycoproteins, and neuraminidases on ^{125}I-choriogonadotropin binding to plasma membranes of bovine corpus luteum*
Aliquots of plasma membranes from bovine corpus luteum were centrifuged at 10000 × *g* for 15 min in the cold and resuspended in Tris buffer at a protein concentration of 1 mg/ml. Aliquots of membranes were incubated with indicated concentrations of various neuraminidase preparations. In the case of experiment 1 and 2, indicated concentrations of mucin, fetuin, bovine serum albumin or mixed brain gangliosides were also added. After incubation at 37 °C for 60 min, 5 ml Tris buffer was added and membranes were sedimented by centrifugation at 10000 × *g* for 15 min. The membranes were washed three times with excess buffer. The resultant membranes were then assayed for ^{125}I-choriogonadotropin binding 5′-nucleotidase or sialic acid content as described in Materials and Methods. The concentration of ^{125}I-choriogonadotropin used was 4 ng (120000 counts/min). Results are ± S.E.M.

Additions	$10^{-3} \times$ ^{125}I-choriogonadotropin bound	Sialic acid
	counts × min^{-1} × mg protein^{-1}	μg/mg protein
Experiment 1		
None	64.4 ± 2.14	
Neuraminidase (100 mU/ml)	114 ± 3.4	
Fetuin (0.1 mg/ml)	78.5 ± 1.29	
Nauraminidase (100 mU/ml) + fetuin (0.1 mg/ml)	75.1 ± 1.9	
Mucin (0.1 mg/ml)	70.7 ± 3.7	
Mucin (0.1 mg/ml) + neuraminidase (100 mU/ml)	77.4 ± 1.0	
Bovine serum albumin (0.1 mg/ml)	75.2 ± 0.8	
Bovine serum albumin (0.1 mg/ml) + neuraminidase (100 mU/ml)	134 ± 4.0	
Experiment 2		
None	84 ± 4.5	
Bovine-brain mixed gangliosides (200 μg/ml)	79 ± 7.8	
Neuraminidase (100 mU/ml)	183 ±14	
Neuraminidase + bovine-brain mixed gangliosides	128 ± 9	
Experiment 3		
None	74.71 ± 4.9	8.47 ± 0.9
Purified *C. perfringens* neuraminidase (Sigma type V, 0.31 U/mg protein)	148 ±10	3.42 ± 0.37
Chromatographically purified *C. perfringens* neuraminidase (Sigma type VI, 5.6 U/mg protein)	139 ± 2	3.55 ± 0.29
C. perfringens neuraminidase purified by affinity chromatography (Sigma type IX, 30–50 U/mg protein)	152 ± 7	3.70 ± 0.24
Purified *C. perfringens* neuraminidase (U.S. Biochemical Corp., 0.5 U/mg rotein)	130 ± 6	5.1 ± 0.73
Purified *C. perfringens* neuraminidase (Boehringer-Mannheim, Corp., 0.6 U/mg dry powder)	128 ±13	5.3 ± 0.33
Experiment 4		
None	127 ± 4	11.68 ± 0.26
C. perfringens neuraminidase (0.1 U/ml)	196 ± 1.8	5.6 ± 0.27
V. cholerae neuraminidase (1 U/ml)	167 ± 2.2	6.39 ± 0.6
Influenza virus neuraminidase (1 U/ml)	137 ± 3.9	11.34 ± 0.37
A. ureafaciens neuraminidase (0.1 U/ml)	175 ± 5.5	7.13 ± 0.30

Effect of Various Glycoproteins, Gangliosides and Different Preparations of Neuraminidase on Gonadotropin Receptor Activity

Since sialic-acid-containing glycoprotein and gangliosides are suitable substrates for neuraminidase, it was of interest to see if coincubation of membrane with these substances could block subsequent enzyme-stimulated binding activity. Results presented in Table 1 show that fetuin and mucin effectively blocked the stimulatory effect when added along with neuraminidase. Addition of mixed gangliosides (bovine brain) partially reversed the stimulatory effect of neuraminidase on ^{125}I-choriogonadotropin binding to plasma membranes although this effect was less pronounced compared to mucin or fetuin. By contrast, addition of these agents or different concentrations of *N*-acetylneuraminic acid (up to 200 μg/ml) to plasma membranes pretreated with neuraminidase did not affect neuraminidase-stimulated activity (data not shown). Both chromatographically purified preparation and the enzyme purified by affinity chromatography stimulated ^{125}I-choriogonadotropin binding and hydrolysis of sialic acid to the same extent (Table 1, experiment 3). These results were comparable to the results obtained with the enzyme purified by salt fractionation (Sigma, type V). In contrast, two other preparations of *Clostridium perfringens* neuraminidase, although enhanced gonadotropin binding and sialic acid hydrolysis, were slightly less effective when compared to the type-V enzyme. The effect of neuraminidases isolated from *C. perfringens*, *Vibrio cholerae*, influenza virus and *Arthobacter ureafaciens* on ^{125}I-choriogonadotropin binding and sialic acid hydrolysis is shown in Table 1 (experiment 4). Among these preparations, the *C. perfringens* enzyme was most effective followed by the *V. cholerae* and *A. ureafaciens* enzymes. Contrary to its stimulation of binding activity, the *A. ureafaciens* enzyme, however, released less sialic acid from plasma membranes compared to the *V. cholerae* as well as the *C. perfringens* enzyme. Neuraminidase purified from influenza virus showed no significant effect either on the binding or on the hydrolysis of plasma-membrane-bound sialic acid.

Effect of Increasing ^{125}I-Choriogonadotropin Concentrations on Neuraminidase-Stimulated Gonadotropin Receptor Activity

Results presented in Fig. 3 demonstrate Scatchard plot [41] analysis of ^{125}I-choriogonadotropin binding to plasma membranes previously exposed to neuraminidase. It is evident that the neuraminidase treatment unmasked and increased the gonadotropin-binding sites without any significant effect upon the affinity of receptor for the hormone. The buffer-treated membranes contained 70.9 fmol binding sites/mg protein while neuraminidase treatment increased the number of binding sites to 101 fmol/mg protein. The dissociation constants (K_d) for buffer-treated and neuraminidase-treated membranes were 1.04×10^{-10} M and 0.8×10^{-10} M, respectively.

Lack of Effect of Neuraminidase on Glycoprotein Enzymes, 5′-Nucleotidase and ATPase

To rule out the nonspecific effect of neuraminidase on gonadotropin receptor activity, we also studied the effect of this enzyme on 5′-nucleotidase and ATPase. 5′-Nucleotidase activity was extremely susceptible to concanavalin A and almost 80% of the activity was inhibited by the addition of 10 μg/ml of this lectin. Higher concentrations of wheat-germ

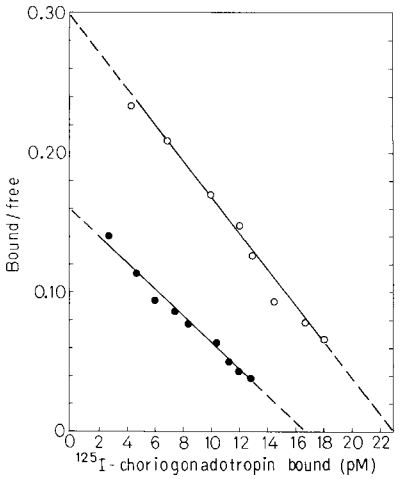

Fig. 3. *Scatchard plot of gonadotropin binding to buffer-treated and neuraminidase-treated membrane in the presence of increasing concentrations of ^{125}I-choriogonadotropin.* Plasma membranes (≈ 1 mg/ml) were incubated without or with 100 mU/ml of *C. perfringens* neuraminidase (Sigma, type V) for 60 min at 37 °C. Following washing of membranes, suitable aliquots (≈ 100 µg membrane proteins in triplicate) were incubated with increasing concentrations of ^{125}I-choriogonadotropin (starting from 10000 – 150000 counts/min) at 37 °C for 60 min. Other details were similar to that described in Materials and Methods. The values bound and free were then utilized to construct a Scatchard plot [41]. (●——●) Buffer-treated membranes; (○——○) neuraminidase-treated membranes

agglutinin also inhibited the enzyme activity although the extent of inhibition was less pronounced compared to concanavalin A. Addition of specific sugars α-methyl-D-pyranoside (50 mM) and *N*-acetyl-D-glucosamine (50 mM) completely reversed the inhibitory action of concanavalin A and wheat-germ agglutinin, respectively. By contrast, 5′-nucleotidase activity was not affected by neuraminidase treatment. ATPase activity was also partly inhibited by concanavalin A. Different concentrations of neuraminidase from 10 mU/ml to 1000 mU/ml failed to modulate 5′-nucleotidase activity (data not shown).

Neuraminidase-Mediated Hydrolysis of Glycoprotein-Bound and Ganglioside-Bound Sialic Acid from Plasma Membrane

Results presented in Table 2 compare the sialic acid content of ganglioside and glycoprotein fractions isolated by different procedures. The percentage of sialic acid recovered in glycoprotein and ganglioside fractions isolated by four different procedures was more or less comparable. However, the procedure of Tettamanti et al. [32] was of choice because of its simplicity and it requires much lower amount of membranes (10 mg protein) compared to the other procedures (at least 50 mg protein). Isolated fractions were also characterized by thin-layer chromatography using the procedure described earlier from this laboratory [24]. Thin-layer chromatography of the ganglioside fraction demonstrated that more than 95% of total sialic acid applied was recovered in separated gangliosides. In contrast, thin-layer chromatography of the glycoprotein fraction failed to show the presence of ganglioside.

Results presented in Table 3 show the distribution and hydrolysis of sialic acid in glycoprotein and ganglioside components of plasma membranes. The ganglioside fraction contained approximately 2.5-times more sialic acid than the glycoprotein fraction. Treatment of plasma membranes with neuraminidase, however, hydrolyzed 50 – 60% sialic acid from

Table 2. *Comparison of various extraction procedures on the recovery of sialic acid ganglioside and glycoprotein fractions of plasma membranes from bovine corpus luteum*
Total sialic acid content of isolated corpus luteum plasma membrane was 13.75 ± 0.47 µg/mg protein ± S.E.M. The results are means of three different experiments

Procedure according to	Sialic acid	
	Gangliosides	Glycoprotein
	µg/mg protein	
Suzuki [34]	7.94	3.25
Ledeen et al. [25]	8.32	3.43
Harth et al. [36]	8.81	3.83
Tettamanti et al. [32]	8.70	3.74

Table 3. *Stimulation of gonadotropin receptor activity and hydrolysis of ganglioside-associated and glycoprotein-associated sialic acid of plasma membranes from bovine corpus luteum by neuraminidase*
Plasma membranes were treated with *C. perfringens* neuraminidase as described in Table 1. The gangliosides and glycoprotein fraction were isolated and assayed for sialic acid as described in Materials and Methods. The concentration of ^{125}I-choriogonadotropin used was 4 ng (80000 counts/min). Results are ± S.E.M.

Additions	Sialic acid			^{125}I-choriogonadotropin bound
	total	gangliosides	glycoprotein	
	µg/mg protein			counts × min^{-1} × mg protein^{-1}
None	12.09 ± 0.82	7.81 ± 0.43	2.97 ± 0.22	85.6 ± 7.8
Neuraminidase	5.37 ± 0.26	3.93 ± 0.12	1.32 ± 0.08	154 ± 11.7

both fractions (Table 3). Under identical experimental conditions, ^{125}I-choriogonadotropin-binding activity was enhanced from 85.6 ± 7.8 × 10^{-3} counts bound × min^{-1} × mg protein^{-1} to 154 ± 11.7 × 10^{-3} counts bound × min^{-1} × mg protein^{-1}.

Role of Glycoprotein-Associated Sialic Acid in Gonadotropin-Receptor Interaction

Since in the above experiments (Table 3) neuraminidase treatment released 50 – 60% sialic acid from glycoprotein and ganglioside components, further experiments were performed to investigate which of the component plays a role in gonadotropin-receptor interaction. Since wheat-germ agglutinin also interacts with the terminal sialic acid [43 – 46] in addition to *N*-acetyl-D-glucosamine [42], we made use of this lectin to declineate the role of ganglioside and/or glycoprotein sialic acid in hormone-receptor interaction. Results presented in Table 4 demonstrate the effect of pretreatment of plasma membranes with wheat-germ agglutinin, concanavalin A, or peanut agglutinin on subsequent hydrolysis of sialic acid and the stimulation of ^{125}I-choriogonadotropin-binding activity by neuraminidase. Wheat-germ agglutinin treatment of membranes significantly blocked (57%) the hydrolysis of glycoprotein sialic acid while it only slightly reduced the extent of hydrolysis of ganglioside sialic acid. Under identical conditions, interestingly, wheat-germ agglutinin also partially pre-

Table 4. *Effect of various plant lectins on sialic acid hydrolysis from plasma membranes of bovine corpus luteum by neuraminidase*
Aliquots of plasma membranes (1 mg/ml) were first incubated with or without lectins and incubated at 30 °C for 30 min. At the end of incubation *C. perfringens* neuraminidase was added and incubation continued at 37 °C for 60 min. After the second incubation one set of membranes was processed for extraction and quantification of ganglioside-associated and glycoprotein-associated sialic acid. The other set of membranes was then incubated either with no sugar or with α-methyl-D-mannoside (50 mM for concanavalin A), *N*-acetyl-D-glucosamine (50 mM) along with *N*-acetylneuraminic acid (10 mM both for wheat-germ agglutinin) or galactose (50 mM for peanut agglutinin) to remove plasma-membrane-bound lectins. The sugar-treated membranes were then assayed for ^{125}I-choriogonadotropin binding as described in Materials and Methods. The concentrations of ^{125}I-choriogonadotropin used was 4 ng (100000 counts/min). Sialic acid is expressed as a percentage of the sialic acid released from control membranes not preexposed. ^{125}I-choriogonadotropin is expressed as a percentage of that bound from control membrane not preexposed to lectins

Additions	Lectin concentration	Sialic acid		^{125}I-choriogonadotropin bound
		glycoprotein	gangliosides	
		%		
Neuraminidase	0	100	100	100
Wheat-germ agglutinin	750	43 ± 6	86 ± 4	46 ± 3
Concanavalin A	1000	87 ± 5	91 ± 6	92 ± 2
Peanut agglutinin	750	98 ± 1	97 ± 2	100

vented the stimulatory action of neuraminidase on ^{125}I-choriogonadotropin-binding activity. By contrast, α-methyl-D-mannoside (concanavalin A) and galactose (peanut agglutinin) specific lectins were without any effect either in the hydrolysis of membrane-associated sialic acid or on gonadotropin-receptor activity. These studies thus establish a direct relationship between sialic acid content of glycoprotein and gonadotropin receptor activity. Furthermore, it is also evident from these studies that sialoglycoprotein(s) play a major regulatory role in gonadotropin-receptor interactions.

DISCUSSION

Earlier studies have described physico-chemical properties of gonadotropin-receptors of ovarian tissues [4–11]. However, precise knowledge about the function of gonadotropin receptors within the membrane environment and the regulatory role exerted by major membrane constituents in gonadotropin-receptor interaction is not established. Previous studies from this laboratory suggest that phospholipids are required for structural integrity as well as for maximal activity of gonadotropin receptors associated with the membrane of bovine corpus luteum [26,27]. By contrast, we have shown that gangliosides play no major role in gonadotropin interaction to ovarian plasma membranes and/or in receptor-mediated and tropic-hormone-induced responses [22–24]. Thus, incubation of ovarian plasma membranes or cells with various gangliosides failed to modulate ^{125}I-choriogonadotropin binding or production of cyclic AMP and progesterone. Under identical experimental conditions the action of cholera toxin which specifically interacts with G_{M1} [47–49] was blocked by coincubation with gangliosides [22–24]. Although gangliosides were previously shown to inhibit thyrotropin, lutropin and choriogonadotropin binding to target cell membranes [17–21] the subsequent studies by Pacuszka et al. [25] suggest that this phenomenon may not have any physiological significance. They were able to observe ganglioside-mediated inhibition of ^{125}I-choriogonadotropin binding to testicular plasma membranes when binding studies were performed under nonphysiological conditions (low ionic strength buffer, 0.025 mM Tris/acetate buffer pH 6; 0.6% bovine serum albumin) and 2500-fold excess unlabeled choriogonadotropin did not block ^{125}I-choriogonadotropin binding. In contrast, when the binding assay was carried out in physiological buffer [50] and where binding was blocked by 250-fold excess choriogonadotropin (designated as 'specific binding') gangliosides were found to be noninhibitory [25]. Thus, studies from our laboratory as well as by Pacuszka et al. [25] support the notion that gangliosides or ganglioside-like structures are not involved in gonadotropin-receptor interaction and do not play a direct role in transmitting the hormonal signals across the gonadal tissue plasma membranes. In the present studies we have used neuraminidase to modify cell-surface sialic acid content in order to identify the role of cell-surface carbohydrates in gonadotropin-receptor interaction. These studies suggest that changes in the plasma-membrane sialic acid (glycoprotein) content lead to modulation of gonadotropin-receptor interaction so as to increase the hormone-binding capacity. We have presented evidence that neuraminidase stimulation of gonadotropin receptor activity was due to intrinsic activity rather than a nonspecific enzymatic contamination. This conclusion was supported by the following points. (a) Different preparations of *Clostridium perfringens* purified to varying extents stimulated ^{125}I-choriogonadotropin binding equally when plasma membranes were exposed to a fixed (100 mU/ml) concentration of these preparations; (b) the stimulatory effect of neuraminidase was blocked by coincubation with the glycoprotein mucin, fetuin or bovine brain mixed gangliosides; (c) the activity of 5′-nucleotidase, a glycoprotein enzyme, was not affected by neuraminidase treatment; and (d) the extent of stimulation of binding activity was followed by parallel hydrolysis of cell-surface sialic acid.

Although *C. perfringens* and *Vibrio cholerae* enzymes were equally effective in unmasking gonadotropin-binding activity, the optimum concentrations of these two were quite different. The requirement for higher amounts of *V. cholerae* enzyme, however, cannot be explained on the basis of differences in enzyme properties since both of these bacterial enzymes have been reported to exhibit similar properties [37,51,52]. The structure of the substrate to some extent can influence the rate, although prolonged incubation and/or higher concentrations of either enzyme could result in the liberation of essentially all of the accessible sialic acid residues [37,51,52]. The rate of hydrolysis of plasma-membrane sialic acid by these two enzymes, therefore, could be influenced not only by the position of sialic acid linkage to the penultimate sugar in the carbohydrate chain [37], but may also be dependent upon the incubation time.

Fractionation of plasma membranes into glycoprotein and ganglioside revealed that neuraminidase released 50–60% of the bound sialic acid from these fractions. The partial release may be due to unique topographical arrangements of gangliosides and glycoproteins in such a way that remaining sialic acid residues are inaccessible to the enzyme or may represent specificity of the enzymes toward certain class of oligosaccharides from glycoconjugates or both. The fact that neuraminidase hydrolysis of sialic acid bound to glycopro-

teins is partially blocked by wheat-germ agglutinin along with corresponding decrease in enzyme-stimulated ^{125}I-choriogonadotropin-binding activity allows us to propose a role for glycoprotein, including sialic acid, in gonadotropin-receptor interaction. The extreme susceptibility of these glycoproteins to neuraminidase leaves little doubt that these are present as external constituents of plasma membranes with exposed oligosaccharide groups. The inability of concanavalin A and peanut agglutinin to protect sialoglycoprotein hydrolysis not only suggests that oligosaccharides that bind concanavalin A and peanut agglutinin are quite separable from that of sialic-acid-containing oligosaccharides, but also suggests a unique regulatory role of sialic acid in gonadotropin-receptor interaction. Previously sialoglycoproteins have been assigned a regulatory role in neurotransmitter [53], cation [54] and wheat-germ agglutinin [31] interaction to their receptors. Similarly, removal of surface sialic acid leads to inhibition of synaptic transmission [55] and neuraminidase treatment of liver plasma membranes is reported to enhance the ability of these membranes to bind cholera enterotoxin [56]. The elegant work of Ashwell and associates have shown that removal of terminal sialic acid residue from serum glycoproteins lead to specific binding of these proteins to liver plasma membranes and subsequent rapid clearance from the circulation [57–60]. Further incubation of thyroid plasma membranes with *N*-acetylneuramic acid was shown to enhance ^{125}I-thyrotropin-binding activity, although neuraminidase removal of plasma membrane sialic acid did not affect hormone binding [61].

We wish to thank Ms Monica Romero for the excellent technical assistance and Ms Juanita Boldt for secretarial services. This work was supported by grant HD 06656 from the National Institutes of Health.

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