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Receptor-mediated gonadotropin action in the ovary

Inhibitory actions of concanavalin A and wheat-germ agglutinin on gonadotropin-stimulated cyclic AMP and progesterone responses in ovarian cells

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Pretreatment of ovarian cells with concanavalin A and wheat-germ agglutinin blocked the gonadotropin-induced cyclic AMP and progesterone responses and this effect was time- and concentration-dependent. Basal production of either cyclic AMP or progesterone, however, was not affected by treatment of cells with lectin. The effect of concanavalin A on gonadotropin-mediated cyclic AMP and progesterone responses was blocked by α-methyl D-mannoside and α-methyl D-glucoside. Similarly the inhibitory effect of wheat-germ agglutinin was reversed by *N*-acetyl-D-glucosamine. Pretreatment of ovarian cells with concanavalin A or wheat-germ agglutinin had no effect on protein synthesis in the ovary as monitored by [^{3}H]proline incorporation studies. Concanavalin A and wheat-germ agglutinin did not affect steroid production in response to dibutyryl cyclic AMP and 8-bromo cyclic AMP, indicating that the inhibitory action of lectin was occurring at a step before cyclic AMP formation. Lectins specific for L-fucose, D-galactose and *N*-acetyl-D-galactosamine, gorse seed agglutinin, peanut agglutinin and *Dolichos biflorus* agglutinin respectively, did not interfere with gonadotropin-induced cyclic AMP and progesterone responses. The present studies suggest that gonadotropin receptors may be glycoprotein in nature or closely associated with glycoprotein structures with the carbohydrate chain containing *N*-acetyl-D-glucosamine, mannose and possibly *N*-acetylneuraminic acid.

In the ovary, gonadotropins regulate several intracellular metabolic processes including steroidogenesis by first binding to specific receptor sites on the external surface of cell membranes (Menon & Gunaga, 1974; Cuatrecasas, 1974; Channing & Tsafriri, 1977). The precise knowledge of the events and the exact mechanism by which gonadotropin receptors function within membrane environment and regulate biological responses is not yet understood. Previously we provided evidence for the regulatory role of membrane phospholipids in gonadotropin–receptor interactions (Azhar & Menon, 1976; Azhar *et al.*, 1976). However, our efforts to demonstrate a role for glycolipid (gangliosides) on gonadotropin binding and subsequent biological responses in ovarian tissues have been so far unsuccessful (Azhar & Menon, 1978, 1979*a*; Azhar *et al.*, 1978). Since gonadotropin receptors 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. In the present studies, we have utilized different sugar-specific lectins of known specificity (Lis & Sharon, 1973; Nicolson, 1974) to determine the potential involvement of carbohydrates on the ovarian cells in gonadotropin-mediated cyclic AMP and progesterone production by using a collagenase-dispersed ovarian cell suspension. Our results demonstrate that concanavalin A and WGA treatment of ovarian cells leads to subsequent inhibition of LH/hCG-induced biological responses.

Abbreviations used: WGA, wheat-germ agglutinin; LH, lutropin ('luteinizing hormone'); hCG, human choriogonadotropin ('human chorionic gonadotropin').

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Materials and methods

Materials

Sprague–Dawley rats (26 days old) were purchased from Spartan Farms, Hazlett, MI, U.S.A.

8-Bromo cyclic AMP, dibutyryl cyclic AMP and

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lectin from *Canavalia ensiformis* (concanavalin A) were purchased from Sigma Chemical Co. Purified hCG (11000units/mg; lot no. CR 119) was kindly supplied by Dr. R. E. Canfield, Columbia University, New York, NY, U.S.A., through the Center for Population Research, NICHD, NIH, Bethesda, MD, U.S.A. Ovine LH (lot no. S18) and bovine LH (lot no. B9) were gifts from the Hormone Distribution Program, NIAMDD, NIH. Eagle's minimum essential medium with Earl's salts (medium 199) was purchased from Grand Island Biological Co. The following lectins were obtained from Calbiochem: gorse seed agglutinin I (extracted from *Ulex europaeus*); lentil agglutinin (extracted from *Lens culinaris*); peanut agglutinin (extracted from *Arachis hypogea*); soya-bean agglutinin (extracted from *Glycine soja*); WGA (extracted from *Triticum vulgaris*); *Dolichos biflorus* lectin. [8-^{3}H]Adenosine 3′:5′-cylic monophosphate (sp. radioactivity 30Ci/mmol) and [1,2,6,7(n)-^{3}H]progesterone (sp. radioactivity 97.9Ci/mmol) were products of New England Nuclear. All other reagents used were of reagent grade.

Preparation of rat ovarian cells

Interstitial cells were prepared from ovaries of 26-day-old rats by collagenase dispersion as described previously from this laboratory (Kawano *et al.*, 1975; Clark & Menon, 1976; Sen *et al.*, 1979). The cells were washed twice and resuspended in medium 100 containing bovine serum albumin (1mg/ml). Cells were counted in a haemocytometer and viability was determined by dye exclusion after staining in 0.02% nigrosin. DNA was estimated by the method described by Burton (1956).

Lectin treatment of cells

Washed cells (1×10^7 cells) were incubated with concanavalin A, WGA or other lectins in a final volume of 1ml of medium 199. Usually cells were incubated for 30min at 37°C in an atmosphere of O_2/CO_2 (19:1, v/v). Details regarding the concentrations of lectin, time periods and temperature are given in the legend of each Table and Figure. After incubation, cells were centrifuged at 600*g* for 3min. The sedimented cells were washed twice with medium 199 and finally suspended in the same medium at a concentration of approx. 1×10^7cells/ml. Samples of cells (approx. 1×10^6 cells) were then used for ^{125}I-labelled hCG binding and hCG-stimulated cyclic AMP and progesterone measurements.

Incubation conditions for cyclic AMP and progesterone measurements

Approx. 2×10^6 ovarian cells (0.1ml) were transferred into test tubes containing 0.3ml of medium 199, 0.1% bovine serum albumin, and, where required, different concentrations of hCG, LH or cyclic nucleotides. The cells were incubated at 37°C in a Dubnoff metabolic shaking incubator for 2h in the presence of O_2/CO_2 (19:1, v/v). After incubation, the reaction was stopped by placing the sample tubes in a boiling-water bath for 3min, after which 0.6ml of water and 10μl (approx. 10000c.p.m.) of [^{3}H]progesterone (to monitor recovery) was added and the samples were left in the cold overnight. Samples were then extracted with light petroleum (b.p.–°C) and assayed for progesterone by radioimmunoassay as described previously (Kawano *et al.*, 1975; Clark & Menon, 1976).

Cyclic AMP determination

Ovarian cells (2×10^6) were dispersed in a final volume of 0.4ml of medium 199 containing 0.1% bovine serum albumin, and, where required, 3-isobutyl-1-methylxanthine, hCG or LH. After incubation, usually for 2h, the reaction was stopped by placing the tubes in a boiling-water bath for 5min, and then transferred to ice, after which 0.5ml of trichloroacetic acid (10%, w/v) and 0.1ml of cyclic [^{3}H]AMP (approx. 2000c.p.m.) were added and the samples were stored overnight in the cold. After centrifugation, the clear supernatant fractions were processed and assayed for cyclic AMP by the procedure of Gilman (1970) as described previously (Menon & Azhar, 1978; Azhar & Menon, 1979*b*).

Measurement of protein synthesis

Protein synthesis in ovarian cells was measured by following [^{3}H]proline incorporation as described previously (Azhar *et al.*, 1981).

Results

Effect of various lectin concentrations on gonadotropin-stimulated steroidogenesis

To determine the effect of lectins on steroidogenesis, ovarian cells were pretreated with increasing concentrations of concanavalin A and WGA. After washing cells with medium 199 to remove free lectins, the cells were incubated with or without hCG or LH to monitor the extent of gonadotropin-stimulated progesterone production. In the absence of lectin pretreatment, both LH- and hCG-stimulated steroidogenesis 30–40-fold over control values (Fig. 1). With increasing lectin concentrations up to 500μg/ml, the maximal response to gonadotropin was progressively decreased. The ED_{50} (median effective dose) of concanavalin A for hCG- and LH-stimulated progesterone production by ovarian cells was 230μg/ml and 86μg/ml respectively. Similarly, the ED_{50} values of WGA for hCG- and LH-induced steroidogenesis were computed to be 66μg/ml and 80μg/ml respectively. In contrast, both lectins

showed no effect on basal production of progesterone (Fig. 1).

Inhibitory action of lectins on steroidogenesis in response to increasing concentrations of LH and hCG

The influence of WGA and concanavalin A on progesterone production in response to different concentrations of hCG or LH is shown in Fig. 2. In a typical experiment, the ovarian cells produced progesterone in response to hCG and LH in a dose-related manner. In the untreated cells, maximal steroidogenesis was achieved at hCG and LH concentrations of 10ng/ml and 100ng/ml respectively. Pretreatment of ovarian cells with WGA and concanavalin A partially blocked the stimulatory effect of LH and hCG. Although WGA- and concanavalin A-treated cells exhibited enhanced steroidogenesis in response to increasing concentrations of hormone, the extent of stimulation was much lower than that observed with corresponding doses of hormone in cells not previously exposed to lectins. Furthermore, even a concentration of 1 μg of gonadotropin/ml failed to overcome the inhibitory action of either lectin.

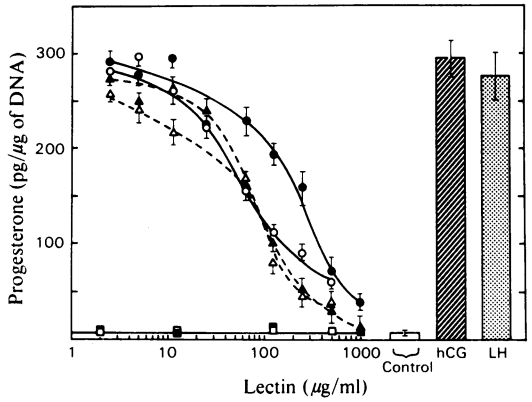

Fig. 1. *Effect of increasing concentrations of WGA and concanavalin A on LH- and hCG-stimulated steroidogenesis*

Incubation conditions were as described in the Materials and methods section. ●, hCG + concanavalin A; ○, hCG + WGA; ▲, LH + concanavalin A; △, LH + WGA; ■, concanavalin A; □, WGA. The concentrations of hCG and LH (lot no. NIH-LH B9) used were 10ng/ml and 100ng/ml respectively.

Effect of various incubation times on LH- and hCG-stimulated steroidogenesis in cells pretreated with lectins

In control cells, addition of maximal concentrations of hCG (10ng/ml) or LH (100ng/ml) resulted in a 15–30-fold increase in steroid production, reaching a maximum at 2–3h. Pretreatment of ovarian cells with concanavalin A or WGA leads to inhibition of LH- or hCG-stimulated steroidogenesis at all time points (60, 90, 120 and 180min) examined. Lectin treatment, however, failed to affect basal progesterone production at all time points examined.

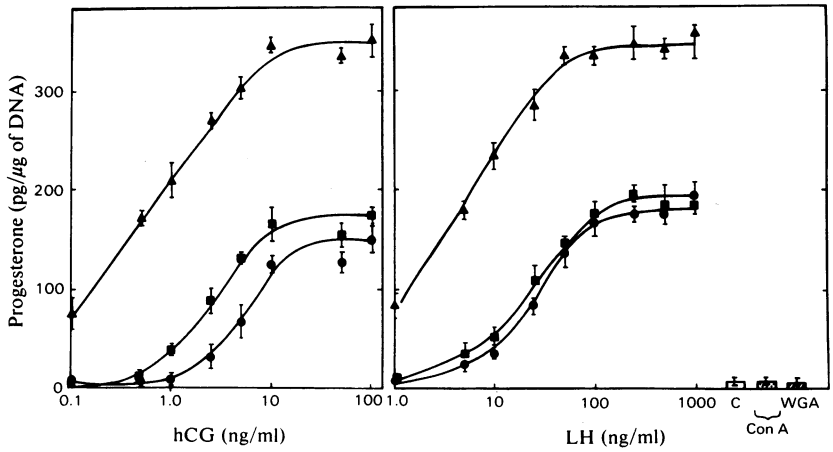

Fig. 2. *Effect of concanavalin A and WGA on progesterone production in response to increasing concentrations of LH and hCG*

Lectin pretreatment and other incubation conditions were as described in the Materials and methods section. The concentrations of concanavalin A and WGA used were 500μg/ml and 250μg/ml respectively. ▲, No lectin; ■, concanavalin A; ●, WGA. The columns on the right of the Figure represent basal production of steroid. The bars represent ±S.E.M. Abbreviations used: C, control; Con A, concanavalin A.

Reversal of inhibitory action of concanavalin A by specific sugars

To test whether the inhibitory action of these lectins was due to interference with gonadotropin receptor function or due to a secondary non-specific effect on cellular metabolism, the following experiments were performed. Cells were pretreated with concanavalin A or WGA and then incubated in the presence of gonadotropins and/or various sugars. Results presented in Table 1 show that α-methyl D-mannoside and α-methyl D-glucoside, the sugars specific for concanavalin A, completely reversed the inhibitory action of this lectin. Sugars that are not specific for concanavalin A, such as L-fucose and D-galactose, were not effective in reversing the inhibitory effect of concanavalin A on hCG- or LH-induced steroidogenesis. In a similar fashion, the inhibitory action of WGA on LH- and hCG-stimulated progesterone production was reversed by *N*-acetyl-D-glucosamine, a sugar specific for WGA. The sugars, D-galactose, L-fucose, *N*-acetyl-D-galactosamine and α-methyl D-mannoside were without effect on WGA-inhibited progesterone synthesis in response to gonadotropins (results not shown).

Lack of effect of concanavalin A and WGA on total cellular protein synthesis

Since gonadotropin-induced steroidogenesis in the ovary is dependent on translational and transcriptional processes (Kawano *et al.*, 1975; Azhar *et al.*, 1981), we tested the possibility that a part of the inhibitory action of these lectins might be due to inhibition of cellular protein synthesis. Pretreatment of ovarian cells with WGA or concanavalin A did not affect total protein synthesis. The incorporation of [^{3}H]proline into total proteins in cells pretreated with concanavalin A (500 μg/ml), WGA (250 μg/ml) or medium alone was 5.2 ± 0.2, 5.8 ± 0.4 and 5.6 ± 0.3 pmol/μg of DNA (mean ± S.E.M.) respectively. Under identical experimental conditions, addition of cycloheximide blocked total cellular protein synthesis about 90% [0.18 ± 0.03 pmol/μg of DNA (mean ± S.E.M.)].

Effect of concanavalin A and WGA on LH- and hCG-stimulated cyclic AMP synthesis

The results reported so far suggest that concanavalin A and WGA inhibition of steroidogenesis is possibly due to interference by these lectins with hormone–receptor interaction. Since the initial event in gonadotropin action is the binding of hormone to ovarian cell surface followed by activation of adenylate cyclase, we investigated the time course of gonadotropin-stimulated cyclic AMP synthesis in cells pretreated with concanavalin A or WGA. Data in Fig. 3 show the inhibitory effect of these lectins on hCG-stimulated cyclic AMP formation. Addition of hCG to control cells caused a rapid increase in cyclic AMP formation, and this stimulatory effect was increased with incubation time, reaching a maximum at 60–90 min. Prior exposure of ovarian cells to concanavalin A and WGA resulted in a diminished cyclic AMP response to hCG. The lectin inhibition was immediate, and this effect persisted throughout the incubation period. In contrast, basal production of cyclic AMP was not affected by lectins as was the case with steroidogenesis.

Data in Fig. 4 show concanavalin A inhibition of cyclic AMP production in response to hCG and LH. Increasing concentrations of hCG- and LH-stimulated cyclic AMP accumulation in a concentration-dependent manner. Treatment of ovarian cells with concanavalin A resulted in inhibition of cyclic AMP response at all concentrations of hCG and LH

Table 1. *Reversal of the inhibitory action of concanavalin A on LH- and hCG-induced steroidogenesis by α-methyl D-mannoside and α-methyl D-glucoside*

After lectin pretreatment, the cells were incubated for 15 min with or without indicated sugars. Steroidogenesis was then initiated by the addition of LH (100 ng/ml) or hCG (10 ng/ml) and incubation was continued for a period of 3 h. The samples were then processed for progesterone measurement by radioimmunoassay. Results are means ± S.E.M.

	Progesterone (pg/μg of DNA)			
Additions	Basal	LH (lot no. NIH-LH B9)	hCG (lot no. CR-119)	LH (lot no. NIH-LH S18)
None	8.2 ± 0.4	415 ± 25	498 ± 29	457 ± 17
Concanavalin A (500 μg/ml)	9.6 ± 0.7	249 ± 24	158 ± 16	274 ± 19
Concanavalin A + α-methyl D-mannoside (50 mM)	13.1 ± 1.4	428 ± 21	465 ± 16	432 ± 33
Concanavalin A + α-methyl D-glucoside (50 mM)	12.8 ± 0.9	432 ± 15	473 ± 25	506 ± 42
Concanavalin A + L-fucose (50 mM)	11.7 ± 1.2	274 ± 14	174 ± 20	258 ± 21
Concanavalin A + D-galactose (50 mM)	12.2 ± 0.8	237 ± 23	166 ± 16	291 ± 11

tested. Even at very high concentrations, LH or hCG could not overcome the inhibitory effect of concanavalin A. Similarly concanavalin A or WGA led to inhibition of gonadotropin-stimulated cyclic AMP production whether the response was measured in the presence or the absence of 3-isobutyl-1-methylxanthine.

Effect of various lectins on gonadotropin-stimulated cyclic AMP and progesterone production

Data in Table 2 demonstrate that, among various sugar-specific lectins, only concanavalin A and WGA were able to inhibit hCG-stimulated cyclic AMP and progesterone responses. The lectins specific for *N*-acetyl-D-galactosamine, D-galactose and L-fucose showed no effect on either response. In contrast with concanavalin A, lentil agglutinin, which is also specific for mannose-type residues, was without any effect. A possible explanation for the

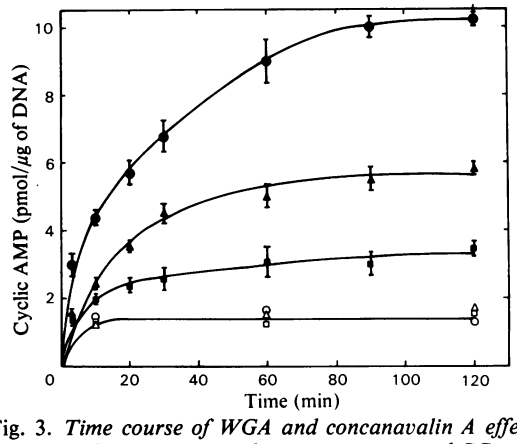

Fig. 3. *Time course of WGA and concanavalin A effect on cyclic AMP accumulation in response to hCG*
Incubation conditions were as described in the Materials and methods section. ●, hCG; ▲, concanavalin A + hCG; ■, WGA + hCG; ○, basal; △, concanavalin A; □, WGA. The concentrations of hCG, concanavalin A and WGA used were 1 μg/ml, 500 μg/ml and 250 μg/ml respectively. Results are means ± S.E.M.

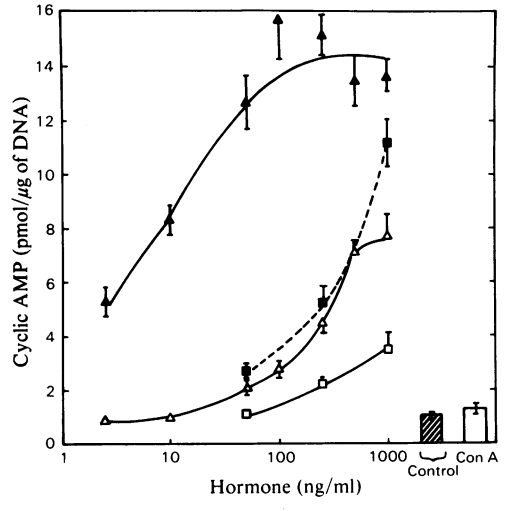

Fig. 4. *Effect of pretreatment of cells with concanavalin A on cyclic AMP accumulation in response to LH and hCG*
Incubation conditions were as described in the Materials and methods section, except that samples were incubated with LH or hCG for 2 h. ▲, hCG; △, hCG + concanavalin A; ■, LH (lot no. NIH-LH S18); □, LH + concanavalin A. The columns on the right of the Figure represent basal production of cyclic AMP. Results are means ± S.E.M. Abbreviation used: Con A, concanavalin A.

Table 2. *Effect of various sugar-specific lectins on cyclic AMP and progesterone production by ovarian cells in response to hCG*
Ovarian cells (1×10^7 cells) were pretreated with indicated concentrations of various lectins as described in the legend to Fig. 1. The lectin-treated cells (approx. 2×10^6 cells) were incubated with and without hCG and processed for progesterone and cyclic AMP determinations as described in the Materials and methods section. The concentrations of hCG used for stimulation of progesterone and cyclic AMP were 10 ng/ml and 1 μg/ml respectively. Results are means ± S.E.M.

	Progesterone (pg/μg of DNA)		Cyclic AMP (pmol/μg of DNA)	
Lectin	Basal	hCG	Basal	hCG
None	15 ± 4	260 ± 18	0.86 ± 0.11	4.16 ± 0.13
Soya-bean agglutinin (200 μg/ml)	7 ± 0.3	232 ± 9	0.82 ± 0.88	3.84 ± 0.06
Peanut agglutinin (200 μg/ml)	9 ± 2	225 ± 10	1.02 ± 0.02	4.34 ± 0.35
Lentil agglutinin (200 μg/ml)	23 ± 2	236 ± 12	1.11 ± 0.04	3.75 ± 0.17
Gorse seed agglutinin I (200 μg/ml)	45 ± 1	331 ± 35	1.03 ± 0.13	3.91 ± 0.35
D. biflorus agglutinin (200 μg/ml)	27 ± 9	269 ± 21	0.84 ± 0.05	3.68 ± 0.17
WGA (200 μg/ml)	21 ± 3	183 ± 23	0.68 ± 0.09	1.48 ± 0.05
Concanavalin A (200 μg/ml)	19 ± 0	168 ± 4	0.89 ± 0.09	2.41 ± 0.17

discrepancy of results observed with these two lectins is discussed in the Discussion section.

Lack of concanavalin A and WGA effect on cyclic nucleotide-stimulated steroidogenesis

Concanavalin A and WGA did not affect steroid production by the cells in response to the addition of dibutyryl cyclic AMP and 8-bromo cyclic AMP, further indicating that the inhibitory action of these lectins was occurring at a step preceding adenylate cyclase activation (results not shown). Under similar experimental conditions, progesterone production in response to hCG could be inhibited by concanavalin A and WGA.

Discussion

The carbohydrate groups in (cell-surface) glycoproteins and glycolipids, by virtue of their heterogeneous nature and external orientation, might play a potential role in receptor-mediated gonadotropin action, including activation of adenylate cyclase and steroidogenesis. Various sugar-specific plant lectins are utilized as a tool in probing the carbohydrate structure of oligosaccharide as well as in the study of membrane glycoproteins (Rabin & Burger, 1974). Although the lectins produce many complex effects (Hughes, 1973; Flowers & Sharon, 1974; Hughes, 1976), the lectin effect is initiated by preferential binding to certain terminal sugars or groups of sugars on carbohydrate chain originating from glycoproteins and glycolipids (Lis & Sharon, 1973; Nicolson, 1974; Brown & Hunt, 1978) of cell membranes.

Pre-incubation of ovarian cells with concanavalin A and WGA caused 50–60% inhibition in LH/hCG-stimulated cyclic AMP and progesterone production. The inhibition of response by these lectins was not due to non-availability of hormone since the addition of high concentrations of gonadotropin failed to overcome the inhibitory action of these lectins. In addition, lectin treatment maximally stimulated ^{125}I-labelled hCG binding 3–5-fold. If subsequent inhibition in responses were due to limited availability of hormone, incubation of lectin-treated cells with 100–1000-fold excess of LH/hCG should have evoked cyclic AMP and progesterone response maximally. Furthermore, inhibition of progesterone synthesis induced by concanavalin A was reversed by α-methyl D-mannoside and α-methyl D-glucoside, which have affinity for specific carbohydrate receptors on concanavalin A (Goldstein, 1976). The specificity of the effect of these sugars was established by the demonstration that L-fucose, D-galactose and *N*-acetyl-D-galactosamine, which do not interact with concanavalin A, did not block the inhibitory action of this lectin. In addition, *N*-acetyl-D-glucosamine, a sugar that binds specifically to WGA (Nagata & Burger, 1974), effectively prevented the inhibitory action of this lectin on steroidogenesis in response to LH/hCG. The specificity of this effect is indicated by the absence of comparable effect with other sugars, including α-methyl D-glucoside and α-methyl D-mannoside. To test the possibility that the inhibitory action of concanavalin A and WGA on steroidogenesis is at a point before the formation of cyclic AMP, the ability of dibutyryl cyclic AMP and 8-bromo cyclic AMP to stimulate progesterone production in cells pretreated with these two lectins was tested. In contrast with lectin inhibition of gonadotropin-induced steroidogenesis, lectin treatment had no effect on cyclic nucleotide-stimulated steroidogenesis. This strongly suggests that the inhibition of ovarian steroidogenic response to LH/hCG is mainly due to lectin action at the cell surface and may, at least in part, be attributed to interference with gonadotropin–receptor interaction. Failure of lectins to affect basal production of progesterone as well as protein synthesis not only rules out the non-specific actions of these lectins, but also indicates that concanavalin A and WGA have no effect on enzymic steps involved in the biosynthetic pathway of progesterone. Similarly, the lack of effect of concanavalin A and WGA on basal production of cyclic AMP is in agreement with the lectin effect being localized at membrane level.

The present studies also demonstrate that various other lectins specific for *N*-acetyl-D-galactosamine, D-galactose and L-fucose residues fail to modify gonadotropin-induced responses. Our results thus indicate the involvement of mannose, *N*-acetylneuraminic acid and *N*-acetyl-D-glucosamine residues in receptor-mediated LH action. The involvement of *N*-acetylneuraminic acid is suggested on the basis of the reported affinity of this sugar for WGA (Greenaway & LeVine, 1973). The differences in the mode of action of concanavalin A and lentil agglutinin suggest that cross-linking of lectin-binding sites by the tetravalent concanavalin A is required for the inhibition of gonadotropin-induced steroidogenesis. In addition, the difference may be due to lower affinity of lentil agglutinin for mannose compared with concanavalin A as reported previously (Edelman *et al.*, 1973).

The observation that gonadotropin-induced responses can be modulated by concanavalin A and WGA opens new routes that could help to increase our understanding of gonadotropin function within membrane environment. On the basis of direct binding studies, it appears that the gonadotropin receptors are glycoprotein in nature (S. Azhar & K. M. J. Menon, unpublished work). The inhibition of the steroidogenic effect of gonadotropins by lectins suggests that they prevent hormone–receptor interaction by binding to the receptor. Since in the

ovarian system only a small fraction of total available receptors needs to be occupied by LH-hCG to elicit maximum responses (Clark & Menon, 1976; Azhar & Menon, 1979*b*), the inhibition by WGA and concanavalin A strongly supports the notion that carbohydrate residues of gonadotropin receptor actively participate in hormone–receptor interaction and subsequent biological responses. Furthermore, at least in the case of concanavalin A, lectin binding may cause clustering of gonadotropin receptors due to the multivalency of concanavalin A. This clustering of receptors, which may be analogous to the patching of concanavalin A receptor sites on the cell surface (Nicolson, 1974; Edelman *et al.*, 1973; DePetris *et al.*, 1973; Yahara & Edelman, 1975), could lead to inhibition of subsequent receptor-mediated gonadotropin action. The possibility that the inhibitory action of these lectins might also be due to the close association of lectin receptors with LH/hCG receptors, thereby preventing hormone–receptor interaction, cannot be completely ruled out. Further studies aimed at selectively removing various cell-surface sugars with glycosidases and the effect of this treatment on subsequent tropic hormone–receptor interaction and biological responses are now required.

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