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GLYCOSYLATED PROLACTIN IN THE MURINE PITUITARY: DETECTION BY A NOVEL ASSAY AND ALTERATION OF CONCENTRATIONS BY PHYSIOLOGICAL AND PHARMACOLOGICAL STIMULI

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SUMMARY: In both rat and mouse pituitary extracts, we detected concanavalin A-binding prolactin immunoreactivity by a lectin-binding radioimmunoassay developed recently. The activity increased in response to estradiol benzoate treatment and lactation, stimuli that augment prolactin secretion, and decreased in response to acute nursing and perphenzine administration, stimuli that cause massive release of prolactin. Western blot analysis revealed a prolactin-immunoreactive band 2,000-3,000 greater in M_r than the main prolactin band that bound to ^{125}I-labeled concanavalin A. These results suggest the existence in the murine adenohypophysis of a glycosylated form of prolactin, which seems to be released under certain physiological states.

PRL, a polypeptide of 197-199 amino acids, has traditionally been recognized as a non-glycoprotein hormone produced in the acidophilic cells of the pituitary gland. Recent work (1, 2) demonstrated that the ovine pituitary also produced a glycosylated form of the hormone, which constituted nearly 15% of total glandular PRL. The glycosylated variant migrated slightly slower than PRL in SDS polyacrylamide gels, with a M_r 2,000-3,000 larger than PRL, and exhibited altered biological and immunological activities (1,3). The site of glycosylation was the asparagine residue at position 31, which formed part of a consensus sequence (Asn-Leu-Ser) for N-linked glycosylation. Since then, the existence of G-PRL has been reported in the human (4) and porcine (5) pituitaries. In pig, it constituted as much as 30-40% of the total pituitary PRL (5). In man, G-PRL appeared to be the major form of the hormone in plasma under resting conditions (6). However, it is not known whether such a form of

The abbreviations used are: PRL, prolactin; G-PRL, glycosylated PRL; IR-PRL, immunoreactive PRL; GH, growth hormone; con A, concanavalin A; 2-ME, 2-mercaptoethanol; BSA, bovine serum albumin; PBS, phosphate-buffered saline; NTC, nitrocellulose; SDS, sodium dodecyl sulfate; RIA, radioimmunoassay.

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PRL is synthesized in the rodent pituitary. Rodent PRL is different from PRLs of these species in its amino acid sequence (7); in particular, it does not have an asparagine at position 31. We, therefore, decided to look for G-PRL in rat and mouse pituitary glands using a novel lectin-binding RIA developed recently (8, 9) and immunoblotting techniques (10, 11). We felt that its detection in laboratory animals would facilitate characterization of its biological significance.

MATERIALS AND METHODS

Animals and tissues. S/A strain rats and S/W strain mice (Simonsen Laboratories, Gilroy, CA) were housed in controlled environment (12 hr light, 12 hr darkness; temperature 24 ± 1°C) and fed Wayne Lab Blox and water *ad libitum*. Anterior pituitary glands were obtained after decapitation and were washed 3-4 times with cold normal saline before being homogenized in Laemmli's sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% 2-ME) in the ratio of 20 mg/ml. The homogenates were heated in boiling water for 3 min and then centrifuged at 1,000 x *g* for 15 min. The supernatant was appropriately diluted with 1% BSA-containing PBS, pH 7.4, for use in the lectin-binding and conventional RIAs.

Physiological experiments. 70-day-old rats were assigned at random to the following groups: a) intact males, (b) intact females, (c) castrated females, (d) castrated females + estradiol benzoate, 10 μg, (d) intact female + progesterone, 5 mg, (e) intact female + estradiol benzoate + progesterone, (f) intact female + testosterone, 500 μg, (g) intact female + CB-154 (2-bromo-α-ergocryptine), a PRL inhibitor, 2 mg. All steroids were dissolved in sesame oil; CB-154 was dissolved in a small volume of absolute alcohol and then diluted with normal saline to 20% alcohol. Castration was performed under light ether anaesthesia. Injections were started 2 weeks after castration and were given once daily, sc, for 2 weeks.

In another experiment, primiparous rats on day 10-12 of lactation were separated from their litters for 15 hr. One half of the mothers were decapitated without nursing; the other half were allowed to nurse their young for 1 hr and then were sacrificed.

In a third experiment, adult female rats were given a single ip injection of perphenazine, 1 μg/g body weight, and were killed at 0, 0.5, 1, and 2 hr after injection.

Measurement of G-PRL. G-PRL in rat pituitary extracts was measured with a lectin-binding RIA described recently (8, 9). A 1:25,000 dilution of a highly specific antiserum to rat PRL (NIH-S_7), kindly provided by the National Hormone and Pituitary Program, NIDDKD, was used as the primary antibody.

Measurement of total IR-PRL. Total IR-PRL in rat pituitary extracts and sera was measured with a conventional RIA (12), using homologous reagents provided by the NIDDKD. Results are expressed in terms of the NIH RP-2 standard.

Western blotting. Rat and mouse pituitary extracts were analyzed for G-PRL by Western blot analysis, as described by Burnett (10). For immunostaining, a rabbit anti-mouse PRL serum that crossreacts with rat PRL as well (12) was used at a 1:1,000 dilution. PRL-immunoreactive bands were visualized with the use of ^{125}I-Protein A (2 to 3 x 10^5 CPM/ml). For lectin staining, con A labeled with ^{125}I (2 x 10^5 CPM/ml) was used, as described (11).

Statistics. Analysis of variance and Duncan's New Multiple Range Test were used for evaluating the significance of differences among treatment means.

RESULTS

Figure 1 presents the results of immunostaining of murine pituitary extracts with an anti-murine PRL serum. As expected, the main PRL band in both mouse and rat pituitary extracts crossreacted strongly with PRL antibodies, as did a number of proteins of smaller M_r, presumably fragments and small M_r variants of PRL. Significantly, a faint band trailing behind the main PRL band (indicated by the half arrow) and approximately 2,000-3,000 greater in M_r than PRL also crossreacted. Since the M_r of main PRL is generally accepted as 22,000-23,000, the apparent M_r of this faint band is tentatively assigned as 24,000.

In order to determine if this band was a glycoprotein, we reacted the pituitary proteins separated in the gel with ^{125}I-con A after blotting them onto NTC paper. As shown in Figure 2, the 24,000 M_r PRL-immunoreactive band exhibited discernible binding to con A, as did another band just behind PRL and an additional band labeled as "25K" PRL (all indicated by half arrows). The band labeled as "25K" PRL has previously been shown by tyrosine peptide mapping to be a PRL-like protein (13), although it does not crossreact with PRL antibodies. These results suggest that the 24,000 M_r PRL-immunoreactive band is most likely a glycoprotein, although it remains to be established beyond doubt, since other proteins co-migrating in the area may bind to con A. It also appears likely from these results that G-PRL migrates as multiple bands in SDS gels as has been found for ovine G-PRL (2), presumably as a result of varying degrees of degradation of the carbohydrate moiety.

We then analyzed rat pituitary extracts with a lectin-binding RIA specific for G-PRL (8). The sensitivity of this assay is between 2-5 ng. As expected, in the presence of con A every gland tested showed a marked dose-related increase in binding (Fig. 3). When the con A was omitted from the reaction tube, most of the binding disappeared, indicating that the response was not due to adsorption

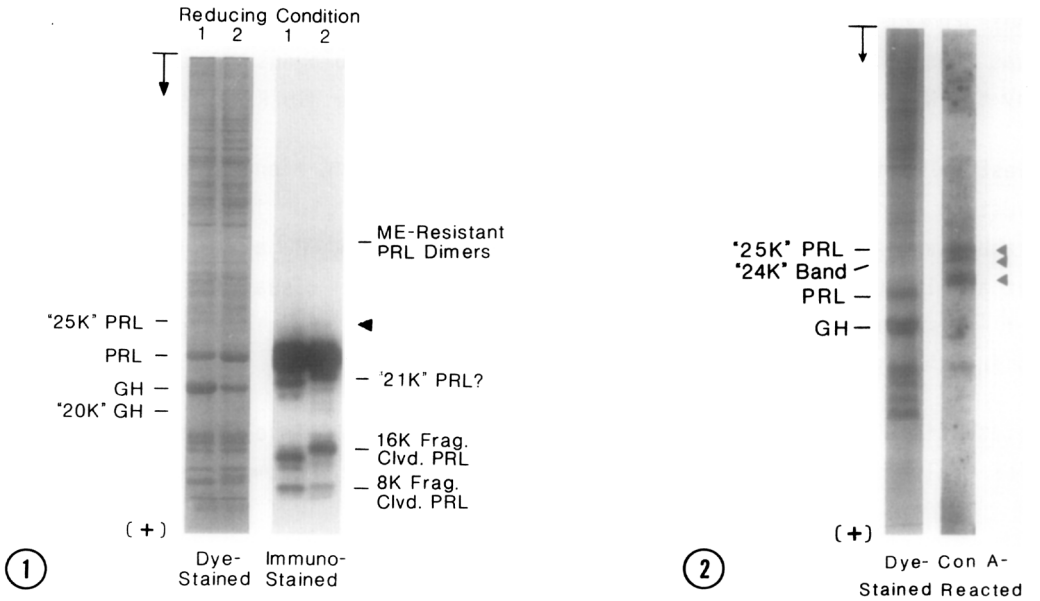

Fig. 1. Immunoblot of mouse (lane 1) and rat (lane 2) pituitary extracts with an anti-mouse PRL serum (1:1,000 dilution). In each case, 0.5 mg equivalent of anterior pituitary tissue from adult females was electrophoresed in 12% acrylamide gel, blotted onto NTC paper, and reacted with the antiserum, followed by ^{125}I-Protein A. The half arrow indicates the 24,000 M_r PRL-IR band that could represent the murine G-PRL, the slight increase in M_r being due to the attachment of the carbohydrate moiety. Frag. = fragment; Clvd. = cleaved.

Fig. 2. Lectin-blot of pituitary extract demonstrating con A-binding of the 24,000 M_r PRL-immunoreactive band seen in Fig. 1. NTC paper containing electrophoretically separated proteins from a 0.5 mg sample of mouse anterior pituitary was reacted with ^{125}I-con A. Besides the 24,000 M_r PRL-immunoreactive band, con A also bound to areas of the gel containing the "25K" PRL and a band just behind the main PRL. Half arrows indicate the con A reactive bands mentioned.

of PRL to the plastic. Purified preparations of rat (NIH-RP-2) and mouse (Lewis 204-36-1) PRL produced no significant binding in the 0-20 ng range (data not presented); neither did extracts of liver, kidney, brain, heart and skeletal muscle.

Figure 4 presents results of lectin-binding RIA of pituitary extracts from rats under different physiological and pharmacological states (serum PRL values for these animals are given in Fig. 5; statistical significance of the data is presented in Table 1). Pituitary G-PRL concentrations in female rats averaged higher than those in males, just as the total IR-PRL concentrations did. Both constituents were without significant change four weeks after ovariectomy. Estradiol benzoate, on the other hand, either alone or in conjunction with progesterone, elicited a marked increase in G-PRL, even greater in magnitude than that for total IR-PRL. Serum PRL concentrations were also greatly elevated in estradiol-treated animals. Progesterone and testosterone injections both significantly increased G-PRL, but not total IR-PRL. CB-154, a PRL inhibitory drug,

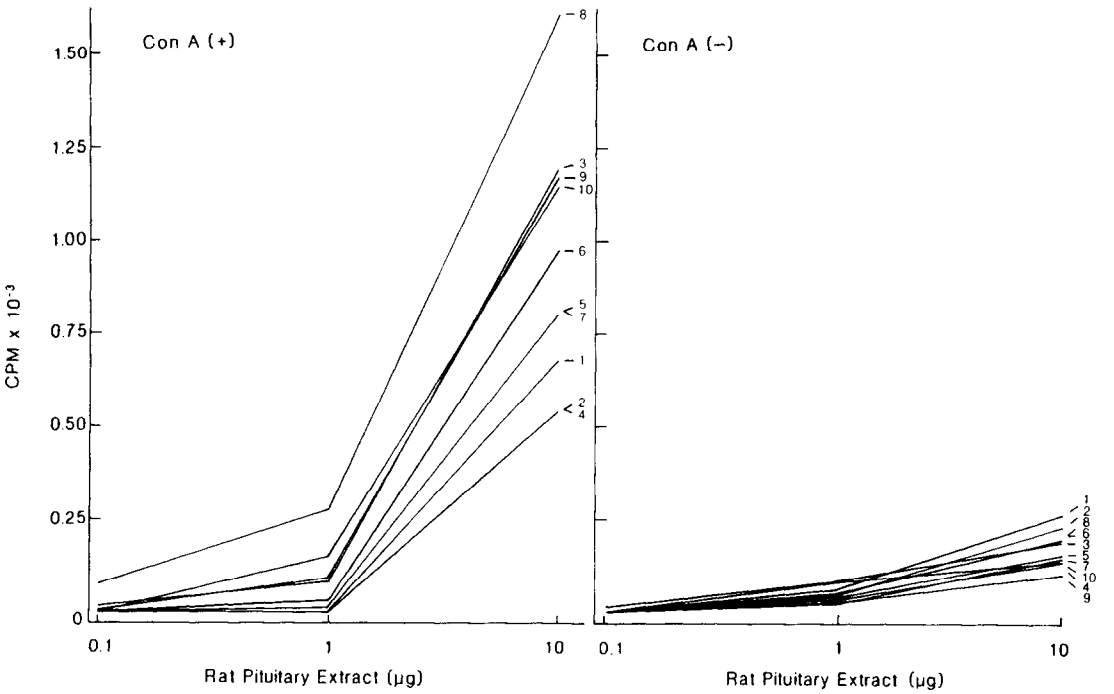

Fig. 3. Dose-response curves of rat pituitary extracts in the lectin-binding RIA. The numbers against the curves identify the glands. Note that the PRL-immunoreactive, con A-binding activity was present in each of the 10 glands tested.

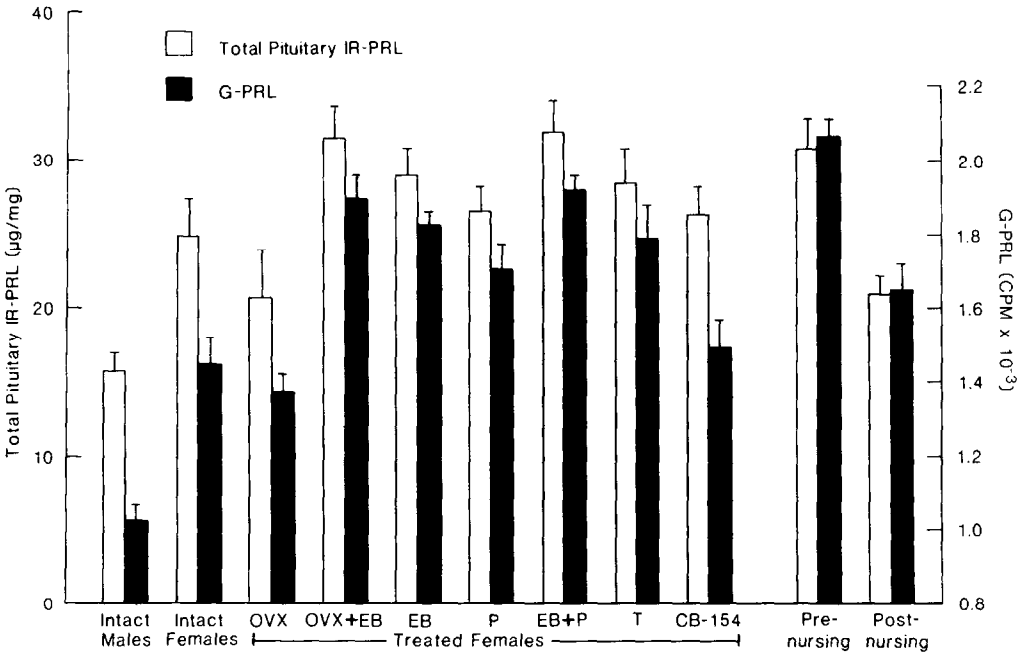

Fig. 4. Effect of some physiological and pharmacological stimuli on G-PRL concentrations of the rat pituitary. OVX = ovariectomized; EB = estradiol benzoate; P = progesterone; T = testosterone; CB-154 = 2-bromo-α-ergocryptine. Values given are mean ± SEM; 10 µg equivalent of pituitary tissue was assayed in each case. n = 10 in each group except OVX which had 8. The percentages and statistical significance of the differences are given in Table 1.

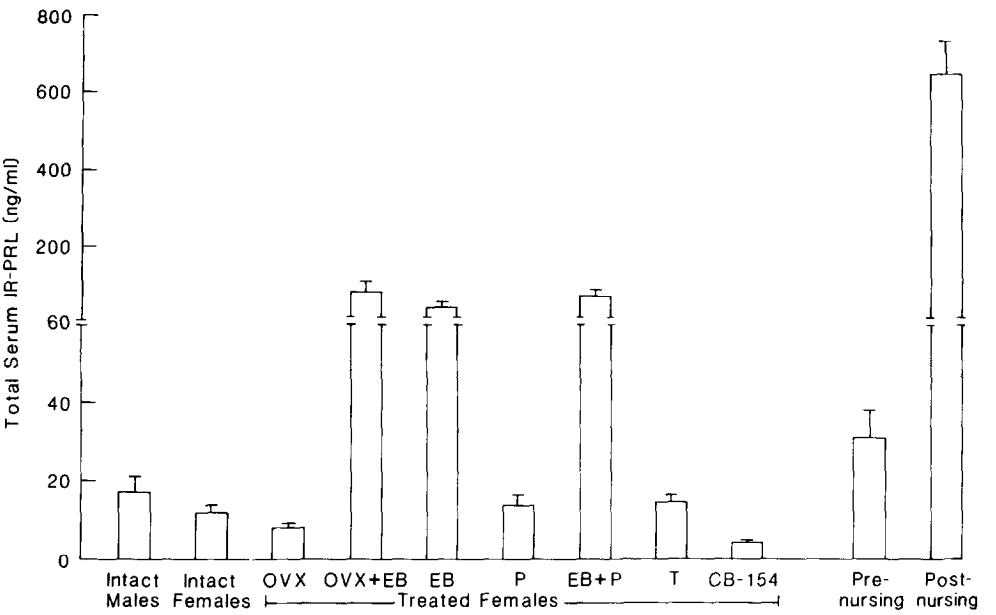

Fig. 5. Serum PRL concentrations of rats presented in Fig. 4. Values given are mean ± SEM. For other details, see legend to Fig. 4.

Table 1

Comparison of G-PRL and Total IR-PRL in the Pituitary Glands of Rats Treated with Various Stimuli

Comparisons	% Change in Pituitary G-PRL	% Change in Pituitary Total IR-PRL	% Change in Serum IR-PRL
1. Male vs Female	+41.2**	+58.0**	-34
2. Female vs OVX	-5.4	-16.5	-35
3. OVX vs (OVX + EB)	+37.8**	+51.7**	+1,844**
4. Female vs EB	+26.1**	+16.9	+806**
5. " vs P	+17.3*	+7.3	+18
6. " vs (EB + P)	+32.8**	+28.2*	+1,083**
7. " vs T	+23.9**	+14.5	+25
8. " vs CB-154	+3.0	+6.4	-61
9. Virgin vs Lactating	+43.0**	+24.2	+171
10. Pre- vs Post-nursing	-20.1**	-31.8**	+2,195**

*$P < 0.05$; **$P < 0.01$; Values without asterisks are not significant ($P > 0.05$). OVX = ovariectomized; EB = estradiol benzoate; P = progesterone; T = testosterone; CB-154 = 2-bromo-α-ergocryptine.

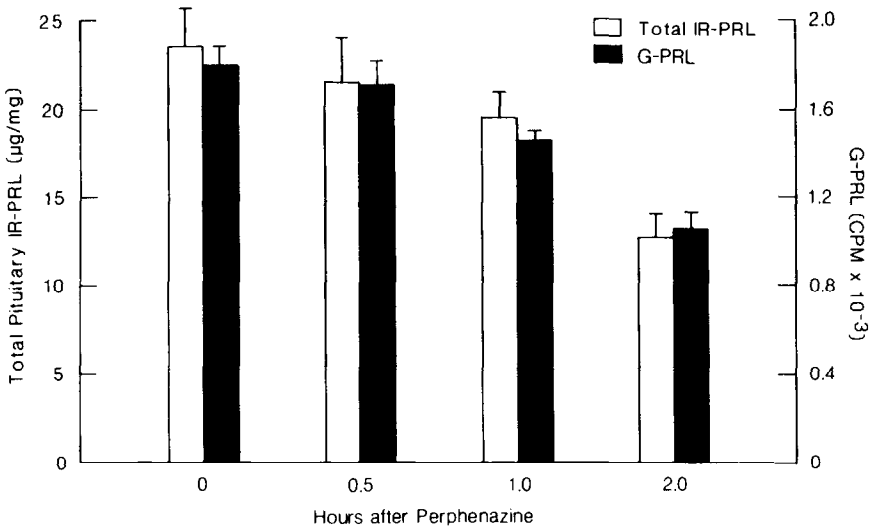

Fig. 6. Effect of perphenazine injection on pituitary G-PRL concentrations. Values given are mean ± SEM. n = 8 in each group. Note that the con A-binding PRL immunoreactivity decreased in parallel to the total IR-PRL concentrations, suggesting release of G-PRL from the pituitary.

produced no significant changes in either component, although it suppressed serum PRL concentrations. Pituitary concentrations of G-PRL averaged higher in lactating females than in virgins; an hour of vigorous nursing markedly decreased the concentrations of both types of PRL.

To assess further whether G-PRL is indeed a secretory protein, we administered perphenazine, a powerful PRL releaser, to a group of rats. Data presented in Figure 6 show a parallel decrease in pituitary G-PRL and total IR-PRL at each of the intervals examined. Serum PRL averaged 12 ± 3, 1,278 ± 209, 797 ± 149 and 660 ± 85 ng/ml at the 0, 0.5, 1, and 2 hr intervals, respectively. The decrease in total pituitary IR-PRL concentrations was obviously a result of a massive release of PRL, as indicated by a nearly 100-fold increase in serum PRL concentrations at the 0.5 hr interval. A similar decrease in pituitary G-PRL concentrations, therefore, strongly suggests that it, too, like PRL, is released in response to perphenazine administration or suckling.

Figure 7 shows immunoblotting analysis of pituitary extracts from some of the groups that had shown, by the lectin-binding RIA, significant increases in G-PRL. Pituitary extracts of all animals within a group were pooled and aliquots from each pool were electrophoresed simultaneously. Two to three PRL-immunoreactive bands of somewhat higher M_r migrating just behind the main PRL band and situated close to each other were seen in most cases (indicated by half arrows). The intensity of these bands appeared higher for females than for males, lower for castrated females than for intact females, and higher for estradiol benzoate-treated females than for non-treated controls, just as the

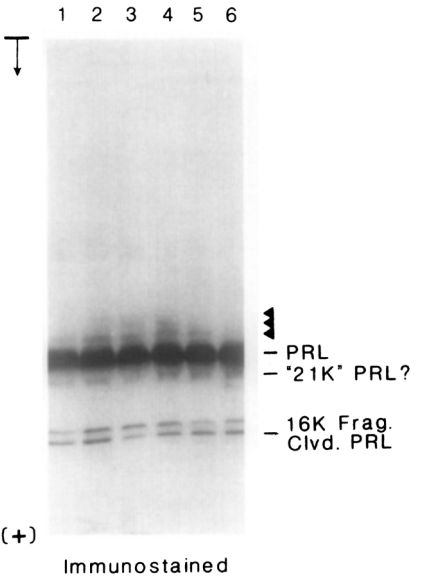

Fig. 7. Immunoblot of pituitaries of rats subjected to some pharmacological stimuli. A 0.5 mg sample of pooled pituitary extract was electrophoresed in each case. Lane 1 = intact male; Lane 2 = intact female; Lane 3 = ovariectomized female; Lane 4 = ovariectomized female treated with estradiol benzoate; Lane 5 = intact female treated with estradiol benzoate; Lane 6 = intact female treated with progesterone. Two to three faint PRL-immunoreactive bands (indicated by half arrows) just behind the main PRL band are visible in most cases. Frag. = fragment; Clvd. = cleaved.

results of the lectin-binding RIA had shown. The concordance in the results of the two assays further suggested that the high M_r PRL-immunoreactive bands seen here and in Fig. 1 most likely represent murine G-PRL.

DISCUSSION

These results clearly show that like the ovine (1, 2), human (4), and porcine (5) adenohypophysis, the murine gland also contains a protein(s) that crossreacts with PRL antibodies and at the same time binds to con A. These properties, together with the fact that its concentration is altered by stimuli known to affect PRL secretion, strongly suggest that the substance is a glycosylated form of murine PRL. The final confirmation will come only when the protein is isolated and sequenced. How much of this protein is present in the murine pituitary is not certain from these results. By immunostaining, the 24,000 M_r band(s) suspected to represent G-PRL appeared weak in comparison to the main PRL band, but this may partly be due to the poor immunological crossreactivity of the protein with the antiserum used. Both ovine (1) and human (4) G-PRLs have been found to have only one-third as much crossreactivity as their non-glycosylated counterparts in conventional RIAs. Furthermore, the murine "25K" PRL band, possibly a member of the G-PRL complex, does not

crossreact with existing PRL antibodies at all (13). And the concentration of this band in rat and mouse pituitaries, as judged from dye-staining, is close to 3-5% of the main PRL band (13). Thus, there may be much more G-PRL in the murine pituitary than appears from the immunoblotting experiments, perhaps as much as 8-10%.

The substance appeared to be a secretory pituitary protein, for its concentration in the gland dropped in response to acute secretory stimuli, such as nursing and perphenazine injection. Furthermore, under some experimental conditions, such as progesterone and testosterone administration, its concentration showed marked increase, even though total pituitary PRL or serum PRL concentrations did not increase. This implies that the variant may have different regulatory and secretory mechanisms.

The amino acid sequence of PRL varies greatly among species (7), and murine PRLs do not contain an asparagine at position 31 within a consensus sequence for N-linked glycosylation. Thus, the site and structural framework of glycosylation has to be different in this species. The glycosylating enzymes are not absolutely specific; glycosylation is known to occur at the asparagine residue in sequence Asn-Ser-Gly and Asn-Gly-Gly (14, 15). Strickland and Pierce (2) suggest that glycosylation may even occur at asparagine in an Asn-X-Cys sequence, where a sulfur replaces the oxygen of the serine side chain. Rat PRL contains two sequences of this type (residues 54-56 and 195-197) and mouse PRL contains one such sequence (residues 54-56).

The biological function of G-PRL is not known. Ovine G-PRL has been reported to have reduced pigeon crop sac-stimulating activity (1) and *in vitro* insulin-like action (3). Porcine G-PRL, on the other hand, has been claimed to have enhanced pigeon crop sac activity (5). Conceivably, glycosylation may even impart an entirely different biological action that has not been looked at yet. The ability to readily measure G-PRL in laboratory animals with the lectin-binding RIA should help elucidate the physiological significance of this new PRL variant.

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