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Down-Regulation of Prolactin Receptors in Rabbit Mammary Gland: Differential Subcellular Localization (41290)

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Abstract. A one-step discontinuous sucrose gradient has been utilized to separate plasma membrane and Golgi fractions in rabbit mammary glands. This procedure produces an enriched plasma membrane fraction (F1) as followed by 5'-AMPase activity and a fraction containing the major portion of the Golgi-rich components, using the enzyme marker galactosyltransferase. There is five times greater specific binding of prolactin compared to insulin in the mammary gland, but the distribution of the two receptors in the various fractions is similar. The intravenous injection of 3 mg ovine prolactin to lactating rabbits leads to an occupation of free, and a down-regulation of total prolactin receptors in the plasma membrane rich fraction (F1) in biopsies removed at the earliest periods after the injection of prolactin, subsequently, followed by a down-regulation of Golgi-associated prolactin receptors. These studies lend support to the view that the down-regulation of prolactin receptors occurs initially at the cell periphery reinforcing the theory of the internalization of the hormone–receptor complexes and their subsequent degradation.

The rabbit mammary gland is a rich source of prolactin receptors, from which they have been biochemically characterized and partially purified (1, 2). We have recently shown that in addition to the well-established up-regulation of prolactin receptors by prolactin, a rapid down-regulation of these receptors occurs both *in vivo* (3) and *in vitro* (4) in glands in explant culture. The mechanism by which the down-regulation occurs probably involves, as has been shown for other hormone receptors, an increased rate of internalization and degradation in lysosomes of hormone receptors (5).

The cellular distribution of prolactin receptors has been studied in rat liver. It has been shown that a major portion of receptors are located in intracellular membranes (6) and that following intraportal injection of labeled prolactin, it can rapidly (within 5–15 min) be localized within the Golgi (7).

The complete separation and purification of plasma membrane and Golgi components in rabbit mammary tissue has proven very difficult. In the present report, we attempt to understand better the subcellular kinetics of the down-regulation phenomenon and, using enriched subcellular fractions, to examine the differences in the rate and amplitude of this process between peripheral receptors (plasma membrane) and those located within the cell.

Materials and Methods. *Animals.* New Zealand rabbits at Day 10 of lactation were treated with 2 mg CB-154 (Sandoz, Basel, Switzerland) injected sc 36, 24, and 12 hr before the animals were sacrificed to increase free prolactin receptor concentrations (8). Three milligrams of ovine prolactin (NIH-P-S13, 30 IU/mg) was injected intravenously and 4-g biopsies were removed at 5 and 15 min and 1, 6, and 24 hr after prolactin injection from animals previously anesthetized with 50 mg/kg sodium pentobarbital as previously described (3).

Subcellular fractionation. The biopsies (4 g) were homogenized in 10 ml of 0.5 *M* sucrose (prepared in distilled water), filtered through three layers of cheesecloth, and applied to the top of a discontinuous sucrose gradient previously prepared, consisting of four densities of 7 ml each at the following sucrose molarities, 0.9, 1.3, 1.7, and 2 *M* representing densities of 1.13, 1.17, 1.23, and 1.25. Tubes were centrifuged in a SW-27 rotor overnight at 96,000*g* at 4°. The cellular components which formed at the interfaces of densities 1.07 and 1.13 formed fraction 1 (F1); 1.13 and 1.17, fraction 2 (F2); 1.17 and 1.23, fraction 3 (F3); and 1.23

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and 1.25, fraction 4 (F4). The various fractions were removed by a straight needle and syringe, diluted in 25 m*M* Tris–HCl buffer, pH 7.5, containing 10 m*M* $MgCl_2$ (Tris buffer), and recentrifuged at 100,000*g* for 1 hr in a 50 Ti rotor. The pellets were resuspended in Tris buffer, an aliquot removed for protein determination, and the remainder frozen at −20°.

Protein and enzyme activities. Protein was determined by the Lowry procedure (9). Galactosyltransferase, a marker for Golgi membranes, was determined in a 20-μl (40 μg protein) sample containing 0.2% Triton X-100, as described, and expressed as nanomoles of *N*-acetyllactosamine formed per hour per milligram of protein (10). Levels of 5′-nucleotidase (5′-AMPase) were measured in 50 μl (100 μg protein) as described by Widnell and Unkeless (11) and expressed as micromoles of P_i formed per hour per milligram of protein.

Hormone-binding assays. Prolactin receptors were measured by incubating 100 μg protein with approximately 100,000 cpm ^{125}I-ovine prolactin, iodinated with Chloramine T at low concentrations (12) to a specific activity of 50 μCi/μg for 16 hr at 22° in duplicate tubes in the absence and presence of 1 μg ovine PRL. The final volume per tube was 500 μl and the assay buffer was Tris buffer containing 0.1% bovine serum albumin. Specific binding was calculated as the difference between total and nonspecific counts per minute and expressed as a percentage of the total counts added. Free and total prolactin receptors were determined by desaturating receptors prior to the assay of total receptor levels by incubating membrane proteins with 4 *M* $MgCl_2$ (12).

Porcine insulin (INS), from Cannaught Laboratories, Toronto, was iodinated in a similar fashion to a specific activity of 80 μCi/μg and specific binding was determined as described for prolactin with the exception that excess insulin was used to displace ^{125}I-labeled insulin and incubation was at 4° for 24 hr.

Results. The method of separation of rabbit mammary gland homogenates on a sucrose density gradient results in the pattern of protein and enzyme markers shown in Fig. 1. Most proteins are located in subcellular fractions which sediment in fractions F3 and F4 having densities >1.17 (Fig. 1A). In contrast, fractions F1 and F2 (density <1.17) are the fractions richest in galactosyltransferase (Fig. 1B) and 5′-nucleotidase (Fig. 1C) which are marker enzymes for Golgi and plasma membrane fractions, respectively. Although fractions F1 and F2 are equally rich in 5′-AMPase activity, the ratio between 5′-AMPase and galactosyltransferase is much higher in F1 (6.5) than in F2 (2.8).

The specific binding of prolactin and insulin in the four fractions is shown in Fig. 2. There is approximately five times greater specific binding of PRL compared to insulin but the distribution is similar for the two receptors. Fractions F1 and F2 contain PRL receptors in the greatest concentrations corresponding to a copurification of marker enzymes and PRL receptors.

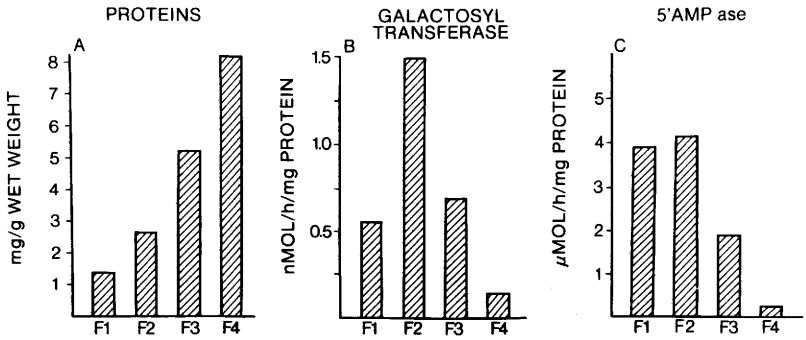

FIG. 1. Concentration of protein (A), galactosyltransferase (B), and 5′-AMPase (C) in the four fractions prepared by discontinuous sucrose gradient ultracentrifugation.

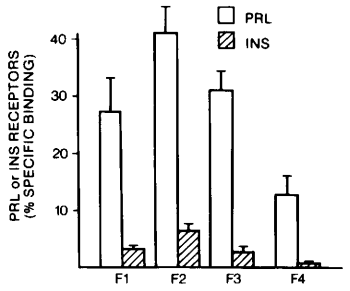

FIG. 2. Prolactin (PRL) and insulin (INS) receptor levels in the four sucrose gradient fractions.

Figure 3 shows the effect of a single intravenous injection of ovine PRL (3 mg) to lactating rabbits on the level of free and total (4 *M* $MgCl_2$-treated membrane) PRL receptors at different times after injection and for different fractions (F1, F2, and F3) from sucrose gradient centrifugation. Occupation is rapid with free receptor levels in F1 being nearly saturated in 5 min while in fractions F2 and F3, maximal saturation occurs at 15 min. Free receptors returned to control levels at 24 hr in F1 and F2 while in F3 they recovered to only 60% of the original level at this time period. The measurement of total receptors in these fractions confirms the phenomenon of down-regulation of PRL receptors, previously described in this same model (3), but the time course and the magnitude of this down-regulation vary with the fraction considered. In fraction F1, down-regulation occurs very rapidly with a reduction of 40% within the first 5 min while in F2 there is only a 15% decline compared to no decrease of total receptors in F3. In all three fractions, an apparent down-regulation occurs which is maximal at 1 hr in F1 and F2 and at 6 hr in F3.

Discussion. The studies presented in this paper support the view that there is a heterogeneity of prolactin receptors within the target cell. Although the initial action of prolactin occurs at the level of the plasma membrane, most of the prolactin receptors are located within the cell rather than at the level of the plasma membrane. This fact may explain why in the past, very little increase in receptor activity has been observed by a purification of marker enzymes associated with the plasma membrane (1).

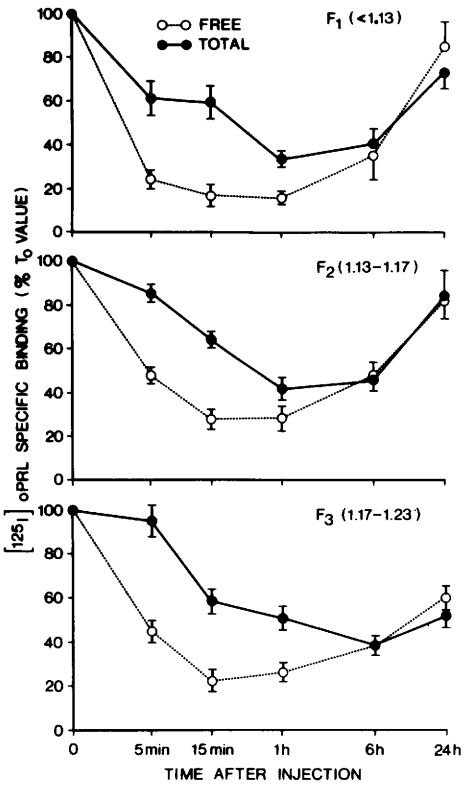

FIG. 3. Free and total prolactin receptor levels in sucrose gradient fractions F1, F2, and F3 prepared from mammary glands of rabbits at various time intervals following a single injection of 3 mg ovine prolactin at time zero.

In addition, the distribution from the gradients utilized in the present report suggest that using a discontinuous gradient from 0.3 and 1.3 *M* (sucrose densities from 1.04 and 1.17) would result in an enriched prolactin receptor preparation. This technique has in fact been utilized to measure receptors in species for which prolactin receptor levels are low, such as in the mammary gland of rat and sheep (manuscript in preparation).

Down-regulation of prolactin receptors has been observed both *in vivo* and *in vitro*. However, the present studies suggest that the rate or amplitude of down-regulation is not the same in the different subcellular fractions. Although the approach utilized in this report does not attempt to attain completely pure plasma membrane or Golgi fractions as has been reported for bovine mammary glands (13), it is possible using this single gradient to differentiate the

major plasma membrane and Golgi components. There is relatively more 5′-AMPase activity in F1 compared to F2 when the ratio between 5′-AMPase and galactosyltransferase is considered. Although there is a similar concentration of 5′-AMPase in the two fractions, due in part to the incomplete separation of plasma membrane and Golgi fractions as well as to inherent 5′-AMPase activity remaining in Golgi membranes (10), in F1 the amount of contamination by galactosyltransferase (Golgi marker) is less than is found in F2, with the result that F1 is the fraction enriched in plasma membrane marker and F2 representing the Golgi-rich fraction.

Both occupation of free and down-regulation of total prolactin receptors appear earlier in the plasma membrane rich fraction (F1) than in either of the other two fractions. This suggests that down-regulation initially affects receptors at the cell periphery, reinforcing the theory of internalization of the hormone receptor complex and their subsequent degradation. The fact that a major portion of receptors in the Golgi-rich fraction (F1) is also rapidly saturated and down-regulated to approximately the same degree as occurs at the level of the plasma membrane indicates a rapid means of access of prolactin to these intracellular binding sites, as has been proposed by Josephberg *et al.* (7).

Although subsequent studies will be required to verify, isolate, and completely purify the various cellular components of the mammary gland, these initial studies demonstrate an important difference in the subcellular distribution during the process of prolactin-induced down-regulation of receptors.

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