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UPTAKE AND PROCESSING OF PROLACTIN BY ALTERNATIVE PATHWAYS IN RAT LIVER

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The uptake and subcellular processing of radiolabelled prolactin has been studied in male and female rats. Analytical subcellular fractionation of liver homogenates from rats injected with ^{125}I-prolactin showed that in female rats the prolactin was primarily internalised to low density (1.12 $g \cdot cm^{-3}$) membranes. Approx. 10–15% of the total label was found in high density membranes, similar in distribution to lysosomal marker enzymes. In the normal male rat, prolactin was internalised solely to lysosomal type membranes. However, in male rats treated with estrogen, the distribution of prolactin was very similar to that seen in the female, indicating that internalisation to low density membrane is dependent on the presence of prolactin receptors. Gel exclusion chromatography showed that the prolactin internalised to the lysosomal membranes was extensively degraded whereas that associated with the low density membrane remained intact. Use of digitonin, to establish the identity of the low density membrane gave inconclusive results, but suggested that the prolactin was associated with membrane bearing NADH pyrophosphatase rather than the classical Golgi marker, galactosyltransferase.

Introduction

Specific binding sites for prolactin are present in the sinusoidal membranes of adult female rat liver but absent from those of the adult male [1–3]. Administration of estrogens to prepubertal or adult male rats, induces the appearance of these sites [4]. Binding of prolactin to these receptors with subsequent translocation of the intact hormone to the Golgi apparatus has been suggested on the basis of ultrastructural and preparative fractionation studies [5] but the precise intracellular location of internalised prolactin is uncertain.

Recent studies in this laboratory [6–8] have shown, by single-step sucrose density gradient centrifugation of whole liver homogenates [9], that it is possible to determine rapidly and quantitatively, the subcellular localisation of internalised membrane-ligand complexes. Thus, studies on the uptake and subcellular processing of glucagon [6] and insulin [7] have shown they are internalised to a population of low density membranes that we have termed ligandosomes [7]. In contrast, IgA is predominantly translocated to high density membranes [8].

In the present study, we follow the uptake and processing of phyisological concentrations of prolactin by rat liver with single-step fractionation procedures and show that the intracellular localisation of the hormone depends upon the estrogen status of the rat. We demonstrate that prolactin is processed by two distinct pathways in adult female rat liver. The major pathway, which proceeds via the specific plasma membrane receptors, shows that the intact labelled prolactin is associated with a population of low density membranes. The minor one, which is the major pathway in the normal male rat liver, shows internalisation of the hormone to high density membranes, most likely of lysosomal origin, with subsequent degradation of the hormone.

Materials and Methods

Young adult female and male Sprague-Dawley rats of about 200 g body weight were used. In certain experiments estrone (Sigma Chemical Co.) was administered subcutaneously daily at a dose of 50 μg for 7 days [4]. Rat prolactin was generously donated by Dr. A.F. Parlow, National Pituitary Hormone Distribution Program (NIH, Bethesda, MD, U.S.A.) and iodinated according to the method of Hunter and Greenwood [10].

Internalisation of radiolabelled prolactin. Rats were aneasthetised with sodium pentobarbitone and the portal vein was exposed through a midline incision. ^{125}I-Labelled prolactin, 10–50 ng, (spec. act. 3.7 GBq/mg) was injected in 0.1 ml of 0.16 M sodium borate-boric acid buffer, pH 7.4, containing 10 g/l bovine serum albumin. The liver was removed at varying time intervals and a portion subjected to single-step analytical subcellular fractionation by sucrose density gradient centrifugation as described by Smith and Peters [9]. The fractions were assayed for radioactivity and for various organelle marker enzymes [9]. Frequency-density histograms were constructed according to the method of Leighton et al. [11]. All histograms presented are of averaged data.

Preparation of a low density membrane fraction. Rat liver homogenate (2.5 ml), prepared as described above from a rat previously injected with ^{125}I-prolactin, was layered onto a discontinuous gradient of 5 ml 1.09 g · cm^{-3} and 10 ml 1.14 g · cm^{-3} sucrose solutions. After centrifugation in a 3 × 25 ml swing out rotor for 2 h at 100 000 × *g*, the low density membrane fraction was aspirated from the 1.09/1.14 g · cm^{-3} interface.

The integrity of the radioactivity associated with the subcellular fractions was examined 10 or 30 min after the injection of ^{125}I-labelled prolactin. The gradient fraction containing the peak of radioactivity was diluted 3-fold with 0.25 M sucrose containing 1 mM Na_2EDTA, pH 7.4 and 22 mM ethanol and centrifuged at 4°C at 100 000 × *g* for 60 min at 4°C. The pellet was suspended in 1.2 ml of 0.01 M HCl containing 1 g/l bovine serum albumin and centrifuged in an Eppendorf 3200 microcentrifuge for 4 min. The supernatant was neutralized with NaOH and 1 ml applied to a Sephadex G75 column (1.5 × 55 cm) pre-equilibrated with 25 mM Tris-HCl, pH 7.4 containing 1 g/l bovine serum albumin. Chromatography of radiolabel released from the low density membranes with digitonin (cytosolic fraction, Fig. 4) was performed on a Sepharose 6B column (2.2 × 70 cm) equilibrated and eluted with 25 mM Tris-HCl, pH 7.4.

Results

Subcellular fractionation studies of internalised radiolabelled prolactin

The distribution of ^{125}I-radiolabel and various membrane marker enzymes was examined after intraportal injection of ^{125}I-labelled prolactin followed 10 min later by fractionation. The results are shown as frequency-density histograms in Fig. 1.

The majority of radiolabel is found in a low density (1.12 g · cm^{-3}) membrane fraction associated with both galactosyltransferase (Golgi marker) and part of the alkaline phosphodiesterase (ligandosome marker) activity. In addition, a small shoulder, similar in modal density to that of *N*-acetyl-β-glucosaminidase (lysosomal marker), is found at density 1.20 g · cm^{-3}.

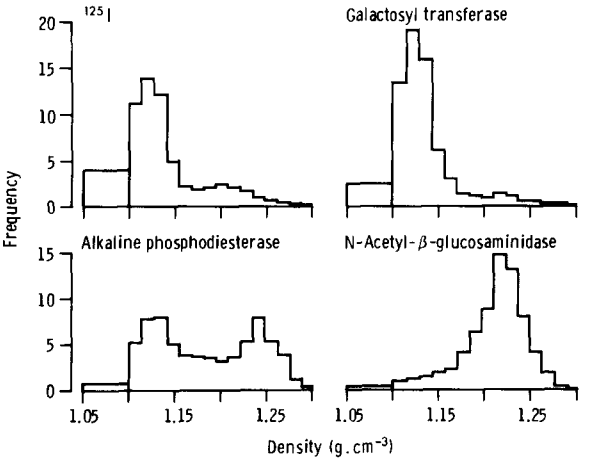

Fig. 1. Distribution of internalised prolactin in female rat liver. A female rat was injected via the portal vein with ^{125}I-labelled prolactin ($3.7 \cdot 10^4$ Bq). After 10 min the liver was removed, fractionated and the fractions assayed for radioactivity and various organelle marker enzymes. The results are expressed as frequency-density histograms and are the average of two separate experiments.

Time course of hepatic prolactin uptake

The time course of uptake of ^{125}I-labelled prolactin into both the homogenate and the low density membrane fraction of female rat liver is shown in Fig. 2. At all time points examined, radioactivity was especially concentrated in the low density membrane fraction since the specific activity of this fraction (cpm/mg protein) was much greater than that of the homogenate. From 2 to 30 min after injection, 50 to 60% of the total radioactivity in the homogenate was recovered in the low density membrane fraction with maximum labelling at approx. 10 min as shown by the relative specific activity of low density membrane (Fig. 2, insert).

The specificity of uptake of radiolabelled prolactin in female rat liver was evaluated by co-injecting 5 μg unlabelled and ^{125}I-labelled prolactin. Prolactin was very effective in apparently inhibiting the uptake of radioactivity (70% to 80% inhibition).

Internalisation of radiolabelled prolactin in male rats

In contrast, in the male rat (Fig. 3, thick line) the low density peak found in the female rat is absent but the peak at high density is still present with a similar absolute level of radioactivity to that found in the corresponding gradient region in female rats. The distribution of ^{125}I radiolabel in the liver of estrogen-treated male rats was examined (Fig. 3, fine line). The distribution profiles are similar to those obtained with female rats but strikingly different to that seen in the control animals. The distributions of the lysosomal, plasma membrane and ligandosome marker enzymes are similar in male, estrogen-treated male and female rat livers.

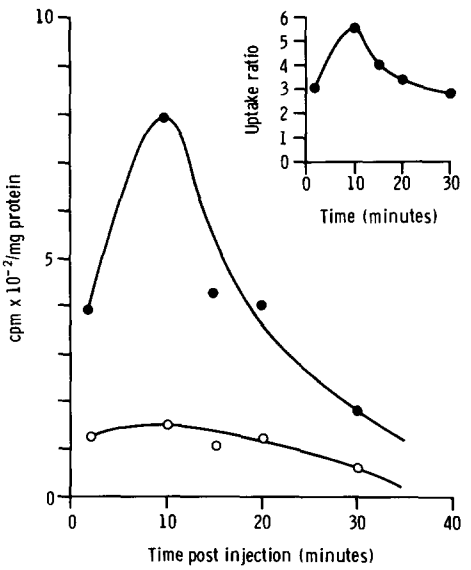

Fig. 2. Time-course of prolactin uptake. The uptake of radiolabel after injection of ^{125}I-prolactin into homogenate and low density membrane is shown as a function of time. The uptake is expressed as cpm per mg protein. The insert shows the variation with time of the ratio of incorporation into low density membrane compared with liver homogenate.

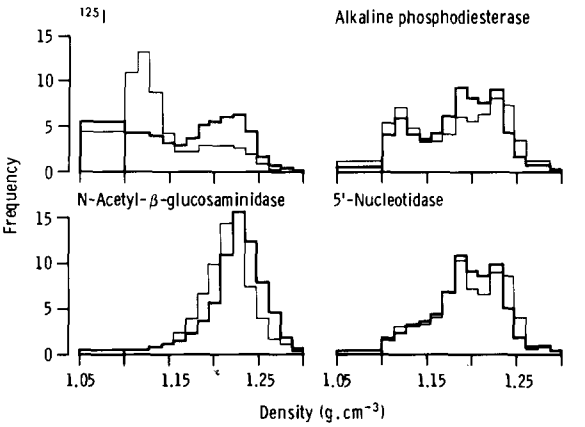

Fig. 3. Distribution of internalised prolactin in male rat liver. The figure compares the distribution of internalised prolactin in normal (thick line) and oestrone-treated (fine line) male rats. Radiolabelled prolactin was administered, and the liver fractionated as described for Fig. 1. Rats were oestrone treated by subcutaneous injection of oestrone (50 μg per day) for 7 days. The experiments were performed on the 8th day.

In order to distinguish between a prolactin localisation to either Golgi or ligandosome, use was made of the selective membrane perturbant, digitonin. Liver from a female rat, that had received intravenously radiolabelled prolactin 10 min previously, was homogenised in sucrose medium containing 0.81 mM digitonin. The distribution profiles of radioactivity and some enzyme markers are shown in Fig. 4. The prolactin was released from the membrane fraction and is recovered in the cytosolic fraction in contrast to the behaviour of galactosyltransferase (slight density shift) and alkaline phosphodiesterase (considerable density shift). Chromatography of the soluble fraction on Sepharose 6B resulted in the elution of the radio-

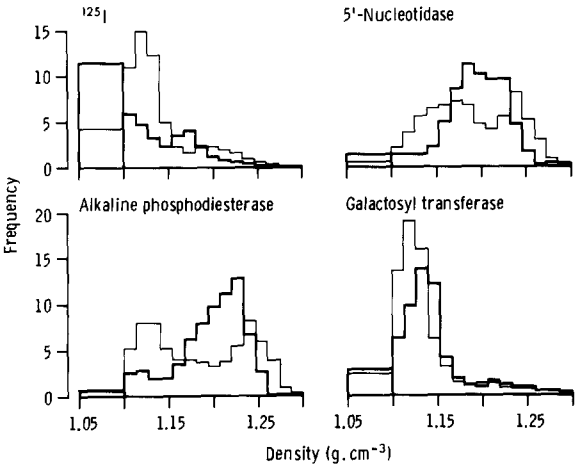

Fig. 4. Effect of digitonin on the distribution of internalised prolactin in the female rat. The effect of digitonin (thick line) on the distribution of internalised radiolabel in female rat liver is compared with distribution under normal (fine line) homogenisation conditions. The experiment was performed under exactly the same conditions as for Fig. 1 except that homogenisation was performed in the presence of 0.81 mmol/l digitonin.

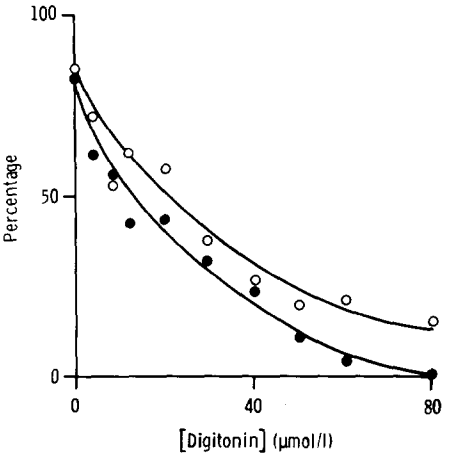

Fig. 5. Effect of digitonin on the latency of NADH pyrophosphatase and the sedimentability of internalised prolactin. The percentage of total NADH pyrophosphatase that is latent (●——●) and the percentage ^{125}I-prolactin that is sedimentable (○——○) are plotted as a function of the digitonin concentration.

activity at the same position as freshly prepared ^{125}I-prolactin.

The effect of digitonin was further investigated using a low density membrane fraction, prepared as described in Methods. Aliquots of this membrane preparation were treated with isotonic sucrose containing various concentrations of digitonin (0–0.08 mmol/l), and were then centrifuged at 100 000 × *g* for 1 h. The percentage sedimentability of the ^{125}I-prolactin and the percentage latency of NADH pyrophosphatase, an alternative expression of alkaline phosphodiesterase activity [7] are shown in Fig. 5. As the concentration of digitonin increases, the latency of NADH pyrophosphatase is progressively diminished. The loss of latency is paralleled by increasing solubilisation of the radiolabelled prolactin.

Integrity of internalised prolactin

The nature of the radioactivity associated with the low density membrane fraction (1.12 g · cm^{-3}) in female rats and the high density membrane fraction (1.20 g · cm^{-3}) in male rats was examined by chromatography on Sephadex G-75. In the female, the majority (90%) of the low density membrane-associated activity was solubilised with dilute acid and had the same retention time as freshly prepared ^{125}I-prolactin, at both 10 and 30 min post-injection (Fig. 6, solid line). In the case of the male rat, the nature of the radiolabel was

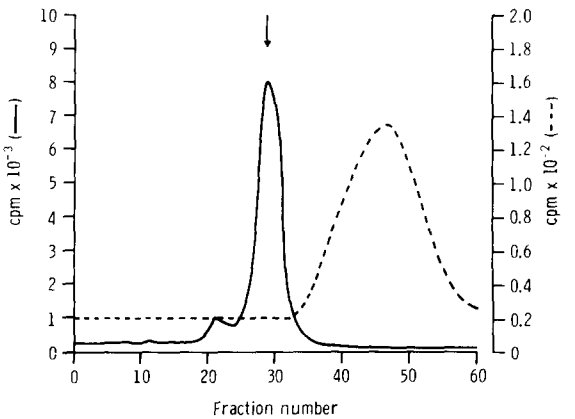

Fig. 6. Elution profile of internalised radiolabel. The radiolabel internalised in female rats to the low density fraction (thick line) was extracted and chromatographed on G-75 Sephadex as described in Methods. Radiolabel internalised in the male rat to the high density membrane (dotted line) was extracted as above following subcellular fraction of liver from a male rat, 30 min after injection of the labelled prolactin. The arrow marks the elution position of ^{125}I-labelled prolactin.

examined only at 30 min post-injection, since the amount of radioactivity accumulated in the fraction over shorter intervals was too small to reliably analyse. Only 60% of the radioactivity was solubilised by dilute acid, but this material was eluted in a much broader distribution (Fig. 6, dotted line) at longer retention than ^{125}I-prolactin indicating degradation of the prolactin.

Discussion

The present study has clearly demonstrated that prolactin can be processed by two distinct pathways in rat liver. The major pathway in female liver, seems to proceed via the specific receptors for prolactin. The evidence for this stems from the fact that this pathway is absent in the normal male, which has no specific receptor for prolactin [1–3] but is found in male rats treated with estrone, a procedure known to induce the specific lactogenic receptors [4]. The hormone (50–60%) is translocated to a low density population of membranes, with maximum uptake at 10 min post-injection. Although the radioactivity associated with this membrane fraction decreased with time, gel permeation chromatography showed that membrane-associated label was intact prolactin. These results agree qualitatively with those obtained by Posner et al. [4]. However at present our results do not allow a definitive assignment to Golgi apparatus or ligandosomes.

Previous experiments on internalised insulin had shown a marked increase in the equilibrium density of the membrane associated insulin on treatment with digitonin, a feature not shared by the traditional Golgi marker, galactosyltransferase, which showed little or no shift with digitonin [7]. With internalised prolactin, solubilisation of radiolabelled material occurred. However, the results obtained on titration of a low density membrane preparation with digitonin, show that solubilisation of prolactin proceeds concomitantly with the loss of latency of NADH pyrophosphatase activity. Since latent NADH pyrophosphatase activity is thought to be associated with the ligandosome [7], this result suggests that prolactin has been internalised to the ligandosome, but is more loosely associated with the membrane than insulin or glucagon.

Chromatography of the soluble fraction on Sepharose 6B indicated that the label was associated with free prolactin and not with the prolactin-receptor complex. This could be interpreted as meaning that a functional lactogen receptor is not present in the vesicle since the dissociation constant of prolactin from its receptor would be expected to be very low [12], and thus limited dissociation would be expected within the time course of the experiment. However, there is increasing evidence [13,14] that receptor-ligand dissociation is an acid pH-mediated event. Under these circumstances, the receptor could still be present but the local environment may favour dissociation of the prolactin-receptor complex. We suggest therefore that, the solubilisation is a result of the detergent properties of digitonin, allowing egress of free prolactin from the vesicle.

The second pathway appears to be of minor importance in the female rat, but is the only route in the normal male rat although the absolute quantity of radiolabel taken up into this population of membranes is essentially the same in the two sexes. This suggests that this pathway is independent of the receptor-mediated step. The equilibrium density of the particulate radioactivity is similar to that of the lysosomal marker, *N*-acetyl-β-glucosaminidase. Chromatography of the solubilised radiolabel indicated that it was associated with low molecular material, showing degradation of the prolactin had occurred. Part of the material associated with the dense membrane appeared to be very tightly bound. The route of uptake is presumably via fluid phase pinocytosis, although previous studies of this pathway with ^{125}I-labelled polyvinylpyrollidone have suggested internalization to a more dense population of membranes [7].

It is also arguable that the high density peak represents material taken up by non-parenchymal cells. However, in the female rat, the percentage of the total internalised label found in this region (10–15%) is in good agreement with the findings of Bergeron et al. [15], who showed 15–18% of the label associated with parenchymal cell lysosomal-like structures.

Our results are in broad agreement with the autoradiographic study of Bergeron et al. [15], who describe the internalisation of ^{125}I-prolactin first to smooth membranous bodies and subsequently

to lipo-protein filled vesicles. Since the distribution profiles shown in our study (Fig. 1) were obtained at 10 min post-injection, our low density membrane may correspond to the smooth-membrane vesicles rather than the lipoprotein filled vesicles. However, fractionation performed at 30 min post injection (results not shown) gave no indication of significant alteration in the modal density of the major peak.

Bergeron et al. [15] suggest that the ultimate fate of the internalised prolactin is to be transferred to secondary lysosomes and be degraded, whereas as discussed above, we interpret the evidence as suggesting two separate pathways. We suggest that the pathway in the normal male corresponds to the minor pathway in the female and represents internalization and degradation of the hormone. The major pathway in the female would seem to be via specific receptors and considering that the hormone appears to remain intact, may result in physiologically significant events.

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