SUBCELLULAR LOCALIZATION, CHARACTERIZATION AND REGULATION OF PROLACTIN RECEPTORS: STUDIES ON THE RAT LIVER AND THE RABBIT MAMMARY GLAND

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Prolactin receptors in rat liver and rabbit mammary gland

GIANTS' CLASH

The most incomprehensible thing about the universe
is that it is comprehensible

Albert Einstein

The universe is not only queerer than we imagine,
but it is queerer than we can imagine

J.B.S. Haldane

If I have seen farther than others,
it has been by standing on the shoulders of giants

Sir Isaac Newton

To all those who are dedicated
to a better understanding of this universe

ABSTRACT

Experiments were carried out in order to increase our knowledge and understanding of prolactin receptor (PRL-R) internalization and fate, following PRL binding to target organs. As a first appproach, plasma membrane, Golgi and lysosomal fractions of rat liver were prepared, in which PRL-R were identified and characterized. Typical secondary lysosomes contained very little intact PRL-R, which were greatly increased by chloroquine treatment of the animals. There was also a subclass of lighter vesicles, morphologically resembling lysosomes ("prelysosomes"), with elevated basal PRL-R activity. When ^{125}I-oPRL was injected into rats, maximum incorporation in the total liver homogenate and in the Golgi fraction occured at 15 min. In contrast, peak incorporation in lysosomal fractions was at 30 min.

In cultured rat hepatocytes, PRL and PRL-R antibodies were able to regulate PRL-R levels. Up-regulation by nanomolar concentrations of PRL could be partially counteracted by cycloheximide, but actinomycin D had little or no effect, suggesting that PRL influences primarily translation. The mimicking effect of the PRL-R antibodies suggests that the PRL molecule is not required beyond its interaction with the receptor to elicit these actions.

We finally attempted to directly visualize PRL-R with immunocytochemistry. Very sparse labelling was observed in the endoplasmic reticulum region, but never in the Golgi or in lysosomes.

RESUME

Ce travail vise à apporter une meilleure compréhension des événements relatifs au récepteur de la prolactine (R-PRL), faisant suite à la liaison de cette hormone aux tissus cibles. Dans un premier temps, nous avons identifié et caractérisé des R-PRL dans des préparations de membranes plasmiques, de Golgi et de lysosomes de foie de rat. Les lysosomes secondaires contiennent normalement peu de R-PRL, mais leur capacité de liaison est grandement augmentée par un traitement des animaux à la chloroquine. Une sous-classe de vésicules moins denses, ressemblant morphologiquement aux lysosomes ("prélysosomes") et très riches en R-PRL a également été identifiée. L'injection intraveineuse de ^{125}I-oPRL résulte en une importante incorporation dans le foie, avec un maximum à 15 min dans l'homogénat total et la fraction golgienne, mais à 30 min seulement dans les deux fractions lysosomiales.

Dans des hépatocytes de rat en culture, la PRL et des anticorps dirigés contre son récepteur influencent les niveaux de R-PRL. La régulation positive induite par des concentrations nanomolaires de PRL est partiellement renversée par la cycloheximide, mais l'actinomycine D n'a que peu ou pas d'effet. Ceci suggère que la PRL agit principalement au niveau traductionnel. L'effet de l'anticorps anti-récepteur suggère que le mécanisme normal de la PRL se limite à son interaction avec le récepteur.

Notre dernier but était de visualiser les R-PRL directement, en immunocytochimie. Cette approche n'a donné lieu qu'à peu de marquage dans le réticulum endoplasmique, et pas du tout dans le Golgi ou les lysosomes.

PREFACE

The reader is informed that I chose the option of including manuscripts of original papers published in learned journals as part of the experimental chapters (Chapters 2 and 3) of this thesis. Each one therefore has its own Abstract, Introduction, Materials and Methods, Results, Discussion and References sections. Chapter 4 represents original work not published or to be published elsewhere.

Each of these articles were coauthored by my thesis supervisor, Dr. Paul A. Kelly, and three of them were also coauthored by two French scientists with whom our group has been collaborating for several years, Drs. Jean Djiane and Louis-Marie Houdebine. These persons contributed with fruitful discussions but were not directly involved in this work. On the other hand, Dr. Alzira A.M. Rosa participated in the work and coauthored the papers dealing with cultured hepatocytes.

The list of published papers included in this thesis is as follows:

1. Ferland, L.H., Djiane, J., Houdebine, L.-M. and Kelly, P.A. (1984) The effect of chloroquine on intracellular lysosomal prolactin receptors in rat liver. Endocrinology 115: 1842-1849. (Section 2.1)

2. Ferland, L.H., Djiane, J., Houdebine, L.-M., and Kelly, P.A. (1984) Intracellular transformation of prolactin following internalization into rat liver. Mol. Cell. Endocrinol. 35: 25-31. (Section 2.2)

3. Rosa, A.A.M., Ferland, L.H., Djiane, J., Houdebine, L.-M. and Kelly, P.A. (1985) Maintenance of prolactin (PRL) binding sites in rat liver cells in suspension culture: effects of PRL and of inhibitors of various cellular functions. Endocrinology 116: 1288-1294. (Section 3.1)

4. Ferland, L.H., Rosa, A.A.M. and Kelly, P.A. (1984) Interactions of prolactin (PRL) binding sites with PRL receptor antibodies in rat liver cells in suspension culture: effect of inhibitors of cellular functions. Can. J. Physiol. Pharmacol. 62: 1429-1433. (Section 3.2)

During her stay with our group, Dr. Rosa worked out the methods for hepatocyte isolation and culture and performed the early experiments on prolactin regulation of prolactin receptors and the modulating effects of the various drugs tested. For standardization reasons, however, I had (being given the responsibility of completing these experiments after Dr. Rosa left) to perform all the experiments from the beginning. The results presented in Chapter 3 are those of my own experiments. Dr. Rosa was a post-doctoral fellow of the Fundaçao ao Amparo ao Ensino e Pesquisida do Estado de Sao Paulo, Brasil.

For the work presented in Chapter 4, I received the technical assistance of Dr. Jean-Luc Servely for mammary epithelial cell culture and of Mrs. Lucette Bélair for explant culture, and the fruitful advice from Drs. Michèle Ollivier-Bousquet, Jean Djiane, Claude Tougard and Isabelle Dusanter-Fourt. Also a professional photographer developed the films and made the prints for the electron micrographs. Part of the processing of the organelle preparations for the micrographs shown in Chapter 1 was performed by Dr. Georges Pelletier.

Except for these, I performed all the manipulations for all experiments reported in this thesis. These include:

- lysosome, Golgi and plasma membrane isolations,
- enzymatic assays,
- hormone and antibody iodinations,
- prolactin receptor assays and Scatchard analyses,
- treatments of the rats (s.c. injections of estradiol, bromocriptine mesilate and chloroquine; i.v. injections (jugular vein) of ^{125}I-ovine prolactin; i.p. injections of Surital or Ketalar for anaesthesia; and sacrifice),
- rat hepatocyte isolation and culture, including all pharmacological works (some hepatocyte cultures were performed by Ms. Lise Faucher, then an undergraduate student who worked under my supervision during the summer of 1982),
- receptor localization experiments with all approaches utilized, including processing of the samples for electron microscopy, ultrathin sectioning and operation of the electron and epifluorescence microscopes.
- I also worked out myself the methods for the preparation of the lysosomal fractions and the acid phosphatase assay, and all the protocols for the localization experiments presented in Chapter 4.

ACKNOWLEDGEMENTS

I would like to greatefully acknowledge all the people who helped me directly or indirectly during these past four years, in the process of my Ph. D. work.

In the first place, Dr. Paul A. Kelly who supervised it and provided good advice on every aspect of my work and career. I will always remain deeply indebted to him. Also, Dr. Fernand Labrie, director of the Laboratoire d'Endocrinologie Moléculaire at Le Centre Hospitalier de l'Université Laval (Ste-Foy, Québec), where this work was fisrt undertaken, and Dr. Harry L. Goldsmith, Director of the Division of Experimental Medicine, McGill University.

I also acknowledge the financial support which I received in the form of studentships by La Fondation Université Laval, La Fondation Dr. George Phénix, Le Fonds F.C.A.C. pour l'aide et la soutien à la recherche (Gouvernement du Québec), the Institut National de la Recherche Agronomique (France) and the Coopération Franco-Québécoise dans le Domaine des Biotechnologies; the funding agencies which support our laboratory: the Medical Research Council of Canada, the National Cancer Institute of Canada and the United States Public Health Service; and the National Hormone and Pituitary Program (NIH) for kindly providing ovine prolactin and human growth hormone.

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ABBREVIATIONS

The abbreviations used throughout this thesis (excluding those directly derived from the SI unit system) are listed here. In most cases, they are also defined at their first appearance in each chapter.

ACTH	: adrenocorticotropin
b	: bovine
BSA	: bovine serum albumin
b.w.	: body weight
CB-154	: bromocryptine mesilate
cpm	: counts per minute
E_2	: estradiol
ELISA	: enzyme-linked immunosorbant assay
ER	: endoplasmic reticulum
FSH	: follicle-stimulating hormone
g	: gravitational acceleration constant
G	: glutaraldehyde
GH	: growth hormone
GH-R	: growth hormone receptor
h	: hour, or human
HEPES	: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMW	: high molecular weight
ICC	: immunocytochemistry
i.d.	: inside diameter
ID_{50}	: dose inducing a 50% inhibition
Ig	: immunoglobulin
i.p.	: intraperitoneal
i.v.	: intravenous
IU	: international unit
L-1	: prelysosomal fraction
L-2	: mature lysosomal fraction

LH	:	luteinizing hormone
mAb	:	monoclonal antibody
min	:	minute
o	:	ovine
P	:	paraformaldehyde
PBS	:	phosphate-buffered saline
PBS-S-G	:	PBS supplemented with saponin and gelatin
PM	:	plasma membrane
PRL	:	prolactin
PRL-R	:	prolactin receptor
RSA	:	relative specific activity
r.t.	:	room temperature
SAR	:	serum anti-receptor
s.c.	:	subcutaneous
SEM	:	standard error of the mean
TCA	:	trichloroacetic acid
TSH	:	thyroid-stimulating hormone

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CHAPTER 1

GENERAL INTRODUCTION

1.1: Prolactin: its ubiquitous nature

Prolactin is a pituitary hormone whose primary actions in mammals involve the stimulation of mammary gland development and of milk production. It is a member of a family of lactogenic hormones which also comprises primate growth hormone and placental lactogen.

The first evidence for a role of the anterior pituitary on mammary action dates back to 1928 when Stricker and Grueter (159) demonstrated that administration of bovine anterior pituitary extracts to pseudopregnant rabbits (ovariectomized or not) resulted in the onset of lactation. This was confirmed independently by the studies of Corner (28). The term "prolactin" was introduced by Riddle et al. (131) for a fraction from bovine or ovine pituitary extracts distinct from either growth or "sex maturity" hormones and which was capable of inducing both the development of the crop gland in pigeons and lactation in rodents.

That mammalian prolactin (PRL) would elicit "lactogenic" actions (the crop sac of birds is the organ which produces "avian milk") in species so distant phylogenetically would suggest that the PRL gene is very old and especially well preserved. In fact, PRL has now been detected in all classes of vertebrates, including human, sheep, cattle, pig, rat, rabbit, dog, chicken, fish, horse, whale, cat, guinea pig, hamster, amphibians, reptiles and birds (reviewed in ref. 86).

The mucoproteins which are thought to have been the secretory products of the early adenohypophysis (110) were suggested to have evolved along two separate lines: one giving rise to the glycoprotein hormones thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the other, to simple proteins. Among these,

adrenocorticotropin (ACTH), melanocyte-stimulating hormone (MSH) and the other hormones of the pro-opiomelanocortin family and, on another line, prolactin (PRL) and growth hormone (GH) (110).

Of course, PRL does not induce milk production in all species cited above. Rather, it elicits a number of different actions in various species, as opposed to other pituitary hormones which specialized early in vertebrate phylogeny for the regulation of a single or at the most a few physiological processes. Nicoll and Bern (111) classified over 80 specific actions of PRL in cyclotosomes, teleosts, amphibians, reptiles, birds and mammals, into five categories: reproduction and nurturing of the offspring, osmoregulation, growth, effects on ectodermal structures and synergism with steroids. There seems to be no "common denominator" for this manifold of actions of PRL (not all of which having been demonstrated to be physiologically relevant) except, perhaps, species adaptation to and survival within a given ecological niche. The work presented here is devoted solely to PRL in mammals.

1.2: The control of PRL secretion

As we have seen, the adenohypophysis is a gland which secretes a number of peptide and glycoprotein hormones, and it does so in response to a variety of stimuli. Perhaps the most important effectors involved in the regulation of pituitary hormone secretion are releasing factors secreted by the hypothalamus and that reach the anterior lobe via a unique portal system (129). Among all pituitary hormones, PRL appears to represent a special case, when one considers the mechanisms controlling its secretion, in that it appears to be principally negatively regulated (102,109). This inhibition is exercised

through a prolactin-inhibiting factor (PIF), identified as dopamine (90,91,162 reviewed in ref. 171), which is released tonically into the portal vein. Recently, a 56 amino acid peptide, GAP (GnRH-associated peptide), has been identified, which has prolactin inhibitory activity, and may in fact represent the endogenous PIF (113a).

The pituitary seems to have the inherent ability to secrete PRL, since pituitary explants did so when, for example, implanted under the kidney capsule, that is, removed from hypothalamic influence (23,38). In addition to producing PRL, such transplants failed to secrete FSH, LH, ACTH and TSH (39). When retransplanted in vascular contact with the median eminence, however, PRL secretion again dropped, whereas gonadotropins secretion resumed (113). Other approaches of removing the pituitary from hypothalamic influence, such as median eminence lesion (23,101), in vitro culture of pituitary tissue (119) or pituitary stalk section (40), yielded similar results.

Recently, new insights on the mechanism by which dopamine (DA) inhibits PRL secretion have become available (31, 70). It is known that DA inhibits adenylate cyclase in homogenates of pituitary gland (52) and lowers cAMP levels in enriched populations of lactotrops (8,161). There is also evidence that DA inhibits the Ca^{2+} messenger system (141,163). The work of Delbeke and Dannies (31) suggests that both these actions of DA are involved in its action on PRL release inhibition since pharmacological stimulation of both was required in order to counteract DA-induced inhibition in perifused rat anterior pituitary cells.

Positive effectors of pituitary PRL secretion also exist. Nicoll et al. (112) reported evidence that the rat hypothalamus may contain both PRL-releasing and inhibiting factors. A number of endogenous substances have been reported to stimulate PRL release. Perhaps the one which is the most

documented is thyrotropin-releasing hormone (TRH) (reviewed in ref. 27). However, these authors have argued that TRH stimulation of PRL secretion is probably not physiologically relevant because there are several physiological or experimental states where PRL and TSH are not released together (suckling stimulus, cold or heat exposure, administration of L-DOPA). Also, patients with altered TRH secretion and/or responsiveness to TRH generally have normal PRL secretory patterns.

Serotonin is another agent from the brain which has been shown to increase PRL secretion (reviewed in refs. 27 and 171). It appears to mediate stimulus-evoked PRL release that accompanies suckling, stress, possibly sleep, and the estrogen-induced PRL surge on the afternoon of proestrus in rats. However, since incubation of anterior pituitaries with serotonin has no effect on PRL release (13), this agent appears to act as a neurotransmitter or a neuromodulator rather than as a neurohormone, possibly by stimulating the secretion of a PRL-releasing factor from the hypothalamus (47). Vasoactive intestinal polypeptide (VIP) also has PRL-releasing activity and may in fact be a true hypothalamic factor, since its concentration in portal blood is 19-fold greater than in peripheral circulation (135) and low concentrations of VIP were shown to stimulate anterior pituitary PRL release <u>in vitro</u> in a dose dependant manner (132,144).

PRL release can also be stimulated via an inhibition of dopamine blockage. Such a mechanism has been proposed by MacLeod (89) to explain the effects of estrogens on on PRL secretion, via the hypothalamus. Estrogens, however, also exert direct stimulatory actions at the pituitary level (104).

A number of other agents have been shown to alter PRL secretion, the physiological relevance of which remains to be demonstrated. These include gamma-aminobutyric acid (GABA),

prostaglandin E_1, glucocorticosteroids, thyroxine and triiodothyronine, and, as part of the neural information intergrated by the hypothalamus, endogenous opiates, noradrenergic drugs (in some systems), histamine and acetylcholine (reviewed in refs. 27 and 171). The mechanisms governing the secretion of prolactin therefore appear to be varied and extremely complex.

1.3: Receptors at the plasma membrane level

In order for a molecule delivering information to exert an effect on a target organ, the message it encodes must be recognized. The concept that specific chemical binding sites exist in living cells for such substances was first proposed by J.N. Langley (85) and P. Ehrlich (36) in the early 1900's. The early works on such "receptive substances" were of pharmacological nature but the concept was soon to be extended to the field of endogenous ligands (hormones, neurotransmitters, etc.). Each of these would appear to have its own specific receptors on target cells with, however, some degree of cross reaction. For example, luteinizing hormone (LH) and human chorionic gonadotropin (hCG) are known to share a common receptor to which they bind with comparable K_d (reviewed in ref. 133). More relevant to this work, primate growth hormones are also lactogenic, and exert this action by virtue of their interaction with PRL receptors (PRL-R) (66). This, and the recent findings that some antibodies to hormone receptors can mimic hormonal actions (6,35,68,72; see also chapter 3) argue that it is the receptor system rather than the chemical nature of the ligand that determines cellular response.

The interaction of a ligand with its receptor is much more dynamic than depicted by the classical "lock-and-key" model. Instead, both the ligand and the receptor are thought to undergo conformational changes, resulting in a tight fit

between the two. Several mathematical models have been designed to describe ligand-receptor interactions (reviewed in refs. 5, 12), all of which are related to the mass action law. Perhaps the one which is now the most commonly used is that of Scatchard (137). This graphical analysis method consists of plotting the ratio of bound to free ligand as a function of bound ligand. In the simplest case (simple reversible binding of the ligand to a homogeneous population of receptors), the Scatchard plot is linear and $-1/K_d$ (K_d=dissociation constant) is obtained as the slope and R_o (total number of receptor sites) as the intercept with the abscissa. Other equations were also derived to account for more complex systems that yield curvilinear plots (presence of non-specific binding sites, of two or more classes of receptors or of cooperativity between sites; reviewed, for insulin receptors, in ref. 53).

Thus, the primary role of a receptor is to recognize a message encoded on a specific molecule. Since these messengers come from the exterior of target cells (e.g. blood, synaptic cleft, etc.), it was suggested that receptors are located at the cell surface (71,122). Until recently, it was thought that the ligand bound to its specific surface receptor, thereby activating it, and then came off (5). As is now understood, however, receptor dynamics appear to be much more complex.

Indeed, in addition to receiving a message from the outside, the receptor also has to transmit the message to relevant effectors inside the cell. This phenomenon is known as "transduction", from latin trans [through (the membrane)] and ducere [to conduct, or to carry (the information)]. A "mobile receptor hypothesis" was suggested to account for these dual roles of receptors (reviewed in ref. 69). This hypothesis is based on the understanding that in most systems, these two functions are actually carried out by discrete molecular entities. This provides for an extra degree of freedom to explain

the complexity of receptor occupation versus biological effects in data observed experimentally.

The model suggests that both receptors and effectors (often adenylate cyclase) are able to diffuse freely in the plane of the membrane, with the former facing outward (to be in contact with circulating ligand) and the latter, inward (to transmit the message to intracellular competent molecules, responsible for the biological response). The actual transduction of the information occurs when a stimulated (occupied) receptor "meets" a responsive molecule of effector.

A variety of models based on the mobility of receptors has been suggested (69), including a three-component model for receptor-adenylate cyclase systems, comprising a guanine nucleotide binding, regulatory component (reviewed in refs. 62,154, 155). In addition to their inherent coherence and the fact that such models are compatible with other observed phenomena like selective desensitization (59,160) and the sharing of a common effector (adenylate cyclase) by a variety of receptors (30), direct evidence for receptor movement in the plane of the membrane is now available (100,138,139). A "diffusion coefficient" for a variety of receptors have been computed (reviewed in ref. 69) and have been found to be in the range of most membrane proteins, suggesting that ligand-receptor complexes diffuse about randomly (117).

1.4: Receptor-mediated endocytosis

However, ligands and receptors do not move only in the plane of the membrane. On the contrary, it is now universally accepted that hormone and receptor internalization occurs following binding and other cell surface events (for recent reviews, see refs. 10,20,57,117,127,177,179). Among the several

types of endocytic mechanisms that have been distinguished, the one involving receptor-mediated uptake of ligands, also termed adsorptive endocytosis, is a means for both selecting and concentrating specific extracellular solutes.

Selectivity, of course, is determined by the nature of the receptors present on the surface membrane of a given cell. Concentration of a circulating ligand is also related to its interaction with cell surface specific receptors but also involves the lateral diffusion mechanism mentioned in the previous section. Although a special driving force for such receptor movements does not seem to exist, occupied receptors appear to have a special affinity for restricted regions of the cell membrane, the "coated pits" (179). These specialized regions of the plasmalemma, representing approximately 2% of the total cell surface (2) therefore act as a trap for occupied receptors. It was suggested that immobilization within coated pits could involve a conformational change in the receptor molecule, resulting from ligand binding (118,177). Considering the density of coated pits on the cell surface and the rate of diffusion of receptors, it was calculated that a given receptor molecule should encounter a coated pit every 3 to 5 seconds on most types of cultured cells (100,139,177).

However, two examples contrary to this general model can be cited: 1) There are some cases (such as for the low density lipoprotein receptor, ref. 103) in which receptor occupancy is not required for immobilization of receptors; 2) There are a few systems in which labelled ligand clustering in coated pits has not been observed (7,11,21).

Nevertheless, coated pits were found in almost all animal cells (179) and can be considered to be involved in receptor-mediated endocytosis of a large number of ligands. The "coat" is located on the cytoplasmic side of the membrane and is predominantly constituted of a protein termed clathrin

(120). On electron microscope preparations, it appears as a fuzzy lining on small selected areas of the plasma membrane but was also found on regions of the trans-reticular elements of the Golgi complex and on endocytic vesicles in some cell types.

Although there is general agreement that clustering of ligand-receptor complexes (and also, in some cases, of free receptors) within clathrin-coated pits of the plasma membrane occurs, identification of the specific structures involved in the following steps of internalization remains controversial. Many investigators (20,50,55,64,82) claimed that coated pits that accumulated ligand-receptor complexes pinch off from the plasma membrane and generate intracellular coated vesicles. Such vesicles are thought to loose their clathrin coat shortly after being formed (20,41), thus becoming uncoated endocytic vesicles. Pastan and Willingham (117,118) opposed the view that the coated vesicles observed near the cell surface were actually tangential sections through deep coated pits, which are stable structures, and suggested uncoated vesicles to be generated directly from coated pits.

Both views were convincingly documented. For example, Kolb-Bachofen et al. (83) and Petersen and van Deurs (121) showed coated vesicles very near to or even fused with intracellular vesicles and confirmed these were intracellular by serial sectioning. On the other hand, Willingham and Pastan (176) and Willingham et al. (174,181) interpreted their morphological data as uncoated vesicles actually being formed from the long and tortuous necks of coated pits. A possible solution to this argument could be in sight, however, since Kolb-Bachofen (81) recently reported either coated or uncoated vesicles to be involved in the receptor-mediated endocytosis of lactosylated bovine serum albumin, depending on the cell type studied. She suggested cell shape was important in determining the type of endocytic vesicles.

In addition to the general term "endocytic vesicle", various other names have been given to these organelles, including endosome, receptosome, pinosome or intermediate vesicles (64). The compartment for uncoupling of the receptor and ligand (CURL) described by Geuze et al. (50) could also be related to the endosomal compartment (see below). Newly formed uncoated receptosomes are about 250 nm in diameter (as compared to 150 nm for coated pits), and range in size from 250 to 400 nm (117). They are smooth membrane-bounded vesicles with a protein layer to their inner surface (probably representing ligand-receptor complexes) (117,176). Other characteristic features of receptosomes are that they frequently contain a single intralumenal vesicle, have a fuzzy border along a straightened edge of their membrane and are surrounded by other small vesicular structures with which they may be in continuity (117,176,177,179).

Perhaps one of the most important characteristic of endosomes, however, is that their intralumenal matrix rapidly becomes acidic, within a few minutes of their formation, with an internal pH approaching that of lysosomes (168). However, since they lack lysosomal enzyme activities (32) and appear to selectively avoid fusion with lysosomes (116,177,179), they cannot be considered as part of the lysosomal compartment. Rather, they were observed to fuse only with each other and with the trans-reticular elements of the Golgi apparatus (116, 177). This contrasts with other endocytic components involved in other forms of non-selective endocytosis (not involving the coated pit-endosome pathway) and which were shown to fuse rapidly with mature secondary lysosomes (107,116,140,153,158,175).

Receptors direct ligands through the coated pit pathway, leading to internalization. Since the ultimate fate of most ligands that enter cells via receptor-mediated endocytosis

is to be degraded within lysosomes (55,57,106,156,177), while receptors are generally recycled back to the surface in order to be reutilized (64,73,95,105,142,156,177), a means of separating the two must exist. The acidic environment within endosomes is thought to serve this purpose (48,60,168). Indeed, in some systems, a low pH interferes with ligand-receptor interaction (64,128,134,164). Galloway et al. (46) recently showed acidification of endosomes to be ATP-dependent, probably involving a plasma membrane-derived proton pump (42).

With ligands dissociated from their receptors, sorting of the two becomes conceivable. Geuze et al. (50) recently described a "compartment of uncoupling of receptor and ligand" which was interpreted by Willingham and Pastan (177) to represent trans-reticular Golgi elements. As mentioned above (118, 177), this compartment appears to be a major target for endosome fusion. The group of Geuze, Schwartz and their colleagues, using a clever double labelling method, brought direct morphological evidence for the segregation not only of a ligand (asialoglygoproteins, ASGP) from its receptor (50), but also of different receptors that may be taken up together, depending on their expected fate (e.g. recycling for ASGP receptors or transcytosis for IgA receptors) (51).

That such sorting should occur in the CURL is consistent with results from the group of Willingham and Pastan, who showed such a role for the trans-reticular portion of the Golgi (177). This region of the Golgi apparatus (probably that actually observed by Camillo Golgi in 1898, ref. 56) is also rich in acid phosphatase (54) and was recently shown to have an acidic internal pH (143). Hence, it was also termed the GERL area (Golgi-Endoplasmic Reticulum-Lysosome compartment).

An important morphologic feature of this portion of the Golgi apparatus is that it also contains clathrin-coated pits

(44,114), which are smaller (70 nm, refs. 173,177) than those located on the plasma membrane but appear to have a similar role: the selection and concentration of given macromolecules in order to arrange for proper subsequent routing. Macromolecules concentrated in these small Golgi coated pits appear to be delivered to newly formed lysosomes (172,176,178). It is not clear, however, how ligands that were dissociated from their receptors (see above) are concentrated within these selected areas; aggregation in the plasma membrane coated pits is dependant upon receptors which are able to freely glide in the plane of the membrane and get trapped in coated pits. Since receptors for mannose-6-phosphate and terminal galactose in ASGP (and possibly also receptors for other sugars) accumulate in Golgi coated pits (51,180) and, since the Golgi complex is known to be an important site for glycosylation (54), endocytosed ligands could be glycosylated upon delivery within the trans Golgi and bound by receptors interacting with such carbohydrate moieties and which are capable of clustering in the Golgi coated pits.

While ligands are usually transferred from the transreticular Golgi to nascent lysosomes via the Golgi coated pits, their receptors, which do not concentrate in such structures, are not delivered to lysosomes either. Several indirect lines of evidence suggest that receptors are recycled back to the cell surface (reviewed in refs. 10,55,64,118) and this was convincingly demonstrated for the ASGP receptor (22,142). It is interesting to note that the receptors for lysosomal enzymes cluster in the Golgi coated pits (180),whereas those for other ligands and which are to be recycled, rather than ending up in lysosomes, are segregated differently (50,51). Also, in the few cases where a ligand is to be spared lysosomal degradation (examples are transferrin, ref. 79 and IgA, ref. 51), the ligand-receptor complexes do not dissociate in the endosomes and both are returned together to the cell surface.

Receptor-mediated endocytosis represents a convenient way by which cells can specifically take up macromolecules from the environment. This mechanism can be extremely rapid with clustering of ligand-receptor complexes in coated pits of the plasma membrane occuring within seconds, internalization in endosomes within minutes, delivery to the trans-Golgi elements around 10-15 min. and to the lysosomal compartment at 20-60 min. (see refs. 48,55,100,117,118,139,176,177,179), with some degree of variation, depending on the system studied. Accordingly, receptors are very rapidly recycled to the cell surface, with the round trip sometimes taking less than 5 min. (1, 142). Also, the plasticity of this mechanism is such that different ligands may enter through this pathway (even in the very same coated pit and endosome, refs. 25,99), yet be segregated along the way (51).

Now that the "how" of receptor-mediated endocytosis is beginning to be understood, the "why" still remains open to question. A possible role for such processes is the control of cell surface active components including, of course, hormone receptors. With this respect, internalization could serve either a general "housekeeping" purpose or could be involved in a more specific "turning off" step in the mechanism of hormone action, taking activated (occupied) receptors apart from their cell membrane effectors. A role in the termination of hormone action is quite likely since nearly all ligands studied so far end up in lysosomes and presumably undergo degradation. On the other hand, it would be tempting to propose that such a well controlled uptake mechanism could be involved in targeting informational molecules to intracellular effectors. However, except for the special case of triiodothyronine (115,136), there is as yet no firm evidence for eventual actions of peptide hormones at intracellular sites. On the contrary, and as will be discussed in Chapter 3, there is a great body of evidence to suggest that actions of polypeptide hormones are elicited strictly at the plasma membrane level.

1.5: Prolactin receptors

As is true for other polypeptide hormones, prolactin exerts its action on target organs via interaction with specific cell surface receptors. Prolactin receptors were initially identified in mouse mammary gland in the early 1970's (14,167) and since then, PRL binding has been reported in a wide variety of tissues and species (reviewed in ref. 66). The broad distribution of PRL-specific binding sites is compatible with the ubiquitous nature of this hormone, as reported in Section 1.1.

To date, a true receptor function (recognition, binding and transduction) has been clearly established only in the mammary gland (149), but there is general agreement that PRL binding sites observed in most, if not all other tissues are also physiologically relevant (66,150). Data arguing in favor of this view are the biochemical and immunological similarities found amongst PRL binding sites in most tissues studied (108, 148,150) and the partial blockage of two different actions of prolactin (lactogenic and luteolytic) observed when anti-receptor antibodies were injected into lactating or normal cycling rats, respectively (18). Therefore, in this work, I shall refer to them as PRL binding sites or as PRL receptors.

Some biochemical and physicochemical characteristics of PRL-R are beginning to be elucidated. These studies are complicated by the fact that the receptor molecule has not yet been purified to homogeneity, although much progress has been made during the past few years. A buoyant density of 1.2-1.4 g/ml has been reported (66) which suggests that the receptor is primarily protein material. This view is in good agreement with the fact that proteolytic enzymes abolish PRL binding (152). However, PRL-R also contain carbohydrate moieties, since solubilized receptors bind to concanavalin A (166). In

addition, phospholipids are also required for PRL receptor activity, since phospholipase C reduces PRL binding (152).

There has been contradictory reports on the molecular weight of the prolactin receptor, with values ranging from 32-37,000 (19,87,88) to as much as 320,000 (26). Hughes et al. (66) suggested that the detergent used for solubilization may be responsible for these discrepancies, with nonionic detergents such as Triton X-100 resulting in the formation of high molecular weight aggregates, and zwitterionic detergents like 3-(3-cholamidopropyldimethylammonio)-1-propanesulfonate (CHAPS) possibly breaking apart noncovalently linked subunits of the receptor molecule. Although it has not yet been clearly demonstrated wether PRL-R have a subunit structure [preliminary results by Shiu, not yet published but mentioned by Hughes et al. (66) would suggest so], it appears that the binding polypeptide has a molecular weight in the range of 36-45,000 in the rat, rabbit and mouse (19,67,87,88). Recent results in our laboratory agree with these values (75).

Molecular charge is one physicochemical datum which suggests heterogeneity among PRL-R in different organs. Indeed, experiments using analytical isoelectric focusing yielded pI values between pH 6 and 7 for the rabbit mammary receptor (147) and 5.1 for the hepatic receptor in the same species (169). In the rat, hepatic and mammary PRL-R also differed by their optimal pH for binding, ion requirements and sensitivity to neuraminidase (152). Heterogeneity for insulin receptors has also been reported by several investigators (45, 63,98,183).

Much attention was also addressed to the mechanisms involved in the regulation of PRL-R, with a special emphasis on receptors in the liver and the mammary gland. Regulation of receptors is of primordial importance, in addition to circulat-

ing hormone levels and, possibly, other regulatory processes which may intervene at post-receptor steps, in order to strictly control cellular activity. Actually, PRL-R regulation itself is very complex, with a variety of factors influencing receptor levels in target cells. These have been reviewed extensively (66,108,150,170).

Sex and development are major factors influencing PRL-R levels in mammals, since the most striking effects of prolactin in these animals are the development of the mammary gland and lactation. Therefore, PRL-R levels are similar in livers of young male and female rats (77), but at puberty (around day 40 in the rat), PRL binding capacity is much higher in the female than in the male. Further sex differences are observed regarding the effects of estrogens in PRL-R regulation (see also below), in that these steroids appear to stimulate PRL-R levels in livers of male mice but to reduce them in females (92).

Some physiological conditions may also alter PRL-R levels, such as the phase of the estrous cycle: rat liver PRL binding has been reported to be higher during estrus and diestrus I (76), and this may be related to the PRL, gonadotropin and estradiol surges which occur on the afternoon of proestrus in female rats.

Other physiological states also influence PRL receptors. During pregnancy in rats, PRL-R are greatly increased in both mammary gland (34,65) and liver (77), although a large part of these receptors are occupied by endogenous lactogens. Indeed, it has been shown (78) that the concentration of plasma prolactin and/or placental lactogen (PL) is high throughout pregnancy in many species, and suppression of these by hysterectomy (for PL) or administration of ergot derivatives (for PRL) results in higher estimates for PRL binding.

A further increase in PRL-R levels is observed in rat mammary gland at parturition and during lactation. This would appear to correlate with the rapid decline in progesterone levels occuring just prior to parturition. Progesterone is known to block lactation in this species (84). However, progesterone administration does not block the increase in PRL-R levels in ovariectomized rats (151). Progesterone may elicit its inhibitory effect on lactation by an action on a post-receptor event (170). In support of this view is the demonstration that ovariectomy in pregnant rats results in lactose synthesis (145). Rather, the induction of PRL-R at parturition and during lactation in the rat appears to be dependant upon the suckling-evoked prolactin release (17): not only does removal of the pups abolish this PRL-R increase, but ergocornine administration also blocks it in the presence of the offspring.

The situation may be somewhat different in the rabbit, since progesterone has been shown to block the increase in PRL-R induced by PRL administration in this species (33). Accordingly, in the studies by Kelly et al. (77), pregnancy in the rabbit did not result in increased hepatic PRL-R levels. There was actually no sex difference in lactogenic receptor levels in adult rabbits, but such a difference was observed for growth hormone receptors (GH-R) and these were increased during pregnancy (77). Similarly, in mouse liver, although higher PRL-R levels are seen in the adult female than in the adult male, pregnancy results in an increase in GH-R but not in PRL-R (123). In other studies with guinea pigs, neither GH- nor PRL-R were augmented in the liver during pregnancy (77,125).

These studies would suggest that there is a considerable variation between species in the mechanisms controlling PRL-R levels during pregnancy, but it must also be kept in mind that, as mentioned earlier (see also ref. 150), failure to

observe elevated receptor levels in some of these studies might be explained in part by receptor occupancy by endogenous lactogens, since desaturation of the binding sites has not been systematically carried out.

These differences could represent ways by which different species have tackled the problem of promoting mammary growth during pregnancy, without inducing lactation. Such a hypothesis should be considered in the view of the reported somatogenic actions of prolactin (9,16,24,130) and the ability of placental lactogens of many species to interact with somatogen receptors (165). Therefore, in some species, GH-R rather than PRL-R are augmented during pregnancy, and PRL and/or PL may induce mammary development via interaction with these receptors. In some other species (e.g. rat), prolactin receptors may be able to discriminate between PRL and PL binding, the latter inducing only the growth-promoting effects mediated by this receptor [e.g. PRL induces somatomedin release from liver (43)]. In species not possessing PL (e.g. rabbit), the post-receptor inhibitory action of progesterone is required in order to prevent lactation to occur during pregnancy, but this would be unnessary (and does not occur) in the rat because circulating PRL levels are low at this stage, with the PRL surge occurring just prior to parturition.

Some striking differences in PRL-R regulation are also seen between organs within a given species. In lactating rats, for example, PRL-R levels are high in the mammary gland while they are low in the liver (61). Testosterone decreases PRL binding in the kidney and adrenal (97) and in the liver (146) but increases it in the prostate (80). Prolactin binding is reduced by glucocorticoids in the kidney and adrenal gland, but hepatic receptors remain unchanged (96). Thyroid-stimulating hormone induces PRL-R in the kidney without affecting the adrenal (93).

The physiological significance of these processes remain unclear and better understanding will await further knowledge on the roles of prolactin in these various tissues. If, for example, the reported PRL- (or, maybe PL-) induced generation of somatomedins by the liver (43) is physiologically relevant and is involved in mammary growth during pregnancy, it would be rational to turn off these hepatic receptors after parturition (when the mammary gland is fully developed) but to keep high mammary receptor levels in order to sustain lactogenesis. This, of course is highly speculative, and the identification of the endogenous mediators involved in, and the elucidation of the mechanisms governing such tight regulation in PRL-R levels represent a major challenge for future research.

A great many hormones have been shown to be part of these regulatory processes. Among these, estrogens appear to play a major role and this correlates with PRL-R changes with respect to sex and during the estrous cycle and pregnancy (see above). Also, direct administration of estradiol or estrone results in a marked increase in hepatic PRL binding in both male and female rats, even before puberty (76,125). In addition, ovariectomy reduces lactogen binding in females and antiestrogens block the estradiol-evoked increase in PRL-R (76).

Several lines of evidence suggest that prolactin itself may also play a key role in the regulation of its receptors. First, injection of PRL into rabbits induces mammary receptors (33). Second, hypophysectomy results in decreased PRL binding (125) and administration of high doses of PRL or pituitary implantation under the kidney capsule are able to partially restore PRL-R in these animals (15,29,124,126). Third, the mechanism of estrogen action just mentioned could involve induction of PRL release, as this would agree with the reported

increase in circulating PRL following estrogen administration (104) and the fact that hypophysectomy prevents the ability of estrone to induce PRL-R in rat liver (125). Furthermore, pituitary implants also restored the response to estradiol in hypophysectomized rats (124-126).

In a study by Kelly et al. (74), however, administration of the dopaminergic agonist CB-154 (a less drastic way of blocking PRL secretion than hypophysectomy) failed to alter the response to estradiol. In another study (4), large variations in circulating PRL levels failed to modify the stimulatory effect of castration on hepatic PRL-R levels in male rats. Also, in their aforementioned paper, Kelly et al. (74) showed that higher estradiol doses are required to significantly elevate plasma PRL than to induce hepatic PRL-R in ovariectomized rats. Therefore, although the pituitary is clearly involved in the regulation of PRL-R at least in the mammary gland and in the liver, it would be possible that PRL itself plays only a permissive role. [It must be remembered, however, that there are considerable differences between species or organs, in the effects of PRL on its receptors. For example, PRL appears to be the only inducer of PRL-R in rabbit mammary gland (33) but to have more limited effects in rat liver (15,29). Note that in the latter studies, receptor desaturation has not been carried out prior to PRL-R determination. In vitro evidence for a direct action of PRL on PRL-R levels in cultured rat hepatocytes will be presented in Chapter 3; in this system, estradiol failed to alter PRL binding (unpublished).]

Shiu and Friesen (150) have gone further and suggested that PRL may not be the inducer of PRL-R. They presented some indirect data supporting the view that a "feminizing factor" or "feminotropin" from the pituitary, earlier described by Eneroth et al. (37) and Stenberg et al. (157), could be the true mediator of the change in PRL-R levels observed with hypophysectomy,

pituitary transplants, or other ways of tampering with the pituitary. They also suggested that this factor could have been present as a contaminant in the PRL preparations used for injections, thus explaining the very high doses (2mg per rat) required to induce rat liver PRL receptors.

In an earlier paper (170), on the other hand, the same group had evoked the involvement of other known pituitary hormones, namely thyroid-stimulating hormone (TSH), adrenocorticotropin (ACTH) and growth hormone (GH) in the regulation of hepatic PRL-R. The relevance of these, as well as, possibly, pituitary gonadotropins (via their action on ovarian estrogens), may account for the fact that PRL alone restores only 20-30 % of liver PRL-R levels in hypophysectomized female rats (29).

Thyroxine and estradiol have been shown to be effective in restoring hepatic PRL binding after thyroidectomy and ovariectomy, respectively (49). Although neither ACTH nor bovine GH (non-lactogenic) alone can induce PRL binding in hypophysectomized female rats, they both synergize with low doses of ovine PRL (3). This effect of ACTH is adrenal-dependant but does not involve increased corticosterone release. Rather, Waters et al. (170) suggested this action of ACTH may be mediated by adrenal estrogens and could explain the decrease in PRL-R observed in livers of normal female rats, following adrenalectomy (76). In the male, such an effect of ACTH appears to be largely counteracted by testosterone of both testicular and adrenal origin (170).

The mechanisms governing PRL-R regulation are therefore quite complex and involve a variety of peptide and steroid hormones. The levels of these various factors under specific physiological conditions will determine the level of PRL binding in any given situation with, however, responses varying

considerably between species and tissues. In this section, I documented only PRL-R regulation in the mammary gland, because it is the site of the most studied action of this hormone - lactation - and in the liver, because this organ received most of the attention in studies dealing specifically with PRL-R regulation, including most of the original work presented in this thesis.

However, some data are also available on the regulation of PRL binding in many other tissues. These include the testes, prostate, ovary, adrenal and kidney (reviewed in refs. 150 and 170). These data highlight further differences between tissues, which may relate to the various effects of prolactin in these tissues. For example, one of the functions of prolactin is the control of electrolyte balance (110,111) and it may exert this action via induction of mineralocorticoid output by the adrenal glands (182). Accordingly, salt-loading, which has no effect on kidney PRL-R, specifically increases adrenal PRL binding (94), which may be viewed as a feedback mechanism, classical to hormonal systems. Another example of organ-specific regulatory process for PRL-R relating to PRL function is the unique interdependancy of prolactin and luteinizing hormone in the control of each other's receptor in the ovary (see ref. 170).

The work which I present in this thesis deals exclusively with hepatic and mammary prolactin receptors and is divided into three major topics. When I joined Dr. Kelly's group as an undergraduate apprentice in the summer of 1980, tissue fractionation studies had been undertaken in order to elucidate how and where PRL-R are compartmentalized. Receptors from plasma membrane and Golgi fractions had been identified and were being characterized. My contribution to these studies is related to PRL-R in the lysosomal compartment and is presented in the next chapter. Briefly, PRL-R were found in lysosomal

preparations, charaterized using the method of Scatchard (137) and compared with plasma membrane and Golgi receptors. Experiments were also designed to follow the uptake of ^{125}I-labelled PRL injected in vivo, the degradation of both PRL and PRL-R and the effect of the lysosomotropic agent chloroquine on PRL-R levels in lysosomal sub-fractions.

The second part of my work was devoted to the regulation of PRL-R in rat hepatocytes in primary culture, using a pharmacological approach. In these experiments, which are reported in Chapter 3, I monitored the effects of PRL, alone or in conjunction with various inhibitors of cellular functions (cycloheximide, actinomycin D, dinitrophenol, chloroquine and colchicine), on total cellular PRL-R levels and used these data in conjunction with the putative actions of the drugs utilized to obtain further insight on the mechanism of PRL-R regulation, including the effects of PRL itself. I also studied the effects of a polyclonal antibody to the PRL-R in this system.

Finally, Chapter 4 is addressed to my attempts to directly visualize PRL-R in rabbit mammary gland, using a monoclonal antibody prepared in our laboratory, at the electron microscopic level. The reason why I turned from studying receptors in rat liver to those in rabbit mammary gland, is that when these experiments were undertaken, only monoclonal antibodies to the rabbit PRL-R were available. Also, PRL-R in this tissue were already well characterized, as they had received much attention from a group of our collaborators in Jouy-en-Josas, France. The approach primarily utilized is immunocytochemistry with a second antibody ($F(ab)'_2$ fragment) labelled with horseradish peroxidase.

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CHAPTER 2

LOCALIZATION AND CHARACTERIZATION OF PROLACTIN RECEPTORS IN RAT LIVER: TISSUE FRACTIONATION STUDIES

SECTION 2.1

THE EFFECT OF CHLOROQUINE ON LYSOSOMAL PROLACTIN RECEPTORS IN RAT LIVER

ABSTRACT

Prolactin receptors have been previously identified in purified rat liver plasma membrane and Golgi vesicle preparations. In this study, we report on prolactin receptors located in highly purified lysosome preparations. These lysosomal prolactin receptors were characterized using the Scatchard analysis and compared to other intracellular and cell surface receptors.

We have identified two classes of lysosomes. Lighter lysosome-like vesicles, which are greatly enriched in acid phosphatase activity (the marker enzyme of lysosomes), contain a great deal of binding activity. This PRL binding was only slightly increased by pretreatment of animals with the lysosomotropic agent, chloroquine. In contrast, mature lysosomes showed very little binding activity in control animals, but chloroquine treatment increased binding 7- to 8-fold in these mature lysosomes. We suggest that the lysosome-like structures are immature lysosomes (namely prelysosomes) towards which the hormone-receptor complex is internalized; they appear to bear little proteolytic activity. These structures could play a role in prolactin receptor recycling.

Lysosomal prolactin receptors showed curvilinear Scatchard plots, in contrast to plasma membrane and Golgi counterparts, which were linear over the same range of hormone concentrations. The high affinity site in lysosomes had a K_d comparable to the cell surface and Golgi receptors. The number of binding sites per mg protein in prelysosomes and lysosomes was 3 times greater than that in the homogenate, but Golgi preparations were 3 times as rich as lysosomes. The great number of PRL receptors in prelysosomes could be attributed, in large part, to the low affinity sites.

The internalization of prolactin into rat liver was examined following *in vivo* injection of ^{125}I-oPRL. The labelled hormone was found initially in the plasma membrane fraction, after which it localized preferentially in the Golgi fraction, with maximum incorporation at 15 min. post-injection. Substantial radioactivity was observed in both classes of lysosomes (L-1 and L-2). In contrast to the Golgi fraction, maximum incorporation of ^{125}I-oPRL in lysosomes was at 30 min. This suggests either that during internalization, prolactin first reaches Golgi elements and is then transferred to the lysosomal compartment, or that there are two independent pathways of internalization, one rapid towards the Golgi complex (maybe a path of receptor recycling) and the other, towards lysosomes (probably leading to receptor degradation).

INTRODUCTION

Prolactin receptors have been identified and characterized in purified Golgi and plasma membrane preparations of rat liver (5,25). It is now widely accepted that after binding of hormones to their specific plasma membrane receptors, the hormone-receptor complex is internalized into the cell (1,3,6,10,11, 13,15,16,26,29,32). Nevertheless, the fate of the hormone-receptor complex following internalization remains unclear (6,7,12,16,26). Khan et al. (22) recently reported that insulin and lactogen receptors could be found in a unique vesicle, distinct from Golgi, lysosomes, or other defined subcellular elements.

In this study, we have identified and characterized prolactin receptors in highly purified lysosomes and light, lysosome-like structures and compared them with previously characterized Golgi and plasma membrane receptors. We also studied the internalization of labelled prolactin into liver cells by following its incorporation *in vivo* into subcellular fractions. Our results demonstrate that internalized radioactivity is observed initially in the Golgi and subsequently in the light lysosomes-like and mature lysosomes and suggest a role of lysosomes in the degradation of the hormone-receptor complex.

MATERIALS AND METHODS

1. Materials

Female Sprague-Dawley rats (CRL:CS(SD)BR, 200-250 g) were purchased from Charles River Canada Inc. (St. Constant, Québec). Metrizamide (Analytical grade) was from Accurate Chemical & Scientific Corporation(Westbury, N.Y.) and reagents for enzymatic assays (2-glycerophosphate, 5'-AMP, N-acetyl glucosamine) were from Sigma (St. Louis, MO) except for [^{14}C]-UDP-galactose which was from Amersham (Arlington Heights, IL; CFB-129). 17β-estradiol (Sigma), chloroquine (Sigma) and CB-154 (Sandoz, Basel, Switzerland) for injection into animals were dissolved in physiological saline containing 1% gelatin. Human growth hormone (hGH; NIH-2160E; 1.7 IU/mg) and ovine prolactin (oPRL; NIH-P-S13; 30 IU/mg) were generously supplied by the National Hormone and Pituitary Program (NIH). [^{125}I]NaI was from New England Nuclear (Boston, MA; NEZ-033H).

2. Tissue fractionation

Lysosomes and light, lysosome-like structures (the L-1 fraction from Wattiaux et al., ref. 30) were isolated from estradiol-pretreated rat (5 ug, s.c. twice a day for 7 days) by a modification of the method of Wattiaux et al. (30). Fresh livers were homogenized in 0.25 M sucrose (7 ml/g liver) and centrifuged and washed at 2000 x g for 15 min. The pellet corresponds to the combination of the N (nuclear) and M (mitochondrial) fractions of de Duve et al. (8). The pooled supernatants were further centrifuged and washed at 17,300 x g for 14 min. and the pellets rehomogenized in 0.25 M sucrose (1 ml/3g fresh liver) yielding the L-fraction (light mitochondrial fraction). The pooled supernatants constitute the combination of the P (microsome) and S (soluble) fractions of de Duve et al.(8). L-fraction was mixed with 2 parts of 85.6% metrizamide, 10 ml of

this mixture were placed at the bottom of cellulose nitrate tubes, and discontinuous density gradients were prepared by delicately layering 6 ml of 32.80% metrizamide and 7 ml each of 26.25, 24.43 and 19.70% metrizamide (densities from bottom to top: 1.329, 1.181, 1.145, 1.135 and 1.109 g/ml). All metrizamide solutions had been previously adjusted to pH 7.4 and densities were verified by optical refraction using a Fisher Scientific Refractometer (Fisher Scientific, Waltham, MA). After centrifugation at 100,000 x g in a SW-27 swinging bucket rotor for 3 hours (particles at equilibrium), L-1 fraction and secondary lysosomes (L-2) as well as fractions L-3 and L-4 were removed from interfaces of the gradients using a syringe fit with a 1.2 mm i.d. needle. Plasma membranes were isolated by the method of Ray (27) and Golgi fraction by a modification of the method of Bergeron et al. (5): rats were not given ethanol and the density gradient had only three stages (densities 1.20, 1.15 and 1.03 g/ml), so that all three subfractions described were mixed together.

3. Protein and enzymatic assays and hormone iodination

Protein determinations were made by the method of Lowry et al. (23) using bovine serum albumin as standard. Assays for 5'-nucleotidase (marker of plasma membrane) and galactosyl transferase (marker of Golgi) were as described previously (31, 4). Assay for acid phosphatase (marker of lysosomes) was as follows. Membranes (20 to 200 ug protein) were incubated for 10 min. at 37°C in a medium containing 250 mM 2-glycerophosphate and 200 mM sodium acetate, pH 5.0, in a total volume of 500 µl. The reaction was stopped on ice by the addition of 500 µl 10% trichloroacetic acid. Tubes were centrifuged at 2500 x g for 20 minutes and phosphate was determined in 500-µl aliquots of supernatant in 3.5 ml water: 4 ml of a mixture containing 1.2 N sulfuric acid, 0.5% ammonium molybdate and 2% ascorbic acid was incubated with the 4 ml samples for 30 minutes at 60°C. After

cooling, absorbance was read at 820 nm and compared with that of standards ranging from 0 to 10 µg phosphate. oPRL and hGH were iodinated using a modification of the chloramine-T method as described previously (19).

4. Prolactin receptor determination and characterization

In the first experiment, the presence of prolactin receptors in lysosomes was demonstrated by radioreceptor assay using ^{125}I-hGH (specific activity approx. 80 uCi/µg) as the labelled hormone. Human GH was used instead of PRL because it was shown to be more stable than labelled PRL with similar lactogenic activity in a number of target organs (20,24,25). In order to measure total binding activity, receptor assays were performed after desaturation of binding sites by 3 M $MgCl_2$ (19). All the animals had been treated with estradiol for 7 days (5 µg, s.c. twice a day) and some were further treated with chloroquine (7.5 mg/100 g b.w., 2h and 5.0 mg/100 g b.w., 1 h before sacrifice). The estradiol treatment was shown to enhance PRL receptor number without altering its other characteristics (17,20,24), thus making the tissue most suitable for these studies. We used 200 µg protein samples for lysosomal subfractions, plasma membranes and Golgi, and 400 µg for other fractions and total homogenate.

Prolactin receptors were characterized using Scatchard analysis calculated from competition curves (28) on lysosomal fractions isolated from estradiol- and chloroquine-treated female rats. The same amounts of receptor preparations as for the radioreceptor assay were incubated for 16 hours at room temperature with 100,000 cpm (20-40 fmol) ^{125}I-hGH and unlabelled oPRL (0 to 1000 ng) in 500 µl of a medium containing 25 mM Tris (pH 7.4), 10 mM $MgCl_2$ and 0.1% BSA.

5. Subcellular localization of prolactin receptors after injection of ^{125}I-oPRL in vivo

Estradiol-treated rats, further treated with the dopaminergic agonist, CB-154 [bromocriptine mesilate; 500 ug 24, 12 and ½ hour before Surital anaesthesia (40 mg/kg)] (19), were injected with 30 x 10^6 cpm ^{125}I-oPRL (approximately 0.2 ug) via the jugular vein. To assess non-specific uptake, other animals were given 500 ug unlabelled prolactin in addition to the labelled hormone. Animals were killed by perforating the heart and lungs at 5, 15, 30 or 45 min. postinjection and livers were rapidly removed, cut into pieces, and processed for organelle isolation as described above. Samples were pooled from three animals per group, such that 12 g were frozen and stored at -80°C for 24 hours for the preparation of Golgi fractions, 4 g samples stored at -80°C for 48 hours for the preparation of plasma membranes, and 10 g processed immediately for the preparation of lysosome and L-1 fractions. Freezing of the liver pieces did not alter the biochemical characteristics (marker enzymes contents) of Golgi or plasma membrane preparations as it does for lysosome and L-1 fractions.

RESULTS

1. Organelle preparations

Lysosomal fractions L-1 and L-2, although containing a very small percentage of total homogenate protein, bore somewhat larger proportions of homogenate acid phosphatase activity (see Table 1). L-2 had the highest relative specific activity (RSA; ratio of specific activity of the fraction over that of the homogenate), and L-1 and L-3 were also considerably enriched in acid phosphatase. Although chloroquine had no significant effect on protein distribution (Table 1), it markedly reduced acid phosphatase activity in all lysosomal fractions but not in other fractions.

Contamination of purified fractions of all four organelles studied by marker enzymes was relatively low as shown in Table 2. Purified lysosome fraction (L-2) was enriched 46-fold in acid phosphatase activity, with very little enrichment in 5'-nucleotidase or galactosyl transferase. Purified plasma membranes (9-fold) and Golgi fractions (28-fold) were also specifically enriched in their respective marker enzymes. The L-1 fraction was enriched 22-fold in acid phosphatase, but also about equally rich in 5'-nucleotidase as was the plasma membrane preparation. Electron microscopic observations (Fig. 1) confirmed these results: the L-1 fraction (Fig. 1A) consisted largely of lysosome-like structures that were rather small in comparison with those of the more purified L-2 fraction (Fig. 1B). This L-2 fraction was almost identical in morphology to the L-2 fraction of the original isolation method (30). Plasma membrane preparations showed many desmosomes, with occasional mitochondria and nuclei (Fig. 1C). Finally (Fig. 1D), the Golgi fraction consisted of typical very low density protein-containing vesicles, comparable to the preparations previously described (5).

TABLE 1

Protein and acid phosphatase activity distribution in the fractions from the isolation of lysosomes

FRACTION	PROTEIN		ACID PHOSPHATASE			
			Distribution		R S A	
	Control	Chloroquine	Control	Chloroquine	Control	Chloroquine
Homogenate	195.1 ± 2.2	215.5 ± 4.1	100	100	1.00	1.00
NM	31.8 ± 0.2	32.2 ± 0.8	23.6 ± 1.2	31.2 ± 0.4	0.67 ± 0.02	1.14 ± 0.02
L-Total	8.9 ± 0.2	7.4 ± 0.4	19.9 ± 0.4	10.1 ± 0.2	3.18 ± 0.05	1.08 ± 0.05
L-1	0.26 ± 0.01	0.26 ± 0.03	3.3 ± 0.1	1.36 ± 0.03	24.7 ± 0.4	3.91 ± 0.01
L-2	0.14 ± 0.01	0.16 ± 0.01	2.88 ± 0.03	0.68 ± 0.02	46.1 ± 0.8	4.26 ± 0.01
L-3	0.20 ± 0.01	0.18 ± 0.01	1.66 ± 0.03	0.34 ± 0.01	25.9 ± 0.8	1.83 ± 0.01
L-4	2.04 ± 0.06	1.16 ± 0.01	1.76 ± 0.05	0.34 ± 0.01	0.74 ± 0.02	0.17 ± 0.01
PS	62.2 ± 1.1	68.2 ± 0.6	26.1 ± 0.8	39.6 ± 0.9	0.82 ± 0.04	1.03 ± 0.02
Recovery %	112.9 ± 1.5	107.8 ± 1.8	69.6 ± 2.4	80.9 ± 1.5		

Lysosomes were prepared as described under "Materials and Methods", yielding the fractions listed. Proteins are expressed as milligrams per g fresh liver for the homogenate and as the % of the homogenate content for the fractions. Distribution of the acid phosphatase activity is expressed as the % of the specific activity of the homogenate, which was 3.15 ± 0.04 umol phosphate produced per hour per mg protein in control (estradiol-treated) animals and 2.85 ± 0.04 umol/h/mg for chloroquine-treated animals. Relative specific activity (RSA) is the ratio of the specific activity in each separate fraction to that of the homogenate. All values are expressed ± SEM and are the average of six separate preparations. NM, nuclei and mitochondria (unpurified); L-total, light mitochondrial fraction from De Duve et al (8); L-1 to L-4, subfractions from L-total, including purified secondary lysosomes (L-2); PS, microsomes and soluble matrix (unpurified).

TABLE 2

Enzymatic characterization of subcellular fractions

FRACTION	PROTEIN	ACID PHOSPHATASE	5'-NUCLEOTIDASE	GALACTOSYL TRANSFERASE
Homogenate	219. ± 17 (7)	1.00	1.00	1.00
L-1	0.35 ± 0.08 (6)	21. ± 2 (5)	8.0 ± 0.7 (2)	4.7 ± 0.3 (2)
L-2	0.24 ± 0.01 (6)	41. ± 2 (2)	3.2 ± 0.1 (3)	3.08 ± 0.04 (2)
Plasmalemma	1.68 ± 0.05 (3)	0.28 ± 0.03 (3)	9.2 ± 0.3 (3)	4.1 ± 0.8 (3)
Golgi	0.203± 0.004(3)	1.9 ± 0.2 (3)	1.32 ± 0.06(3)	34 ± 17 (2)

Lysosomes were isolated and acid phosphatase was assayed as described under "Materials and Methods". Other organelle preparations and enzymatic assays were performed as described (4,5,27,31). Proteins are expressed as milligrams per g fresh liver and enzymatic activities as RSA (see text and Table 1). Enzymatic activities in the homogenate were 2.6 ± 0.2 and 2.4 ± 0.4 umol phosphate producted per hour per mg protein for acid phosphatase and 5'-nucleotidase (± SEM of 6 experiments), respectively, and 8.2 ± 1.8 nmol galactose transferred per hour per mg protein for galactosyl transferase (± SEM of 6 experiments). All numbers are expressed ± SEM except when only 2 values were available (± half-range) and are the average of the number of experiments indicated in parentheses. L-1 and L-2 are lysosomal subfractions.

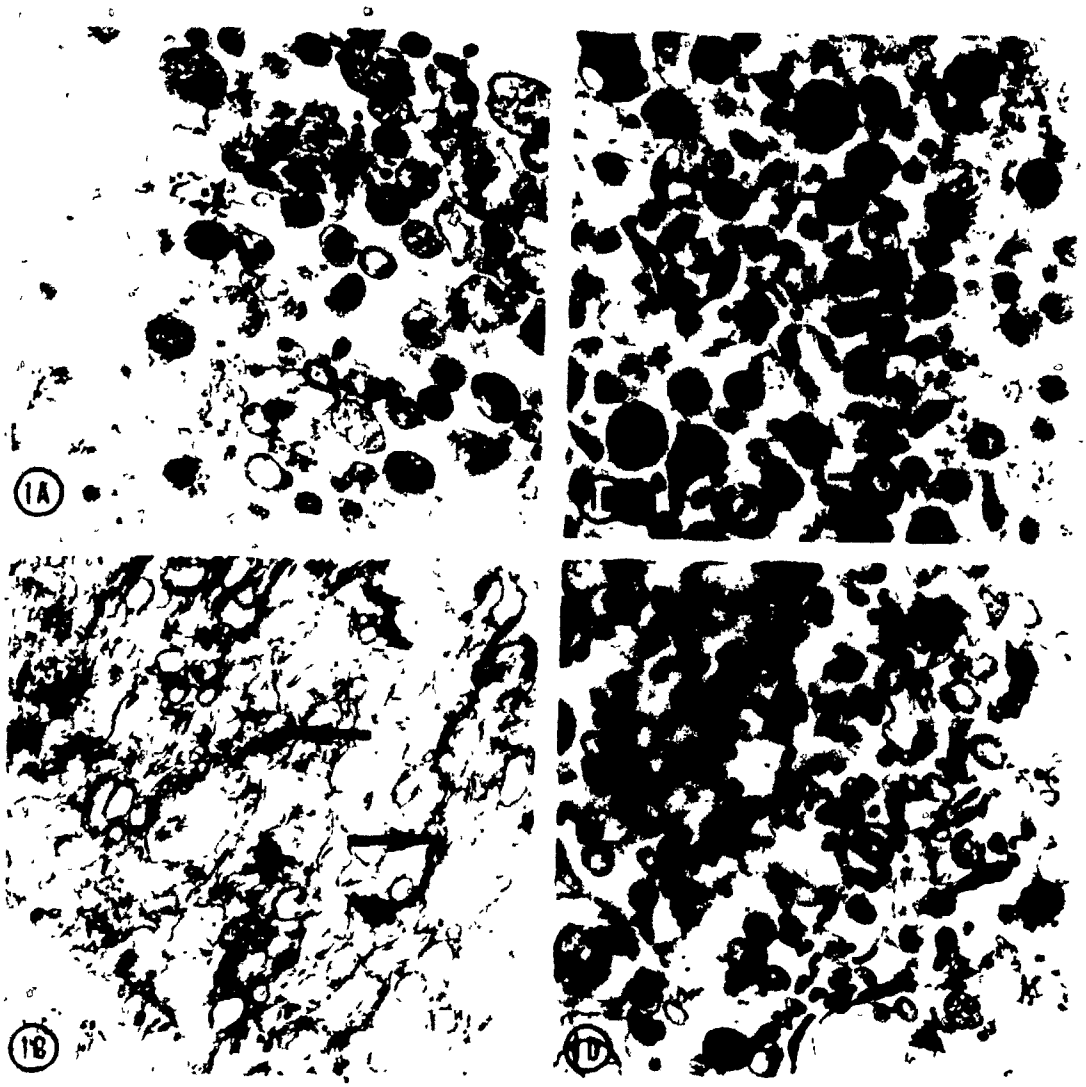

Fig. 1 Electron microscopic view of the four purified organelle preparations. A) The L-1 fraction consists largely of dense and multivesicular bodies that correspond to the description of lysosomes, but most vesicles are smaller than the typical lysosomes shown in B. Some undefined long-shaped structures are also seen. B) Mature lysosomal fraction shows the structures expected: (1) rounded, (2) elongated and (3) ring-shaped dense bodies, (4) residual bodies and (5) multivesicular bodies. C) Plasma membrane preparations show empty vesicles and a great many desmosomes (arrows), the hall-mark of plasmalemma. Occasional mitochondria or nuclei (not shown here) were also observed. D) Typical Golgi fraction was observed with the structures of the three subfractions described by Bergeron et al (5): very low density lipoprotein-containing vesicles of various shapes and sizes and flattened saccules (dumbbell-shaped elements).

2. Lysosomal binding and Scatchard analysis of lysosomal prolactin receptors

Figure 2 shows hGH binding to prolactin receptors in fractions from the isolation of lysosomes. $MgCl_2$ had little effect on receptor levels in any fraction, probably due to the CB-154 treatment of the animals. On the other hand, chloroquine increased hGH binding to prolactin receptors in lysosomal fractions, with the maximum effect in the most purified fraction (L-2), probably the result of reducing degradation of lysosomal receptors. The L-1 fraction was high in prolactin binding activity in control animals, while other lysosomal fractions were very low.

Receptors were further characterized using Scatchard analysis (24). They showed curvilinear plots (Fig. 3), suggesting negative cooperativity between sites or the existence of two different classes of binding sites. When analyzed as a two-site model, both sites showed remarquable constancy for dissociation constants in all fractions (except for L-1 fraction; see Table 3 and Fig. 3), but sites were more concentrated in fractions L-1 and L-2. Chloroquine treatment of the animals had no effect on dissociation constants compared to control animals (data not shown).

3. ^{125}I-oPRL uptake in the liver after injection in vivo

We examined ^{125}I-oPRL uptake in various organelles from rat liver, after injection of 30 x 10^6 cpm into the jugular vein. Maximum incorporation in the homogenate was observed at 15 min. as previously reported by Josefsberg et al. (ref. 16; see also Fig. 4A). It has been previously shown that intracellular receptors could account for as much as 70 % of the receptors of the total liver cell (5). In this study, we demonstrated that most of the labelled hormone was internalized into the Golgi

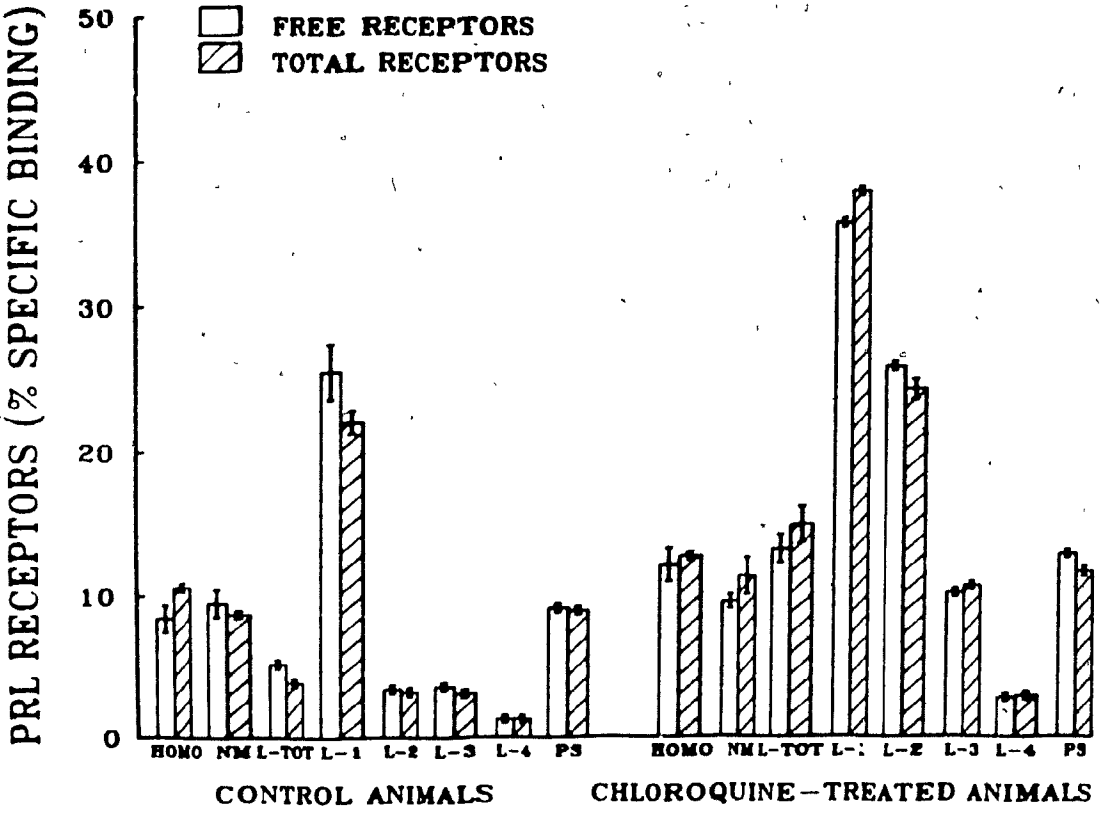

Fig. 2. Livers from female rats treated with estradiol and CB-154 (control animals) and further treated or not with chloroquine were processed for lysosome isolation. Each fraction and subfraction was assayed for prolactin receptors as described previously (19), using 400 µg protein for the homogenate and nuclei and mitochondria (unpurified; NM), light mitochondrial fraction of de Duve et al. (8) (L-TOT), microsomes and soluble matrix (unpurified; PS) fractions, and 200 µg protein for subfractions L-1 to L-4. All values are expressed per 200 µg protein. $MgCl_2$ treatment (19) was performed on separate aliquots of each fraction to desaturate the binding sites for measurement of total receptors. Values are means ± one half the range of 2 determinations. HOMO, total liver homogenate; L-1 to L-4, subfractions of L-TOT, including purified secondary lysosomes (L-2).

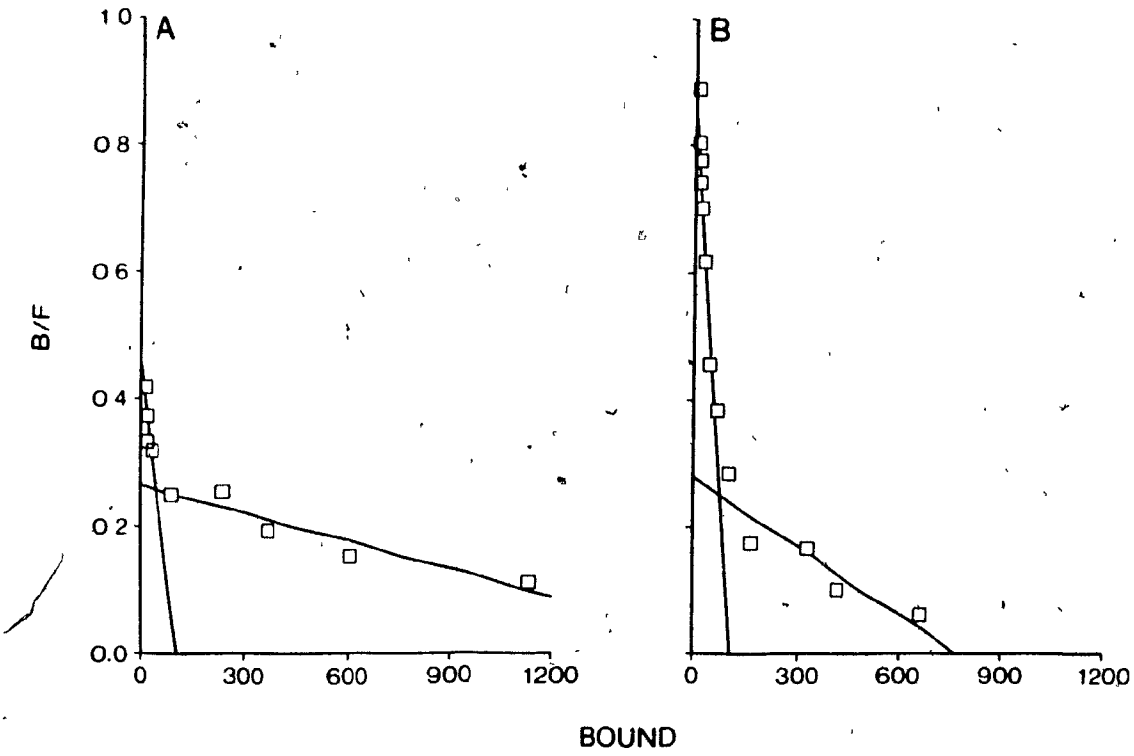

Fig. 3 Scatchard plots of prolactin receptors in lysosomal fractions L-1 and L-2 isolated from livers of estradiol- and chloroquine-treated female rats. The assay was made as described under "Materials and Methods" and results analyzed as representing two distinct classes of sites. (A) L-1 fraction, (B) L-2 fraction (lysosomes). See also Table 3. B/F, bound to free ratio.

TABLE 3

Characterization of prolactin receptors in the fractions from the isolation of lysosomes

FRACTION	HIGH AFFINITY SITE		LOW AFFINITY SITE	
	K_d (nM)	No sites (fmol/mg)	K_d (nM)	No sites (fmol/mg)
Homogenate	0.23 ± 0.01	180 ± 22	2.6 ± 1.6	990 ± 500
NM	0.15 ± 0.01	150 ± 46	3.9 ± 1.0	820 ± 240
L-Total	0.23 ± 0.02	310 ± 86	5.0 ± 1.4	1900 ± 640
L-1	0.50 ± 0.31	560 ± 240	14.0 ± 3.3	9200 ± 3800
L-2	0.25 ± 0.04	550 ± 270	5.6 ± 1.3	3900 ± 1300
L-3	0.28 ± 0.03	150 ± 43	2.0 ± 0.4	600 ± 140
L-4	0.23 ± 0.04	50 ± 14	3.8 ± 0.3	300 ± 160
PS	0.27 ± 0.03	210 ± 55	5.9 ± 1.3	1420 ± 480

Livers from estradiol-treated rats further treated with chloroquine were processed for the isolation of lysosomes as described under "Materials and Methods", yielding the fractions and subfractions listed. 400 ug protein from each separate fraction or 200 ug protein from subfractions L-1 to L-4 were used for Scatchard analysis by competition of prolactin receptors as described (24). Curvilinear plots (Fig. 3) were analyzed as a two-site model. These results are representative of three separate experiments. For the high affinity site, there was no significant difference in the K_d or number of sites among all the samples. For the low affinity site, K_d and number of sites for L-1 fractions were significantly different from all the others ($p < 0.01$). Abbreviations are defined in Table 1.

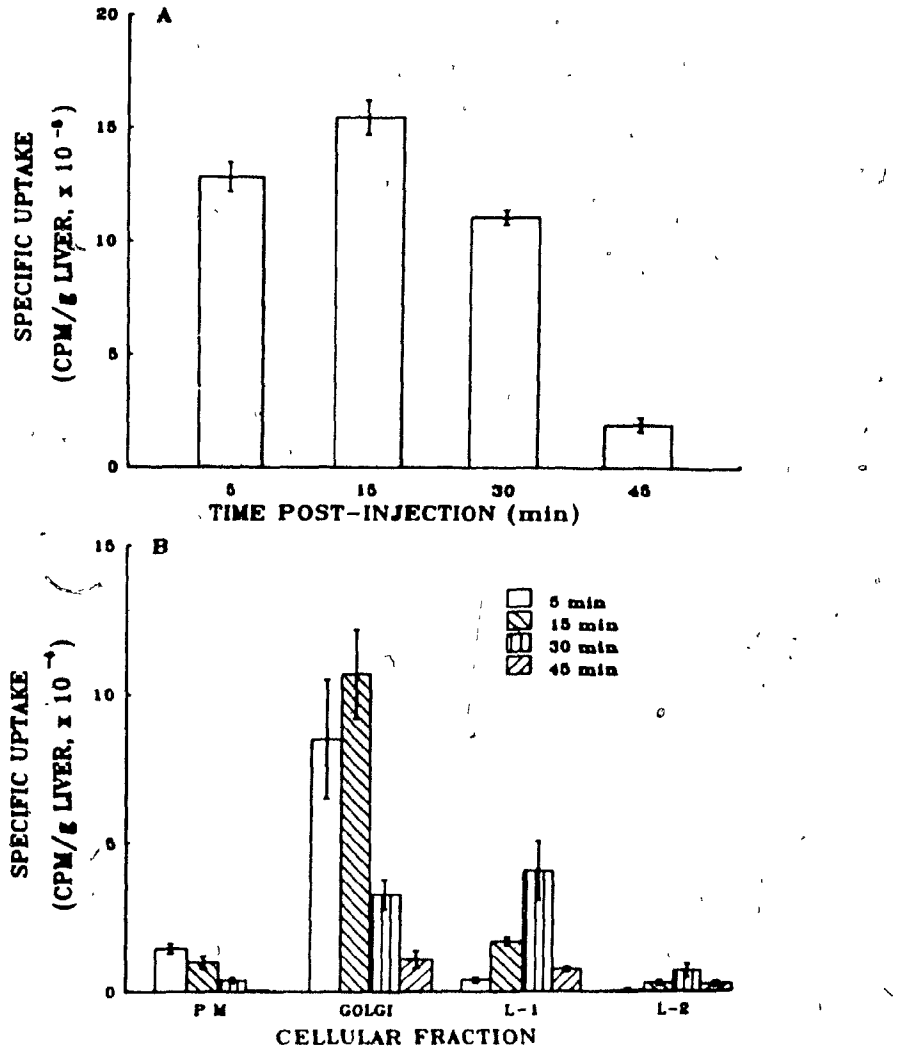

Fig. 4 Uptake of ^{125}I-oPRL in liver homogenate (A) and purified fractions (B) following injection of 30 x 10^6 cpm ^{125}I-oPRL (approx. 7.5 pmoles) via the jugular vein. Female rats treated with estradiol and CB-154 (see "Materials and Methods") were anaesthetized with Surital, the jugular vein was exposed, and the labelled hormone injected in a volume 0.2 to 0.4 ml. Other animals were given 500 µg unlabelled prolactin in addition to ^{125}I-oPRL to determine the level of nonspecific uptake. At the times indicated, livers were rapidly removed, cut into pieces, weighed, and processed as described (1, 19, "Materials and Methods"). Values are expressed as the counts per liver specifically bound to the homogenate or to fractions, and are means ± SEM of 4 separate experiments. P.M., plasma membranes; Golgi, golgi membranes; L-1, L-1 fraction; L-2, mature, secondary lysosomes.

(Fig. 4B). About half of the injected radioactivity was taken up by the liver at 15 min., 10% of which was concentrated in the Golgi. The internalized material could well be in the form of hormone-receptor complexes since large amounts of hGH-binding sites have been shown to be present in those vesicles (5). In contrast to what was observed in the homogenate and in the Golgi fraction, maximum incorporation in lysosomes and in L-1 fraction was seen at 30 min.

DISCUSSION

Prolactin receptors have been identified and characterized from the Golgi complex and the plasma membrane of rat liver (5, 25). The aim of this study was to increase our knowledge of intracellular prolactin receptors by characterizing those located in highly purified lysosomal fractions and comparing them with other intracellular or cell surface receptors. Prolactin receptors have been previously observed in rat liver lysosomes and tritosomes (22). In the present study, two classes of lysosome-like vesicles were identified. The L-1 fraction was rich in acid phosphatase and contained many intact receptors. Binding activity of L-1 fraction was only moderately augmented by chloroquine treatment of the animals, suggesting that these vesicles bear little degradative activity. Typical secondary lysosomes (L-2 fraction) were twice as rich in acid phosphatase as L-1 fraction, yet very poor in receptors in control animals. The fact that chloroquine treatment considerably enhanced receptor levels in secondary lysosomes (7-8 fold) suggests that degradative processes may occur in these organelles.

Khan et al. (22) and more recently, Baenzinger and Fiete (2) reported insulin, lactogen and asialoglycoprotein receptors to be internalized into uncoated vesicles with a density higher than that of Golgi vesicles and comparable to that of plasma membrane. Khan et al. (21) reported these vesicles to have lysosome-like features. They could correspond to the small, homogeneous lysosomes with which receptosomes appear to fuse, about 30 min. after internalization of α_2-macroglobulin (30). The L-1 fraction in the present studies appears to closely resemble these vesicles. Nevertheless, despite the lysosome-like morphology of this fraction, it cannot be defined simply as light lysosomes, since it is only half as enriched in acid phosphatase activity as the true lysosomal fraction, yet about 7

times richer in prolactin receptors in control animals and resistant to the action of chloroquine. We suggest it may consist of immature lysosomes, or prelysosomes, in which proteolytic activity would be less than that of mature, secondary lysosomes (fraction L-2). Such a hypothesis is supported by the facts that the L-1 fraction is enriched in 5'-nucleotidase and less rich in acid phosphatase activity than secondary lysosomes, that prolactin receptors remain largely intact, and that receptors are only minimally affected by treatment of the animals with the lysosomotropic agent chloroquine. In contrast, the mature lysosomes (L-2 fraction) are very rich in acid phosphatase activity (and presumably in general proteolytic activity), poor in intact prolactin receptors in control animals (due to degradative processes occuring within lysosomes) and rich in prolactin receptors following blockage of lysosomal degradation by chloroquine. Because the L-1 fraction consists largely of lysosome-like vesicles, it seems likely that receptor activity in this fraction is related to receptor activity measured in the lysosomal compartment.

Hisuka et al. (14) pointed out that lysosomotropic agents such as NH_4Cl and chloroquine increased cell-associated ^{125}I-hGH in cultured lymphocytes by a mechanism that involves inhibition of degradation. The present study shows that not only the hormone, but also the internalized receptors that reach the lysosomal compartment are protected by chloroquine.

In contrast to what was observed in previous studies on prolactin receptors from the plasma membrane and Golgi fractions (18), Scatchard plots of lysosomal receptors showed a curvilinear pattern. When analyzed as a two-site model, the high affinity site had a dissociation constant similar to that of the single site observed in Golgi and plasma membrane fractions. It is not excluded that the low affinity binding site might arise in prelysosomes and lysosomes as a result of partial alteration

of the high affinity site even if the animals were treated with chloroquine. The high affinity sites were concentrated in purified prelysosomes and lysosomes rather than in fractions of nuclei and mitochondria or microsomes and soluble matrix (2- to 3-fold), but purified Golgi fractions were still 3 times as rich as purified lysosomes (about 1500 fmol/mg protein, ref. 32).

As can be seen in Table 3, the increased number of prelysosomal prolactin receptors may be attributable in large part to the low affinity site. It is possible that receptor-ligand dissociation may occur in these structures and recycle prolactin receptors to the cell surface, as has been suggested for asialoglycoprotein receptors (2).

Iodinated prolactin was taken up <u>in vivo</u> by the liver (maximum incorporation at 15 min. in the homogenate). It was found to be associated with both Golgi, as first reported by Josefsberg et al. (16), and with lysosomes (22). The kinetics of internalization differed between Golgi and lysosomes, with a maximum at 15 min. in the Golgi fraction and at 30 min. in both prelysosomes and lysosomes. This suggests either that ^{125}I-PRL is sequentially internalized into Golgi and then reaches the lysosomal compartment, as suggested by Khan et al. (21) for ^{125}I-insulin internalization or, alternatively, that there exist two independent paths of internalization, one towards the Golgi complex (for receptors to be recycled) and the other going directly, although less rapidly, towards the lysosomes (for the receptors to be degraded). Such a model has been suggested by Geisow (9) and is supported by the high content of plasma membrane marker enzyme found in prelysosomes. Further studies will have to be carried out in order to determine the fate of internalized prolactin and prolactin receptors.

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SECTION 2.2

INTRACELLULAR TRANSFORMATION OF PROLACTIN FOLLOWING INTERNALIZATION INTO RAT LIVER

ABSTRACT

We investigated prolactin (PRL) degradation in rat liver lysosomes both *in vivo* and *in vitro*. In previous studies, we showed that in addition to the Golgi apparatus, PRL is internalized towards lysosomes and light, lysosome-like vesicles which we identified as "prelysosomes". Injected ^{125}I-oPRL that localized in lysosomes and prelysosomes at times varying from 0 to 45 min showed significant differences from fresh and plasma membrane- (PM) or Golgi-bound hormone. First, it was more easily dissociable by 3M $MgCl_2$ than Golgi- but less than PM-bound ^{125}I-oPRL. Second, it was only in lysosomal fractions that, as time following injection increased, a significant part of dissociable radioactivity became non-TCA-precipitable. When $MgCl_2$-extracted ^{125}I-oPRL was subjected to gel filtration on a Sephadex G-75 fine column, some of the radioactivity, and especially that extracted from prelysosomal or lysosomal fractions, eluted as a high molecular weight (HMW) entity, most co-migrated with fresh ^{125}I-oPRL and a little was found in small fragments. Only the central peak had any rebinding activity, which was comparable to that of fresh hormone.

In an *in vitro* study, we incubated ^{125}I-hGH with lysosomal fractions for 16h at 25°C. After centrifugation, an aliquot of supernatant hormone was assayed for its binding capacity to standard receptor preparations, and the rest subjected to gel filtration. Peak fractions were also tested in binding assay. ^{125}I-hGH that had been in contact with prelysosomes lost almost all of its ability to bind to standard receptors and totally migrated in the HMW peak, at the void volume of the column. Hormone incubated with lysosomes bound about 65% as well as the fresh hormone and, correspondingly, 35-40% of the radioactivity was found in the HMW peak and a little as small fragments. It is possible that in lysosomes, and especially in prelysosomes, binding of the hormone to its receptor becomes irreversible and the hormone-receptor complex may become partially solubilized.

INTRODUCTION

It is now established that, after binding of prolactin to its cell surface receptor, the hormone is translocated inside the cell (13,21,25). The demonstration by Clark and Harrison (5) that insulin binds covalently to its receptor, suggests that it is internalized in the form of a ligand-receptor complex. This point of view is shared by Felhman et al., (9). The results presented in section 2.1 and studies by other investigators (17,22) showed that in rat liver, prolactin locates preferentially in the Golgi apparatus and also in lysosomes and in light, lysosome-like vesicles which we termed "prelysosomes" (see Section 2.1). In this study, we investigated the integrity of lysosome-bound prolactin either after injection of the labelled hormone in vivo or after incubation with lysosomal or prelysosomal preparations in vitro.

MATERIALS AND METHODS

1. Materials

Female Sprague-Dawley rats (CRL:CD(SD)BR; 200-250 g) were purchased from Charles River Canada Inc. (St-Constant, Québec). 17β-estradiol (Sigma, St. Louis, MO) and CB-154 (Sandoz, Basel, Switzerland) for injection into animals were dissolved in physiological saline containing 1 % gelatin to retard absorption. Acid phosphatase assays were performed using 2-glycerophosphate (Sigma) as substrate. Human growth hormone, (hGH; HS-2160E; 1.7 IU/mg) and ovine prolactin (oPRL; NIH-P-S13; 30 IU/mg) were generously supplied by the National Hormone and Pituitary Program (NIH). [^{125}I]NaI was from New England Nuclear (Boston, MA; NEZ-033H).

2. Assays, iodinations and organelle preparations

Protein determinations were made by the method of Lowry et al., (19) using bovine serum albumin as standard. Enzymatic assays for acid phosphatase (Section 2.1), 5'nucleotidase (28) and galactosyl transferase (1) were used to assess lysosome, plasma membrane (PM) and Golgi preparations purity, respectively. Typical preparations were described in Section 2.1. Binding assays were performed as described earlier (15); standard receptors refer to rabbit mammary gland microsome preparation produced in this laboratory. Iodination of oPRL and hGH were performed using low concentration of chloramine-T (475 ng and 600 ng in the 55 µl incubation mixture for hGH and oPRL, respectively) as described previously (15). Specific activities were 80-100 µCi/µg for both hormones. Plasma membranes (PM) were isolated using the method of Ray (26), Golgi fractions by a modification of the method of Bergeron et al. (2) and lysosomes as well as prelysosomes by a modification of the method of Wattiaux et al. (27) as described in Section 2.1.

3. In vivo experiments

Estradiol-treated rats (5 ug, s.c. twice a day for 7 days), further treated with the dopaminergic agonist CB-154 (500 ug, s.c. at 24, 12 and ½h before Surital anaesthesia, 40 mg/kg) to reduce endogenous PRL circulating levels, were injected with 30 x 10^6 cpm ^{125}I-oPRL (approx. 0.2 ug) via the jugular vein. At times varying from 0 to 45 min, animals were sacrificed by perforating the heart and lungs and the livers rapidly removed and processed for organelle isolation as described in Section 2.1. In short, for each time point, we used 6 animals, 3 of which were injected with a large excess of unlabelled prolactin (500 ug) in addition to the labelled hormone in order to assess for non-specific uptake. The livers from the 3 animals in each group were pooled, cut into pieces, and 12 g samples were frozen at -80°C for 24 h before the preparation of Golgi fractions, 4 g samples thus frozen for 48 h before the preparation of PM fractions and 10 g samples processed immidiately for the preparation of lysosomal and prelysosomal fractions. As mentioned in Section 2.1, freezing of the liver pieces did not alter the biochemical characteristics (marker enzyme contents) of Golgi or PM preparations. Final pellets from PM, Golgi, prelysosomal and lysosomal fractions were homogenized in 3 M $MgCl_2$ to dissociate hormone from the receptors (15) and the suspensions counted, centrifuged at 100,000 x g for 20 min and the supernatant and pellet counted again. An aliquot of the supernatant was assayed for precipitability in 5 % trichloroacetic acid (TCA) and the remainder eluted on a Sephadex G-75 fine column (0.9 x 100 cm). The peak fractions were assayed for their ability to bind to a standard prolactin receptors preparation.

4. In vitro experiments

In the in vitro experiments, human growth hormone was used instead of prolactin because it has been used in previous stud-

ies by various groups and ^{125}I-hGH was shown to be more stable than labelled PRL and to have similar lactogenic activity in a number of target organs (16,23,24). 400 µg protein samples of lysosome or prelysosome preparations (as well as L-3 and L-4 fractions) isolated from estradiol- and CB-154-treated rats were incubated with 800,000 cpm ^{125}I-hGH for 16 h at room temperature in a 25 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$ and 0.1 % BSA, in a total volume of 1 ml. Membranes were centrifuged, aliquots of the supernatants were assayed for binding ability on standard receptors and the rest processed for gel filtration on a Sephadex G-75 fine column followed by binding assay of peak fractions. The four "lysosomal" fractions were derived from the "Light Mitochondrial" fraction of De Duve et al. (7) with L-1 subfraction consisting mainly of small lysosome-like vesicles (prelysosomes; see Section 2.1), L-2 fraction consisting of typical, secondary lysosomes and L-3 and L-4 containing increasing proportions of mitochondria and decreasing lysosome content and acid phosphatase activity (see Section 2.1, or ref. 27 for a detailed description of these fractions).

RESULTS

1. In vivo experiments

Injected ^{125}I-oPRL had different characteristics, depending on what organelle it was bound to. In addition to being unevenly distributed inside the liver cell (Section 2.1), it was generally more firmly bound to prelysosomes and lysosomes than to PM and a little less than to Golgi as shown in Fig. 5. There was little variation with time, the radioactivity extractable with 3 M $MgCl_2$ ranging between about 20-35 % from the Golgi, between 45-55 % from both lysosomal and prelysosomal fractions and 60-75 % from the PM fraction. (There were two individual exceptions: prelysosomes at 45 min and PM at 15 min post-injection that diverged from these values).

Figure 6 shows that nearly all of the $MgCl_2$-extracted radioactivity was TCA-precipitable for every organelle at every time except for prelysosomes and lysosomes at 45 min, suggesting that degradation of ^{125}I-oPRL has begun at this time in these vesicles.

Elution patterns of the $MgCl_2$-extracted radioactivity (not shown here) featured a small high molecular weight (HMW) peak (Table 4). This peak was highest in lysosomes, where it accounted for 10-12 % of the total extracted radioactivity, moderate in prelysosomes (6-10 %), smaller in Golgi (3-9 %) and not observable in the radioactivity extracted from PM. For a given organelle, the height of the HMW peak relative to the total radioactivity remained relatively constant at all times assayed. There was very little radioactivity eluting at the total volume (peak III), suggesting eventual degradative fragments may rapidly leave the liver. This is supported by the rapid clearance of injected labelled oPRL from rat liver which was shown in Section 2.1, and by the short half-life of plasma prolactin reported by

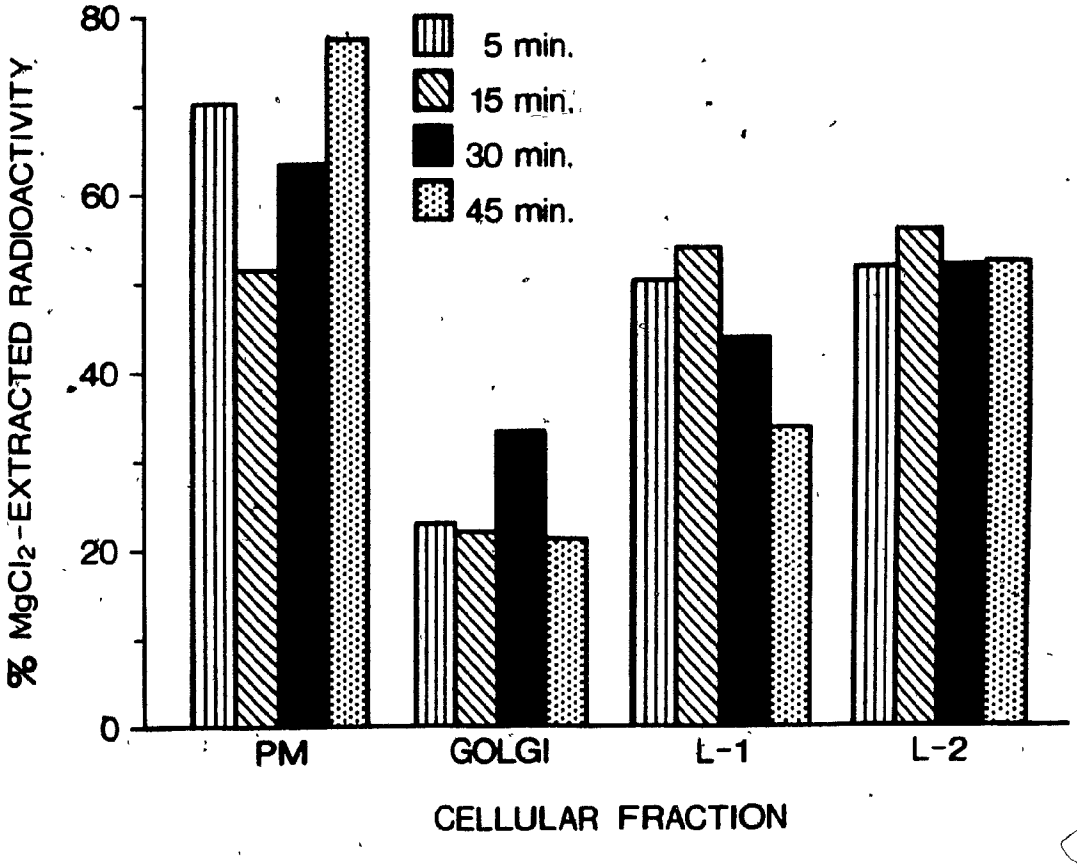

Fig. 5 Radioactivity extractable from purified organelles following the injection of 30 x 10^6 cpm ^{125}I-oPRL *in vivo*, via the jugular vein. Animals were sacrificed at indicated times post-injection, their livers rapidly removed and processed for organelle isolation as described in Section 2.1. Final pellets were resuspended and homogenized in 3 M $MgCl_2$, the total suspension counted, centrifuged and the supernatant and pellet counted again. The sum of the supernatant and pellet counts varied from 81 to 102 % of the total suspension counts. Values are expressed as % of the radioactivity in the $MgCl_2$ supernatant as compared to the sum of supernatant and pellet.

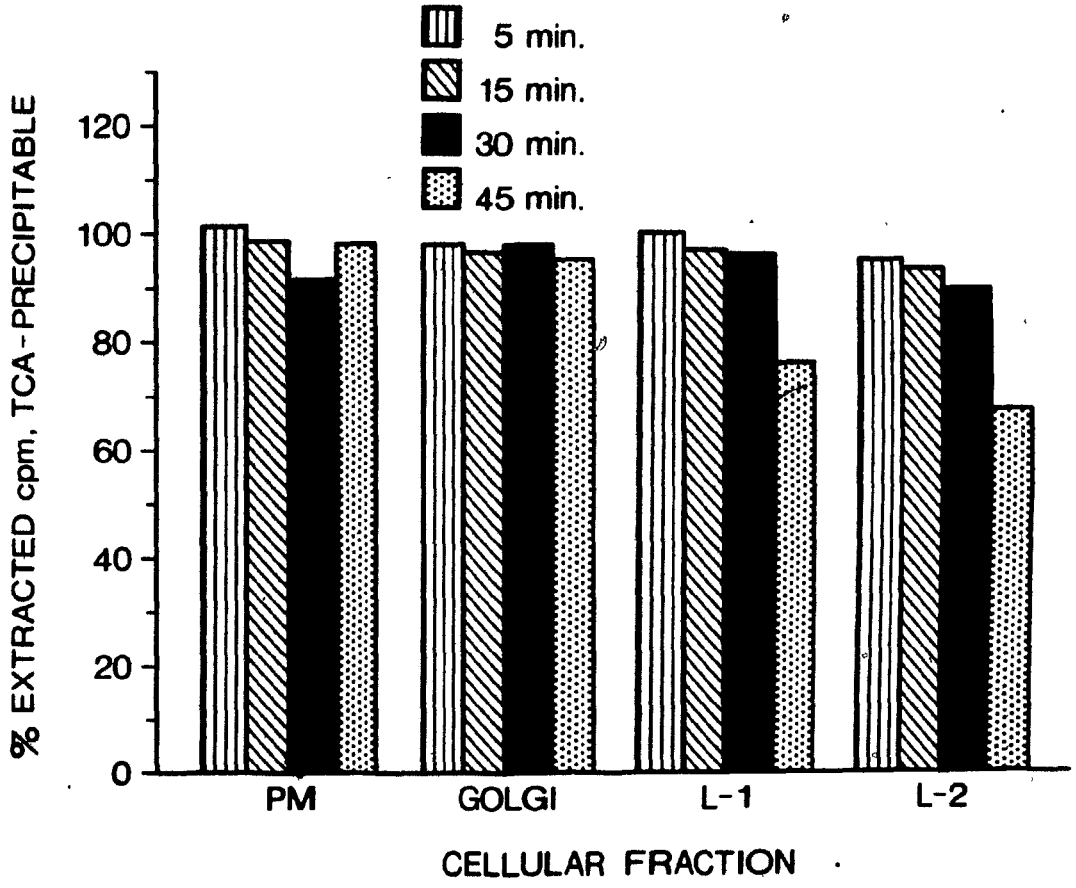

Fig. 6 A 500 ul aliquot of the $MgCl_2$ supernatant, obtained as described in the legend to Fig. 5, was mixed with an equal volume of 10 % trichloroacetic acid (TCA). Tubes were counted, centrifuged and the supernatant and pellet counted again. Values are expressed as percent radioactivity in the TCA-precipitated pellet as compared to the radioactivity initially present in the aliquot.

TABLE 4

Gel filtration distribution of $MgCl_2$-extracted ^{125}I-oPRL that was bound to various liver organelles following in vivo injection.

Organelle	Time post-injection (min)	Radioactivity (cpm) Peak I	Peak II	Peak III
PM	5	BKG	41,430	BKG
	15	BKG	12,580	BKG
	30	BKG	14,150	960
	45	BKG	BKG	BKG
Golgi	5	55,330	596,300	7,090
	15	10,090	133,520	15,990
	30	8,840	322,970	1,530
	45	1,700	17,130	BKG
L-1	5	6,380	50,720	3,310
	15	16,480	151,190	6,210
	30	8,550	131,760	4,340
	45	1,270	13,770	2,340
L-2	5	1,340	9,700	BKG
	15	2,300	15,250	1,200
	30	3,230	26,230	2,820
	45	BKG	BKG	BKG

Female rats were injected with 30×10^6 cpm ^{125}I-oPRL (approx. 7.5 pmol) via the jugular vein. At the times indicated, the animals were sacrificed and their livers removed, homogenized and processed for organelle preparation as described in Section 2.1. Radioactivity from the final pellets was extracted with 3 M $MgCl_2$ (15) and processed for gel filtration on a Sephadex G-75 fine column. Peak I, void volume; Peak II, fresh ^{125}I-hGH peak; Peak III, total volume. PM, plasma membrane fraction; Golgi, Golgi fraction; L-1, pre-lysosome fraction; L-2, mature lysosomé fraction. BKG, background level (approx. 100 cpm).

Grosvenor et al. (11). When there was enough radioactivity, material from the peaks was assayed for its ability to bind to a standard receptor preparation (data not shown). Radioactivity from the HMW peak or from peak III (total volume) of any cellular fraction at any time was totally unable to bind to standard receptors, but material which co-migrated with ^{125}I-oPRL always yielded binding comparable to the fresh oPRL.

2. In vitro experiments

^{125}I-hGH that had been in contact with a prelysosomal preparation for 16h at 25°C lost nearly all of its ability to bind to standard receptors as depicted in Table 5, but about 55 % of binding capacity remained after incubation with mature lysosomes. Chloroquine did not protect the hormone from this *in vitro* transformation (not shown). There was no significant decrease in binding capacity when the hormone had been in contact with lysosomal fractions L-3 or L-4.

All of the radioactivity in the supernatant from the incubation with prelysosomes eluted as a single high molecular weight (HMW) peak (Fig. 7) and radioactivity from this peak was totally unable to bind to standard PRL receptors (Table 5). In contrast, and according with remaining binding ability figures, 55-60 % of supernatant radioactivity from the incubation with mature lysosomes comigrated with and had comparable binding ability to fresh ^{125}I-hGH (Fig. 7 and Table 5), about 40 % formed the HMW peak (and was unable to bind to standard receptors) and a little eluted at the total volume of the column (possibly free ^{125}I produced by deiodination of the labelled hormone or ^{125}I-iodotyrosine or other low molecular weight degradation products). Elution patterns of the hormone incubated with other lysosomal fractions were similar to that of the fresh hormone (not shown) as is confirmed by the integrity of the ability to bind to standard receptors (Table 5). Again, radioactivity from peaks other than ^{125}I-hGH showed no binding activity.

TABLE 5

Binding ability of ^{125}I-hGH that has been in contact with lysosomal fractions.

Fraction	% Specific Binding		
	Supernatant	Peak fractions	
		HMW peak	hGH peak
Fresh hGH	35.8	-	36.5
L-1	0.3	1.3	-
L-2	19.2	4.2	33.4
L-3	33.4	n.d.	n.d.
L-4	34.6	n.d.	n.d.

^{125}I-hGH was incubated with lysosomal subfractions (800,000 cpm corresponding to approx. 200 fmol ^{125}I-hGH with 400 ug protein in a total volume of 1.0 ml) for 16 h at 25°C. Tubes were centrifuged and an aliquot of each supernatant was used as labelled hormone for prolactin receptor assay as described (15) on standard rabbit mammary gland receptors (second column). The remainder of the supernatants from L-1 and L-2 incubations as well as fresh ^{125}I-hGH were processed for gel filtration on a Sephadex G-75 fine column (see Fig. 7) and radioactivity from the high molecular weight (HMW) peak as well as that co-migrating with fresh hGH was also assayed for its ability to rebind to standard receptors. L-1, prelysosomes; L-2, mature lysosomes; L-3 and L-4, other lysosomal fractions (see text and Section 2.1 or ref. 27); n.d., not determined.

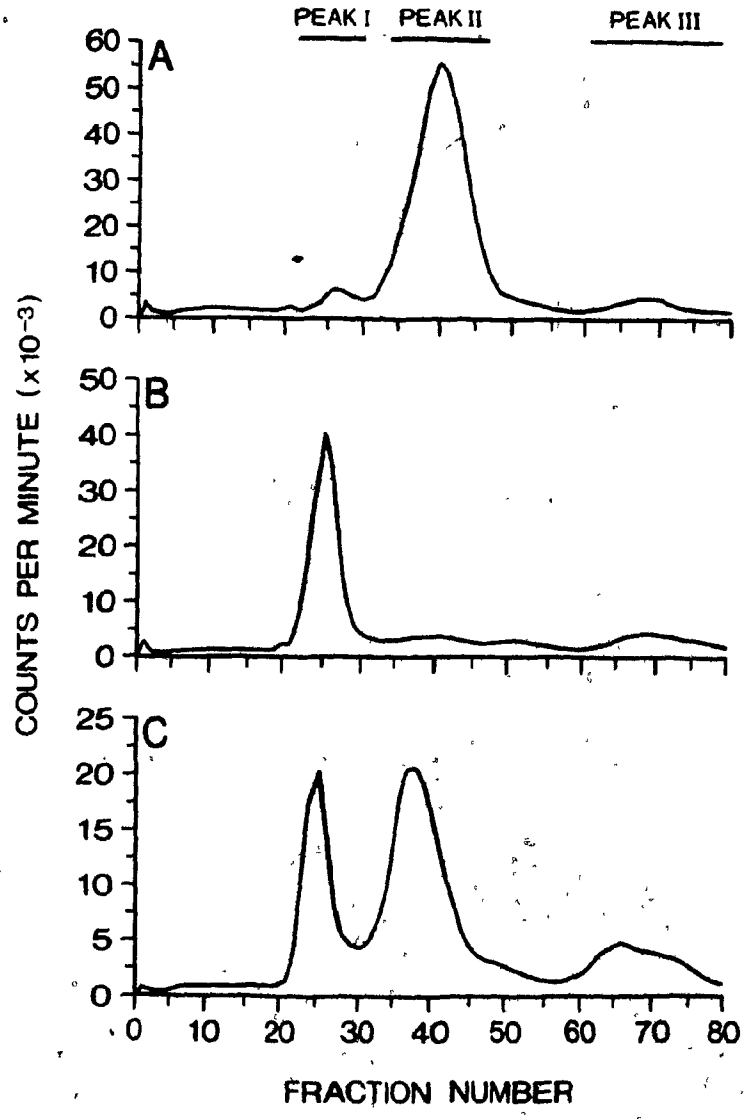

Fig. 7 Elution profiles of (A) fresh ^{125}I-hGH, (B) ^{125}I-hGH incubated with the prelysosomal (L-1) fraction or (C) the mature lysosomal (L-2) fraction. 400 ug protein and 800,000 cpm (approx. 200 fmol) ^{125}I-hGH were incubated for 16 h at 25°C in a total volume of 1.0 ml. Tubes were centrifuged and the supernatants were processed for gel filtration on a Sephadex G-75 fine column (0.9 x 100 cm) and fractions of 0.5 ml were collected. When there was enough radioactivity, the four fractions forming the top of the peak were pooled and assayed for binding ability on a standard receptor preparation (see Table 5). The profiles presented here are representative of four separate experiments.

DISCUSSION

It is now widely accepted that after binding of prolactin to its plasma membrane receptor, it is internalized into the cell (6,12,13,17, and Section 2.1). Internalized PRL has been localized in Golgi, lysosomes and prelysosomes of rat liver and PRL receptors have been localized in these organelles as well as in another, as yet undefined lysosome-like structure, that appears to be distinct from classical Golgi or lysosomes (17).

Our previous *in vitro* studies (14) showed that PRL binding to either PM or microsome receptors was not freely reversible with the hormone being more firmly bound as association time increased. Similar results have been reported for hGH binding (8,12). In the studies with labelled PRL (14), it was shown that PRL was more dissociable from PM than from microsomal receptors for both short (1 h) and long (10 h) association times. This was confirmed in the present *in vivo* study at very short times, with more radioactivity (that proved to be mainly intact ^{125}I-oPRL) being $MgCl_2$-extractable from PM than from Golgi, lysosomes or prelysosomes (Fig. 5). This is in good agreement with reports from other investigators (3,8,20) who demonstrated hGH and insulin receptor heterogeneity and suggested a conversion of low affinity (fast dissociating) cell surface receptors to high affinity (slow dissociating) form, following binding of the hormone. Krupp and Livingston (18) actually separated three distinct insulin binding components from rat liver plasma membrane, all with different affinities and association to insulin degradation.

Olefsky et al., (20) suggested that insulin receptors protect insulin from degradation before conversion to the high affinity form, and that they mediate such degradation afterwards. Again, this is consistent with results reported here for PRL, since hormone bound to intracellular receptors (in particu-

lar to lysosomes which are thought to represent the main site of hormone degradation as reviewed by Chertow, ref. 4) was less readily dissociable than that bound to PM, and kinetics of association to PM was much more rapid as compared to lysosomes, as shown in Section 2.1).

In addition to studying PRL binding strengh in various organelles following injection of ^{125}I-oPRL *in vivo*, we examined the integrity of the extracted hormone using TCA precipitation and gel filtration. A significant amount of radioactivity became non-TCA-precipitable only in prelysosomes and lysosomes at 45 min post-injection (Fig. 6), suggesting that the process of degradation has started in these organelles at that time. This is correlated with the height of peak III of radioactivity extracted from L-1 fraction, relative to total radioactivity eluted (Table 4). (There was not enough radioactivity extractable from L-2 fraction at 45 min post-injection to carry out a similar test). On the other hand, a significant amount of radioactivity extracted from intracellular organelles eluted at the void volume of the column (Table 4), indicating that part of the injected ^{125}I-oPRL is in a high molecular weight (HMW) form.

A similar HMW form was also observed by *in vitro* incubation of ^{125}I-hGH with prelysosomes and lysosomes (Fig. 7; Table 5), prelysosomes altering the molecular form of the ligand to a point such that it was no longer recognized by its receptors. We do not believe this HMW material to simply consist of aggregated ^{125}I-hGH molecules, as higher receptor binding could be expected from such aggregates. These HMW peaks may represent solubilized hormone-receptor complexes, a fact that would explain the almost complete lack of binding ability of material from these peaks.

The significance of very tight hormone-receptor complexes remains unclear. The association of tight binding with degradation (20) agrees with our finding of such complexes in prelyso-

somes and lysosomes, but we failed to observe degradation products after incubating the hormone with lysosomal fractions for 16 h. Our lysosomal fractions might be altered as a result of the isolation technique or possibly, for lysosomal degradation to occur, extralysosomal factors might be required.

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CHAPTER 3

STUDIES ON THE REGULATION OF PROLACTIN RECEPTORS IN CULTURED RAT HEPATOCYTES

SECTION 3.1

MAINTENANCE OF PROLACTIN BINDING SITES IN RAT LIVER CELLS IN SUSPENSION CULTURE: EFFECTS OF PROLACTIN AND OF INHIBITORS OF VARIOUS CELLULAR FUNCTIONS

ABSTRACT

An *in vitro* method to study the regulation of prolactin receptors has been established using adult rat liver cells cultured in a continuous suspension in L-15 medium. Prolactin binding averaged 28.2 ± 1.8% of the added labelled hormone per 10^6 cells in freshly isolated liver cells prepared from female rats treated with 17β-estradiol. When these cells were incubated at 37°C, binding rapidly declined by 20-50 % at 10 hours and 80-90 % at 48 hours. This rapid decline could be counteracted by the inclusion of ovine prolactin (50 nM), which maintained initial prolactin receptor levels up to 48 hours of culture. Higher concentrations of prolactin (2.5 μM) induced a rapid down-regulation, apparent at 2 and 10 hours of culture. Cycloheximide (50 μg/ml) induced a slight diminution of control prolactin receptor levels and partially reversed the effect of 50 nM prolactin. Approximately 60% of the prolactin receptors were resistant to the effect of cycloheximide. On the other hand, actinomycin D (10 μg/ml) had no effect on prolactin receptor levels in control, and only a very slight effect in prolactin-treated cells. Dinitrophenol, which blocks metabolic oxidation, also partially reversed the effect of 50 nM prolactin although it was without any significant effect on control levels. Chloroquine (100 μM) and colchicine (1 μM) failed to alter prolactin binding either in the absence or presence of 50 nM prolactin. Our results suggest that the existence of regulatory factors occurring *in vivo*, which are absent in the culture medium, could be responsible for the decline in prolactin receptor levels in the control hepatocytes. Prolactin itself could be one of these factors. On the other hand, and in agreement with the putative actions of the drugs utilized, the mechanism of the prolactin-induced maintenance of receptor levels appears to lie in part with an effect on receptor synthesis at the translational (ribosomal) level but to be independent of internalization or of lysosomal degradation.

INTRODUCTION

Prolactin binding sites in rat liver have been shown to have similar characteristics to prolactin receptors in rabbit mammary gland (35,40). Hepatic prolactin binding is sensitive to the endocrinological milieu with hypophysectomy resulting in an almost complete loss of binding sites (25).

Prolactin itself has been shown to have a stimulatory or up-regulatory effect on prolactin binding sites in rat liver (22,30,34) as well as in mammary tissue (10), and to play an important role in the overall mammary gland development and in the variation of PRL receptor levels observed in this tissue during pregnancy and lactation (6,11). More recently, a pituitary factor under hypothalamic control has been implicated in the regulation of PRL binding in the liver (33).

In addition to the up-regulatory effect of prolactin, which requires days for its maximal effect to be attained, a rapid down-regulation of prolactin receptors has been observed in vivo in rat liver and rabbit mammary gland (7) as well as in mammary explants (8). This process of down-regulation of prolactin receptors appears to involve an increased rate of degradation of prolactin receptors in lysosomes (12), as has been shown for other hormone-receptor systems (1,4,18).

Following binding of polypeptide hormones to receptors located on the plasma membrane, there is an internalization of the hormone-receptor complex (5,17,39). Prolactin receptors have been localized inside rat liver cells in Golgi membranes (2,20) in lysosomes (Chapter 2) and in another, yet undefined subcellular element (27). Accordingly, intact prolactin can be found in these organelles within 5-30 minutes following injection of the labelled hormone intravenously (2, and Chapter 2) as well as within the Golgi of mammary cells in lactating rats (32).

Although numerous studies have been conducted on prolactin binding in freshly isolated rat liver cells, our previous attempts to establish primary monolayer cultures of liver cells have proven unsatisfactory (unpublished observations). In a preliminary study (37), we have shown that either prolactin or prolactin receptor antibodies were capable of increasing prolactin receptors in rat liver cells in culture. In this report, we utilize this *in vitro* approach to study the regulation of prolactin binding sites in rat liver cells cultured in continuous suspension. The response of these cells to various concentrations of prolactin as well as to various cellular inhibitors was examined.

MATERIALS AND METHODS

1. Procedures for cell isolation

Female rats weighing 200 to 250 g were treated for 7 days twice a day with 17β-estradiol (5 µg) to enhance PRL receptor numbers (22,25,35) and for a 24-hour period with the dopaminergic agonist, CB-154 (bromocryptine mesilate; 500 µg, every 12 hours) to lower circulating levels of prolactin (11). These were injected s.c. in 0.9% saline containing 1% gelatin.

Rats were anesthetized with Surital (40 mg/kg). From this point on, all procedures were performed under sterile conditions, in a laminar flow hood. Following routine convenient aseptic procedures, the abdomen was opened, the liver exposed and the hepatic portal vein was canulated with a butterfly No. 19G (Abbott Diagnostics, North Chicago, IL). The liver was perfused using a pump roller (Travenol Inc., Deerfield, IL) with 700 ml of Ca^{2+} free HEPES buffer (10 mM, pH 7.4, containing 142 mM NaCl and 7 mM KCl) at 37°C to break desmosomes and to remove as much blood as possible. In order to avoid distension and subsequent rupture of the liver, just after the first drops of HEPES buffer entered the liver, the superior cava vein was cut above the diaphragm, and the rest of this solution was allowed to pass through the liver, over a 10-min. period. Cell dissociation was induced by the perfusion of 300 ml of collagenase solution (30,000-40,000 IU/rat in 10 mM Hepes buffer, pH 7.4, containing 50 mM $CaCl_2$ and 120 mM NaCl) over a 15-min. period. This method is a modification of the technique of Berry and Friend (3). The liver was removed and placed in a Petri dish containing cold Eagle's minimum essential medium supplemented with 32 mM Tricine and 26 mM $NaHCO_3$ (pH 7.4). The liver capsule was dissected and with the help of light agitation, the hepatocytes were dissociated in a vial containing supplemented Eagle's minimum essential medium.

Three centrifugations were performed at 500-1000 x g for 5 minutes each. After the first, the pellet was gently resuspended in serum-free L-15 culture medium modified with glutamine and supplemented with 2.5 mM succinic acid, 10 mM glucose, 100 IU/ml penicillin and 50 µg/ml streptomycin and buffered to pH 7.4 with 150 mM HEPES. Cells were counted using a hemacytometer and diluted in supplemented L-15 medium to a concentration of 3.3 x 10^6 cells per ml.

Ten ml of the cell suspension were placed in 50 ml Erlenmeyer flasks with untightened screw caps and incubated at 37°C, with normal aeration under continuous shaking (Junior Orbit Shaker, Lab-Line Instruments Inc., Melrose Park, IL). Tryptan blue exclusion test revealed greater than 80% cell viability at the outset of culture with viability only slightly reduced (approximately 5%) and cell shape slightly altered at 24 and 48 hours of culture. Aliquots of cells were removed after various times of incubation and frozen at -20°C until receptor assay.

2. Cell concentration

The effect of varying cell concentration in the incubation medium on the maintenance of prolactin receptors was investigated. Incubations at 37°C were performed at cell concentrations of 0.83, 3.3 and 13.2 x 10^6 cells/ml.

3. Hormone iodination

Human growth hormone (hGH) was labelled with ^{125}I (New England Nuclear, Boston, MA; or Atomic Energy of Canada, Ottawa, Ontario) using low concentration of chloramine T as previously described (24). Typical specific activity was 60-80 µCi/µg. hGH was used for PRL receptor assay because it was shown to be more stable than labelled PRL with similar lactogenic activity (including binding to PRL receptors) in a number of target organs (35).

4. Receptor assay

Frozen cells were allowed to thaw and the tubes were centrifuged at 1500 x g for 15 minutes to remove the incubation medium. This step proved to be important, especially when elevated concentrations of prolactin were included in the incubation. Following removal of the supernatant, the pellet was resuspended in Tris buffer (25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) and homogenized in a teflon-glass homogenizer at medium speed for 10 seconds.

Total cellular binding was measured after desaturation of the receptors: the homogenized cells were centrifuged at 2300 x g for 15 minutes, the supernatant removed by decantation and 0.5 ml of 3M $MgCl_2$ was added to the tubes. Samples were then vortexed, 4 ml of Tris buffer containing 0.1% bovine serum albumin (BSA) was added and the tubes centrifuged as previously described (24).

Cell homogenate equivalent to 300 µl of the original incubation, representing 10^6 cells (or approximately 300 µg protein measured using BSA as standard; ref. 29) were incubated in Tris-buffer containing 0.1% BSA at room temperature (22°C) for 16-18 hours, with approximately 100,000 cpm [^{125}I]-labelled human growth hormone, in the presence or absence of excess (1 µg) unlabelled ovine prolactin (oPRL), in a total volume of 500 µl. The reaction was stopped with 3 ml of cold Tris buffer containing 0.1% BSA. Bound and free hormones were separated by centrifugation for 15 minutes at 2300 x g. The pellet was counted in a LKB 1270 Rackgamma scintillator from LKB Instruments, Inc., (Rockville, MD) (66.5% efficiency). Specific binding was calculated as the difference in cpm bound, in the absence or presence of an excess of unlabelled oPRL, and was expressed as a percentage of the total radioactivity added to the tube. The results are presented as a percentage of the time zero specific binding (prior to incubation of the cells).

Statistical analyses were performed using Duncan-Kramer's multiple range test after logarithmic transformation to correct for heterogeneity of variance (28).

5. Chemicals, drugs and hormones

HEPES and collagenase (type Specific Use or Type IV) were from Sigma Chemical Company (St.Louis, Mo). Eagle's minimum essential medium (modified) and L-15 (modified with glutamine) were from Flow Laboratories (Rockville, MD). 17ß-estradiol was from Sigma and CB-154 (bromocryptine mesilate) from Sandoz (Basel, Switzerland).

All drugs tested were from Sigma; chloroquine, colchicine and cycloheximide were dissolved in water but actinomycin D and dinitrophenol (DNP) were dissolved in ethanol (1% ethanol, final concentration in the incubation media). They were compared with appropriate control and PRL-treated incubations. Cycloheximide and dinitrophenol were renewed after 24 hours of incubation because their effect appeared to be short term.

Human growth hormone (hGH; HS2160E, 1.7 IU/mg) and ovine prolactin (oPRL; NIH-P-S13, 30 IU/mg) were generously supplied by the National Hormone and Pituitary Program (NIH). The final concentration of ovine prolactin present in the hepatocyte culture medium, up to 48 hours of culture was determined by radioimmunoassay (21).

RESULTS

1. Prolactin receptor maintenance

The loss of prolactin binding sites in rat liver cells in continuous suspension culture as a function of time is shown in Fig. 8. Time zero binding was 28.2 ± 1.8% which represents 330 fmol per 10^6 cells, as evaluated using Scatchard analysis by competition (38). A rapid reduction of total binding sites was observed, with values declining to 50-80 % at 10 hours and 10-20 % of time zero binding at 48 hours of culture. A similar loss of binding sites was seen in rat liver cells cultured in monolayer in L-15, although the rate of decline is 4-5 fold more rapid (23). The rate of decline observed in suspension culture is very similar to that observed in vivo, following hypophysectomy (25).

Varying the concentration of cells in the incubation medium affected the maintenance of prolactin binding in rat liver cells in culture. The rate of loss of receptors was high when cell concentration was less than 1×10^6 cells/ml, whereas at concentrations of 3.3×10^6 cells/ml or greater, the maintenance as well as the response to prolactin (see below) was improved (data not shown).

In a recent study (37), we have reported the "stimulatory" effect of nM concentrations of prolactin on prolactin binding in rat liver cells in suspension culture. Fig. 8 shows the maintenance effect of the optimal dose of prolactin (50 nM) on total prolactin receptor levels. This effect began to become apparent at 10 hours of culture ($p < 0.05$ and $p < 0.01$ from 24 hours on) when compared to control values. The effect was specific to lactogenic hormones, since 50 nM hGH (equipotent to oPRL in the rabbit mammary gland) also maintained PRL receptors to time zero values throughout the 48 hour incubation period. No effect was

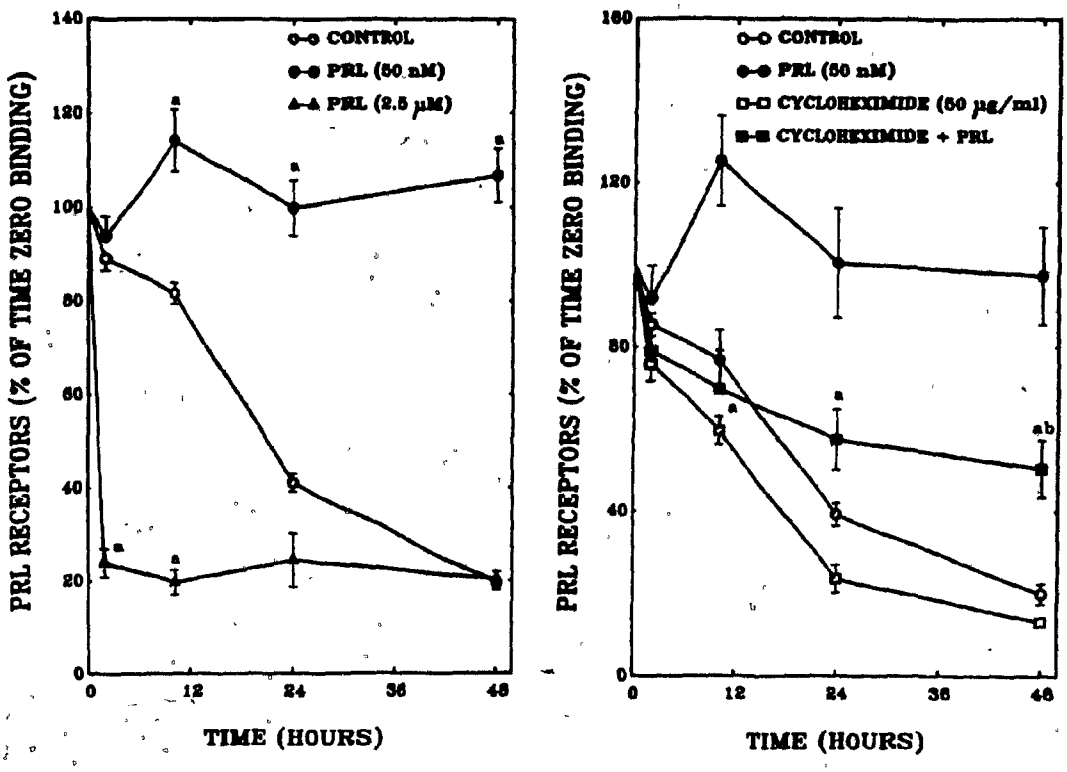


Fig. 8 (Left) Prolactin binding in rat liver cells in suspension culture. Values are expressed as percent of time zero binding (just before the incubation) and are means ± SEM of 13 (control and PRL 50 nM) or 4 (PRL, 2.5 μM) independent cultures. Percent specific binding at time zero was 21.8 ± 1.4 and 24.4 ± 4.2 per 10^6 cells, respectively. a: $p < 0.01$ vs control.

Fig. 9 (Right) The effect of cycloheximide (50 μg/ml) or a combination of cycloheximide with the optimum up-regulatory concentration of prolactin (50 nM) on total prolactin binding in rat liver cells in suspension culture. Values are expressed as percent of time zero binding and represent means ± SEM of five independent cultures. Time zero binding of control was 20.6 ± 2.1% per 10^6 cells, representing approximately 330 fmol binding sites/10^6 cells. a: $p < 0.01$ vs PRL; b: $p < 0.01$ vs control.

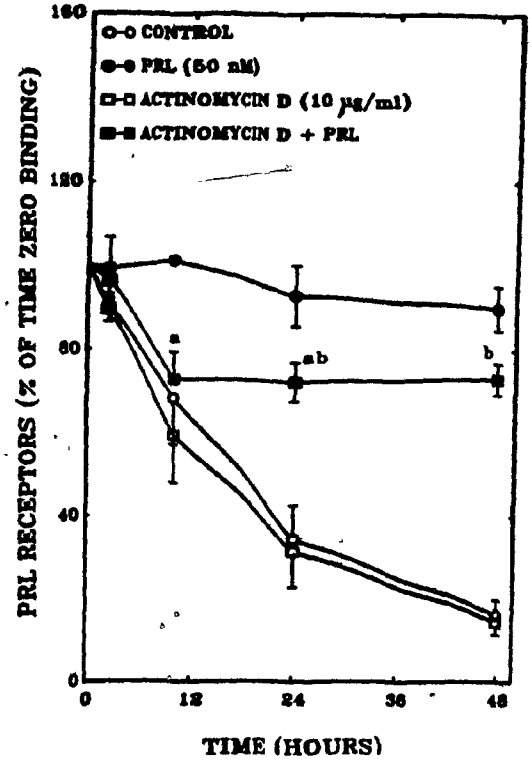
observed with the nonlactogenic hormone bGH, nor with any other pituitary hormone tested (TSH, LH, FSH and ACTH; data not shown).

In order to induce a down-regulation of prolactin receptors in vitro, the concentration of prolactin in the medium must be increased. As shown in Figure 8, prolactin (2.5 µM) produced a dramatic diminution of prolactin receptor levels at 2 hours of culture, and at 10 hours, this effect was still significant ($p < 0.01$) No PRL binding sites became measurable in the medium during the process of down-regulation (not shown). The down-regulated levels were observed throughout the 48 hours incubation but were not significantly different from control values from 24 hours on. The short duration of the measurable down-regulatory effect is in good agreement with previous reports of this effect of prolactin in liver and mammary gland (7,8).

The actual prolactin concentration present in the culture medium at various times during the 48 hours of culture was measured by specific radioimmunoassay (data not shown). At all prolactin concentrations utilized from 2.5 uM (115 ug/ml) to 0.1 nM (2.3 ng/ml), a pattern of declining prolactin concentration from 2 to 48 hours of culture was noted. A maximal degradation to about 50% of initial values was observed, with the degradation being most noticeable at the lower prolactin doses (0.1 and 1 nM).

2. Effect of blockers of cellular functions

Cycloheximide (50 µg/ml) had a slight inhibitory effect on PRL receptors in control hepatocytes, as shown in Fig. 9. The effect of cycloheximide was most noticeable when used in combination with 50 nM PRL and compared with the effect of PRL alone: from 10 hours on, cycloheximide reversed the PRL-maintained levels first, down to the control levels (at 10 and 24 hours)



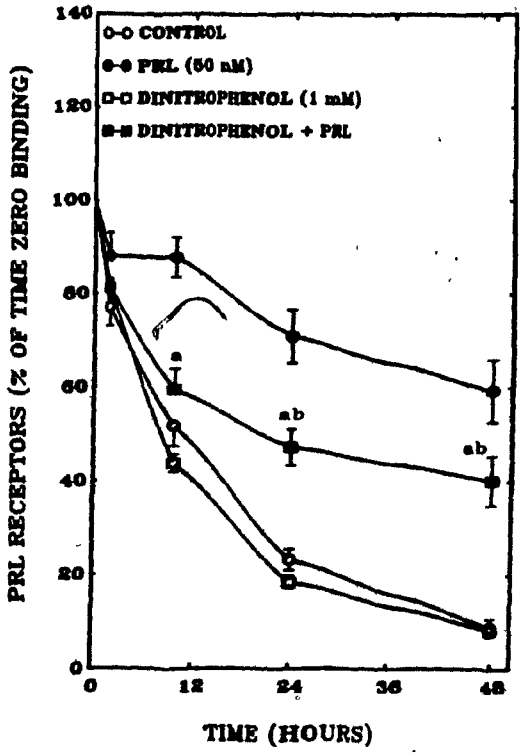


Fig. 10 (Left) The effect of actinomycin D (10 µg/ml) or a combination of actinomycin D and prolactin (50 nM) on total prolactin binding in rat liver cells in suspension culture. Values are expressed as percent of time zero binding and represent means ± SEM of four independent cultures. Time zero binding of control was 18.9 ± 1.4% per 10^6 cells. a: $p < 0.05$ vs PRL; b: $p < 0.01$ vs control.

Fig. 11 (Right) The effect of dinitrophenol (DNP; 1 mM) alone or in association with prolactin (50 nM) on total prolactin receptors in rat liver cells in suspension culture. Values are expressed as percent of time zero binding and represent means ± SEM of 8 independent cultures. Time zero binding of control was 19.8 ± 1.4% per 10^6 cells. a: $p < 0.01$ vs PRL; b: $p < 0.01$ vs control.

but only partially (60%) at 48 hours of culture ($p < 0.01$ for cyclo + PRL *vs* PRL at 10, 24 and 48 hours and cyclo + PRL *vs* control at 48 hours).

On the other hand, and as shown in Fig. 10, actinomycin D (10 µg/ml), which blocks protein synthesis at the transcriptional level, had a significant effect ($p < 0.05$) only at 10 and 24 hours of incubation on the PRL-maintained PRL receptor levels, and no effect on control cells.

The effect of prolactin (50 nM) on PRL binding can also be blocked by 1 mM dinitrophenol, a metabolic uncoupler, as is seen in Fig. 11. The reversal was complete at 10 hours but only partial at 24 and 48 hours ($p < 0.01$ for DNP + PRL *vs* PRL at 10, 24 and 48 hours and for DNP + PRL *vs* control at 24 and 48 hours). Finally, we observed no effect with the lysosomotropic drugs, chloroquine (100 µM) or NH_4Cl (10 mM) nor with colchicine (1 uM), a potent microtubule-disrupting agent (data not shown). The possible significance of these negative results is discussed below.

DISCUSSION

The decline in prolactin binding in control cultured hepatocytes suggests the lack of a physiological factor (probably from the pituitary, see ref. 33) responsible for the regulation of PRL receptor levels in the rat liver. Indeed, this observation is in good agreement with the decline observed in hypophysectomized animals (25) which first demonstrated the importance of the pituitary in the maintenance of liver prolactin receptors. In vivo, prolactin has been shown to be the factor responsible for the induction of hepatic prolactin receptors, first by the transplantation of a pituitary under the kidney capsule (34) and later by direct administration of ovine PRL to rats (22,30).

Using the present in vitro model, prolactin alone was able to completely maintain (up to 48 hours of culture) the level of PRL binding present at the outset of the culture. This model does not permit us to rule out the possibility that one or many other pituitary or extrapituitary factors are involved in the maintenance of prolactin receptors. Human GH, for instance, which is both somatogenic and lactogenic, when administered in vivo by continuous infusion, also restored hepatic prolactin binding sites in hypophysectomized rats (33) and was as potent as PRL on the cultured hepatocytes (not shown). Moreover, we used ovine prolactin with rat hepatocytes and our optimal concentration (50 nM, equivalent to 1.1 µg/ml) far exceeds physiological circulating PRL concentration in the normal adult female rat, although it represents levels of placental lactogen seen during pregnancy (26).

We also showed a reduction of prolactin receptor levels at high prolactin concentrations. The down-regulation is of short duration, apparent only at 2 and 10 hours (Fig. 8), probably due to the fact that receptor synthesis is occurring concomitantly with receptor loss. This effect is similar to the down-regula-

tion observed in rat liver or rabbit mammary gland models *in vivo* (7) or *in vitro* (8). It would appear that simple occupation of the binding sites was not responsible for such down-regulation, since receptor assays were performed after desaturation of the receptors using 3M $MgCl_2$ (24). However, we showed in Chapter 2 that following binding of the hormone to its receptor at the plasma membrane level, a portion of the internalized prolactin was concentrated in lysosomes. This material, when dissociated by 3M $MgCl_2$, partly eluted on Sephadex G-75 column, as a high molecular weight peak (perhaps the solubilized hormone-receptor complex that could not be dissociated by $MgCl_2$ treatment). Therefore, the down-regulation observed in cultured hepatocytes using high concentrations of PRL could result from the formation of such $MgCl_2$ nondissociable hormone-receptor complexes. It is possible that lower concentrations of prolactin induced a similar effect, although to an extent too small to be detected only by measuring total cellular binding. Measurement of PRL receptors in a purified microsomal or lysosomal fraction would be required to show if this is the case.

Studies were conducted with various inhibitors of cell functions. At the doses used, the effects observed are usually those which are sought, although non-specific effects cannot be entirely excluded. Cycloheximide, an inhibitor of protein synthesis at the translational level, reduced prolactin binding by approximately 40%. Effects of a similar magnitude were observed in the mammary gland in explant culture (8,9). Part of the maintenance induced by prolactin appears to be due to synthesis of new receptors since cycloheximide reversed 60% of this effect (Fig. 9). A similar effect was observed with DNP, a metabolic uncoupler which indirectly inhibits ATP-consuming processes, such as protein synthesis. On the other hand, actinomycin D, which blocks nuclear transcription, had very little (approximately 15%) effect on PRL receptor levels so that the involvement of protein synthesis in PRL receptor maintenance by PRL appears

to lie principally at the translation level. There may be pre-existing pools of PRL receptor mRNA (which would suggest a long half-life for this mRNA, ref. 9) or of a receptor preprotein that needs only to be processed to become active.

Recently, it has been reported that the addition of cycloheximide to astrocytoma cells treated with isoproterenol to down-regulate the β-receptors did not prevent the recovery of receptors (13), suggesting receptors may not be completely degraded following down-regulation. These studies also demonstrate that receptor synthesis may not be essential for an increase in receptor number to be observed. Also, evidence has recently been presented demonstrating the recycling of insulin receptors in isolated rat adipocytes (31). In any case, some complementary mechanism or mechanisms must be operating to explain the 40-60% of PRL receptors that are resistant to cycloheximide in the cultured hepatocytes (Fig. 9).

Lysosomotropic drugs have been shown to have an important role in blocking the degradation of PRL receptors in rabbit mammary tissue (9,12) where the rate of degradation is less than the rate of occupation and endocytosis of receptors. In the rat liver, we showed that chloroquine injected *in vivo* was able to considerably enhance PRL receptor concentration in the lysosomal compartment (Chapter 2). Nevertheless, chloroquine showed no effect on total PRL receptor numbers in cultured hepatocytes or in our previous *in vivo* study (Chapter 2; data not shown). Therefore, the absence of effect on total binding in the hepatocytes is consistent with our suggestion that when lysosomal action is blocked by chloroquine, PRL receptors that are routed towards these vesicles are either not degraded to any observable extent, or replaced at the same rate as they are degraded.

There is substantial evidence supporting the notion that colchicine inhibits endocytotic processes by blocking microtu-

bule assembly (36). It was thus surprising that it had no effect on concentration of PRL receptors in hepatocytes, especially since transmission signals from peripheral binding sites to the interior of the cell was suggested to involve microtubules (14). In particular, colchicine has been shown to block some of the prolactin-induced actions in the mammary gland such as casein synthesis (19). Moreover, in rat hepatocytes, this drug reduces insulin degradation and increases insulin receptors (41). Therefore, the effects of colchicine appear to depend not only on the tissue, but also on the system examined. As for PRL receptors in rat hepatocytes, regulation of the receptor levels appears to be independent of microtubule assembly or of internalization.

In conclusion, the results from this study show a direct action of prolactin on rat hepatocytes, responsible for the maintenance of its receptors in serum-free medium. This maintenance seems to involve, in part, synthesis of new receptor molecules with little synthesis of its mRNA, but to be independent of internalization or lysosomal degradation.

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SECTION 3.2

INTERACTION OF PROLACTIN (PRL) BINDING SITES WITH PRL RECEPTOR ANTIBODIES IN RAT LIVER CELLS IN SUSPENSION CULTURE: EFFECTS OF INHIBITORS OF CELLULAR FUNCTIONS

ABSTRACT

We have recently demonstrated that prolactin is able to maintain the level of its receptors in cultured rat hepatocytes. This effect could be modulated by various inhibitors of cellular functions. We report here that an antibody developed against a partially purified prolactin receptor preparation can mimic this effect of the hormone (although to a lesser extent) and that drugs can modulate it in a similar manner. In particular, cycloheximide (50 µg/ml), which reduced basal receptor levels by approximately 40%, totally reversed the maintenance induced by the antireceptor serum. Actinomycin D (10 µg/ml), another protein synthesis inhibitor (at the transcriptional level), had no effect on basal receptor concentration, but counteracted by about one-half the antiserum-induced maintenance. This effect of actinomycin D is much clearer here than the effect previously observed on prolactin-induced receptor levels in rat liver cells in culture. The effect of dinitrophenol (1mM) on basal levels was of limited amplitude but maintenance was again partly reversed by this drug. In accordance with previous results obtained with prolactin, chloroquine (100 µM) and colchicine (1 µM) failed to alter prolactin binding either in the absence or presence of 5% antireceptor serum. The effect of the antiserum indicates that prolactin itself is not required beyond the membrane for its effect on receptor regulation to be attained. These results also confirm our previous results with prolactin maintenance of prolactin receptor levels in rat liver cells in culture, that the mechanism of receptor maintenance appears to be due in part to a stimulation of receptor synthesis but to be independent of internalization or of lysosomal degradation.

INTRODUCTION

Prolactin receptors have been shown to be under regulatory processes, PRL itself having a stimulatory effect on the rat liver (20,24,25) as well as in the mammary tissue (7). We have recently reported (29) that PRL and an antibody developed against a partially purified PRL receptor preparation could both prevent receptor loss in rat hepatocytes in continuous suspension. In the previous section, we characterized the PRL-induced maintenance of PRL receptors (with 50 nM PRL) and these studies have provided new insights in the mechanism of action of PRL.

Djiane and al. (8) have reported prolactin-like activity of anti-prolactin receptor antibodies on casein and DNA synthesis in the mammary gland, and there is abundant literature suggesting that anti-insulin receptor antibodies can mimic insulin action on the uptake and oxidation of glucose (1,11,14,15,17, 18). The activity of those antireceptor antibodies suggests that the strict action of these hormones could be limited to the interaction with their binding sites.

Experiments were thus designed to characterize the stimulatory effect of anti-prolactin receptor serum and confirm the various effects of inhibitors of cellular functions.

MATERIALS AND METHODS

1. Materials

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and collagenase (type IV) were from Sigma Chemical Company (St. Louis, MO), MEM (Eagle's Minimum Essential Medium, modified) and L-15 (Leibovitz medium), modified with glutamine were from Flow Laboratories (Rockville MD). 17β-estradiol (E_2) was from Sigma and CB-154 (bromocryptine mesilate) from Sandoz (Basel, Switzerland). These last two were dissolved in minimum ethanol and then in 0.9% saline solution containing 1% gelatin for injection into the animals. Female Sprague-Dawley rats (CRL: CD(SD)BR) weighing 200 to 250 g were purchased from Charles River Canada Inc. (St. Constant, Québec).

All drugs tested were from Sigma; chloroquine, colchicine and cycloheximide were dissolved in water but actinomycin D and dinitrophenol (DNP) were dissolved in ethanol (1% ethanol, final concentration in the culture media). They were compared with appropriate control incubations. Cycloheximide and DNP were renewed after 24 hours of incubation because their effect appeared to be short-term. [^{125}I] NaI was from Atomic Energy of Canada (Ottawa, Ontario).

Ovine prolactin (oPRL; NIH-P-S13; 30 IU/mg) and human growth hormone (hGH; HS2160E; 1.7 IU/mg) used in the binding assay were generously supplied by the National Hormone and Pituitary Program (NIH). Prolactin receptors for production of the antiserum were partly purified from crude microsomal fractions of lactating rabbit mammary glands, using hGH (HS 19340, 2.6 IU/mg) bound to Affigel-10 (BioRad) as discribed (19,21,31). The partly purified receptor fraction was injected at monthly intervals into male sheep at a concentration of 50 µg antigen per injection in Freund's complete adjuvant and animals were bled at monthly invervals, 7-10 days after the booster immunization.

2. Liver cell preparation and culture

Rat hepatocytes were isolated by a modification of the method of Berry and Friend (9) as described in detail in Section 3.1. In short, female rats pretreated with E_2 (5 µg s.c., twice a day for 7 days) and CB-154 (500 µg at 24, 12 and ½ h before anaesthesia with 40 mg/kg Surital) had their hepatic portal vein canulated and the liver perfused successively with 700 ml of Ca^{2+}-free HEPES buffer (10 mM, pH 7.4, containing 142 mM NaCl and 7 mM KCl) and 300 ml of a collagenase solution (30,000-40,000 IU/rat in 10 mM HEPES buffer, pH 7.4, containing 50 mM $CaCl_2$ and 120 mM NaCl). The perfusion was carried out at 37°C using a peristaltic pump, under sterile conditions in a laminar flow hood. The liver was removed, washed with cold MEM supplemented with 32 mM Tricine and 26 mM $NaHCO_3$. The liver capsule was then dissected and the hepatocytes harvested by centrifugation at 500-1000 x g for 5 minutes. The pellet was washed twice in serum-free L-15 medium modified with glutamine and supplemented with 2,5 mM succinic acid, 10 mM glucose, 100 IU/ml penicillin and 50 µg/ml streptomycin and buffered to pH 7.4 with 150 mM HEPES. Cells were counted using a hemacytometer and diluted in supplemented L-15 medium to a concentration of 3.3×10^6 cells/ml.

Ten ml samples of cell suspension were incubated for 48 hours in 50 ml Erlenmeyer flasks with untightened screw caps, at 37°C with normal aeration and under continuous, gentle shaking, in the presence or absence of the serum anti-receptor (SAR) or drugs to be tested. Control cells viability (assessed by Tryptan blue exclusion) was approximately 80% at the outset of culture and 75% after 48 hours of incubation. Drugs concentrations used were: SAR, 5%; cycloheximide, 50 µg/ml; actinomycin D, 10 µg/ml; dinitrophenol, 1 mM; chloroquine, 100 µM and colchicine, 1 µM (all final concentrations in the culture media). Three

hundred ul aliquots were taken at the outset of the culture in the control incubation and at 2, 10, 24 and 48 hours in each flask, and frozen at -20°C until receptor assay. Receptors are stable at -20°C for several weeks.

3. Hormone iodination

hGH was labelled with ^{125}I using a low concentration of chloramine T as previously described (22). Typical specific activity was 60-80 uCi/ug. hGH was used for PRL receptor assay because it was shown to be more stable than labelled PRL with similar lactogenic activity in a number of target organs (26).

4. Receptor assay

PRL receptor assays were carried out as described earlier (22). The handling of samples was described in Section 3.1. In short, frozen cells were allowed to thaw and then were centrifuged at 2300 x g for 15 minutes to remove the incubation medium. The pellet was resuspended in Tris buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.1% bovine serum albumin) and homogenized in a teflon-glass homogenizer. Receptors were desaturated using 3 M $MgCl_2$ (22) in order to measure total binding sites. Cell homogenates equivalent to 300 ul of the original incubation (representing 10^6 cells) were incubated in Tris buffer at room temperature for 16 hours, with approximately 100,000 counts per minute (cpm) ^{125}I-labelled hGH, in the presence or absence of excess (1 ug) unlabelled oPRL, in a total volume of 500 ul. The reaction was stopped with 3 ml of cold Tris buffer. Bound and free hormone were separated by centrifugation for 15 minutes at 2300 x g.

Specific binding was calculated as the difference in cpm bound in the absence or presence of excess unlabelled oPRL and is expressed as a percentage of the total radioactivity added to

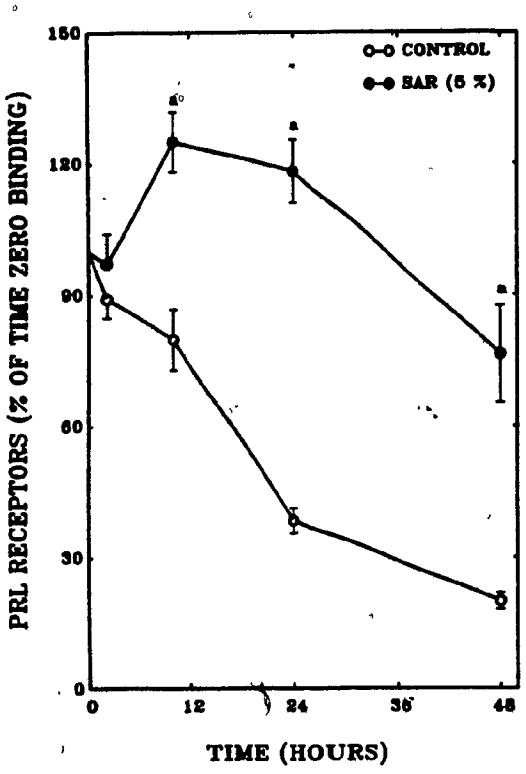
the tube. The results are presented as the percentage of time zero binding (just prior to incubation). Statistical analyses were performed using Duncan-Kramer's multiple range test (23) after logarithmic transformation.

RESULTS

The serum containing anti-prolactin receptor antibodies can prevent part of the loss of PRL receptors in cultured hepatocytes (fig. 12), thus mimicking the effect of PRL itself (Section 3.1). Maintenance was complete up to 24 hours but significant decrease ($p < 0.05$) occured at 48 hours with a loss of 25% of the binding activity originally present.

The following figures show the effects of the various drugs tested, alone or in combination with the optimum maintening concentration of SAR (5%, see ref. 29). Cycloheximide (50 µg/ml, Fig. 13), a drug that blocks protein synthesis at the ribosomal level, lowered basal levels of PRL binding by about 40% at 10 hours of culture ($p < 0.01$). It also completly counteracted the maintenance induced by the antiserum, reducing binding to control values. Actinomycin D is another inhibitor of protein synthesis, which acts on nuclear transcription. Fig. 14 shows that at 10 µg/ml, it had no effect on basal levels of PRL receptors but that it provoked the loss of about one half of the binding activity that was protected by the action of the antiserum. Dinitrophenol (DNP) is a metabolic uncoupler which indirectly inhibits ATP-consuming processes such as protein synthesis. Fig. 15 shows the effect of 1 mM DNP on PRL binding activity in the cultured liver cells: no inhibition (40% at 48 hours) of basal receptor levels was observed but a partial reversal of the SAR-induced maintenance of PRL receptors was seen ($p < 0.01$ at 10 and 24 hours and $p < 0.05$ at 48 hours; values at 24 and 48 hours were also significantly different from control, $p < 0.01$).

On the other hand, and in accordance with previous results (Section 3.1), chloroquine, which was shown to protect PRL and PRL receptors from lysosomal degradation *in vivo* (Chapter 2) had no effect (at 100 µM) on basal nor on SAR-protected levels of PRL binding (not shown). Colchicine (1 µM), a potent microtubu-



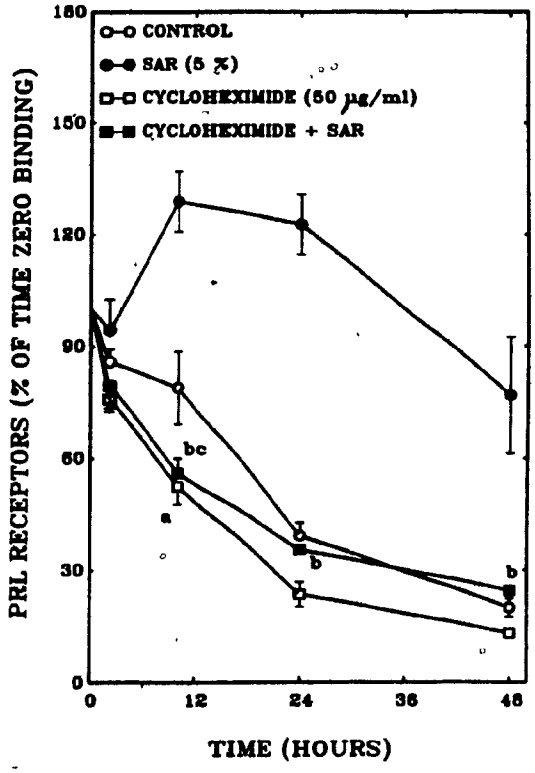


Fig. 12 (Left) The effect of the serum anti-receptor (SAR) on prolactin binding in rat liver cells in suspension culture. Values are expressed as percent of time zero binding (just prior to incubation) and are means ± SEM of 5 independent cultures. Time zero specific binding was 21.9 ± 1.6 per 10^6 cells. a: $p < 0.01$.

Fig. 13 (Right) The effect of cycloheximide (50 µg/ml), SAR (5%) or a combination of both on total PRL binding in rat liver cells in suspension culture. Values are expressed as percent of time zero binding and represent means ± SEM of 4 independent cultures. Time zero specific binding was 21.9 ± 2.1% per 10^6 cells. a: $p < 0.05$ vs control; b: $p < 0.01$ vs SAR; c: $p < 0.05$ vs control.

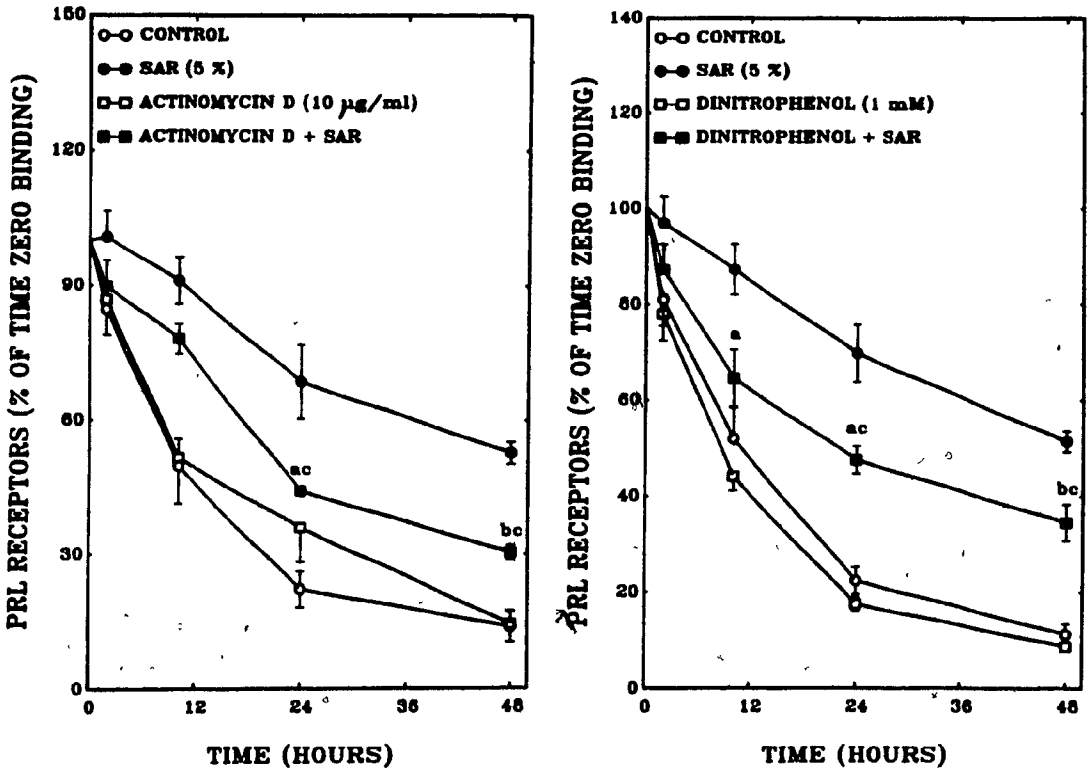


Fig. 14 (Left) The effect of actinomycin D (10 µg/ml), SAR (5%) or a combination of both on total PRL binding in rat liver cells in suspension culture. Values are expressed as percent of time zero binding and represent means ± SEM of 4 independent cultures. Time zero specific binding was 18.9 ± 1.4% per 10^6 cells. a: $p < 0.01$ vs SAR; b: $p < 0.05$ vs SAR; c: $p < 0.05$ vs control.

Fig. 15 (Right) The effect of dinitrophenol (DNP; 1 mM), SAR (5%) or a combination of both on total PRL binding in rat liver cells in suspension culture. Values are expressed as percent of time zero binding and represent means ± SEM of 4 independent cultures. Time zero specific binding was 22.0 ± 2.1% per 10^6 cells. a: $p < 0.01$ vs SAR; b: $p < 0.05$ vs SAR; c: $p < 0.01$ vs control.

le disrupting agent which is thought to interfere with hormone internalization (28) was also without effect. It is important to indicate that ethanol alone (the vehicle for actinomycin D and DNP) interfered with both basal and SAR-induced receptor levels (data not shown), but results for those drugs have been compared with controls and SAR incubations containing the same concentration of ethanol.

DISCUSSION

In a recent paper (29), we have identified PRL and SAR maintenance effects on PRL receptor levels in cultured hepatocytes. Control and PRL-containing incubations have been described in detail in Section 3.1. Control incubations featured a rapid decline in binding activity as opposed to the total maintenance of receptor levels observed up to 48 hours in incubations containing 50 nM oPRL.

The present results confirm the action of the anti-receptor serum in mimicking the effect of PRL. It is consistent with the demonstration by Djiane et al. (8) of the prolactin-like activity of anti-prolactin receptor antibodies on casein and DNA synthesis in the mammary gland which suggest that the prolactin molecule is not required beyond the initial binding to its receptor for its message to reach the genome.

In both models, lysosomotropic agents do not alter the response to PRL (ref. 8 and Section 3.1) nor to SAR (ref. 8 and these results), suggesting that lysosomal degradation bears no important role for PRL action on either stimulation of casein gene expression or regulation of its own receptor. In addition, lysosomes appear not to be involved in the loss of binding activity in control cells. However, we have shown in Section 2.1 that, when administered in vivo, chloroquine could stimulate levels of hepatic PRL receptors; Djiane et al. (9) also showed a stimulatory effect of chloroquine on PRL receptors in the cultured pseudopregnant rabbit mammary gland. The effect of chloroquine on PRL and PRL receptor degradation therefore appears to vary from one model to another.

The response to colchicine between rat liver cells in culture and rabbit mammary explants also differs. Colchicine had no effect on PRL or SAR-induced PRL receptor regulation (Section

3.1 and the present results), while it blocked their action on casein and DNA synthesis (8). In any case, although it is now clearly demonstrated that for many hormone systems, including prolactin and insulin, internalization of the hormone-receptor complex occurs following binding at the membrane level (2,12,13, 16,27) and that there is considerable evidence suggesting that microtubules are involved in the endocytotic processes (28), this translocation towards the interior of the cell appears not to be important for a number of actions of these two hormones.

Cycloheximide, a translation inhibitor, decreased basal receptor levels by about 40% (Fig. 13), suggesting permanent synthesis of new receptors; continuous synthesis and degradation (nonlysosomal) could be organized as a dynamic equilibrium. Similar effects of cycloheximide have been observed in mammary gland in explant culture (5,6). In addition to its action on basal receptor levels, cycloheximide completly reversed the SAR-induced maintenance, suggesting that the action of SAR consists of a stimulation of the translation of the PRL receptor mRNA. It should be noted that in the previous section, cycloheximide only partially reversed the stimulation by 50 nM PRL, which led us to the conclusion that there could be another mechanism involved in increasing binding activity in hepatocytes, in addition to stimulation of translation.

Actinomycin D partially reversed the stimulated levels, indicating that nuclear transcription may play a role in the maintenance of PRL receptors. This effect is much clearer than previous results obtained with 50 nM PRL-induced maintenance. The fact that reversal is only partial in this case might be inferred by a relatively long half-life of the receptor mRNA as is suggested by the absence of effect of actinomycin D on basal PRL receptor levels. The uncoupling action of DNP is less defined and the mechanism of its inhibitory effects on both basal and SAR-maintained receptor levels could involve interferences

with many metabolic processes, including protein synthesis. It should be noted that Costlow and Hample (4) have observed an increase in plasma membrane prolactin binding induced by DNP in rat mammary normal and tumor cells. However, they measured only cell surface receptors and this effect was not observed in the liver.

In conclusion, the results from this study show that an antibody directed against PRL receptors can mimic the action of prolactin on the maintenance of its own receptor levels in rat hepatocytes. In accordance with results obtained with PRL, the SAR-induced maintenance seems to involve, at least in part, a stimulation of the synthesis of new receptor molecules, but to be independent of internalization or lysosomal degradation.

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CHAPTER 4

STUDIES ON THE LOCALIZATION OF PROLACTIN RECEPTORS AT THE ULTRASTRUCTURAL LEVEL IN RABBIT MAMMARY GLAND

INTRODUCTION

Having gained some insight into the intracellular localization of PRL-R and its regulation and movement following PRL binding, it was of interest to try to directly visualize receptors. Very few reports of hormone receptor localization exist and they are even fewer for ultrastructural localizations.

The first immunocytochemical demonstration of prolactin receptors (PRL-R) in the rat mammary gland by Nolin and Witorsch (33) was only indirect, in that they showed an increased labelling of mammary sections with a PRL antibody when the sections were preincubated with exogenous PRL. More recently, other investigators (1,8,13,15,31,56) used similar protocols and stained various tissues for PRL-R. These include mouse adrenal, human breast tissue and prostate, rat kidney, liver, adrenal, testis and choroid plexus and dog prostate and mammary gland. There is only one report on PRL-R localization using a polyclonal antiserum raised against the receptor itself (14). The tissue studied was the rat ovary and PRL and PRL-R were found to colocate under all physiological conditions tested, at the optical microscopic level. The ultrastructural localization of PRL-R, however, has never been reported, nor has the use of monoclonal antibodies (mAbs) for PRL-R localization studies.

In contrast, several papers have reported the localization of receptors for other endogenous substances, using mAbs. A great deal of the attention has been directed towards the estrogen receptor (17,24,25,28,29,38,40-42,50,51), but some work was also completed on receptors for progesterone (37), transferrin (55), gamma-aminobutyric acid and benzodiazepine (47), acetylcholine (2,3), glycine (52a), nerve growth factor (45), epidermal growth factor (4) and insulin (54). Among all these,

however, ultrastructural studies were performed only for estrogen (41), transferrin (55), glycine (52a) and EGF (4) receptors.

Monoclonal antibody techniques provide an important advantage compared to their polyclonal counterparts, in that they represent homogeneous antibody preparations, even when raised against an impure antigen (PRL-R has not yet been purified to homogeneity), thus eliminating any cross-reaction with contaminants that may be present in the antigen preparation. These reagents should therefore be much more specific than polyconal antibodies preparations and especially well suited for localization studies. (This, however, is not always the case; see the comment on the incidence of shared epitopes in Discussion section.)

Three mAbs to the rabbit PRL-R have been produced in our laboratory that were available for the localization of receptors at the ultrastructural level in the rabbit mammary gland. All three are directed against the binding site of the receptor molecule, as assessed by their ability to inhibit PRL binding. They have, however, different ID_{50} values which range from 0.25 nM for M110 to 2.49 nM for A82 (21). In addition, A917 has the interesting feature that it can mimmic PRL actions on casein and DNA synthesis in rabbit mammary gland explants (12), just as the antireceptor serum mentioned in Chapter 3 could mimmic PRL action on PRL-R levels in rat hepatocytes.

M110, with its extremely high affinity (actually higher than that of PRL itself), was the first choice as a tool for the localization studies. We used immunocytochemistry as the primary approach, with a second antibody coupled to horseradish peroxidase (HRP). Such techniques have been reported to give very precise localization of antigens with good sensitivity. In our case, however, since the number of PRL-R is very low

(approximately 1200-2000 receptor molecules per cell have been reported for cells isolated from the mammary gland of lactating rabbits, and as low as 600-800 for pregnant animals[1]; refs. 2a,51a), some experiments with autoradiography, which has a better sensitivity but is less precise, as well as immunofluorescence (highest sensitivity but only at the optical microscopic level) were also carried out.

This work was performed under the supervision of Drs. Michèle Ollivier-Bousquet and Jean Djiane at the Laboratoire de Physiologie de la Lactation, INRA, Jouy-en-Josas, France.

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[1] These figures appear to be much lower than that for rat liver, which had a binding capacity of 330 fmol per 10^6 cells in cultured hepatocytes (Chapter 3), representing 200,000 PRL-R per cell.

MATERIALS AND METHODS

1. Chemicals

Hanks' balanced salts solution and Medium 199 used for explant culture were from Gibco (Paisley, England). Medium 199 for cell cultures was purchased from BioPro (Strasbourg, France). Chloroquine, saponin and diaminobenzidine (DAB) were purchased from Sigma (St Louis, Mo.), as well as bovine insulin (for explant culture). Porcine insulin (Actrapic MC) from Novo (Paris, France) was used for cell cultures. For both explant and cell cultures, cortisol was from Roussel-UCLAF (Paris, France). Bromocryptine mesilate (CB-154) was from Sandoz (Basel, Switzerland) and was injected as a suspension in 2% gelatin in physiological saline.

Paraformaldehyde (polyoxymethylene) was from Rhône-Poulenc (Paris, France), glutaraldehyde (as a 25% aqueous solution) from Taab Laboratory Equipment (Reading, England) and osmium tetraoxyde (OsO_4, solid) from Labosi (Paris, France). Finally, Epon 812, dodecenyl succinic anhydride, nadic methyl anhydride and the DMP-30 accelerator were purchased from Polaron Equipment Ltd. (Waterford, England).

2. Antibodies

With all approaches, the mAb M110 described earlier (12,21) was used as the first antibody. For autoradiographic studies, M110 was first iodinated following a modification of the chloramine T procedure described elsewhere (22). Two different HRP-conjugated second antibodies were used. The first was a sheep IgG to mouse IgG and IgM, H and L chains (IgGαM-HRP) purchased from Institut Pasteur Production (Paris, France). The second was goat F(ab)'2 with the same specificities

(F_2αM-HRP) purchased from Bioart (Meudon, France). I also used the fluorescein isothiocyanate conjugate of this $F(ab)'_2$ preparation for the immunofluorescence experiments.

3. Biological materials

Most of the localization experiments were performed with fresh lactating rabbit mammary glands between 14-22 days of lactation. At this stage, the animals yielded 160-230 g of milk on the morning of sacrifice, as determined by their weight before and after feeding the pups. In a few cases, lactating rabbits were given the dopaminergic agonist CB-154 (2 mg/ animal, s.c. at 36, 24 and 12 h before sacrifice) in order to depress circulating PRL levels and therefore, PRL-R occupancy. This 36 h treatment is not long enough to down-regulate the PRL-R but has been shown to induce a 3-fold increase in measurable PRL-R levels in this tissue (11). However, it also affects the ultrastructural morphology (34).

Some immunoperoxidase experiments were performed on pseudopregnant rabbit mammary gland explants in culture. Lactating tissue could not be used because it is fully developed and does not grow well in culture. The explant culture system has been described in detail (9). In brief, fresh mammary glands from female rabbits at about day 15 of pseudopregnancy were cut into small pieces (approximately 1 mm^3), placed on stainless steel grids and cultured for 24 h at 37°C under 5% CO_2, in Medium 199 supplemented with 5 µg/ml insulin and 0.5 µg/ml cortisol, in the absence or presence of 1 µg/ml of the lysosomotropic agent, chloroquine. Cultured explants were subsequently handled the same way as fresh tissue.

The third type of biological specimen used was isolated mammary epithelial cells. For the same reason as above, only freshly isolated cells could be used when prepared from lactat-

ing animals. However, mammary cells isolated from pseudopregnant animals were cultured for 48 h on a collagen matrix in the same medium and under the same conditions as explants. The collagenase dispersion followed by a pre-culture on plastic dishes yielded epithelial cell preparations virtually devoid of fibroblasts. These procedures are described in detail elsewhere (4a,25a).

4. Experimental procedures for immunocytochemistry (ICC)

The general procerure for ICC experiments is outlined in Table 6. Tissue fragments from lactating animals were first incubated in oxygenated Hanks' balanced salts solution for 20 min at 37°C. This procedure has been shown to increase the responsiveness of mammary epithelial cells to PRL in previous studies (Michèle Ollivier-Bousquet, unpublished observation), presumably by dissociating bound PRL and increasing the availability of cell surface receptors. This step was important because M110 recognizes the binding site on the receptor molecule (21). The use of high ionic strength chaotropic ions which is an effective means of desaturating bound receptors (22; see also Chapters 2 and 3) was obviously not appropriate for these ultrastructural localization studies. This initial step was omitted when tissues from either CB-154-treated animals, cultured explants or isolated cells were used.

The entire ICC procedure was performed at room temperature, under light agitation. Tissues were fixed for 2 h in a mixture of 2% paraformaldehyde (P) and 0.05% glutaraldehyde (G) (or, in some instances, 4% P alone or 2% P + 2% G) in 0.1 M phosphate buffer, pH 7.2. During this period, the fragments were cut into smaller pieces approx. 0.1 mm^3. After fixation, the specimens were rinsed in the same buffer (2 x 20 min) and then in 0.01 M phosphate buffered saline (PBS; 2 x 10 min).

TABLE 6

Procedure for immunocytochemical experiments

Day 1

1. Fragments of fresh rabbit mammary gland
2. Incubation in Hanks' BSS
3. First aldehyde fixation; cut fragments into smaller pieces
4. Incubation in 50 mM NH_4Cl
5. Incubation in 0.2 % gelatin
6. Permeabilization with 0.03 % saponin
7. Incubation with first antibody (monoclonal)
8. Incubation with second antibody (HRP conjugate)
9. Second aldehyde fixation
10. Storage overnight in isotonic rinse buffer

Day 2

11. Revelation of HRP with DAB and H_2O_2
12. Post-fixation with OsO_4
13. Dehydration in graded ethanol
14. Impregnation in graded propylene oxide:Epon 812

Day 3 and following days

15. 48 h polymerization
16. Ultrathin sectioning
17. Observation under the electron microscope

BSS, balanced salts solution; HRP, horseradish peroxidase; DAB, diaminobenzidine

Free aldehyde groups from the fixatives were deactivated by amidation with 50 mM NH_4Cl for 30 min. This was followed by saturation of electrostatic charges with 0.2 % gelatin for 30 min in order to minimize non-specific binding of the antibodies. Membranes were permeabilized with 0.03% saponin as described by Tougard et al. (52). All three solutions were prepared in PBS.

The fragments were divided (controls and samples) in multiwell plates (10-20 fragments per well) and all the following steps up to dehydration took place in a volume of 500 µl and in the dark. Fragments were placed in fresh wells after each step. Samples were incubated with the first antibody (or the vehicle for controls) for 5 h in PBS containing 0.03% saponin and 0.2% gelatin (PBS-S-G). Addition of saponin in the incubations with the antibodies was necessary because its effect on permeabilization was shown to be short-termed and reversible (52). M110 concentrations ranging from 0.1 ng/ml to 250 µg/ml were tried, but most of the experiments were performed at a concentration of 10 µg/ml.

After rinsing in PBS-S-G (3 x 20 min), the specimens were incubated for 4 h with the second antibody in PBS-S-G. Dilutions were 1:40 for the IgG from Institut Pasteur Production and 1:100 or 1:200 for the $F(ab)'_2$ from Bioart. Samples were rinsed as above and re-fixed with 1% G in 0.2 M cacodylate buffer, pH 7.2, for 30 min, rinsed 3 x 20 min in the vehicle and were allowed to stand overnight in the same buffer supplemented with 0.15 M sucrose (isotonic). The second fixation is important because, the H_2O_2 used as peroxide donor for the enzymatic reaction deteriorates lightly fixed tissues.

On the second day, the tissue pieces were transfered to Tris-HCl buffer (50 mM, pH 7.6) and incubated for 1 h with 0.05% DAB in this buffer (500 µl), in order to allow for proper

penetration of the substrate into the specimens. At the end of this period, 55 µl of 0.1% H_2O_2 in Tris buffer (0.01% final) were added to each well and the reaction was allowed to proceed for 15 min, still at room temperature, in the dark and under constant agitation.

Following this, the samples were rinsed 3 x 20 min with the same buffer, post-fixed for 1 h with 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2, and rinsed again several times in water. Finally, samples were dehydrated with graded ethanol (70, 90 and 100%; 10 min each), transfered to glass dishes, briefly dipped into propylene oxide and impregnated with graded Epon : propylene oxide mixtures (1:2, 1:1, 2:1; 30 min each). The final impregnation bath in pure Epon lasted at least 5 h at room temperature. The resin was allowed to polymerize for 48 h at 55°C. In some cases, the specimens were stained "en bloc" with 4% uranyl acetate during the 10 min bath in 90% ethanol, in order to increase contrast.

Ultrathin sections (silver to light yellow) were prepared using a LKB Ultrotome III ultramicrotome and observed on a Zeiss EM-10 electron microscope set at 80 kV.

RESULTS

The early experiments were performed on fixed pieces of lactating rabbit mammary gland, using a total IgG (IgGαM-HRP) as second antibody. Only extremely discrete labelling (if any) could sometimes be seen on small intracellular vesicles, with both 4% P alone and 2% P + 0.05% G as fixatives (not shown). In contrast, some intense reaction could be seen at the periphery of epithelial cells on both apical (Fig. 16) and basal (Fig. 17a) membranes, as well as on casein micelles (Fig. 16).

An important observation affecting these and perhaps other experiments is that such peripheral labelling was seen even on samples incubated only with the second antibody (Figs. 16a and 17a). In Fig. 18 are shown basal and apical regions of a specimen incubated in the absence of either the first or second antibody (2% P + 0.05% G fixation). The absence of labelling confirms that the reaction seen on Figs. 16a and 17a was a result of the non-specific binding of the second antibody.

We hypothesized that this non-specific reaction might be due to the interaction of the IgGαM-HRP with Fc receptors (non species-specific), located on the surface of epithelial cells. Therefore, experiments were performed using a bivalent antibody fragment ($F(ab)'_2$; devoid of the Fc region) to mouse IgG, coupled to HRP (F_2αM-HRP). This reagent fulfilled its role perfectly. Fig. 17 shows basal regions of samples incubated with IgGαM-HRP (Fig. 17a) or F_2αM-HRP (Fig. 17b), without prior incubation with M110. The latter did not display the strong reaction seen with IgGαM-HRP, thus supporting our hypothesis.

Using this system, some very sparse intracellular structures were labelled. Fig. 19 shows labelling in the endo-

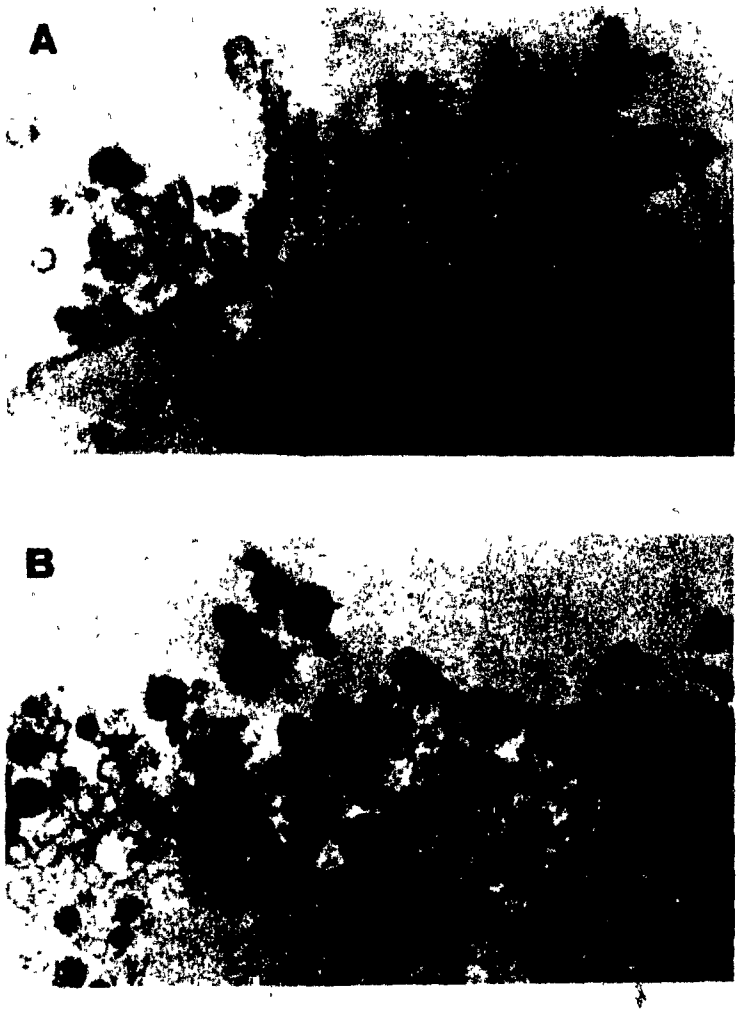

Fig. 16 Intense reaction on the apical side of lactating rabbit mammary epithelium incubated with (A) a total IgG molecule coupled to horseradish peroxidase (IgGαM-HRP) or (B) M110 and IgGαM-HRP. Note the labelling on the digitations and on casein micelles. 4% P fixation. Bar, 0.5 µm.

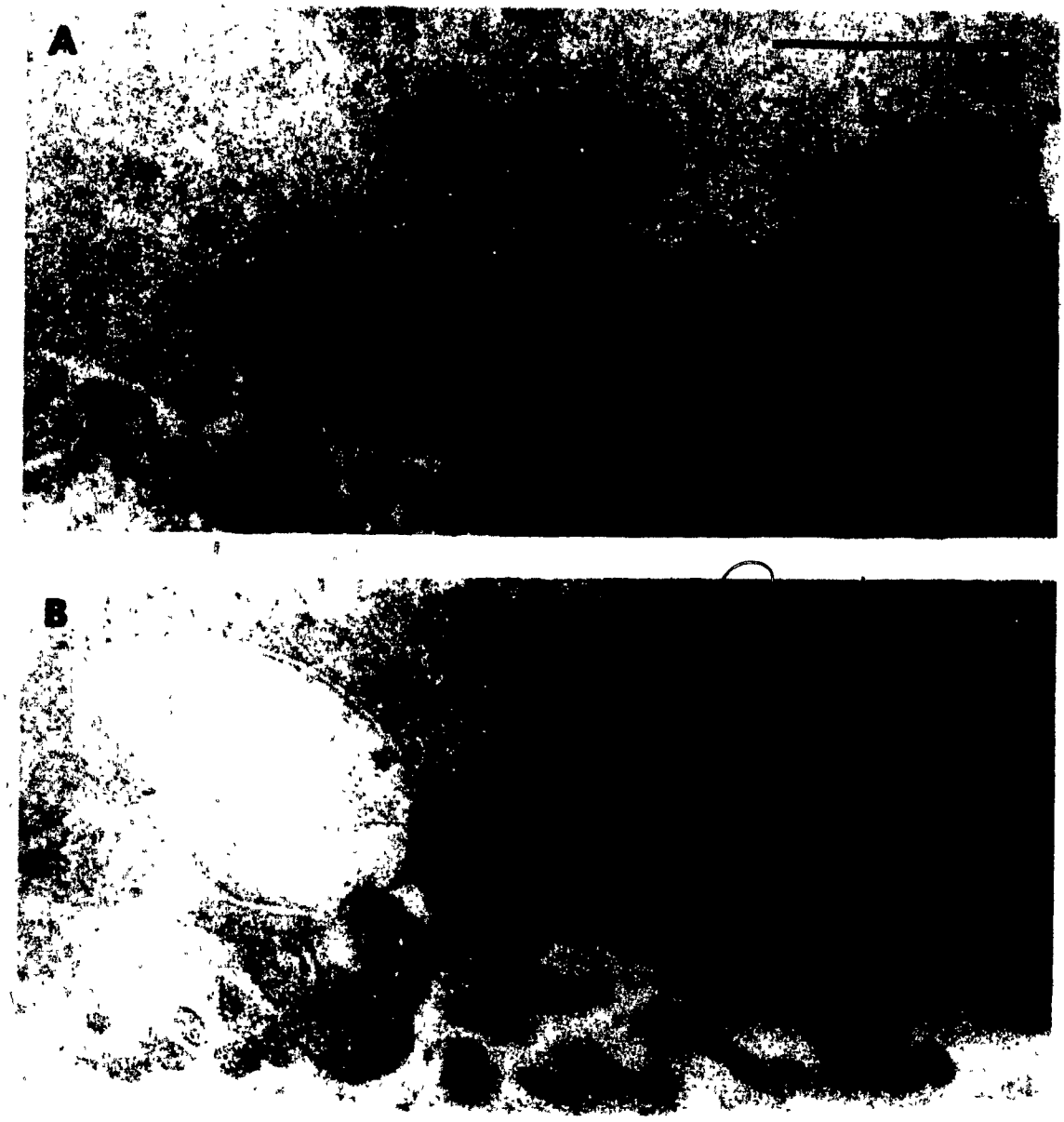

Fig. 17 Comparison of the background reaction observed with (A) a total IgG molecule or (B) a $F(ab)'_2$ fragment coupled to HRP, when used without prior incubation with M110, on the basal side of epithelial cells in fragments of lactating rabbit mammary gland. Note the strong reaction in panel A and the complete absence of such reaction on panel B. A, 4% P fixation; B, 2% P + 0.05% G fixation. Bars, 0.5 µm

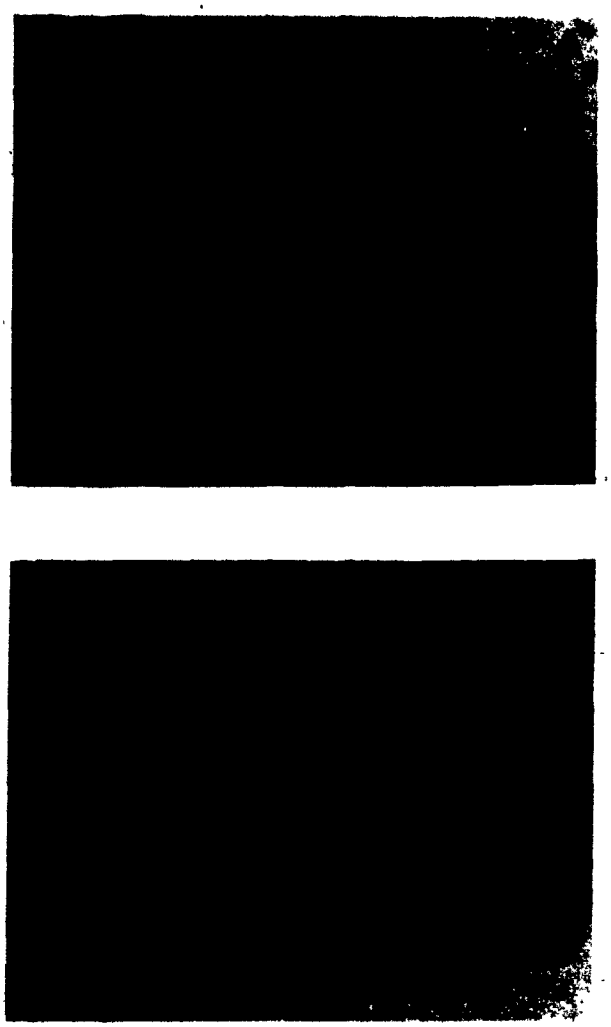

Fig. 18 (A) Apical and (B) basal regions of a fragment of lactating rabbit mammary gland incubated with neither antibody. 2% P + 0.05% G fixation. Bar, 1 μm.

plasmic reticulum (ER) region. Though the morphology is not very good in this particular sample, ribosomes, a zone of smooth membranes (lower right), a mitochondrion (lower left corner) and a multivesicular body (upper edge) are easily recognized. The morphology of the specimen shown in Fig. 20 is much better. The absence of coloration makes the reaction in the vesicle more striking than for the previous figure, but does not allow for the actual identification of ribosomes on nearby vesicles. It has, however, the general appearance of the ER. Another slightly labelled structure (from a different fragment of the same sample) is shown on Fig. 21, still in the ER region (ribosomes are seen on the neighboring membranes) near the apex of the cell.

Labelling was not inhibited by an excess (100 µg/ml) of a heterologous (goat) IgG and can therefore not be attributed to interaction of the first antibody with Fc receptors. In addition, no labelling was observed when M110 was replaced by another mouse mAb, purified from ascites fluid produced by a negative clone (i.e. a hybridoma that did not produce antibodies that give a positive ELISA test using partially purified PRL-R as antigen). Thirdly, no reaction was seen when M110 was incubated in conjunction with an excess (1µg/ml) of ovine PRL. This reaction would thus appear to be a specific labelling of the PRL-R.

In a parallel series of experiments, we used the same ICC protocol on mammary explants from pseudopregnant rabbits. This model has been used for many years for the biochemical study of the regulation of PRL-R levels, and is therefore fairly well characterized. On the other hand, mammary tissue at pseudopregnancy is obviously not as rich in PRL-R as that in lactation.

Fig. 19 Intracellular labelling (endoplasmic reticulum region) of a sample of lactating rabbit mammary gland incubated with M110 and $F_2\alpha$M-HRP. Intracellular organelles are recognized. 2% P + 0.05% G fixation. Uranyl acetate counterstain. Bar, 0.3 µm.

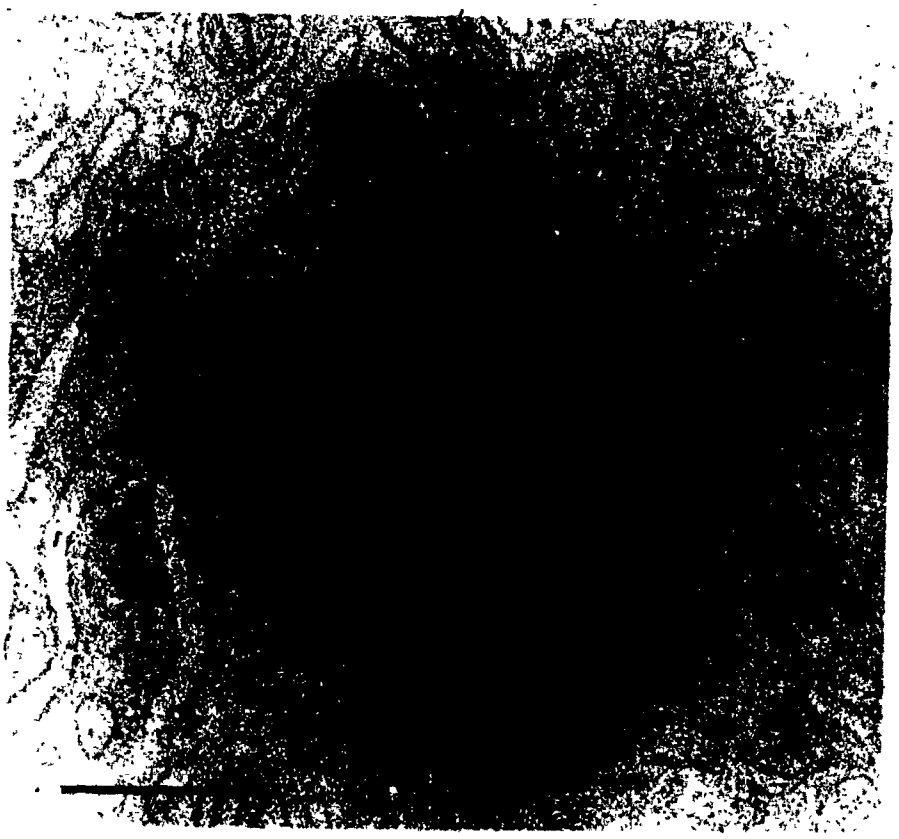

Fig. 20 Sharp intracellular labelling in what appears to be the ER region. Numerous mitochondria are seen, but ribosomes cannot be distinguished. 2% P + 0.05% G fixation. Bar, 0.5 µm.

Fig. 21 Intracellular structure slightly stained (arrow) after incubation with M110 and F_2αM-HRP, in a region of rough membrane, in proximity to the apex. 2% P + 0.05% G fixation. Bar, 0.5 µm.

Fig. 22 shows a general view of an acinus in a control explant (insulin + cortisol) incubated with M110 and $F_2\alpha$M-HRP. The reaction is restricted to membrane invaginations on fibroblasts (one arrow) and the edge of acini, all along the basal membrane (two arrows), and even on myoepithelial cells (pair of arrows to the right). Note that at this stage, the Golgi is poorly developed. As shown on Fig. 23, again, the labelling was very sparse. Many vesicles are seen close to the cell surface of a fibroblast. However, only one is heavily labelled. It might just be closing off. Fig. 24 shows another fibroblast with collagen fibers actually protruding from it (upper right corner). In this case, a labelled vesicle is seen farther from the surface than previously, but serial sections were not performed to verify whether this structure is in contact with the outside.

Other explants were treated with chloroquine. This lysosomotropic agent was shown to result in a 3-fold increase in PRL-R levels in cultured explants (as determined by radioreceptor assay), probably by protecting them from lysosomal degradation (10). Labelling similar to that seen in control explants was observed: on membrane invaginations or small vesicles located near the surface of fibroblasts (not shown) and along the basal membrane of acini (Fig. 25 and 26).

Fig. 25 shows morphological features that appear to be characteristic of chloroquine-treated explants (arrows). These rolled up structures could be attributed to the lysosomal compartment, their content being protected from digestion by the action of chloroquine (acid phosphatase localization was not performed to verify this hypothesis). Fig. 26 shows a higher magnification of the peripheral labelling. Note the total absence of reaction product inside the cell.

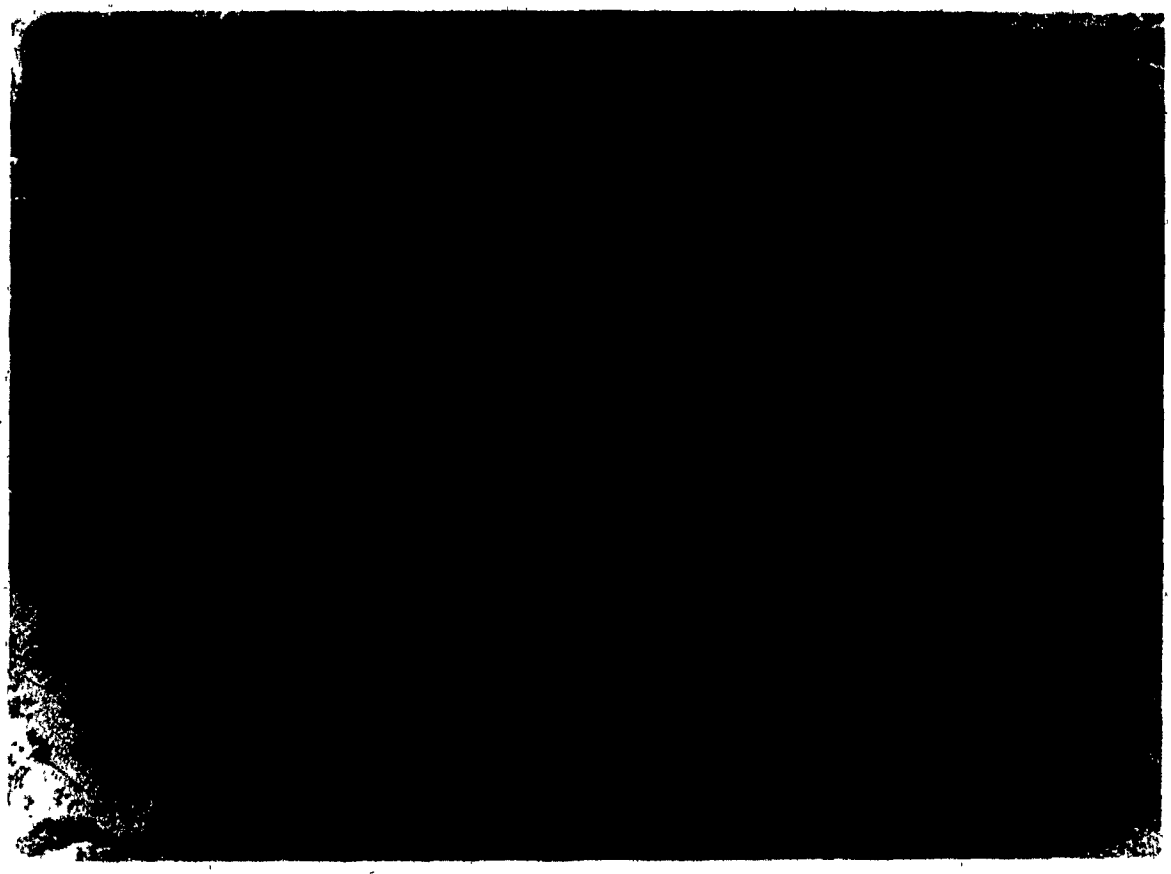

Fig. 22 Explant of pseudopregnant rabbit mammary gland cultured in control medium (insulin + cortisol) and reacted with M110 and $F_2\alpha$M-HRP. Note that the labelling is restricted to a membrane invagination on a fibroblast (arrow) and along the basal membrane (pairs of arrows), at the exclusion of any intracellular labelling. 2% P + 2% G fixation. Bar, 3.0 µm.

Fig. 23 Higher magnification of the fibroblastic labelling in a control pseudopregnant mammary explant like that shown on Fig. 22. Note that the staining is highly regionalized and locates solely in one of these vesicles. 2% P + 2% G fixation. Bar, 0.3 µm.

Fig. 24 Another sharply labelled invagination (or small vesicle near the cell surface) on a fibroblast from the same sample as in Fig. 23. Collagen fibers can be seen protruding from the cell. 2% P + 2% G fixation. Bar, 0.3 µm.

Fig. 25 General view of a pseudopregnant rabbit mammary explant cultured in the presence of 1 µg/ml of chloroquine. Some new morphological features (not seen in the control explants) are observed, as well as fibroblastic (not shown) and basal staining comparable to that observed in explants not cultured with chloroquine. 2% P + 2% G fixation. Bar, 1.0 µm.

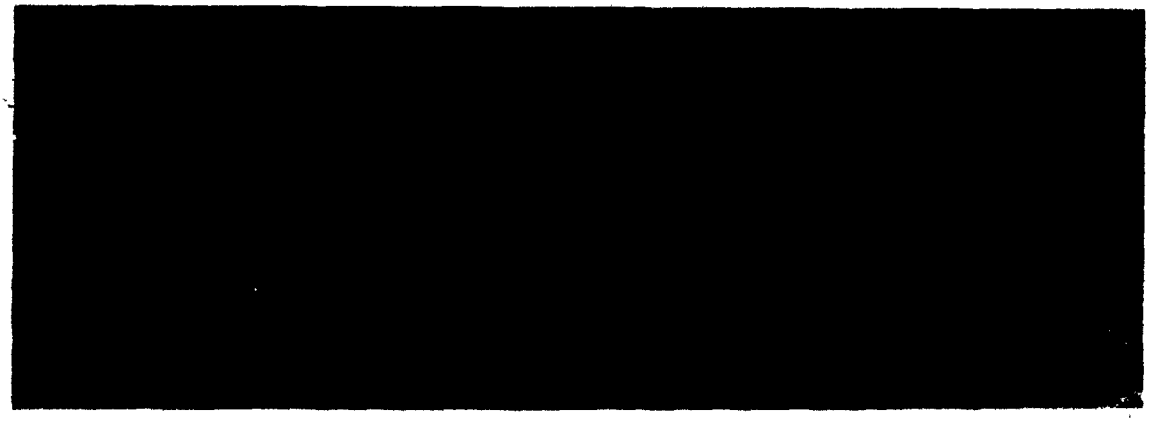

Fig. 26 High magnification of a pseudopregnant rabbit mammary explant treated with chloroquine, showing a reaction all along the basal membrane. Note, however, the complete absence of intracellular labelling. 2% P + 2% G fixation. Bar, 0.5 µm.

In order to retain the best possible morphology, these explants were fixed with 2% P + 2% G. The absence of intracellular labelling could have been due to membranes which, because of the strong fixation, were too rigid and did not permit the reagents to diffuse inside, even in the presence of saponin. Other experiments were therefore performed with the less severe fixation used with fresh lactating tissue (2% P + 0.05% G). Under these conditions, not only did I fail to observe intracellular localization of PRL-R, but also the positive reactions on fibroblasts and basal membranes were absent (not shown). As expected, the control mouse mAb yielded no observable reaction.

Other experiments were performed using different biological materials (fresh mammary glands from lactating rabbits treated with CB-154, isolated mammary epithelial cells from both lactating and pseudopregnant rabbits) and with different approaches (counting radioactivity, autoradiography and immunofluorescence). These approaches and their results are discussed in the next section.

DISCUSSION

Our early results showed that an intact IgG molecule could not be used as a second Ab for the immunocytochemical localization of PRL-R in the mammary gland. This problem had previously been met by other investigators (15,31,33,41,51) who overcame it by preincubating the tissues with a heterologous Ab. This is consistent with the concept that Fc receptors are present on the surface of mammary epithelial cells and is further supported by the absence of non-specific reaction when a $F(ab)'_2$ was used as second antibody. In fact, milk has been shown to contain immunoglobulins that are not produced in the mammary gland (44). Therefore, these Fc receptors could be physiologically relevant in that blood-borne imunoglobulins would be taken up by the mammary epithelium (via interaction of their Fc region with corresponding receptors), endocytosed and cast into the lumen, with other milk constituents.

The importance of this observation is that if this hypothesis is correct, then no antigen can be studied in this tissue using a total Ig molecule as second antibody. There does not seem to be a problem with the first antibody (an excess of a heterologous Ig does not compete for M110 reaction and a control mouse mAb gave no observable reaction), presumably because a much lower concentration is used and these reagents bind preferably (high affinity) to their specific antigen.

The second observation of practical importance is that strong fixation never permitted intracellular labelling. This was shown for pseudopregnant rabbit mammary gland explants (Figs. 22-26), and similar experiments were performed with fresh lactating rabbit mammary tissue with the same result, that is, no labelling was observed either superficially or

intracellularly at the electron microscopic level. It is possible that a strong fixation renders the membranes too rigid to allow for the proper diffusion of the antibodies inside the cells.

On the other hand, loss of antigenicity after fixation is not uncommon and has been shown for a great number of antigens (18,43). A variety of other fixatives (in particular non-aldehyde ones, like Nakane's lysine periodate fixative) should therefore be tried in future experiments on the PRL-R localization. A fixation step, however, remains necessary in order to retain good morphological features and permit identification of the ultrastructures.

Some surface labelling which would appear to be specific for M11O (not seen with the control mAb) was observed, however, in cultured pseudopregnant tissue, with the strong fixation used in this study. The structures with the strongest label were membrane invaginations or small vesicles located near the cell surface of fibroblasts. A positive reaction was also observed along the basal membrane, but not inside the cells. Previous studies (23,39; see also Chapter 2) have shown that PRL-R are mainly located in intracellular organelles, especially the Golgi complex, at least in the liver and the lactating mammary gland. It is, however, possible that during pseudopregnancy, PRL receptors are located principally peripherally since the Golgi apparatus is poorly developed and PRL is not present in large concentrations to stimulate receptor endocytosis.

The fact that peripheral labelling is restricted to certain areas on fibroblast membranes contrasts with the localization on the epithelium which was all along the basal membrane. These observations do not correlate with any physiologically relevant model for PRL-R distribution. Also, no label-

ling was observed in the lightly fixed explants (see Results) and standard binding procedures failed to show any ^{125}I-M110 specific binding to cultured fibroblasts removed during pseudo-pregnancy (not shown).

Nevertheless, the highly regionalized fibroblastic labelling pattern does not seem to represent mere non-specific adherence of the antibodies, especially since such a reaction was not observed with the control mAb. Other investigators (18,26,35,46) recently reported that mAbs may sometimes recognize more than one antigen. The immunofluorescence demonstration by Saegusa et al. (46) that mAbs to various hormones specifically label various structures in tissues where these antigens are not present is particularly convincing. Since mAbs are thought to be specific with regard to their epitope, it was suggested that epitope sharing by various molecules may explain such non-specific reactions. Hollmann et al. (18) and Shaw et al. (49) actually demonstrated the existence of such epitopes, shared by two or more totally unrelated proteins. This could possibly explain the unexpected labelling on fibroblasts observed with M110 in this study.

It would not explain, however, that with the lighter fixation, faint reactions were occasionnaly seen in the ER region (Figs. 19-21), but never in the Golgi complex, lysosomes or lysosome-like vesicles, where PRL-R were shown to be present using other methods (23,39 and Chapter 2). As mentioned earlier, it is possible that the site of the receptor molecule recognized by the mAb was destroyed by fixation, with the nascent receptor molecules in the ER region not being affected to the same degree for some reason. On the other hand, evidence is rapidly accumulating that a monoclonal antibody often recognizes its specific epitope only under certain particular conditions. For example, some mAbs were reported to recognize phytochrome (6,36) or maize leaf nitrate reductase (5) either

only in the native state or when denatured by SDS. In our case too, M110 was found not to bind the denatured PRL-R (unpublished observation). It was also shown that even a physiologically-related transformation of an antigen, e.g. phosphorylation of rhodopsin (32), transformation from inactive to active forms of phytochrome (6,36) or binding of the activated complement component 1 to plasma kallikrein (7) may affect its binding by a mAb. This, however, probably does not account for the absence of labelling with M110 in the present study, since M110 has a demonstrated biological activity, namely, inhibition of PRL binding and of PRL-induced casein and DNA synthesis in rabbit mammary gland explant culture (12). It should also be underscored that very little labelling was observed in the present study, suggesting that the small number of PRL-R, even in the lactating rabbit mammary gland (1200-2000 receptors/cell, refs. 2a,51a), may be close to the detection limit of this immunocytochemical approach. If this is true, it would also imply that the absence a of reaction with the control mouse mAb and with PRL competition does not definitely assure the specificity of this labelling.

Various other approaches were tried to visualize receptors. These include ultrastructural autoradiography using ^{125}I-M110 and, at the optical level, immunofluorescence using a F(ab)'$_2$ to mouse IgG and IgM labelled with fluorescein. In both cases, the reaction was too light and/or the background too high for positive localization.

Both immunoperoxidase and autoradiographic experiments were also performed on lactating rabbit mammary glands from animals treated with CB-154 and on isolated mammary epithelial cells. The use of these biological materials was theoretically promising: mammary glands from CB-154-treated animals represent the richest tissues for PRL-R known to this date and the use of isolated cells would rule out eventual problems of

penetration of the reagents within tissue fragments (the mammary gland has a very tight matrix of connective tissue). Despite those high expectations, no specific labelling could be seen in these materials with either approach.

Overall, there is very little available information on the immunocytochemical localization of an antigen using monoclonal antibodies. It should be pointed out that, as discussed above, the use of mAbs raises some new problems. Nevertheless, it should not be concluded that these reagents are unsuitable for immunocytochemistry since several investigators have used them successfully (2-4,17,19,24,25,27 29,37,38,40-42,45,47,48, 50,51,52a-55). Among these studies, some (4,41,52a,55) were performed at the ultrastructural level.

In conclusion, it is important to note that a high reactivity of a mAb in an ELISA (the most commonly used screening test) or a binding inhibition assay does not always correlate with good reactions in toto. Therefore, in addition to various fixation procedures as discussed above, other hybrids should also be investigated when the immunocytochemical localization of any given antigen is attempted, even clones with lower ELISA reactivities. Eventual sharing of specific epitopes by molecular entities other than the antigen for which localization is attempted should be verified. Finally, some of the problems discussed could possibly be overcome by using a mixture of several monoclonal antibodies.

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CHAPTER 5

GENERAL DISCUSSION

Implicit to the mechanism of receptor-mediated endocytosis delineated in Chapter 1 is the notion that there must exist pools of intracellular receptors. This work addressed the prolactin receptor, its subcellular localization and its intracellular path and fate following binding of the hormone. I also studied the fate of the prolactin molecule itself.

The hypothesis that the process of endocytosis via coated pits and endosomes involves delivery of the ligand to lysosomes (for degradation) and recycling of the receptor to the cell surface (for reuse) following segregation of the two at an early stage, would suggest specific locations for the receptor [plasma membrane, endosomes, trans-Golgi and the proposed compartment for recycling (which could be the same as that for exocytosis, see ref. 43)] to the exclusion of any other (reviewed in refs. 40,41).

Our observation that PRL-R are present in lysosomes (Chapter 2) contrasts with this view. It is true that PRL binding was small in mature lysosomes in control animals (Fig. 2). The 7- to 8-fold increase in chloroquine-treated animals was interpreted as a direct protective effect of the drug at the lysosomal level but recently, acidic vesicles other than lysosomes were shown to acccumulate such "lysosomotropic" weak bases (35). Thus, it would be possible that the neutralization of an acidic compartment distinct from lysosomes could be responsible for this increase. For example, if the sequence of events proposed by Willingham and Pastan (40,41) holds for the prolactin system, neutralization of endosomes [which were shown to be acidic (20,28,30,36)], would prevent the dissociation of hormone-receptor complexes and could alter further routing.

Thus, one of two things may happen: either the receptor follows the ligand in its normal path, or the ligand follows its receptor. The first case would explain the appearance of

PRL-R in putative lysosomes (L-2 fraction, see Chapter 2) when animals are treated with chloroquine. The alternate view would suggest that binding activity observed in lysosomal fractions is actually due to a contaminant found in these fractions, possibly the "unique" vesicles described by Khan et al. (27).

This new compartment remains to be identified. On the ground of their enzymatic contents and morphological features, we tentatively identified the L-1 fraction (not much affected by chloroquine treatment of the animals) as being related to the lysosomal compartment ("prelysosomes") and the L-2 fraction (in which PRL-R are dramatically augmented by chloroquine) as being highly purified, putative secondary lysosomes. This, and the kinetics of *in vivo* uptake of ^{125}I-oPRL in L-1 fraction (see Fig. 4), would agree with the delivery of endocytosed ligand to small uniform lysosomes between 15 and 30 minutes after internalization, as described by Willingham and Pastan (39). However, the lysosomal compartment is morphologically heterogeneous and it was shown that vesicles not containing acid hydrolases can easily be mistakenly identified as lysosomes using such criteria (41).

Another possibility is that these vesicles represent another compartment involved in intracellular routing of endocytosed materials. Because of the kinetics of incorporation of ^{125}I-oPRL within the L-1 fraction (maximum at 30 minutes post-injection, Fig. 4), identification of these vesicles as endosomes can be ruled out. Thus, these vesicles could represent the recycling compartment for PRL-R. A major difficulty with this hypothesis is the presence of the hormone in these fractions, because ligands are expected to be destined for delivery to lysosomes. A third possibility is that they might represent a class of vesicles responsible for the transfer of endocytosed materials from the Golgi complex to lysosomes but this would not be compatible with the view that lysosomes form directly

from the coated pits of the GERL area, where lysosomal enzymes and their receptors (mannose-6-phosphate receptors) were found to accumulate (32,42). In addition, if receptors are to be recycled, they should not be found in such structures.

The real difficulty, then, is that PRL and PRL-R were found to co-locate at a time when the proposed model for receptor-mediated endocytosis would predict dissociation and segregation had already occured. Dunaif et al. (16) also found PRL and its receptor to always co-locate in rat ovary. Although the model has been convincingly demonstrated by the groups of Willingham and Pastan and that of Geuze and Schwartz to hold for a number of ligands, these include only few hormones or growth factors [insulin, triiodothyronine and epidermal growth factor (33) and glucagon (3)]; most of the other examples are various plasma proteins such as α_2-macroglobulin and low density lipoprotein (33,37), immunoglobulins (2, 34), transferrin (6,19,24), serum albumin (11), asialoglycoproteins (38) and interferon (46) or lysosomal enzymes, toxins and viruses (reviewed in ref. 33). Even ferritin, which is a broadly used marker for electron microscopy, was observed to cluster within coated pits (7). On the other hand, this was never observed for prolactin and one should consider the possibility that this ligand does not fit the general model.

The model of Willingham and Pastan has been challenged on aspects other than whether endosomes are coated or not (this point has been discussed in Chapter 1). Some authors (7a,21a, 36a), for example have reported that endosomes fuse directly with lysosomes. Gorden et al. (21a) monitored internalization of ^{125}I-insulin by electron microscopic autoradiography, and found that it is initially bound to the plasma membrane and subsequently associated with lysosomes, but they were unable to show any labelling in Golgi elements, endoplasmic reticulum or nuclei. A likely hypothesis to explain this is that they re-

stricted their localization experiments only to samples exposed to the tracer for 5, 30 or 60 minutes (7a), therefore omitting the intermediate time point (15 min), when ligand accumulation is maximal in the Golgi. It should also be remembered that the model of internalization depicted in Chapter 1 is specific for receptor-mediated endocytosis, at the exclusion of general pinocytosis or phagocytosis. In the work of van Deurs and Nilausen (36a) for example, internalization of cationized ferritin was monitered. This ligand does not have specific receptors but binds to cell surface anionic sites. Accordingly, they developed a model involving a direct membrane shuttle between the plasma membrane and the lysosomal compartment.

Herman and Albertini (22a) directly monitored endosome and lysosome movements by time-lapse video image intensification microscopy. In these studies, they used labelled low-density lipoprotein (LDL; a ligand that most authors agree follows the coated pit-endosome pathway), followed its entry via endocytic vesicles, and identified its end target to be lysosomes (as stained by acridine orange). The most striking result was that the pattern of labelled LDL movement is biphasic along its way to lysosomes. In the first phase, the label undergoes centripetal saltatory motion until, after 20 min, it aligns in a perinuclear area. From there on, it moves more progressively towards the center of the cell and finally locates in acridine orange-positive vesicles.

It would be tempting to conclude on such grounds that endosomes fuse with lysosomes. Such disagreement with the model amply discussed above could, however, arise simply from the formal definition of the term "endosome". In this thesis, my discussion implied a functional definition, that is, I define endosomes as vesicles generated at the cell surface and involved in the actual process of endocytosis (restricted, however, to receptor-mediated phenomena; otherwise, one should refer to

pinocytic or phagocytic vacuoles). The change in the type of motion observed by Herman and Albertini (22a) could represent a change in vesicle type, such that during the second phase of endocytosis, the labelled ligand is no longer in endocytic vesicles, but rather in an intermediate compartment with which endosomes would have fused. Both the time (20 min of exposure with the ligand) and the general region (alignment in a perinuclear area) where this occurs would agree with the general idea that this target compartment is the Golgi complex.

Bergeron et al. (4a) have recently reported that three distinct types of endosomes exist. One class consists of heterogeneous and lysosome-like vesicles which accumulate chloroquine by virtue of their low intraluminal pH. This description agrees very well with that of other groups (see Chapter 1 and refs. therein). The other two classes consist first of small vesicles probably originating from -or close to- the cell surface and possibly including coated vesicles, and secondly of larger structures, often vesicular and not accumulating chloroquine.

It is possible that the identification of these fractions as endosomes arose from the modified difference in the definition of the endosomal compartment by these authors. They identify as endosomes, "all of the intracellular non-lysosomal components involved in the uptake of exogenous substances into cells". This definition is less restrictive than the functional one mentioned above, since it also includes the Golgi complex or any other non-lysosomal compartment with which endocytic vesicles fuse. Indeed, in the original articles to which they refer (also reviewed in ref. 33a), they document insulin and lactogen uptake in fractions termed "Golgi vesicles" which are enriched in galactosyl transferase (GT) and incorporate labelled ligands with a peak around ~15 min. It should be underscored, however, that in the above-mentioned paper (4a), these authors describe a fraction highly enriched in GT (RSA=120) with a max-

imum incorporation of ^{125}I-insulin at 5 min. after i.v. injection, which is totally novel.

There are some data which suggest that PRL-R are recycled to the cell surface after internalization, such as the fact that cycloheximide and actinomycin D only partially reverse the PRL-induced maintenance of its receptors in our experiments (see Figs. 9 and 10). The subpopulation of receptors which is resistant to these drugs could be postulated to represent partial recycling of receptors. However, and as discussed in Chapter 3, the partial resistance to actinomycin D could also be explained by the demonstrated long half-life of the receptor mRNA (13). Resistance of the PRL effect to cycloheximide could be related to unmasking of a pool of cryptic receptors, since such receptors were shown to be present and induced by estradiol in mouse liver (4) (all our animals were primed with estradiol) and, most importantly, to be regulated by PRL in mammary tumor cells (10). In addition, in our hands, lysosomes appear to be the final site of localization (Chapter 2).

Another fact suggesting the absence of PRL-R recycling is their rapid down-regulation in hepatocytes incubated with large concentrations of prolactin (Fig. 8). Under these conditions, receptor levels were already minimal at the first time point and stable until the end of the incubation, with no sign of replenishment. This suggests that following ligand-induced internalization, PRL-R are not reused, which is compatible with the view that they are delivered to and degraded within lysosomes. It is also consistent with the high molecular weight form of PRL observed both after <u>in vitro</u> incubation with lysosomal fractions (Fig. 7) and after <u>in vivo</u> uptake within these organelles (not shown). This high molecular weight material was suggested to represent hormone-receptor complexes which appear to have a tendency to dissociate in mature lysosomes (Fig. 7c) but not in prelysosomes (Fig. 7b), perhaps reflecting the pH

status of these vesicles. In any case, even firm demonstration of PRL-R recycling would not necessarily imply that the general model for receptor-mediated endocytosis is relevant to prolactin and its receptor.

The lack of effect of colchicine and the mimicking effect of the anti-receptor serum reported in Chapter 3 both suggest that prolactin may not be required beyond its interaction with the plasma membrane receptor for its action on PRL-R regulation to be elicited. This raises the question as for a role of internalization in hormonal action. As discussed by Willingham and Pastan (41), there is yet no evidence for such a role of endocytosis in the mechanism of polypeptide hormone action. It is possible that endocytosis actually serves only for the clearance of circulating hormone (including delivery to lysosomes) and regulation of cell surface receptors. On the other hand, colchicine inhibits some actions of PRL other than receptor regulation, such as casein and DNA synthesis in rabbit mammary explants (23).

Another interesting result is the relative stabilization of the prolactin interaction with its intracellular receptors as compared to the cell surface binding sites. This was reported earlier by our group (26) and further evidence is presented in Chapter 2 when less ^{125}I-oPRL was $MgCl_2$-extractable from Golgi or lysosomal receptors than from plasma membrane receptors (Fig. 5). Also, and as mentioned above and in Chapter 3, nondissociable prolactin-receptor complexes, especially after internalization, could explain the rapid down-regulation of PRL-R observed in cultured rat hepatocytes (see Fig. 8). Other investigators have reported covalent binding to occur between insulin and its receptor (8,9) and similar results were recently obtained for PRL binding (45). This represents an additional argument for the view that PRL-R may not recycle as some receptors have been shown to do (reviewed in

refs. 21,22,40,41). On the other hand, there is some reported evidence for insulin receptor recycling (18).

Prolactin receptor regulation appears to be quite complex. Indeed, the mechanism of PRL message transduction across the membane of target cells remains to be elucidated, as investigators are still looking for an intracellullar mediator of PRL actions. The receptor molecule itself is complex, with a binding site consisting of at least two functionally distinct loci: one for hormone recognition and binding and another for the generation of the intracellular signal.

This point is reinforced by the discrepancy between the biological activities of the various monoclonal antibodies to the binding site of the PRL-R which were produced in our laboratory (14). All three clones studied were able to occupy the receptor, as assessed by their ability to inhibit PRL binding (25), but only one was capable of "activating", leading to biological responses expected from the hormone (14). Also, there is a difference in the extent of the action of cycloheximide on PRL- or antireceptor serum-induced maintenance of receptor levels in cultured hepatocytes: this agent totally blocks the action of the antiserum (Fig. 13) but only partially inhibits that of prolactin (Fig. 9). Prolactin effects on receptor levels were therefore interpreted to involve, in addition to stimulation of de novo synthesis of receptor molecules, either recycling of internalized receptors or, as previously discussed, unmasking of a cryptic pool of PRL-R. Thus, it can be suggested that the stimulation of the receptor by the antiserum turns on only part of the PRL-induced mechanisms, which would also point out the complexity of the transduction mechanism.

As mentioned above, the physiological significance of internalization or of intracellular receptors remains unclear. Although a large proportion of PRL-R were shown to be intracel-

lular (5), precise localization is still lacking because of the indirect approaches used, namely tissue fractionation (5,27 and Chapter 2) or morphological experiments using the labelled ligand (1,12,15,17,29,31,44). Clearly, a system was needed to directly and precisely localize the receptor.

To our knowledge, there is only one report of PRL-R visualization using an antibody directed against the receptor itself (16) and these experiments were carried out at the optical microscopic level, thus not permitting precise identification of the compartments where these receptors preferentially localize. Our attempts to localize this antigen at the electron microscopic level using monoclonal antibodies to the receptor are reported and thoroughly discussed in Chapter 4. This represents a novel method which still has to be refined before it can yield the expected results.

Work in this direction is still in progress which should provide definite data on prolactin receptor localization and new insights in receptor movements and compartmentation in various physiological or experimental conditions. Ultimately, these studies should help to better understand the overall mechanism of prolactin action.

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CLAIMS TO ORIGINAL RESEARCH

The following is a list of the novel observations and findings which arose from the studies presented in this thesis.

1. Identification and characterization of prolactin receptors (PRL-R) in lysosomes of rat liver. (Figs. 2-4, Table 3).

2. Protective effect of chloroquine administration on lysosomal PRL-R. (Fig. 2)

3. Differential kinetics of incorporation of ^{125}I-oPRL by Golgi and lysosomal compartments of rat liver. (Fig. 4)

4. Identification of a subclass of small, light lysosomes (prelysosomes) which also contain PRL-R and take up circulating PRL. (Figs. 2-4, Table 3)

5. Differential strength in PRL binding with respect to intracellular compartment. (Fig. 5). There had been a previous report that PRL binds more firmly to rat liver microsomes than to plasma membrane; however, work on purified Golgi and lysosomal or prelysosomal fractions is original.

6. Intracellular transformation of PRL following uptake from the circulation by the liver, and incorporation into the lysosomal compartment. (Figs. 5,6; Table 4). Likewise, *in vitro* transformation of ^{125}I-oPRL by lysosomal and prelysosomal fractions. (Fig. 7, Table 5)

7. Total maintenance of PRL-R levels by a direct action of oPRL on cultured rat hepatocytes. (Fig. 8). Evidence for the involvement of *de novo* synthesis (especially translation) of receptor molecules in this action of PRL. (Figs. 9,10).

8. Further evidence for heterogeneity between tissues, species or mechanisms of the different actions of PRL: absence of effect of colchicine on the PRL-induced regulation of PRL-R in rat hepatocytes (Section 3.1), in contrast to its previously reported counteracting effect on PRL stimulation of casein synthesis in rabbit mammary gland.

9. Mimicking effect of PRL action by antibodies to PRL-R, in the regulation of its receptors. (Fig. 12). Such a mimicking effect had previously been observed for PRL-induced casein and DNA synthesis in rabbit mammary explants.

10. Close resemblance of the modulatory actions of various pharmacological agents on PRL- and receptor antibody-evoked maintenence of PRL-R. (Chapter 3).

11. First attempt to localize PRL-R at the ultrastructural level, using a monoclonal antibody to the receptor. Identification, along with several other authors, of some of the specific problems which may arise from such experiments. (Chapter 4).