# Lipid-depleted diet perturbs membrane composition and intracellular transport in lactating mammary cells[1]

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**Abstract** When rats were fed a control or a lipid-depleted diet for five generations, reproduction was not disturbed but pup growth was affected. The membrane organization and the secretory activity of mammary epithelial cells from these lactating rats were investigated. This diet induced a large decrease in the level of polyunsaturated fatty acids of membrane phospholipids (26.6% versus 44.0%). The level of 20:4 (n–6) was strongly decreased, mainly in phosphatidylethanolamine. Annexin VI, which interacts preferentially with this phospholipid, accumulated at the periphery of the cell and was largely associated to the hydrophobic region of the bilayer as compared to control membranes. Casein synthesis and casein secretion measured in incubated explants, after pulse-chase metabolic labeling, were both reduced by about 60% in lipid-deprived cells. The secretory ratio (radioactive secreted caseins in %) was not modified, suggesting that the mechanism of basal secretion was not mainly affected. On the contrary, the secretagogue effect of prolactin disappeared. The intracellular transport of the hormone was considerably slowed down by the diet and prolactin did not reach the lumen of the acini after 1 h of chase, in contrast to what occurred in control cells. Addition of 20:4 (n–6), in vitro, to mammary fragments from lipid-deprived rats restored the localization of annexin VI, increased synthesis and secretion of caseins as well as intracellular transport of PRL. Together, these data underline the importance of the level of 20:4 (n–6) in membrane phospholipids for exocytic and endocytic transport in lactating mammary epithelial cells.—**Ollivier-Bousquet, M., F. Lavialle, P. Guesnet, D. Rainteau, and G. Durand.** Lipid-depleted diet perturbs membrane composition and intracellular transport in lactating mammary cells. *J. Lipid Res.* 1997. **38:** 913–925.

**Supplementary key words** mammary epithelial cell • arachidonic acid • casein • prolactin • annexin • secretion

In the mammary epithelial cell (MEC), the secretion of milk proteins and lipids is associated with a large flow of membranes (1). In this cell, as in other cell types, phospholipid composition of membranes and interactions between membrane phospholipids and proteins are important in controlling endocytic and exocytic events (2). These membranes exhibit a very specific fatty acid composition. The phospholipids are as rich in polyunsaturated fatty acids (PUFA) as those of other tissues, but the proportion of total (n–3) PUFA is low. Consequently, the (n–6):(n–3) ratio, which reaches a value of 12, is higher than that described in other tissues and organs in which it varies between 1 and 6 (3, 4). The importance of PUFA of the (n–6) family in the regulation of the secretory activity of MEC has been strongly suggested. Casein secretion is stimulated by prolactin (PRL) in vitro. One of the early events, after binding of PRL to its receptor is a transient release of 20:4 (n–6) in 1–5 min (5). Exogenous $PLA_2$ induces the same release of 20:4 (n–6) and mimics the secretagogue effect of PRL (6). Inhibitors of the metabolism of 20:4 (n–6) by the lipoxygenase pathway suppress this effect (5). Consequently, it was hypothesized that 20:4 (n–6) could play a role in transmitting the secretagogue effect of PRL.

To test this hypothesis, initial experiments were carried out to deplete MEC membranes of PUFA which are considered as essential because mammalian cells are

Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; EDTA, ethylenediamine-tetraacetic acid; FITC, fluorescein isothiocyanate; Mb, membrane fraction; MEC, mammary epithelial cell; MUFA, monounsaturated fatty acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; $PLA_2$, phospholipase $A_2$; PRL, prolactin; PUFA, polyunsaturated fatty acid; S, soluble fraction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SFA, saturated fatty acid.

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unable to synthesize them. In rats fed with deficient (n–3) and (n–6) PUFA diets, modifications of the PUFA content of membrane phospholipids of lactating MEC were observed and the PRL secretagogue effect was abolished. However, the basal level of casein secretion was not greatly modified and compensation by various lipids included in these diets could be suggested (4). In order to establish a model system to study the role of (n–6) PUFA on the secretory function of MEC at the cellular level, rats were fed for five generations without added lipids. In these conditions, compensation by other PUFA was minimized.

Four points were addressed. *i*) Effects of a lipid-depleted diet on the composition of membrane phospholipids were analyzed. *ii*) As it is known that interactions between membrane phospholipids and proteins are of primary importance for the control of cellular events, the localization of a protein from the annexin family, annexin VI, that may participate in exocytic and endocytic events (7–9) was compared in the MEC from control and lipid-deprived rats. *iii*) Effects of the diet on basal and PRL-regulated secretion of MEC were measured. *iv*) It has been previously shown that PRL does not exert its secretagogue effect at low temperature (25°C) in vitro and that at the same time the transport of the hormone across the cell is disturbed (10). The question, therefore, arose about a relationship between endocytosis of the hormone and possible intracellular sites involved in transmission of the hormonal signaling. We investigated whether the intracellular pathway by which PRL is carried across the cell to the milk was affected in MEC from lipid-deprived rats.

Concerning these four points, results showed that the MEC underwent alterations. Consequently, to specify the role of PUFA of the (n–6) family in the secretory function of MEC, the ability of 20:4 (n–6) to restore altered functions in vitro was studied.

## MATERIALS AND METHODS

### Animals and diets

Experiments were performed on two groups of rats originating from our laboratory. For 5 consecutive generations control animals received a balanced diet containing 6% lipids derived from rapeseed and peanut oils. This provided an adequate supply of PUFA [1.2 g of 18:2 (n–6) and 0.2 g of 18:3 (n–3)/100 g diet] (3). Lipid-deprived animals received a diet without addition of lipids for five successive generations. However, small traces of lipids, from casein and corn starch, were still present. This corresponded to 12 mg of (n–6) PUFA and 2 mg of (n–3) PUFA/100 g diet, i.e., only 1% of the recommended supply. Detailed diet compositions are reported in **Table 1** and **Table 2.**

TABLE 1. Diet composition

| | Control | Lipid-Deprived |
|---|---|---|
| | *g/kg* | |
| Casein | 220 | 220 |
| DL-methionine | 1.6 | 1.6 |
| Corn starch | 432.3 | 472.3 |
| Saccharose | 216.1 | 236.1 |
| Cellulose | 20 | 10 |
| Agar–agar | — | 10 |
| Mineral mixture[a] | 40 | 40 |
| Vitamin mixture[b] | 10 | 10 |
| Oils[c] | | |
| Peanut | 32.4 | — |
| Rapeseed | 27.5 | — |

[a]Composition of the mineral mixture (g/kg): $CaHPO_4 \cdot 2H_2O$, 380; $K_2HPO_4$, 240; $CaCO_3$, 180; NaCl, 69; MgO, 20; $MgSO_4 \cdot 7H_2O$, 90; $FeSO_4 \cdot 7H_2O$, 8.6; $ZnSO_4 \cdot H_2O$, 5; $MnSO_4 \cdot H_2O$, 5; $CuSO_4 \cdot 5H_2O$, 1; NaF, 0.8; $CrK(SO_4)_2 \cdot 12H_2O$, 0,5; $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 0.02; KI, 0.04; $CoCO_3$, 0.02; $Na_2SeO_3$, 0.02.

[b]Composition per kg of mixture, triturated in dextrose): *dl* α-tocopherol acetate, 5.0 g; L-ascorbic acid, 10.0 g; choline chloride, 75.0 g; D-calcium pantothenate, 3.0 g; inositol, 5.0 g; niacin, 4.5 g; para-aminobenzoic acid, 5.0 g; pyridoxine HCl, 1.0 g; riboflavine, 1.0 g; thiamine HCl, 1.0 g; menadione, 0.1 g; folic acid, 0.2 g; retinyl acetate, 0.5 g; D-biotin, 20.0 mg; ergocalciferol, 6.25 mg; cyanocobalamin, 1.35 mg.

[c]Total dietary lipids added to the control diet: 60 g/kg (6%).

Mating was scheduled when animals were 10 weeks old. At parturition, dams and their entire progenies (live and stillborn) were weighed and litters were made equal to 9 pups. At 14 days post-partum, dams and their progenies were weighed. Six dams of each group, randomly chosen, were killed by decapitation and the mammary gland was taken bilaterally. Part of the tissue was rinsed in physiological saline, wiped, cooled to −80°C, and freeze-dried until phospholipid extraction. The remaining part was immediately used for protein analysis, metabolic studies, and immunofluorescence studies.

### Lipid and protein analysis

Total lipids were extracted from freeze-dried mammary tissues (11). Phospholipids were separated on a silicic acid column (12). Fatty acid methyl esters, obtained by transmethylation with 10% boron trifluoride ($BF_3$) in methanol (13), were extracted in hexane. After evaporation of hexane, the methyl esters were solubilized in chloroform. Methyl esters of milk lipids were kept in hexane to prevent loss of methyl esters from short chain fatty acids ($C \leq 12$). Methyl esters were separated on a Carlo Erba chromatograph (Carlo Erba 4180, Fisons Instruments, France) equipped with an on-column injector and a capillary column (length 50 m,

TABLE 2. Fatty acid composition of dietary lipids

| Fatty Acid | Control | Lipid-Deprived |
|---|---|---|
| | *mg/100 mg fatty acids* | |
| 8:0 | ND | 0.4 |
| 10:0 | ND | 3.0 |
| 12:0 | ND | 4.3 |
| 14:0 | ND | 11.7 |
| 15:0 | ND | 1.7 |
| 16:0 | 7.7 | 32.2 |
| 17:0 | ND | 0.6 |
| 18:0 | 2.8 | 10.3 |
| 20:0 | 1.1 | 0.2 |
| 22:0 | 1.7 | 0.2 |
| 24:0 | 0.9 | ND |
| Σ SFA | 14.2 | 64.6 |
| 16:1n–9 | ND | 0.4 |
| 16:1n–7 | 0.1 | 1.8 |
| 18:1n–9 | 57.3 | 21.5 |
| 18:1n–7 | 1.9 | 2.3 |
| 20:1n–9 | 1.3 | 0.3 |
| Σ MUFA | 60.6 | 26.4 |
| 18:2n–6 | 21.6 | 6.3 |
| 18:3n–6 | ND | 0.3 |
| 20:3n–6 | ND | 0.3 |
| 20:4n–6 | ND | 0.4 |
| 22:4n–6 | ND | 0.3 |
| Σ n–6 PUFA | 21.6 | 7.6 |
| 18:3n–3 | 3.6 | 0.7 |
| 18:4n–3 | ND | 0.4 |
| 20:5n–3 | ND | 0.1 |
| 22:5n–3 | ND | 0.2 |
| Σ n–3 PUFA | 3.6 | 1.4 |
| Σ n–6 + n–3 | 25.2 | 9.0 |
| n–6/n–3 | 6,0 | 5.4 |
| Lipids, g/100 g of diet | 6.33 | 0.35 |
| Fatty acids, g/100 g of lipids | 89 | 46 |
| PUFA, mg/100 g of diet: | | |
| Σ n–6 | 1218 | 12 |
| Σ n–3 | 203 | 2 |

ND, not detected.

diameter 0.3 mm, Chrompack, France). Chromatographic peaks were identified using standards and quantification was performed using Nelson 2600 software (SRA, Lyon, France). After separation of the different classes of mammary membrane phospholipids by HPLC (Beckman System Gold 126, Beckman Instruments France), methyl esters of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were identified as detailed above.

Protein composition of mammary cell membranes was analyzed after homogenization of 1 g of mammary tissue in 30 ml of buffer A (10 mM HEPES, 10 mM EDTA, 150 mM NaCl; pH 7.4) supplemented with a cocktail of proteases. Debris and nuclei were removed by centrifugation at 600 *g* for 15 min. Membranes (Mb) and soluble (S) material were fractionated by centrifugation at 105 000 *g* for 1 h. The membrane pellet was resuspended in buffer A and centrifuged at 105.000 *g* for 1 h. Proteins were quantified by the method of Peterson (14) and resolved by SDS-PAGE (12% acrylamide) according to Laemmli (15). After transfer on nitrocellulose using a semi-dry procedure (10 mA per $cm^2$ for 1 h), annexin VI was identified after incubation in the presence of rabbit anti-annexin VI IgG prepared in the laboratory (1 h, dilution 1:2000 in PBS/0.3% Tween 20) and horseradish peroxidase-conjugated goat anti-rabbit IgG (45 min, 1:1000 in PBS/0.3% Tween 20). Results were visualized by enhanced chemi-luminescence (ECL Amersham, France).

### Metabolic labeling of proteins

Immediately after sampling, connective and adipose tissues were removed from the pieces of mammary gland taken from each animal. The pieces were cut into 0.1–0.2 mg fragments (total weight of each sample: 10–30 mg). Fragments from each animal were incubated separately in Hanks' medium, pH 7.4, at 37°C, in an atmosphere of 95% $O_2$, 5% $CO_2$. After 30 min of preincubation, fragments were pulse-labeled for 3 min with 0.74 MBq/ml of L-[3,4,5-$^3$H]leucine (ICN, Orsay, 17.76 TBq/mmol), extensively rinsed in the same medium, and chased for 60 min in the absence or presence of 5 µg/ml of rat PRL kindly provided by NIDDK, Bethesda, MD. To verify whether 20:4 (n–6) added in vitro to MEC from lipid-deprived rats could restore altered functions of these cells, fragments were pre-incubated for 30 min with or without 100 µM 20:4 (n–6) (Sigma) then pulse-labeled as described above.

The labeled tissue proteins and the labeled caseins secreted in the medium were assayed using the method previously described (4, 10). The percentage of radioactive caseins secreted in the incubation medium, which mirrors the secretion process (secretory ratio), was expressed as: radioactive secreted caseins (%) = 100 × radioactivity of secreted caseins (Bq/mg of tissue) × [radioactivity of secreted caseins (Bq/mg of tissue) + radioactivity of tissue proteins (Bq/mg of tissue)]$^{-1}$.

### Endocytosis and transport of bio-PRL

Biotinylated PRL (bio-PRL) was prepared using a biotinylation kit (Amersham, UK) as described previously (10). After 15 min preincubation in Hanks' medium at 37°C, tissue fragments from control and lipid-deprived rats were incubated in the same medium in the absence or presence of 100 µM 20:4 (n–6) at 37°C for 30 min then at 15°C for 30 min in the presence of 50 µl/ml of PBS containing bio-PRL (corresponding approximately to 25 µg of bio-PRL). After extensive washing at 15°C, tissue fragments were incubated for 5 and 60 min at 37°C, then fixed with paraformaldehyde 2% in 100 mM sodium cacodylate buffer, infused with 40% sucrose in

PBS, frozen in liquid nitrogen and sectioned in 2-μm sections at −35°C with a Cryocut Reichert Jung (Leica, Rueil, France). Sections were incubated with fluorescein isothyocyanate (FITC)-conjugated avidin 1:400 in 0.01 M PBS (Sigma), mounted in Mowiol 4–88 (Calbiochem, La Jolla, CA) and observed with a Reichert-Jung Polyvar microscope equipped for fluorescence.

### Endocytosis and transport of Au-PRL

Gold-labeled PRL (Au-PRL) was prepared with colloidal gold (15 nm) from Janssen (Beerse/Belgium) as described previously (10).

Aggregates of MEC from control and lipid-deprived rats were separated by collagenase treatment (140 IU/mg of tissue) at 37°C in Hanks' medium, pH 7.4, for 90 min, washed, and incubated at 15°C in the presence of Au-PRL (final dilution of the stock solution 1/6.5) for 30 min, and washed and incubated again at 37°C in Hanks' medium. After 5, 15, 30, and 60 min of chase, aggregates were fixed in 2% glutaraldehyde in 100 mM cacodylate buffer, post-fixed in 1% $OsO_4$ in the same buffer, dehydrated and embedded in Epon.

The number of gold particles was counted on micrographs at 22,000 magnification. In each experiment corresponding to one animal and at each time interval, 10 to 20 cells were analyzed. Very few gold particles were detectable in control MEC after 1 h chase (about 150 gold particles), probably due to a quick transport of the hormone and its release in the incubation medium. Many gold particles were detectable in MEC from lipid-deprived rats at all time intervals (about 1,000 gold particles) probably due to the accumulation of the hormone. The number of particles associated with each cell compartment was expressed in percent of the total number of particles. Results were obtained on five control and five lipid-deprived rats.

### Immunofluorescence

Fragments of mammary gland from control and lipid-deprived rats and fragments of mammary gland from lipid-deprived rats incubated in the absence or presence of 100 μM 20:4 (n–6), as described above, were fixed for 4 h with 2% paraformaldehyde in 100 mM sodium cacodylate buffer, infused for 17 h with 40% sucrose in PBS, frozen in liquid nitrogen, and cut in 2-μm-thick sections at −35°C with a 2800 Frigocut Reichert Jung (Leica, Rueil, France). Sections were sequentially incubated in 50 mM $NH_4Cl$ in PBS (45 min), 1% BSA in PBS (45 min), sheep serum (1 h, 1:10 in PBS/1% BSA), rabbit anti-annexin VI IgG (2 h, 1:10 in PBS/1% BSA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (30 min, 1:200 in PBS/1% BSA). Sections were mounted in Mowiol 4-88 and observed with a Reichert-Jung Polyvar microscope equipped for fluorescence.

### Statistical analysis

Data on reproduction, pup growth, and fatty acid analysis were compared by one-way analysis of variance (ANOVA) StatView SE + graphics™ (Abacus Concepts Inc., ASD Meylan, France). Comparisons of diet group means were made by Scheffe's test.

For the secreted casein assay, each experiment was conducted using the mammary gland of one rat. In one experiment, control and treated fragments differed only by the addition of PRL. The pairs of values from the same animal were compared using the paired Student's *t*-test. Differences were considered significant for $P < 0.05$.

## RESULTS

### Lipid-depleted diet does not significantly perturb reproduction but affects pup growth

As shown in **Table 3,** the mean number of pregnant females, the mean number of pups per litter and the mean weight of litter at birth were smaller in lipid-deprived rats as compared to controls although these differences were not statistically significant. In both groups, the weight of pups at birth was similar and the mortality was very low. On the other hand, the weight of 14-day-old lipid-deprived pups was 23% lower ($P < 0.01$) than that of controls. From weaning to mating, scheduled when females were 10 weeks old, the weight of lipid-deprived females remained lower than that of control animals. During lactation, the weight of control females increased by 13% ($P < 0.01$) while that of lipid-deprived rats did not vary.

### Mammary cell membranes of lipid-deprived rats have perturbed fatty acid composition and display modified annexin VI localization

*Fatty acid composition of phospholipids.* **Table 4** shows that the lipid-depleted diet significantly affected the fatty acid content of total phospholipids. Quite interestingly, SFA and MUFA contents increased by 8% and 61%, respectively, when PUFA content (n–6) + (n–3) decreased by 40%. Within the SFA group, these changes did not concern all fatty acids to the same extent; 16:0 increased by 20% although neither 14:0 nor 18:0 was modified. In the MUFA group, the lipid-depleted diet affected the three major fatty acids [16:1 (n–7), 18:1 (n–9), 18:1 (n–7)]. In the (n–6) PUFA group, long chain fatty acids 22:4 (n–6) and 22:5 (n–6) increased

TABLE 3. Effects of lipid-deprived diet on reproduction and postnatal growth of rats

| Variable | Control | Lipid-Deprived |
|---|---|---|
| Number of females | 10 | 10 |
| Body weight to the mating (g) | 188 ± 12 | 169 ± 11[b] |
| Number of pregnant rats | 9 | 8 |
| Number of pups per litter | 10.6 ± 1.8 | 9.1 ± 1.4 |
| Mortality (%) | 3.1 | 2.8 |
| Litter weight at birth (g) | 55.3 ± 7.6 | 48.9 ± 10.0 |
| Pup weight at birth[a] (g) | 5.2 ± 0.5 | 5.4 ± 0.7 |
| Dam weight after parturition (g) | 221.6 ± 11.5 | 194.8 ± 11.5[b] |
| Pup weight at 14 days (g) | 27.2 ± 2.0 | 21.0 ± 1.6[b] |
| Dam weight at 14-day post partum (g) | 250.8 ± 10.4[c] | 199.3 ± 14.6[b] |

Values are means ± SD.

[a]The mean birth weight of pups was evaluated taking into account the weight of each litter and the corresponding number of pups.

[b]$P < 0.01$ relative to the control diet.

[c]$P < 0.01$ relative to the dam weight after parturition.

although their precursor 18:2 (n–6) decreased by 56%. On the other hand, 20:4 (n–6) was rather less affected, its level decreasing by 27%. In the (n–3) family, which represents only 3.5% of total phospholipids, both 20:5 (n–3) and 22:6 (n–3) decreased by 58%. Eicosatrienoic acid [20:3 (n–9)] level was significantly higher in the lipid-deprived rats than in controls.

The fatty acid composition of the two main classes of phospholipids (PC and PE), which represented, respectively, 58% and 28% of the total phospholipids, was studied (Table 4). It appeared that the lipid-depleted diet affected PC and PE in a different way: 14:0 and 16:0 significantly increased in PE but remained stable in PC. In the (n–6) PUFA family of lipid-deprived rats, 18:

TABLE 4. Fatty acid composition of total phospholipids (PL), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) of mammary membranes of rats at 14 days lactation (g/100 g fatty acids)

| | PL (n = 6) | | PC (n = 3) | | PE (n = 3) | |
|---|---|---|---|---|---|---|
| Fatty Acid | Control | Lipid-Deprived | Control | Lipid-Deprived | Control | Lipid-Deprived |
| 14:0 | 3.3 ± 1.1 | 3.1 ± 0.5 | 3.0 ± 0.3 | 3.1 ± 0.1 | 0.5 ± 0.1 | 1.5 ± 0.2[a] |
| 16:0 | 17.2 ± 0.7 | 20.6 ± 1.4[b] | 25.1 ± 0.3 | 29.6 ± 0.9 | 7.5 ± 0.9 | 17.4 ± 1.7[a] |
| 18:0 | 14.4 ± 1.7 | 14.1 ± 2.3 | 16.7 ± 0.2 | 14.2 ± 0.4 | 21.9 ± 0.5 | 21.1 ± 3.1 |
| Σ SFA | 36.4 ± 1.9 | 39.3 ± 1.2[a] | 46.0 ± 0.1 | 47.2 ± 1.2 | 30.5 ± 1.1 | 41.0 ± 3.7[a] |
| 16:1n–9 | 0.2 ± 0.0 | 0.5 ± 0.2 | 0.6 ± 0.2 | 0.6 ± 0.3 | 0.2 ± 0.0 | 0.6 ± 0.0[a] |
| 16:1n–7 | 1.2 ± 0.2 | 4.7 ± 0.6[b] | 1.1 ± 0.2 | 5.0 ± 0.3[a] | 1.4 ± 0.3 | 7.0 ± 1.2[a] |
| 18:1n–9 | 14.4 ± 0.9 | 18.8 ± 2.2[b] | 13.5 ± 0.5 | 17.3 ± 0.3[a] | 21.8 ± 0.7 | 22.8 ± 2.3 |
| 18:1n–7 | 3.0 ± 0.2 | 6.0 ± 1.4[b] | 1.8 ± 0.1 | 4.1 ± 0.3[a] | 1.9 ± 0.3 | 3.1 ± 0.4 |
| 20:1n–9 | 0.3 ± 0.0 | 0.5 ± 0.2 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1[b] |
| Σ MUFA | 19.2 ± 0.8 | 31.0 ± 2.0[b] | 17.8 ± 0.9 | 27.9 ± 0.7[a] | 26.5 ± 1.2 | 34.7 ± 3.4 |
| 18:2n–6 | 20.9 ± 2.1 | 9.0 ± 1.3[b] | 21.1 ± 0.3 | 12.0 ± 0.4[b] | 10.6 ± 0.9 | 8.3 ± 1.2 |
| 20:3n–6 | 0.3 ± 0.1 | 1.4 ± 0.2[b] | 1.3 ± 0.1 | 2.0 ± 0.2 | 1.0 ± 0.2 | 1.3 ± 0.3 |
| 20:4n–6 | 17.4 ± 2.1 | 12.7 ± 1.7[b] | 10.4 ± 0.9 | 7.0 ± 0.6[a] | 19.5 ± 1.1 | 6.2 ± 1.3[b] |
| 22:4n–6 | 0.4 ± 0.0 | 0.8 ± 0.1[b] | 0.2 ± 0.1 | 0.2 ± 0.1[b] | 0.8 ± 0.3 | 0.6 ± 0.3 |
| 22:5n–6 | 0.3 ± 0.1 | 0.8 ± 0.1[b] | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.7 ± 0.3 | 0.4 ± 0.1 |
| Σ n–6 PUFA | 40.5 ± 1.9 | 25.1 ± 1.6[b] | 33.7 ± 1.0 | 21.7 ± 0.8[b] | 32.8 ± 1.6 | 17.0 ± 1.6[a] |
| 18:3n–3 | 0.1 ± 0.1 | ND | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.1 |
| 20:5n–3 | 1.7 ± 0.2 | 0.7 ± 0.2[b] | 0.9 ± 0.1 | 0.3 ± 0.2[a] | 1.3 ± 0.1 | 0.4 ± 0.1[a] |
| 22:5n–3 | 0.4 ± 0.1 | 0.3 ± 0.2 | 0.2 ± 0.0 | 0.2 ± 0.1 | 0.8 ± 0.2 | 0.1 ± 0.1[a] |
| 22:6n–3 | 1.2 ± 0.2 | 0.5 ± 0.1[b] | 0.7 ± 0.1 | 0.2 ± 0.1[a] | 1.6 ± 0.1 | 0.3 ± 0.1[b] |
| Σ n–3 PUFA | 3.5 ± 0.3 | 1.5 ± 0.3[b] | 2.0 ± 0.1 | 0.8 ± 0.1[a] | 3.9 ± 0.2 | 0.9 ± 0.3[a] |
| n–6 + n–3 | 44.0 ± 1.8 | 26.6 ± 1.7[b] | 35.7 ± 1.8 | 22.5 ± 1.1[a] | 36.7 ± 2.0 | 17.9 ± 1.9[a] |
| n–6/n–3 | 11.6 ± 1.4 | 16.7 ± 1.7[b] | 16.8 ± 1.5 | 27.1 ± 2.2 | 8.4 ± 1.1 | 18.9 ± 1.6 |
| 20:3n–9 | 0.3 ± 0.0 | 3.1 ± 0.7[b] | 0.3 ± 0.1 | 2.2 ± 0.5[a] | 0.5 ± 0.1 | 4.6 ± 1.3[a] |

Values are means ± SD; ND, not detected.

[a]$P < 0.05$.

[b]$P < 0.01$.

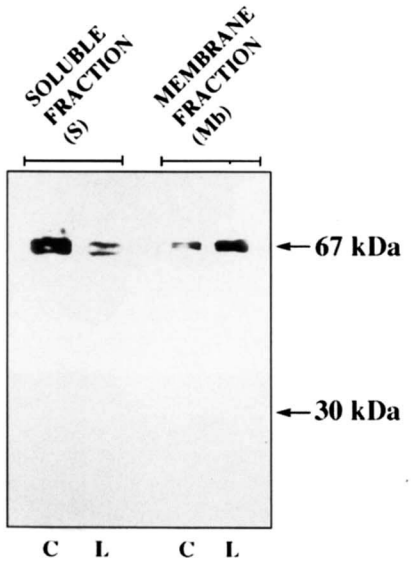


**Fig. 1.** Detection of annexin VI in mammary tissue from control (C) and lipid-deprived (L) rats by immunoblot assay. S and Mb were fractionated by centrifugation at 105 000 *g* for 1 h; 10 μg was loaded per well.

2 (n–6) decreased to a larger extent in PC than in PE. In contrast, the decrease in 20:4 (n–6) was more pronounced in PE than in PC.

*Annexin VI localization.* Annexin VI interacts preferentially with PE. Its localization was studied in the two types of tissues. After homogenization of tissue fragments of control and lipid-deprived rats, a pool of soluble proteins (S), which included cytosolic proteins and proteins involved in electrostatic interactions with membranes, was separated from the pool of membrane proteins (Mb). S and Mb contained, respectively, 74% and 24% of the total proteins. **Figure 1** displays the signals obtained after immunoblotting using anti-annexin VI antibody. In the S fraction of both groups of rats it shows the characteristic doublet of annexin VI (16). Interestingly, only the upper band of the doublet was detected in the pool of membrane proteins. Quantitative computer analysis of the ECL signals indicated that, in control rats, the S fraction was enriched in annexin VI as compared to Mb (84% versus 16%). On the other hand, annexin VI was almost equally distributed between S and Mb fractions recovered from lipid-deprived rats (40% versus 60%).

Annexin VI was localized by immunofluorescence in MEC of both groups of rats. In control rats, a bright punctuate staining was detectable within the cell and on small portions of the basal, lateral and apical membranes (**Fig. 2a**). In lipid-deprived animals, a strong continuous staining surrounded the cells of all acini. An important diffuse cytoplasmic labeling was also detectable (Fig. 2b). As the annexin VI content of post-nuclear supernatants was quite similar in both groups of rats (immunoblots, not shown), such a strong signal intensity may stem from an enhanced accessibility of the antigen.

When fragments from mammary gland of lipid-deprived animals were incubated for 30 min in the presence of 20:4 (n–6), localization of annexin VI by immunofluorescence was strongly modified in most of the acini compared to fragments incubated in Hanks' medium. Annexin VI accumulation was still detected in mammary fragments from lipid-deprived rats after incubation in Hanks' medium (**Fig. 3a**). On the other hand, in fragments incubated in the presence of 20:4 (n–6), localization of annexin VI was similar to that observed in mammary fragments from control rats (Fig. 3b).

### Basal and PRL-stimulated secretion of caseins is perturbed in lipid-deprived rat mammary cells

Fragments from the mammary glands of the two groups of rats were pulse-labeled and then chased for 60 min. Total radioactive proteins measured in the mammary gland fragments at the end of the chase included intracellular radioactive proteins, plus the radioactive milk proteins secreted in the lumen of the acini and retained in the fragments. **Figure 4a** shows that the amount of total radioactivity, which reflects the protein synthesis activity of the cells, decreased by 60% in the lipid-deprived group as compared to the control group. The radioactivity associated with caseins measured in the incubation medium after a 1 h chase reflected the secretory activity of these cells. Secretion of newly synthesized caseins in the incubation medium from the lipid-deprived group was decreased by 65% compared to the control group (Fig. 4b). It could be hypothesized that the remaining radioactive proteins measured in the incubation medium are not caseins but soluble proteins of milk. However, as attested by electron microscopy, in MEC from lipid-deprived rats, casein micelles were present in intracellular secretory vesicles and inside the lumen of the acini (**Fig. 5**). Moreover, gel electrophoresis showed that milk from control and lipid-deprived rats displayed quite similar electrophoresis patterns (not shown). Thus, in MEC from lipid-deprived rats, caseins are still synthesized but in small amounts, and the decrease in casein secretion paralleled the decrease in synthesis of total proteins. Consequently the secretory ratio was not affected (Fig. 4c).

Addition of PRL, after the pulse, in the chase medium allowed us to measure the secretory response of these cells to the hormone. In these experimental conditions, PRL did not modify total radioactive proteins measured in the mammary gland fragments of both groups of rats (Fig. 4a). As previously shown (4), PRL increased the amount of newly synthesized caseins se-

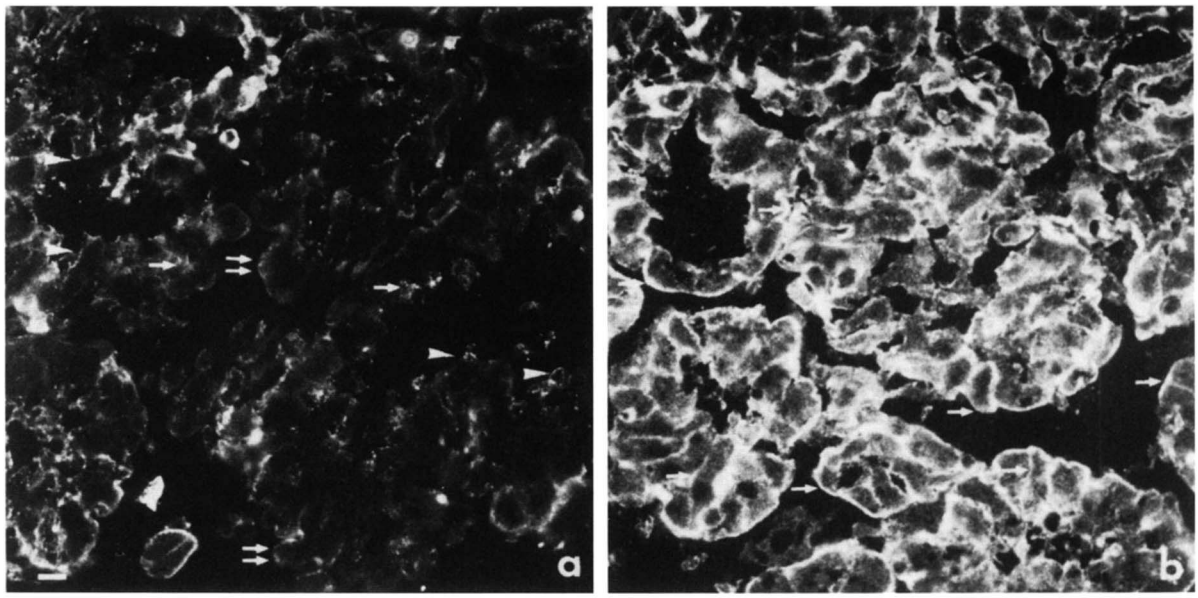


**Fig. 2.** Immunofluorescence localization of annexin VI in mammary tissues from control and lipid-deprived rats. Tissues were fixed and frozen. Sections were stained with rabbit anti-annexin VI antibody followed by FITC-conjugated anti-rabbit IgG. (a) In MEC from control rats labeling is detectable in cytoplasm (arrow), associated to the apical membrane (arrowhead) and to short portions of the basal membrane (double arrow). (b) In MEC from lipid-deprived rats, an important labeling is located on the whole basolateral and apical membrane (arrow). Bar = 10 μm (a, lower left).

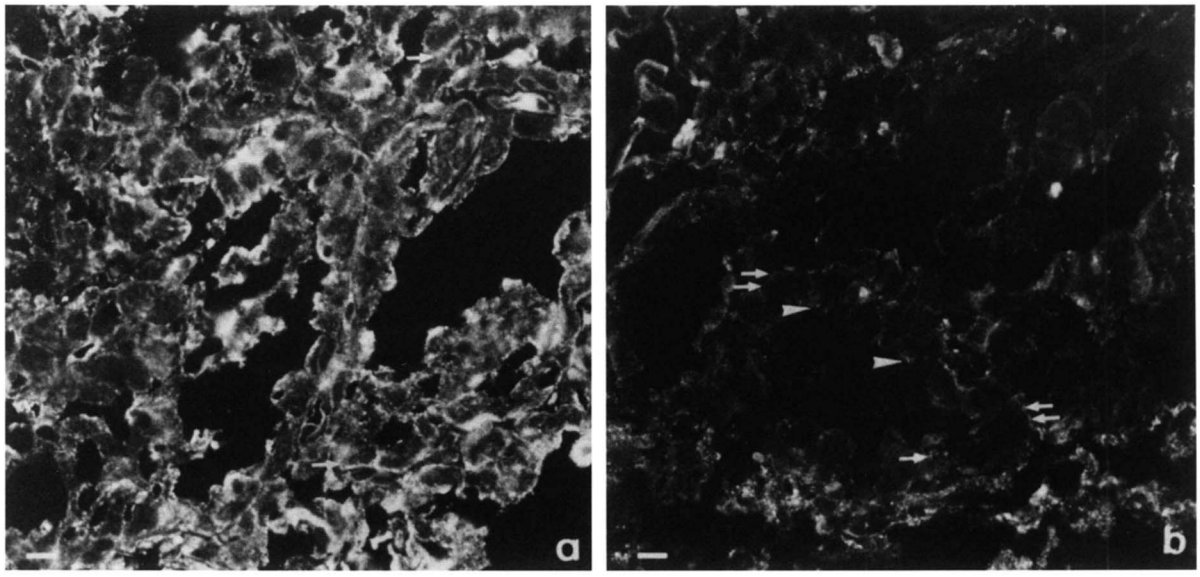


**Fig. 3.** Effect of 20:4 (n–6), on the localization of annexin VI by immunofluorescence in mammary tissues from lipid-deprived rats. Mammary tissues were incubated in Hanks' medium in the absence (a) or presence (b) of 100 μM 20:4 (n–6) for 30 min. Tissues were fixed and frozen. Sections were stained with rabbit anti-annexin VI antibody followed by FITC-conjugated anti-rabbit IgG. (a) In MEC from lipid-deprived rats, incubated for 30 min in Hanks' medium, the localization of annexin VI was not modified compared to samples fixed without incubation (compare with Fig. 2b). (b) After 30 min of incubation in the presence of 100 μM 20:4 (n–6), labeling detectable in the cytoplasm (arrow), associated with apical membrane (arrowhead) and portions of the basal membrane (double arrow) is strongly reduced and comparable to the labeling detectable in control rat MEC (compare with Fig. 2a). Bar = 10 μm.

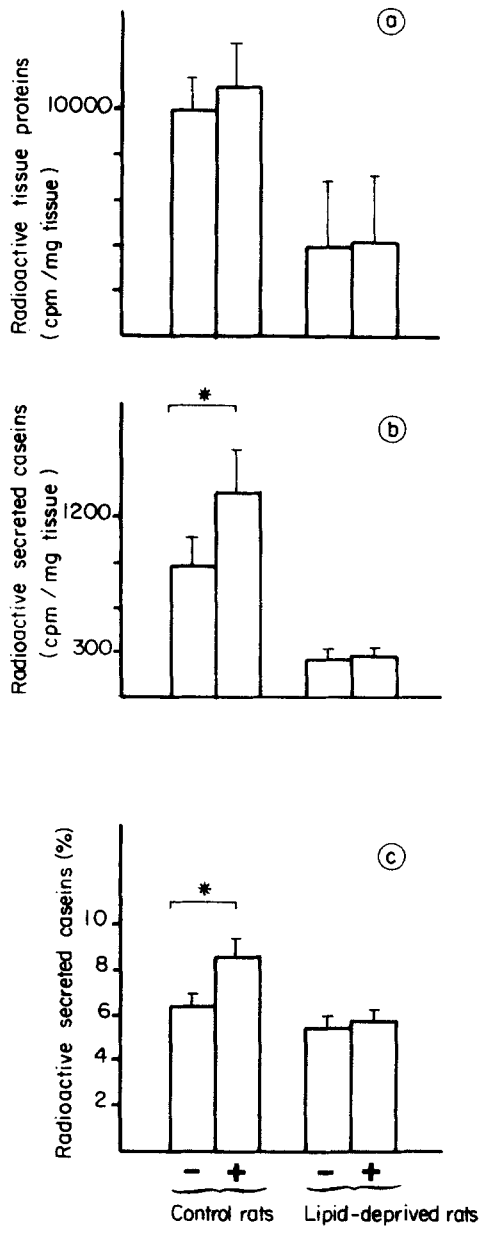


**Fig. 4.** Effect of a lipid-deprived diet on the synthesis and secretion of milk proteins by MEC. Mammary fragments from control and lipid-deprived rats were incubated in Hanks' medium, pulse-labeled by L-[$^3$H]leucine for 3 min and chased in the absence (−) or presence (+) of 5 μg/ml PRL during 60 min after the beginning of the pulse. At the end of the incubation, radioactive proteins and radioactive caseins were quantified as described in Material and Methods. (a) Radioactive tissue proteins. (b) Radioactive caseins secreted in the incubation medium. (c) Secretory ratio (radioactive secreted caseins in %) calculated as described in Material and Methods. Means ± SEM; 9 control rats; 23 lipid-deprived rats; $*P < 0.05$.

creted in the incubation medium by mammary fragments of control rats. By contrast, the hormone did not exert its secretagogue effect on mammary fragments of lipid-deprived rats (Fig. 4b). The secretory ratio of caseins, which was increased by the hormone in mammary fragments of control rats, remained unchanged in mammary fragments of lipid-deprived rats (Fig. 4c).

In order to verify whether 20:4 (n–6) added in vitro to the cells is able to restore altered secretory function, MEC from lipid-deprived rats were preincubated with this PUFA before the pulse-chase labeling. **Figure 6** shows that after preincubation with 100 μM 20:4 (n–6), the radioactivity of tissue proteins was strongly increased. Similarly, the radioactivity of secreted caseins was more than twice that of cells preincubated without the PUFA. It can be concluded from these results that 20:4 (n–6) is able to partially restore the secretory function of the cell.

### Intracellular transport of PRL is disturbed in lipid-deprived rat MEC

When fragments from the mammary gland of control rats were incubated in the presence of bio-PRL at 15°C for 30 min, washed, then chased at 37°C for 60 min, PRL was detected predominantly in the lumen of the acini (**Fig. 7a**). When fragments from the mammary gland of lipid-deprived rats were incubated in the same conditions in the presence of bio-PRL, a strong accumulation of PRL was detected in the cytoplasm of MEC (Fig. 7b). When these fragments were previously incubated in the presence of 20:4 (n–6) for 30 min, bio-PRL was located mainly in the lumen of the acini (Fig. 7c). These results clearly illustrated that the intracellular transport of PRL was considerably slowed down in MEC from lipid-deprived rats and that this transport could be restored by 20:4 (n–6) added in vitro.

In order to specify the sites where this transport was perturbed, the intracellular transport of PRL was monitored in MEC from the two types of rats, at the electron microscope level.

When aggregates of epithelial cells from control rats were incubated in the presence of Au-PRL at 15°C for 30 min, washed, and then chased at 37°C for different time intervals, gold particles were located in the different compartments involved in endocytosis and exocytosis. The vesicles and the tubulo-vesicular organelles close to the basal membranes were considered as endosomes. Vesicles containing small vesicles were considered as multivesicular bodies. Secretory vesicles were identified by their typical content of casein micelles. Basal membranes were characterized by the close proximity of the extracellular matrix and collagen and the apical membranes were characterized by the presence of microvilli, tight junctions, and by the secretory vesi-

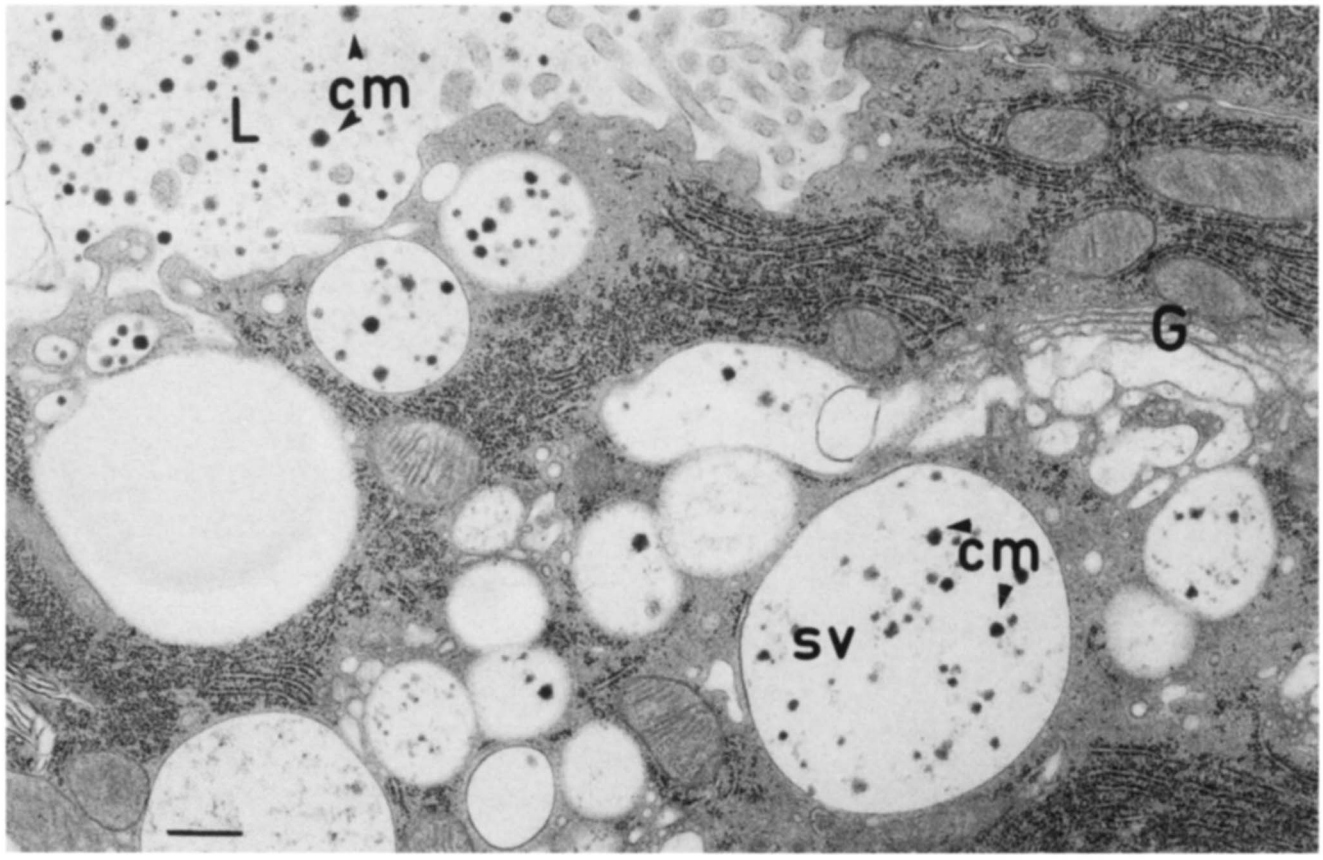


**Fig. 5.** Morphology of the apical region of a mammary epithelial cell from a lipid-deprived rat. Lumen (L), Golgi apparatus (G), secretory vesicle (sv), casein micelle (cm). Bar = 0.5 µm.

cles in the exocytotic process. In agreement with previous data (10), after 5 min of chase at 37°C, Au-PRL was detectable at the base of the cell (**Fig. 8a**) in endosomes and in multivesicular bodies. After 30 min chase, Au-PRL was detectable inside the secretory vesicles containing the casein micelles (Fig. 8b) and in the lumen of the acini. On the other hand, in aggregates of MEC from lipid-deprived rats, the labeling accumulated at the base of the cell and in endosomes after 5 min of chase at 37°C, revealing that the hormone was internalized (Fig. 8c). In addition, Au-PRL remained accumulated in endosomes, in tubulo-vesicular organelles, and in multivesicular bodies after 30 min of incubation at 37°C (Fig. 8d). It must be noted that multivesicular bodies can be observed in the basal region of the cell but also in the region containing secretory vesicles (Fig. 8d).

Quantification of the distribution of Au-PRL during the 1-h chase confirmed that Au-PRL transport was strongly slowed down in lipid-deprived rats (**Fig. 9**). In control MEC, the labeling of endosomes, which was about 40% of total labeling after 5 min chase, decreased to 5% after 60 min chase. During the same time, labeling of secretory vesicles increased from 5% after 5 min to 35% after 60 min. An increased labeling appeared in the lumen of the acini after a 30-min chase. In MEC of lipid-deprived rats, about 35% and 30% of total labeling, respectively, remained in endosomes and multivesicular bodies, during the whole chase. Labeling never exceeded 10% in secretory vesicles and 2% in the lumen during the 60-min chase.

## DISCUSSION

These results show that a lipid-depleted diet induced a strong decrease of the level of PUFA of the (n–6) family in phospholipids, coupled with an increase in either SFA or MUFA. These compositional changes could be associated with a perturbed localization of annexin VI in MEC from lactating rats. Functional consequences of these modifications were perturbations of basal and PRL-regulated secretion as well as perturbations of intracellular transport of this hormone. These altered functions were in part restored when 20:4 (n–6) was

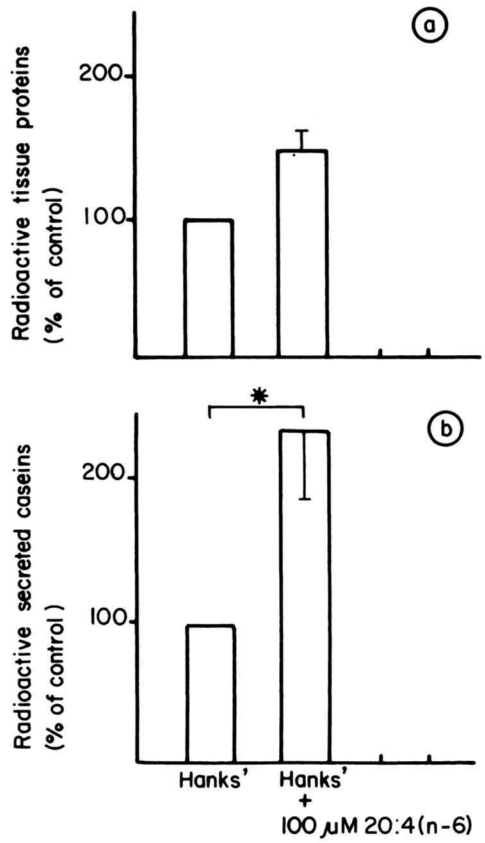


**Fig. 6.** Effect of 20:4 (n–6) on the synthesis and secretion of milk proteins by MEC from lipid-deprived rats. Mammary fragments from lipid-deprived rats were incubated in the absence or presence of 100 µM 20:4 (n–6) for 30 min then pulse-labeled by L-[$^3$H]leucine for 3 min and chased in the absence or presence of 100 µM 20:4 (n–6) during 60 min after the beginning of the pulse. At the end of the incubation, radioactive proteins and radioactive caseins were quantified as described in Material and Methods. (a) Radioactive tissue proteins expressed as percentage of control. Means ± SEM of radioactivity of tissue proteins were 2614 ± 370 cpm/mg of tissues, for tissues incubated in Hanks' medium and 4186 ± 1000 cpm/mg of tissues for tissues incubated in the presence of 20:4 (n–6). (b) Radioactive caseins secreted in the incubation medium. Means ± SEM of radioactivity of secreted caseins were 160 ± 63 cpm/mg of tissues for tissues incubated in Hanks' medium and 283 ± 86 cpm/mg of tissues for tissues incubated in the presence of 20:4 (n–6). Means ± SEM; 5 lipid-deprived rats; $*P < 0.05$.

previously added in vitro to incubation medium of MEC from lipid-deprived rats. It is strongly suggested that the level of this fatty acid in membrane phospholipids of lactating MEC is of primary importance for optimal secretory function.

Membrane composition of lactating MEC showed important repercussions depending on the diet. It mainly affected the content in (n–3) and (n–6) PUFA of membrane phospholipids, but this was compensated for by an increase in SFA, MUFA, and 20:3 (n–9). Surprisingly, the decrease in (n–6) PUFA content was very similar to that previously described (4) although the diet used in the present study supplied much less linoleic acid (12 mg versus 100 mg/100 g of diet). As it was reported for 22:6 (n–3) in rats fed a diet deficient in (n–3) PUFA (17), the lipid-deprived rats may conserve (n–6) PUFA by decreasing the turnover of these fatty acids. Under such conditions, whatever the diet supply

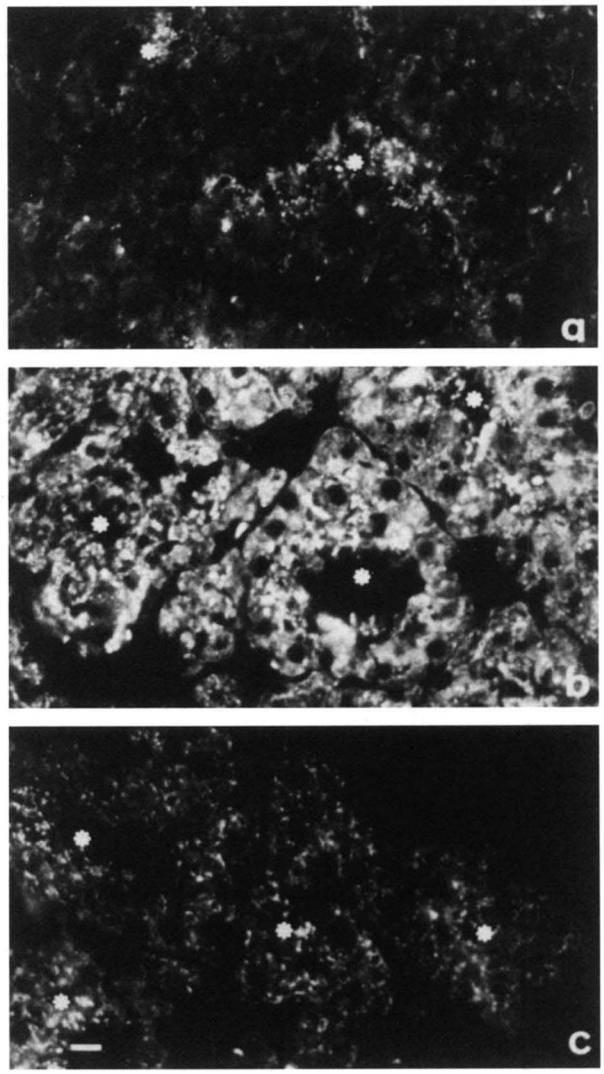


**Fig. 7.** Effect of 20:4 (n–6) on the cytochemical localization of bio-PRL in lactating mammary cells. Mammary fragments from control and lipid-deprived rats were preincubated in Hanks' medium for 30 min at 37°C in the absence (a, b) or presence (c) of 20:4 (n–6), then labeled for 30 min at 15°C with bio-PRL. After washing, mammary fragments were chased for 60 min at 37°C, fixed, frozen, and treated for cytochemical detection of bio-PRL. a) Mammary fragments from control rats preincubated and chased in Hanks' medium. Few labeling was detectable in the epithelial cells. Labeling accumulated in the lumen of acini (asterisk). b) Mammary fragments from lipid-deprived rats preincubated and chased in Hanks' medium. Epithelial cells are strongly labeled. Contents of the lumen were not or faintly labeled (asterisk). c) Mammary fragments from lipid-deprived rats preincubated and chased in the presence of 20:4 (n–6). Epithelial cells were faintly labeled and labeling accumulated in the lumen (asterisk). Bar = 10 µm.

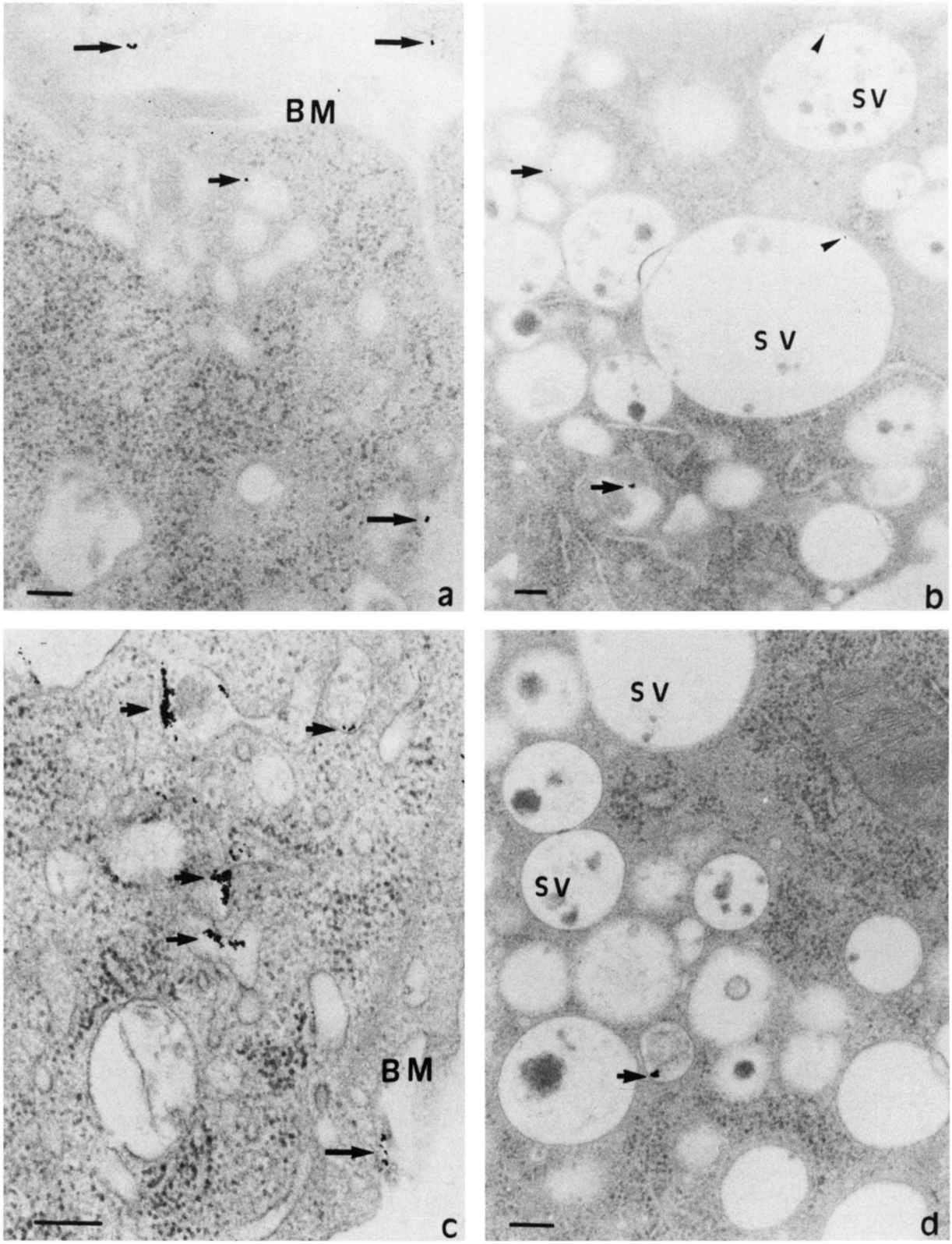


**Fig. 8.** Localization of Au-PRL in enzymatically dissociated aggregates of rat MEC, incubated at 15°C for 30 min in the presence of Au-PRL, washed, and chased at 37°C. (a) MEC from control rats after incubation at 37°C for 5 min. Au-PRL is located on the basal membrane (BM) (long arrow) and in endosome (short arrow). (b) MEC from control rats after incubation at 37°C for 30 min. Au-PRL is detectable in endosome and multivesicular body-like vesicle (short arrows) and in secretory vesicles (SV) (arrowhead). c) MEC from lipid-deprived rats, after incubation at 37°C for 5 min. Au-PRL is located on the basal membrane (BM) (long arrow) and is accumulated in tubulo-vesicular organelles (short arrows). d) MEC from lipid-deprived rats, after incubation at 37°C for 30 min. Au-PRL is detectable in a multivesicular body (short arrow) close to nonlabeled secretory vesicles. Bar = 0.2 μm.

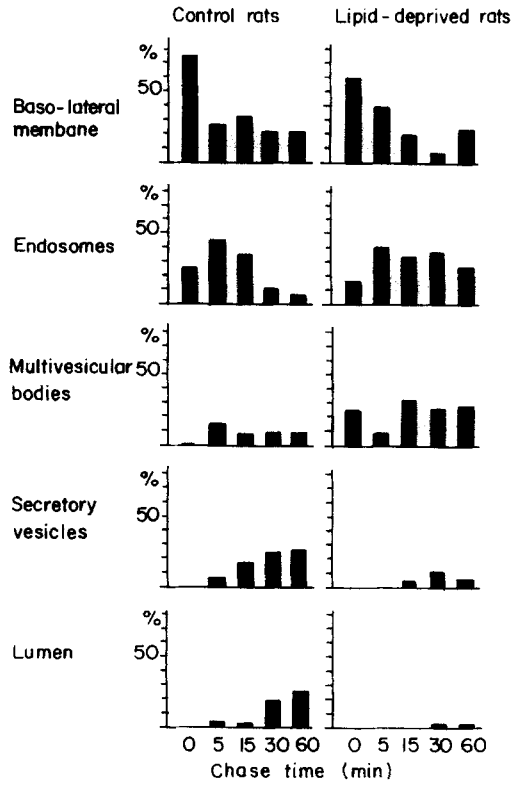


**Fig. 9.** Distribution of Au-PRL in aggregates of mammary fragments from control and lipid-deprived rats, incubated at 15°C for 30 min in the presence of Au-PRL and chased at 37°C. The percentage of gold particles was calculated after 5, 15, 30, and 60 min of chase in basolateral membrane, endosomes, multivesicular bodies, secretory vesicles, and lumen as described in Material and Methods.

of (n–6) PUFA (≤100 mg/100 g diet), comparable effects on the fatty acid composition must be observed. Interestingly, the decrease in the level of (n–6) PUFA mostly concerns the 20:4 (n–6) content of PE. This is of utmost interest for the two transport pathways described in this study.

First, concerning the casein secretion, as attested by the non-modified secretory ratio, the mechanism of basal secretion while depressed does not seem to be fully perturbed. Thus, despite a 65% decrease of the amount of casein synthesized, perturbations in membrane phospholipids do not prevent membrane events involved in the vesicular transport of small amount of caseins during the process of basal secretion. The question remains as how such a severe perturbation of membrane composition still allows basal secretion to occur. It is, however, clear that replenishment of membrane in vitro with 20:4 (n–6) increases synthetic activities of the MEC. In these experimental conditions, PRL did not exert any additional effect on casein secretion (not shown). This can be easily explained by the presence of a large amount of 20:4 (n–6) metabolites in the cells before the addition of the hormone. The secretagogue effect of PRL is abolished in the diet-modified MEC. The compensation by 20:3 (n–9), SFA, and MUFA could allow a part of the basal secretion but not the stimulating effect of PRL. These results confirm previous data obtained with (n–6)-deficient PUFA diet (4). As PRL has been shown to induce a release of free 20:4 (n–6), probably by the activation of $PLA_2$ (5), it can be hypothesized that the decrease of the level of 20:4 (n–6) induced by the diet could be responsible for the lack of response to PRL. As it has been shown that PE is preferentially utilized by $PLA_2$ to directly yield free 20:4 (n–6) (18), this phospholipid is a good candidate to provide substrate to $PLA_2$, after PRL activation. Decrease of the level of 20:4 (n–6) in PE could strongly reduce the substrate available. However, a significant amount of 20:4 (n–6) (27%) is still present in PE of lipid-deprived rats. The increase in 16:0 in PE decreases membrane fluidity and thus the accessibility of the 20:4 (n–6) (Table 4). Only PE shows compensation of lost PUFA with SFA (PC,PL compensate with MUFA). Phospholipid asymmetry has been shown to play an important role in modulating the activity of phospholipases toward biological membranes. Pancreatic $PLA_2$ hydrolyzes PE present in the internal layer of membranes (19). In MEC, perturbations of the membrane composition due to the diet could modify the phospholipid asymmetry and consequently the hydrolysis of PE. Finally, it should be noted that annexins possess the ability to inhibit $PLA_2$ activity in vitro (9).

Second, the importance of the composition of PE for transport of PRL can also be hypothesized. It is known that among the proteins interacting with the membrane phospholipids, annexin VI exhibits a special affinity for PE (20, 21). Annexin VI, which has been previously localized on the external surface of lactating mouse alveoli (22), has been shown to be required for budding of clathrin-coated pits in SV 589 cells (23) and has been detected in endosomes of hepatocytes (24), suggesting that this protein could be implied in endocytic events. As we show that in MEC of lipid-deprived rats, annexin VI localization is modified concurrently to modification of PE composition and that, in this group of rats, PRL accumulates in multivesicular bodies, we suggest that PE-annexin VI interactions could participate in the transport of PRL. The slowing down of the intracellular transport of PRL has also been observed in MEC incubated at low temperature (10). At 25°C, PRL accumulated in multivesicular bodies. Under these conditions, basal casein secretion still occurred. In contrast, PRL was not able to increase the secretion. It is presently difficult to correlate these two events except by suggesting a common effect on the membrane dynamics. As Au-PRL is always able to bind to the basal membrane

and to be endocytosed in MEC from lipid deprived rats, it can be supposed that the lack of secretagogue effect is not directly related to the absence of the plasma membrane receptors. Moreover, the signaling induced by the binding of PRL on its receptor, on casein gene expression, is always efficient as casein synthesis still occurs. Thus, the decrease in the level of (n–6) PUFA specifically induced perturbations on the secretagogue response to PRL.

In conclusion, the present results underline the importance of (n–6) PUFA composition of membrane for optimum functioning of the endocytic and exocytic pathways in the lactating MEC. They clearly show that 20:4 (n–6) plays a role in these mechanisms. The questions remain unanswered whether a relationship exists between endocytosis of PRL and regulation of exocytosis of caseins and whether this relationship is controlled by the level of the fatty acid. However, the model system obtained with lipid-deprived rat MEC will enable us to study these events at the cellular level.

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