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ARACHIDONIC ACID, BRADYKININ AND PHOSPHOLIPASE A_2 MODIFY BOTH PROLACTIN BINDING CAPACITY AND FLUIDITY OF MOUSE HEPATIC MEMBRANES

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Summary: The objective of this study was to determine if arachidonic acid, a precursor of prostaglandin synthesis, bradykinin, a decapeptide known to stimulate membrane phospholipid methylation, arachidonic acid release and prostacyclin synthesis, and enzyme phospholipase A_2, capable of liberating arachidonic acid, alter the fluidity of hepatic membranes which could in turn modify the functionality of prolactin receptors. Liver homogenates of adult C_3H female mice incubated at 28°C for various times with 1-20 μg/ml arachidonic acid, 1-100 μg/ml bradykinin or 0.26-0.00026 U/ml phospholipase A_2 provided the 100,000 x g membrane pellets for subsequent ovine prolactin binding and membrane fluidity studies. Membrane microviscosity was determined by fluorescence polarization techniques using the lipid probe 1,6 diphenylhexatriene. Arachidonic acid, bradykinin and phospholipase A_2 stimulated specific oPRL binding, in a dose-related fashion, with maximum increases of 73%, 21% and 46%, at 4 μg/ml arachidonic acid, 5 μg/ml bradykinin and 0.026 U/ml PLA_2, respectively. This induction, occurring within 30 min of incubation, was found to be due to an increase in the number of receptor sites. Under the same conditions, arachidonic acid, bradykinin and PLA_2 induced 22%, 16%, and 18% decreases in membrane microviscosity, respectively. These data suggest that prostaglandin synthesis modifying agents may modulate the number of prolactin receptors in vivo by changing the lipid fluidity of the target cell membranes by either of their known effects: arachidonic acid release from the phospholipid matrix, synthesizing appropriate prostaglandins at correct concentration or methylation of membrane phospholipids.

Modulation of hormone receptor levels within the cell membranes by various agents is well documented (1-7). Prolactin is one such agent and is known to modulate its own receptors under various physiological and pathological conditions. Recently we reported that prolactin receptors are also modulated by prostaglandin I_2 and an inhibitor of prostaglandin synthesis (8,9). It appears that the changes in prolactin binding capacity of membrane are under the particular influence of physical state of the membrane, their functionality and detectability presumably being modified by the ease with which receptor proteins move within the lipid bilayer. Mouse hepatic

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microsomal membranes with high microviscosity, determined by measuring the mobility of a fluorescent probe inserted into the lipid domain of the membrane, were found to have less prolactin binding capacity whereas more fluid membranes had greater numbers of prolactin receptors (8-10).

These studies imply a causal relationship, that the prolactin receptors may be controlled directly or indirectly via changes in the membrane microviscosity and further suggest that the products of the prostaglandin cascade are involved. These observations prompted the experiments reported herein: to determine if the prostaglandin precursor arachidonic acid and bradykinin, a decapeptide known to stimulate membrane phospholipid methylation and arachidonic acid release (11,12), would modulate the fluidity of target cell membranes and change the detectability of prolactin receptors. Furthermore, earlier studies (13) had shown that a selective modification of the lipid bilayer by various phospholipase altered the ability of the hepatocyte to bind prolactin. Therefore, studies were also undertaken to observe the effects of phospholipase A_2 on fluidity and prolactin binding capacity.

MATERIALS AND METHODS

Reagents: Ovine prolactin (oPRL, NIH-S-13, 35 I.U./mg) was provided by the National Pituitary Agency. Arachidonic acid (Na-salt), bradykinin (lysine), phospholipase A_2 (bee venom) and indomethacin were purchased from Sigma Chemical Company, St. Louis, MO, and 1,6-diphenylhexatriene (DPH) was purchased from Aldrich Chemical Company, Milwaukee, WI. Ham's F-12 medium with 50 U penicillin and 50 µg streptomycin per ml, without glutamic acid, calcium or magnesium, was purchased from the National Institutes of Health media unit. Other chemicals were of reagent grade.

Iodination: oPRL was iodinated by a lactoperoxidase method as described elsewhere (14).

Animals: C_3H female mice, obtained from Charles River Breeding Laboratories, at 3-4 months of age, were maintained in a 12-hr light-dark cycle with water and Purina rat chow available ad lib.

Tissue Preparation: Animals were decapitated and the livers were immediately frozen in liquid nitrogen and then pulverized. They were then homogenized (Polytron, Brinkmann) at a setting of 6 for 1 min at 4°C in approximately 10 vol of 0.3 M sucrose buffered to pH 7.6 with 25 mM Tris-HCl. Liver homogenates were then incubated either in 25 mM Tris-HCl buffer containing 10 mM $MgCl_2$ at pH 7.6 with graded concentrations of bradykinin (1-100 µg/ml) and arachidonic acid (1-20 µg/ml) or in Ham's F-12 medium with graded concentra-

tions of phospholipase A_2 (0.26-0.00026 U/ml) in a final volume of 5.0 ml for various time intervals at 28°C. The incubations were terminated by adding 1.0 ml of ice-cold buffer and placing the tubes in ice. These suspensions were then centrifuged at 4°C at 15,000 x g for 20 min. The supernatants from this preliminary centrifugation were then centrifuged at 100,000 x g for 1 hr. The membrane pellets, resuspended in buffer, were used for oPRL binding and membrane fluidity studies. The oPRL binding activity was determined as described in previous studies (14).

Membrane microviscosity was studied by fluorescence polarization at 24°C with the lipid probe 1,6-diphenylhexatriene. Membrane preparations containing 50 µg of protein per 1.5 ml were incubated for 1 hr at 25°C with 1.5 ml of 2 µM 1,6-diphenylhexatriene dispersed in phosphate-buffered saline (pH 7.1). These samples were then subjected to polarization analysis by measuring the emission fluorescence between 380 and 460 nm with the excitation wavelength kept constant at 366 nm. The polarization constant, proportional to the membrane microviscosity, was calculated according to the equation $P = (I_V - TI_H)/(I_V + TI_H)$ in which I_V and I_H are the relative fluorescence intensities measured at an angle of 90° to the incident beam with the emission polarization in the vertical and horizontal positions, respectively, and $T = I_V/I_H$ measured with the excitation polarization in the horizontal position (15).

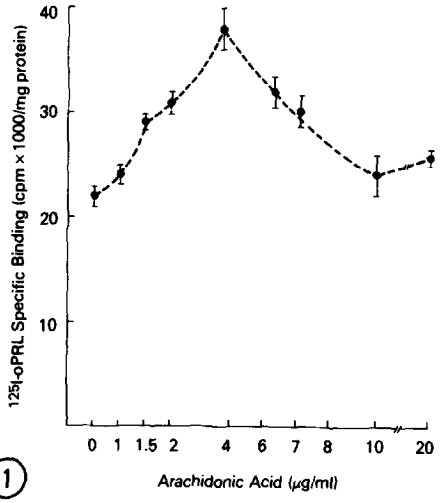
RESULTS

Stimulation of oPRL Binding by Arachidonic Acid:

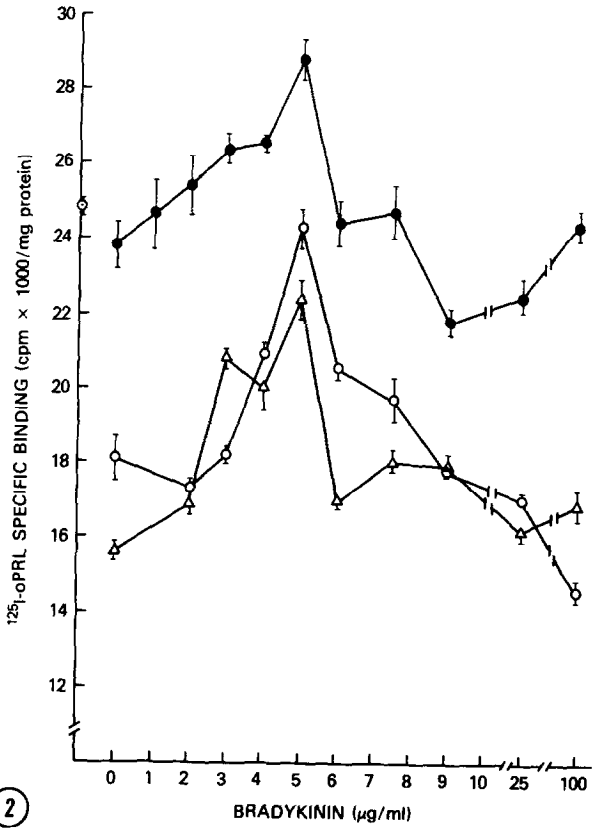
Incubation of liver homogenates at 37°C for 30 min resulted in a 15-25% loss in the oPRL binding capacity of membrane preparations, whereas incubation at 28°C resulted in only a 5-10% decrease. Subsequent incubations were performed at 28°C to minimize this spontaneous loss of oPRL binding.

To determine the effect of arachidonic acid on oPRL binding to the liver membrane preparations, liver homogenates were incubated with graded concentrations of arachidonic acid at 28°C for 30 min. The subsequent 100,000 x g membrane fractions were assayed for oPRL binding capacity (Figure 1). Arachidonic acid was initially dissolved in a small quantity (50 µl) of 95% ethanol and then made up to 5.0 ml with 25 mM Tris-HCl buffer to make a stock solution prior to further dilution in buffer. Incubation tubes with equivalent amounts of ethanol served as a control for arachidonic acid-treated group. The incubation mixture consisted of 0.5 ml of arachidonic acid solution and 2.0 ml of Tris-HCl buffer (pH 7.4) added to 2.5 ml of liver homogenate.

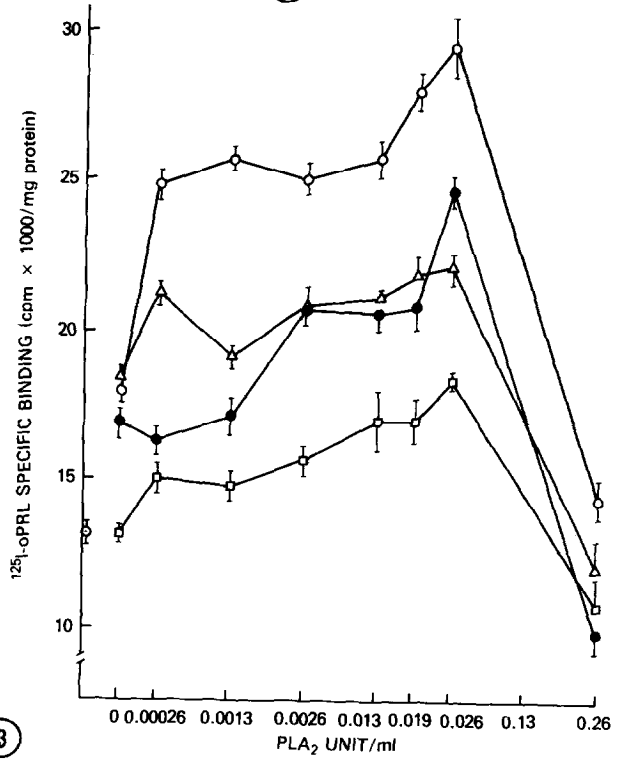
Incubation of liver homogenates with a range of arachidonic acid concentrations of 1-20 µg/ml showed an inductive effect on the oPRL binding capacity. This effect was found to be dose-dependent with the maximal effect at


^{125}I-oPRL Specific Binding (cpm x 1000/mg protein)
40
30
20
10
0 1 1.5 2 4 6 7 8 10 20
Arachidonic Acid (μg/ml)

(1)


^{125}I-oPRL SPECIFIC BINDING (cpm x 1000/mg protein)
30
28
26
24
22
20
18
16
14
12
0 1 2 3 4 5 6 7 8 9 10 25 100
BRADYKININ (μg/ml)

(2)


^{125}I-oPRL SPECIFIC BINDING (cpm x 1000/mg protein)
30
25
20
15
10
0 0.00026 0.0013 0.0026 0.013 0.019 0.026 0.13 0.26
PLA$_2$ UNIT/ml

(3)

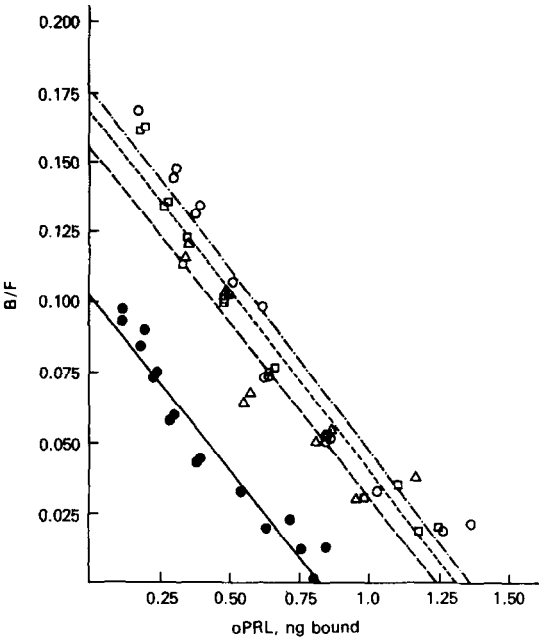


Figure 4
Scatchard plot of ^{125}I-oPRL binding to liver membranes from C_3H female mice. Data are plotted according to the method of Scatchard (29). The abscissa depicts the amount of oPRL bound to the liver membranes; the ordinate depicts the ratio of bound to free hormone. ●——, control;△— —, □---,○—·—, liver homogenate treated with 4 µg/ml arachidonic acid, 5 µg/ml bradykinin and 0.026 U/ml PLA_2, respectively, for 30 min at 28°C.

4 µg/ml arachidonic acid being 73% higher than control values observed. Higher concentrations of arachidonic acid resulted in a return of this induction to the control levels. In repeated experiments, the peak in induction remained at 4 µg/ml of arachidonic acid concentration and the values at this point varied from 135-173% of the control values.

Scatchard analyses of data obtained from oPRL binding to membrane prepared from untreated liver homogenates or from homogenates treated with 4 µg/ml arachidonic acid at 28°C for 30 min are presented in Figure 4. The

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Figure 1-3

Induction of specific ^{125}I-oPRL binding to a 100,000 x g membrane preparation of C_3H female mice after the liver homogenate was incubated with arachidonic acid (Figure 1, 1 to 20 µg/ml), bradykinin (Figure 2, 1 to 100 µg/ml) and phospholipase A_2 (Figure 3, 0.00026 to 0.26 U/ml) for 15 min (□—□), 30 min (●—●), 1 hr (○—○) and 2 hr (△—△) as described in text.
Values are mean of 6 observations ± S.E.

apparent affinity constants, K_a, were not significantly different, the values being 1.41 nM and 1.47 nM in control and treated membranes, respectively. The total number of binding sites, however, increased from 175 fmol/mg protein in control membranes to 196 fmol/mg of protein in the treated membranes.

Measurements of fluorescence polarization of membrane preparations obtained from the liver homogenates treated with and without arachidonic acid (4 μg/ml) are presented in Table I. A significant decrease in fluorescence polarization was observed in the membranes obtained from the liver homogenates treated with arachidonic acid, values being 78% of control values. This decrease in polarization value indicates that the membrane lipids became more fluid as a result of treatment with arachidonic acid.

Stimulation of oPRL Binding by Bradykinin:

The 100,000 x g membrane fractions obtained from liver homogenates, incubated with graded concentrations of bradykinin at 28°C, were assayed for oPRL binding capacity (Figure 2). Incubation of liver homogenates with a range of bradykinin concentrations showed an increase in oPRL binding capacity with a maximal effect at 5 μg/ml. Any further increase in concentration of bradykinin above this level resulted in a decline in binding capacity. Repeated experiments showed a similar pattern with maximum induction ranging from 18-36% after 30 min incubation. Increasing the time of incubation to 1 hr and 2 hr also resulted in 34% and 44% increases in oPRL binding capacity, respectively, when compared with membranes obtained from homogenates incubated for appropriate time but without the addition of bradykinin. However, when compared with zero time values, a maximum induction was obvious only after incubating liver homogenated with 5 μg/ml bradykinin for 30 min since a spontaneous loss in oPRL binding capacity of membranes was noted when the incubation period was increased beyond 30 min. Therefore, subsequent studies of fluorescence polarization and Scatchard analyses were performed using a 30-min incubation period.

TABLE I

Effects of Arachidonic Acid, Bradykinin and Phospholipase A_2 on Fluorescence Polarization of Hepatic Membranes from Female C_3H Mice

Treatment	Polarization Constant (P) at 24°C
Control	0.193 ± 0.0110
Arachidonic Acid (4 µg/ml)	0.151 * ± 0.0068 (78%)
Bradykinin (5 µg/ml)	0.162 * ± 0.0167 (84%)
Phospholipase A_2 (0.026 U/ml)	0.158 * ± 0.0079 (82%)

Liver homogenates were incubated in either Tris buffer or Ham's F-12 media, as described in text, with 4 µg/ml arachidonic acid; 5 µg/ml bradykinin or 0.026 U/ml PLA_2 for 30 min at 28°C. These suspensions were centrifuged, and 15,000-100,000 x g pellets were subsequently obtained. Pellets were resuspended in PBS (pH 7.2) and fluorescence polarization (with 1 µM 1,6-diphenylhexatriene in phosphate-buffered saline) were measured at 24°C.
Values are mean of 3-6 observations ± S.D.
Values in parentheses are % of control values.
*Values significantly different from control values, $p < 0.001$.

Scatchard analyses of oPRL binding data using liver homogenates treated with or without 5 µg/ml bradykinin at 28°C for 30 min are presented in Figure 4. The apparent affinity constants, K_a, were not significantly different, the values being 1.41 nM and 1.50 nM in control and treated membranes, respectively. However, the total number of binding sites was increased to 222 fmol/mg protein in the treated membranes from control values of 175 fmol/mg protein.

Measurements of fluorescence polarization of membranes from the liver homogenates with and without bradykinin are presented in Table I. A 16% decrease in P value was observed in membranes obtained from the liver homogenates treated with 5 µg/ml bradykinin.

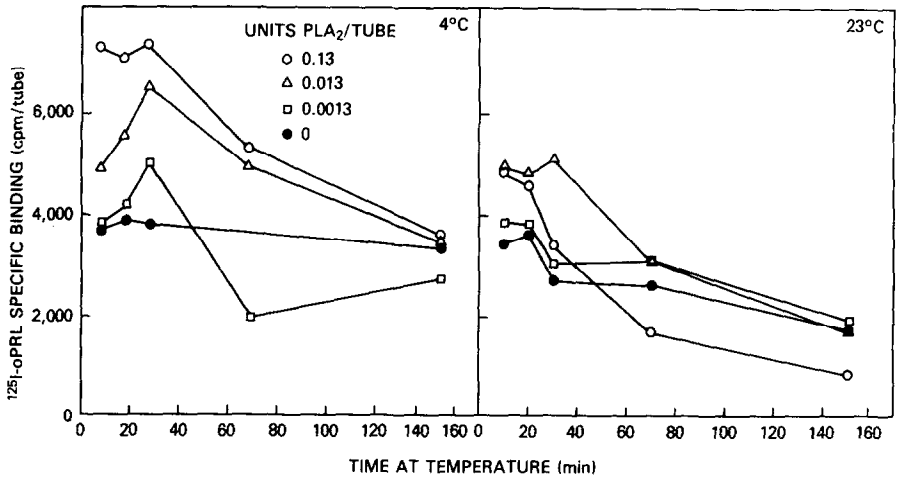

Figure 5
Incubation of isolated hepatocytes from C_3H female mice with PLA_2 in Ham's F-12 media alters their ability to bind ^{125}I-oPRL. Low concentrations of PLA_2 increase the specific binding, whereas high concentrations suppress the detectable binding. This occurs as a function of time and temperature with maximal effects being observed after 30 min of incubation at 4°C.

Stimulation of oPRL Binding by Phospholipase A_2:

Incubation of isolated hepatocytes from C_3H female with various phospholipase for different time intervals at 4°C, 23°C, and 37°C resulted in selective modifications of prolactin-binding capacity (13). A marked induction in prolactin-binding capacity was observed by incubating the hepatocytes with 0.13, 0.013 and 0.0013 units of phospholipase A_2 at 4°C, the maximum effect being observed after 30 min incubation (Figure 5). A smaller induction was noted by increasing the incubation temperature to 23°C; however, this effect was not evident at 37°C with a progressive loss in binding capacity occurring with time. Incubation with phospholipase C and D did not affect oPRL binding (data not shown).

Therefore, to determine the effect of PLA_2 on oPRL binding capacity and fluorescence polarization of liver membrane preparations, liver homogenates were incubated with graded concentrations of PLA_2 at 28°C for various time periods. The subsequent 100,000 x g membrane fractions were assayed for oPRL binding capacity (Figure 3).

Incubation of liver homogenates with PLA_2 showed an inductive effect on the oPRL binding capacity with the maximal effect observed at 0.026 U/ml concentration. A dramatic decline below control levels was observed when the PLA_2 concentration was increased to 0.26 U/ml at all time intervals studied. This induction was also noticeable after 15 min incubation with a maximum increase in oPRL binding capacity, 63%, that was observed after incubating the homogenates with 0.026 U/ml PLA_2 for 1 hr. However, only prolongation of the incubation to 2 hr resulted in a lower increase, 21%, in oPRL binding. Since earlier studies of the PLA_2 effect on isolated hepatocytes were made by incubating hepatocytes with phospholipase in Ham's F-12 media, in the present studies on PLA_2 liver homogenates were incubated with PLA_2 in Ham's F-12 media.

Scatchard analyses of data obtained from oPRL binding to membranes from untreated liver homogenates or from homogenates treated with 0.026 U/ml PLA_2 are presented in Figure 4. The apparent affinity constants, again, were not significantly different; the values were 1.41 nM and 1.43 nM in control and treated membranes, respectively. However, the total number of binding sites was increased from 175 fmol/mg protein in control to 220 fmol/mg protein in the treated membranes.

As shown in Table I, measurements of fluorescence polarization again demonstrated a significant decrease in membranes obtained from liver homogenates treated with 0.026 U/ml of phospholipase A_2.

DISCUSSION

Cell membranes exist as a dynamic matrix which responds to various physiological or pathological conditions by modifying its physical state. This allows movement or conformational changes of membrane-associated proteins in a specific fashion. The concept that alteration of the physical characteristics of the membrane lipid bilayer results in changes in the detectability of membrane-associated receptors and enzyme proteins has now been widely accepted. The membrane-associated receptors may, therefore, be

considered to float within the lipid matrix with their movements being modified by changes in the lipid microviscosity of the membrane. This thesis is supported by data correlating the activity of membrane enzymes with the fluidity of membranes (16-18).

The microviscosity measurements described within this study represent the averaged measures of changes taking place throughout the membrane. Changes in specific areas of the membrane may be much more extensive than these averaged values. Such changes may be induced by modifications of the phospholipids to cholesterol ratio (19-21), concentrations of specific phospholipid class or the ratio of lipid to protein (20,22) and degree of unsaturation and length of the fatty acyl moieties within the phospholipids in membrane bilayer (23,24).

Dietary and physiological changes have been reported to modify membrane fluidity and prolactin receptors (25-27). Hepatic membranes of animals maintained on diets lacking the essential fatty acids and aged animals had both decreased fluidity and prolactin-binding capacity, whereas female mice during pregnancy had both increased fluidity and prolactin-binding capacity.

In a recent study carried out in this laboratory (10,28), it was observed that liver membranes obtained from hypophysectomized female rats, treated with PMSG/hCG/DES and injected with graded amounts of oPRL, exhibited a dose-related modification in prolactin-binding capacity and fluidity with simultaneous maxima being observed when the animal had been replaced with physiologic amounts of oPRL. Granulosa cells obtained from these animals exhibited similar maximal increases in the rates of synthesis of both PGE and $PGF_{2\alpha}$. Another series of experiments also demonstrated that prostacyclin increased both the fluidity and oPRL-binding capacity of mouse liver microsomal membrane preparations in vitro (8). This suggested that alterations within the prostaglandin cascade might be the mechanism through which prolactin exerted physiological effects and that changes in the membrane fluidity occur via prostaglandin cascade.

The present study supports such a thesis and shows that in vitro treatment of membranes with the precursor of PG synthesis, arachidonic acid, and agents which induce arachidonic acid release result in simultaneous changes in the membrane fluidity and prolactin binding capacity. Treatment of liver homogenate with bradykinin might have a dual action, either release of arachidonic acid from the membrane phospholipids or methylation of membrane phospholipids, both of which have been shown to change membrane fluidity (22). Similarly, the inductive effects of PLA_2 treatment might also be either due to release of arachidonic acid or resulting lysophospholipids. Studies carried out in this laboratory have shown that addition of mono-oleoyllecithin (0.25 mg/ml), a product of membrane exposure to PLA_2, increases the specific oPRL binding to 80% and 100% when incubated for 4 hr at 4°C and 37°C, respectively (13). However, exposure to dipalmitoyllecithin, phosphatidylethanolamine, phosphatidylserine or di-oleoyllecithin did not show such an augmentation of oPRL receptors.

The data presented herein demonstrate that the modulation of the prostaglandin cascade may be a mechanism by which prolactin induces functionality within its own receptor. It is, therefore, suggested that such increases in prolactin binding are due to emergence of receptor sites within the membrane secondary to fluidization of the lipid matrix by prostaglandins. It is also suggested that compositional modifications of the lipid environment are also of prime importance in modulation of the prolactin receptor sites. Such effects of prostaglandin precursors may be a result either of a well-balanced and favorable localized prostaglandin level which may have direct action upon the membranes or of activation of other biochemical pathways which, in turn, modify the phospholipid composition of the membrane.

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