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Cell Biology

Phospholipid methylation stimulates lactogenic binding in mouse mammary gland membranes

(*S*-adenosyl-L-methionine/human growth hormone)

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ABSTRACT **Addition of the methyl donor *S*-adenosyl-L-methionine to membranes prepared from mammary glands of lactating mice results in increased binding of ^{125}I-labeled human growth hormone to the lactogenic receptors. This stimulation is dose dependent and specific for *S*-adenosyl-L-methionine and is partially inhibited by simultaneous addition of *S*-adenosyl-homocysteine to the reaction. Pretreatment of the membranes with *S*-adenosyl-L-methionine for 30 min at 37°C is sufficient to cause enhanced binding. Scatchard analysis shows that treatment with *S*-adenosyl-L-methionine results in an increase in the number of lactogenic binding sites without changing the apparent affinity constant for ^{125}I-labeled human growth hormone. The increase in the number of binding sites is believed to be due to alteration in the phospholipid composition of the membrane because methylation of phospholipids is observed under these conditions.**

Lactogenic binding sites have been modulated by altering the lipid composition and viscosity of artificially reconstituted vesicles into which the lactogenic receptors are incorporated (1). Membranes prepared from either rat erythrocytes or bovine adrenal medulla contain two enzymes that synthesize phosphatidylcholine from phosphatidylethanolamine by using *S*-adenosyl-L-methionine (AdoMet) as the methyl donor (2, 3). These two enzymes, their substrates, and products are localized within the membranes in such a way as to be implicated in maintaining the asymmetric environment of the phospholipids in the membranes (3). Binding of L-isoproterenol to β-adrenergic receptors in rat reticulocyte ghosts causes an increase in phospholipid methylation and translocation in these preparations. This, in turn, decreases membrane viscosity and enhances coupling of the β-adrenergic receptors to adenylate cyclases (4).

To investigate the possibility that these enzymes are involved in control of lactogenic receptors, we methylated lipids in membranes from mammary glands of lactating mice by using AdoMet as methyl donor. As a result, the binding of human growth hormone (somatotropin, hGH) to the membranes was enhanced. This is due to an increase in the number of lactogenic binding sites with no significant change in the apparent affinity constant for the hormone.

MATERIALS AND METHODS

Preparation of Membranes and Assay for Binding Sites. Female C3H/HeN mice, 10–12 days lactating after their first pregnancy, were used throughout this study. Animals were killed by cervical dislocation and mammary glands were immediately excised. The tissue was minced and then homogenized at 4°C in 8 vol of 25 mM Tris·HCl (pH 7.4) containing 0.3 M sucrose by using a Polytron PCU-Z-110 set at 7. The homogenate was then centrifuged at 15,000 × *g* for 15 min at 4°C. Membranes were collected by centrifugation of the resulting supernatant at 100,000 × *g* for 60 min at 4°C. The pellet was resuspended in a 0.1 M phosphate buffer (pH 7.4) at a concentration of 10–20 mg of membrane protein per ml.

The binding assays were carried out by a modification of the method of Shiu *et al.* (5). Membranes (250–350 μg of protein) were resuspended in a total volume of 0.5 ml containing binding buffer (25 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/0.1% bovine serum albumin), ^{125}I-labeled hGH (^{125}I-hGH) (60,000–80,000 cpm, 50–80 μCi/μg, 1 Ci = 3.7 × 10^{10} becquerels), and unlabeled ovine prolactin (oPRL). The reactions were incubated for 16–18 hr at room temperature and terminated by addition of 1.0 ml of cold binding buffer. The membranes were centrifuged at 1500 × *g* for 20 min at 4°C and the supernatant was aspirated. The pellet was washed once with cold binding buffer and radioactivity was determined in a Packard autogamma spectrometer. Nonspecific binding was determined by the addition of 1 μg of oPRL to the reaction tubes. Unless otherwise indicated, nonspecific binding was subtracted from total binding to yield specific binding.

Determination of Phospholipid Methylation. The methylation reaction was carried out under conditions similar to those of the binding reaction. The total reaction volume of 100 μl contained 25 mM Tris·HCl (pH 7.4), with 10 mM $MgCl_2$, 0.023 μmol of AdoMet, 2 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]-methionine (Ado[^{3}H]Met), and lactating mammary gland membranes (approximately 250 μg of protein). The tubes were incubated at 37°C for various lengths of time. The reactions were stopped by addition of 3.0 ml of chloroform/methanol, 3:1 (vol/vol). As described (2), the tubes were vigorously shaken with 2.0 ml of 0.1 M KCl in 50% methanol and centrifuged at 2000 × *g* for 10 min. The aqueous layer was discarded and the chloroform layer was washed once with 2.0 ml of methanolic KCl. The chloroform layer was evaporated to dryness and radioactivity was measured in a lipid scintillation spectrometer.

Materials. Human growth hormone (hGH HS243) and ovine prolactin (oPRL P-S-12) were a gift from the Hormone Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases. AdoMet, *S*-adenosylhomocysteine (AdoHcy), 5′-AMP, and L-methionine were purchased from Sigma. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (43 Ci/mmol) was obtained from Amersham. Other chemicals were of reagent grade. hGH was iodinated by a lactoperoxidase method as described (6).

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; Ado[^{3}H]Met, *S*-adenosyl-[*methyl*-^{3}H]methionine; AdoHcy, *S*-adenosylhomocysteine; hGH, human growth hormone (somatotropin); oPRL, ovine prolactin.

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RESULTS

Stimulation of Lactogenic Binding by AdoMet. Lactogenic binding sites, in membranes from lactating mammary glands, bind both ^{125}I-hGH and ^{125}I-oPRL (7, 8). Because both of these hormones have superimposable competitive binding curves, either can be used to assay lactogenic binding sites (7–10). Because AdoMet acts as a specific methyl donor for transmethylation reactions (2), the effect of methylation of membranes on lactogenic binding sites was studied by the addition of AdoMet to the binding reaction. Fig. 1 shows that increased binding of ^{125}I-hGH to lactogenic receptors occurred with increasing concentrations of AdoMet. At 0.46 μmol of AdoMet per ml, over 50% stimulation of total binding was obtained. The maximum extent of stimulation with different preparations of membranes varied from 40 to 300% (data not shown).

Because AdoMet was present throughout the incubation in the above experiment, it was conceivable that it participates in the binding reaction itself. To eliminate this possibility, we pretreated membranes with various concentrations of AdoMet for 30 min at 37°C, after which the membranes were again isolated and washed twice. Subsequently the membranes were assayed for binding of ^{125}I-hGH to the lactogenic binding sites. Table 1 shows that such pretreatment with AdoMet resulted in an increase in hormone binding. Increased binding was observed with AdoMet concentrations up to 0.46 μmol/ml (61%). Above this concentration no increase in binding was observed and frequently a slight decrease was detected. These results suggested that AdoMet stimulates binding of lactogenic hormones to mammary gland membranes by causing changes in the membrane, which persist even after the AdoMet has been removed. Since this was the case, in subsequent experiments, AdoMet was present throughout the binding reactions.

Specificity of Stimulation by AdoMet. If the observed stimulation of lactogenic binding is due to membrane methylation, the effect should be specific for AdoMet but not for other analogs. Moreover, AdoHcy, a specific competitive inhibitor of the transmethylation reaction (2, 11), should at least partially suppress the effects of AdoMet on binding. Table 2 shows the effect of different analogs of AdoMet on lactogenic binding. 5′-AMP, AdoHcy, and L-methionine did not significantly alter the total binding. However, when AdoHcy was added with AdoMet, the stimulatory effect of AdoMet was partially suppressed. These observations indicate that the effect is specific for AdoMet and that the enhanced binding of ^{125}I-hGH may be due to alterations in the membranes caused by transmethylation reactions using AdoMet as substrate.

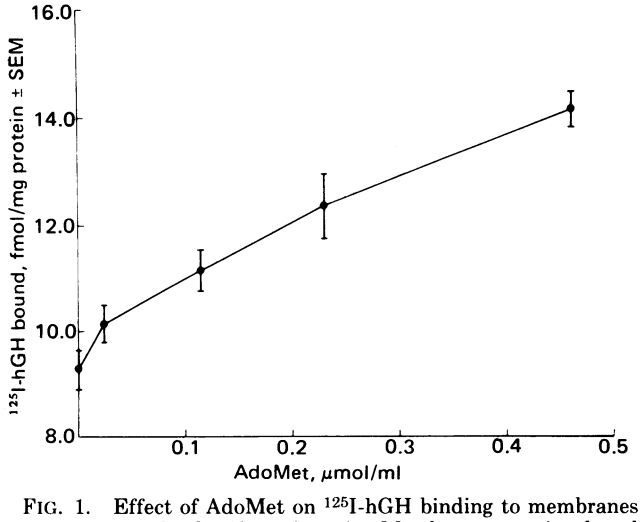

FIG. 1. Effect of AdoMet on ^{125}I-hGH binding to membranes from mammary glands of lactating mice. Membranes were incubated with ^{125}I-hGH in binding buffer (25 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/0.1% bovine serum albumin) containing various concentrations of AdoMet. After 16 hr, membranes were collected and samples were processed. Nonspecific binding was determined for each concentration of AdoMet by addition of 1 μg of unlabeled oPRL to the reaction. Specific binding was obtained by subtracting nonspecific from total binding.

Table 1. Effect of pretreatment with AdoMet on ^{125}I-hGH binding to membranes from lactating mouse mammary glands

Additions	^{125}I-hGH bound, fmol/mg protein ± SEM	% binding
None	23.0 ± 0.92	100
AdoMet		
0.23 μmol/ml	32.66 ± 0.90*	142
0.46 μmol/ml	37.03 ± 0.75*	161
0.69 μmol/ml	34.73 ± 1.35*	151

Membranes from lactating mouse mammary glands were incubated with various concentrations of AdoMet at 37°C in 2.0 ml of 25 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$. After 30 min, the membranes were centrifuged at 1500 × *g* for 20 min. The supernatant was discarded and the membranes were washed twice with 2.0 ml of incubation buffer. Subsequently, ^{125}I-hGH binding to the membranes was determined.

* $P < 0.001$ compared to reaction without AdoMet.

Scatchard Analysis of Binding Sites in Membranes Treated with AdoMet. An increase in hormone binding to receptors can be due to either an increase in the affinity for the hormone or an increase in the number of binding sites or both. These two effects can be distinguished by a Scatchard analysis of the binding reaction (12). Fig. 2 shows that the apparent affinity constants, as calculated from the slopes of the straight lines, did not significantly change in the presence of AdoMet (0.44×10^9 M^{-1} without and 0.33×10^9 M^{-1} with AdoMet). Neither was the nonspecific binding significantly changed. However, a significant change in the total number of specific binding sites was observed (20.5 fmol/mg of protein without and 62 fmol/mg of protein with AdoMet). This unmasking of binding sites may be a result of physical and chemical changes in the membranes.

Table 2. Effect of different analogs of AdoMet on ^{125}I-hGH binding to membranes from lactating mouse mammary glands

Additions	^{125}I-hGH bound, fmol/mg protein ± SEM	% binding
Exp. I		
None	9.5 ± 0.75	100
AdoMet	13.2 ± 0.47*	139
5′-AMP	8.26 ± 0.34	87
L-Methionine	10.83 ± 0.35	114
AdoHcy	9.88 ± 0.62	104
AdoMet + AdoHcy	11.87 ± 0.39†	125
Exp. II		
None	12.47 ± 0.53	100
AdoMet	22.6 ± 1.0	179
AdoMet + AdoHcy	18.3 ± 0.8‡	145

^{125}I-hGH binding assay was performed as described in *Materials and Methods*. The indicated chemicals were added at a final concentration of 0.3 μmol/ml and were present throughout the binding reaction. Where indicated, AdoMet and AdoHcy were added simultaneously at a final concentration of 0.3 μmol/ml each (Exp. I) and at 0.3 μmol of AdoMet and 3 μmol of AdoHcy per ml (Exp. II).

* $P < 0.01$ compared to no additions.

† $P < 0.1$ compared to no additions.

‡ $P < 0.05$ compared to AdoMet alone.

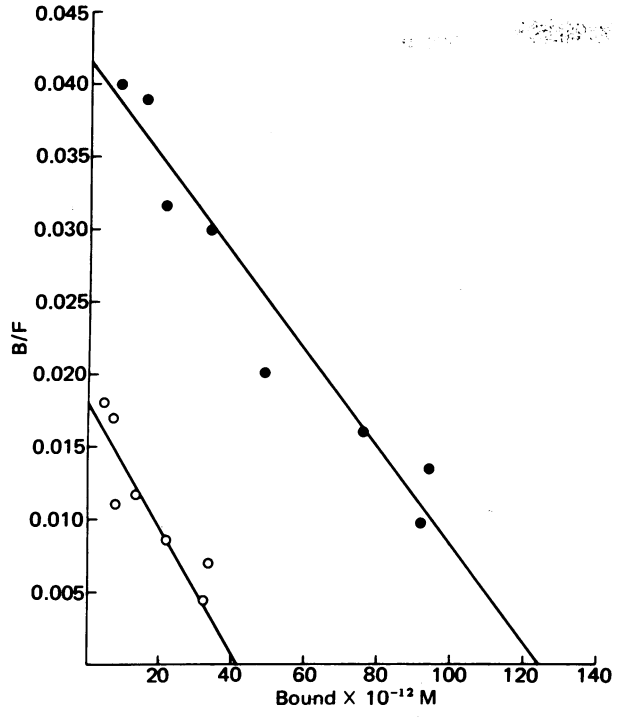

FIG. 2. Scatchard plot of ^{125}I-hGH binding. AdoMet (0.5 μmol/ml) was added to the binding reaction along with ^{125}I-hGH and various concentrations of unlabeled oPRL. The data are plotted according to the method of Scatchard (12). Ordinate depicts the ratio of bound/free hormone; the abscissa depicts the amount of hGH bound to the lactating mammary gland membranes. ○, No additions; ●, AdoMet.

Incorporation of [^{3}H]Methyl Groups into Membrane Phospholipids. The methyltransferases described in other systems specifically methylate phospholipids by using AdoMet as methyl donor (2, 3). Therefore, we examined the incorporation of [^{3}H]methyl groups from Ado[^{3}H]Met into phospholipids of mammary membranes under conditions that result in increased binding of ^{125}I-hGH (see Table I). Membranes were incubated in the presence of Ado[^{3}H]Met for various lengths of time. At the end of the reactions, the phospholipids were extracted and the amount of [^{3}H]methyl groups incorporated was determined. Table 3 shows that maximum incorporation was observed after 30 min at 37°C. With increasing time, the level of [^{3}H]methyl groups found in the phospholipid fraction decreased, presumably due to phospholipase action. At no time was significant radioactivity found in the protein fraction (data not shown).

Table 3. Methylation of membrane phospholipids with AdoMet

Time at 37°C, min	[^{3}H]Methyl groups incorporated, pmol/mg protein
15	1.68
30	9.35
45	4.90
60	3.50

Membranes were incubated at 37°C for the indicated times in 0.1 ml of buffer (25 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$) containing 0.23 μmol of AdoMet per ml and 2 μCi of Ado[^{3}H]Met. At the end of the reaction, the lipids were extracted and radioactivity was determined. Blank values, obtained by incubations at 0°C, were subtracted in each case.

DISCUSSION

Binding of lactogenic hormones to receptors in target tissues is regulated by many hormones (6, 9, 13–17). The mechanisms by which this occurs are not known although alterations in membrane lipid composition with resulting changes in fluidity have been implicated in some systems (1, 14, 18). This is based on the observation that the number of hepatic lactogenic binding sites is significantly diminished in mice fed a diet deficient in an essential fatty acid (18). In addition, the induction of hepatic lactogenic receptor activity in Snell dwarf mice by bovine growth hormone does not require protein synthesis (14). Triiodo-L-thyronine, a lipolytic hormone, enhances the binding of lactogenic hormones to receptors in mouse mammary tissue in organ culture (17). This increase does not require protein synthesis (17), suggesting that the hormone may act by altering the lipid environment of the membranes. Moreover, the extent to which lactogenic binding activity can be reconstituted into artificial lipid vesicles varies with different types of phospholipids (1).

The methyltransferases present in membranes prepared from several animal tissues catalyze the synthesis of phosphatidylcholine from phosphatidylethanolamine (2, 3). This, in turn, results in rapid translocation of the phosphatidylcholine to the external surface of the membranes (3, 4) and alteration in membrane microviscosity and charge (4, 11). Such changes influence the rate of movement and activity of membrane proteins. Binding of β-adrenergic agonists to rat reticulocyte ghosts enhances phospholipid methylation which, in turn, decreases membrane viscosity and enhances coupling between the hormone receptor and adenylate cyclase (4). Thus, membrane methylation may be a fundamental metabolic process in cellular regulation.

We have shown that the addition of the specific methyl donor AdoMet to the lactogenic binding reaction in membranes from mammary glands results in a significant increase in the number of binding sites. This effect is specific for AdoMet and is partially suppressed by AdoHcy, a competitive inhibitor of the membrane-bound methyltransferases. Similar effects were seen when AdoMet was present only during a preincubation period, suggesting that in this system also, enzymatic methylation of phospholipids has occurred. When membranes were examined after treatment with Ado[^{3}H]Met, label was exclusively found in the lipid fraction.

Note Added in Proof. Since this paper was submitted, Strittmatter *et al.* (19) have reported a similar unmasking of cryptic β-adrenergic receptors in rat reticulocyte membranes after phospholipid methylation in the presence of *S*-adenosyl-L-methionine. Thus, metabolic control of methylation reactions and subsequent alteration of membrane phospholipid composition may be an important mechanism for the regulation of sensitivity of target tissues to various hormones.

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