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METABOLIC INHIBITORS INCREASE PROLACTIN BINDING TO CULTURED MAMMARY TUMOR CELLS

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SUMMARY: We determined the effects of metabolic inhibitors on ^{125}I-labeled prolactin binding in monolayers of cultured rat mammary tumors. Chemical agents that blocked energy production increased binding by 8-20 fold, as did lowering the temperature from 37°C to 4°C. This difference was not due to blocking degradation of the hormone and inhibitors of degradation (lysosomotropic amines, bacitracin) did not increase binding. In the presence of a metabolic inhibitor at 37°C, binding reached a steady state within 3 h and had an apparent dissociation constant of $\sim 6 \times 10^{-10}$ M. Studies with fresh tumor slices produced comparable results. The findings indicate that the level of metabolic energy in mammary tumor cells can regulate prolactin binding.

Studies with certain polypeptide hormones (i.e., insulin, human chorionic gonadotropin and epidermal growth factor) and low density lipoprotein indicate that the binding to cell surface receptors is followed by a rapid uptake of the ligand (presumably with the receptor) and subsequent degradation (1,2). The recent immunocytochemical localization of prolactin within mammary cells (3) and the observation that injected prolactin rapidly binds with intracellular membranes in rat liver (4) suggest that prolactin may be internalized after binding to the cell surface. It is not clear whether uptake and degradation are required for response to the hormone; however, uptake of receptors may be related to the phenomenon of "down-regulation" in which target cells become unresponsive to further hormonal stimulation. Some of the evidence for hormone uptake is based on the effects of known metabolic inhibitors. We have recently developed a method for maintaining primary monolayer cultures of 7,12-dimethylbenzanthracene-induced rat mammary tumors. These cells retain prolactin receptors and are responsive to the hormone (5). Using these cells, we have begun to investigate whether prolactin uptake

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occurs after prolactin binds to mammary tumors. We have tested the effect of metabolic inhibitors on prolactin binding to monolayers of mammary tumor cells and we report some unusual consequences of metabolic inhibition on prolactin binding.

MATERIALS AND METHODS

Cell culture. Mammary tumor cell suspensions were prepared from 7,12-dimethylbenzanthracene-induced rat mammary tumors as previously described (5), except that the wash and enzyme-dissociation media contained 5% fetal bovine serum (instead of bovine serum albumin) and insulin, progesterone, estradiol, triiodothyronine and corticosterone (concentrations given below). Lot #49A068 of Worthington CLS III collagenase was used for these studies. Cell suspensions were placed into plastic flasks (25 sq cm, surface area; Costar) and grown for 4 to 7 days in Leibowitz L-15 medium that contained 1% charcoal-stripped fetal bovine serum, 10^{-9} M insulin, 10^{-7} M progesterone, 10^{-9} M corticosterone, 10^{-9} M triiodothyronine, 10^{-10} M estradiol, ovine prolactin (200 ng/ml), gentamycin (25 μg/ml) and 2 mM glutamine (complete medium). Cells were fed on day 1 and every other day thereafter. A monolayer of cells usually formed within 4 days. Two days prior to the start of an experiment, the cells were switched to complete medium without prolactin.

Prolactin binding assay. Ovine prolactin (NIH PS12) was iodinated in the presence of lactoperoxidase and then purified by DEAE cellulose chromatography and stored at -20°C, as described previously (6). The average specific activity was 110 μCi/μg. On the day of an experiment, the ^{125}I-labeled prolactin was chromatographed on Sephadex G-100 (25 x 1 cm) that had been equilibrated in assay medium (Earle's balanced salt solution with 20 mM HEPES buffer and 1.5% bovine serum albumin; pH 7.4). The fractions that contained monomer were pooled and then diluted in assay medium. For determinations of prolactin binding to cell monolayers, the growth medium was drained from the flasks and replaced by assay medium with or without the indicated agent(s). The cells were preincubated at 37°C for 1 h and then the medium was replaced by assay medium that contained ^{125}I-labeled prolactin with or without the agent and with or without unlabeled prolactin (2 μg/ml). Following the last incubation, the flasks were cooled to 4°C, drained and then washed with ice-cold assay medium that contained ovine prolactin (1 μg/ml) (wash medium). The cells (in about 1 ml of wash medium) were then scraped from the flasks with perforated cellophane and placed in butadiene styrene polymer tubes (12 x 75 mm). The flasks were rinsed with about 1 ml of wash medium and the pooled cell suspension was sedimented at 20,000 x g for 10 min. The supernatant was discarded and the amount of bound isotope and the DNA content (7) of each cell pellet was determined. Since cultured tumor cells are derived from tumors with inherently different levels of prolactin binding (5), the level of binding in different experiments will vary.

RESULTS

When cultured tumor cells were incubated with ^{125}I-labeled prolactin, binding was low at 37°C and much higher at 4°C (Table 1). Incubating the cells with ^{125}I-labeled prolactin for less or more than 1 h did not increase binding at 37°C. At 4°C a steady state was reached by 48 h (data not shown).

Table 1: Effect of Inhibitors on Prolactin Binding to Monolayers of Mammary Tumor Cells

Additions	Specific prolactin binding (cpm/μg DNA)	
	37°C, 1 h	4°C, 28 h
None	17	400
Chloroquine (0.5 mM)	45*	0*
Bacitracin (1 mM)	0*	0*
2,4 dinitrophenol (1 mM)	140	460
2,4 dinitrophenol (1 mM) - without glucose	210	520

Tumor cells (Tumor N_8) were incubated for 1 h at 37° or 4°C in assay medium with the indicated additions (2 ml). The flasks were then drained and ^{125}I-labeled prolactin (1,890,000 cpm; 14.5 ng) ± 4 μg unlabeled prolactin in assay medium (2 ml) was added along with the indicated compounds. After incubation specific prolactin binding and DNA were determined. Results are the mean values for duplicate flasks (mean range, 16%). *Nonspecific binding accounted for ∿ 85% of total binding. Under all other conditions, the nonspecific binding level was 53 cpm/μg DNA. The average DNA content was 65 μg DNA/flask.

The addition of bacitracin [which inhibits degradation of insulin (8) and vasoactive intestinal peptide (9)] or chloroquine, an inhibitor of lysosomal hydrolases, did not increase specific binding at 37°C, but did interfere with the binding assay by greatly increasing the level of nonspecific binding at both temperatures. By contrast, 2,4 dinitrophenol, a potent uncoupler of oxidative phosphorylation, increased binding at 37°C by about 8-fold. Binding was further increased by deleting glucose from the assay medium. Even at 4°C, 2,4 dinitrophenol (minus glucose) had a modest stimulatory effect. Although the metabolic inhibitors will eventually kill cells, trypan blue staining did not increase appreciably at 37°C during a 4 hr incubation.

The ability to stimulate prolactin binding was shared with other chemically unrelated metabolic inhibitors. Table 2 shows that the effect of 2,4 dinitrophenol on prolactin binding could be produced by another uncoupler of oxidative phosphorylation (sodium arsenate) and by inhibitors of electron transport. Sodium malonate (which inhibits succinate dehydrogenase) or

Table 2: Effect of Metabolic Inhibitors and Lysosomotropic Amines on Prolactin Binding to Monolayers of Mammary Tumor Cells

Additions	Specific prolactin binding (cpm/μg DNA)
None	19
NaN_3 (5 mM)	410
KCN (5 mM)	310
2,4 dinitrophenol (1 mM)	280
Sodium arsenate (without PO_4) (2 mM)	210
Sodium malonate (50 mM)	52
2-Deoxy-D-glucose (0.2%)	13
Glucose (1 mg/ml) + NH_4Cl (5 mM)	23
+ Chloroquine (50 μM)	30
+ Methylamine (10 mM)	57

Tumor cells (Tumor P_8) were incubated in assay medium (minus glucose) with the indicated additions at 37°C for 1 h. The flasks were drained and ^{125}I-labeled prolactin (901,000 cpm; 6.3 ng) ± 2 μg unlabeled prolactin (in 1 ml) was added along with the indicated compounds. After incubation for 2 h at 37°C, specific prolactin binding and DNA were determined. Results are the mean values for duplicate flasks (mean range, 15%). The mean level of nonspecific binding was 50 cpm/μg DNA, with an average of 125 μg DNA/flask.

2-deoxy-D-glucose (which blocks glycolysis) had little effect. Several amines that presumably act as lysosomotropic agents (10) but may also affect membrane mobility (11) only slightly stimulated binding. Fig. 1 shows the time course of prolactin binding in the presence of 2,4 dinitrophenol. A steady state in binding was reached by 3 h. Scatchard analysis (12) of prolactin binding (in cells treated with KCN) disclosed a single class of receptors with an apparent dissociation constant (Kd) comparable to that estimated for cultured tumor cell homogenates (5) and membranes prepared directly from tumors (14) (Fig. 2)

To determine whether the low level of binding at 37°C (without a metabolic inhibitor) was due to inactivation of prolactin, we measured the level of ^{125}I-labeled prolactin binding in receptor-containing liver membranes (15) after the prolactin had been incubated with tumor cells. There was no

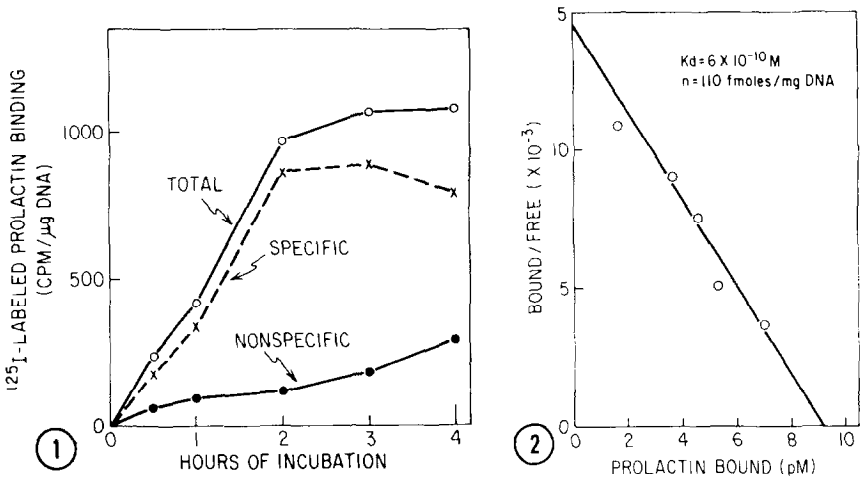

Figure 1. Time course of ^{125}I-labeled prolactin binding to cultured tumor cells (Tumor Q8). Cells were preincubated at 37°C with 2,4 dinitrophenol for 1 h. At time 0, we then added medium (+2,4 dinitrophenol) that contained ^{125}I-labeled prolactin (798,000 cpm; 3.8 ng) ± unlabeled prolactin (2 μg/ml). Results are the mean values for duplicate flasks. (Average range, 23%; average DNA content, 34 μg/flask) Without 2,4 dinitrophenol specific binding at 4 h was 25 cpm/μg DNA.

Figure 2. Scatchard analysis of prolactin binding to cultured tumor cells (Tumor Y8) treated with KCN. Cells were incubated at 37°C for 4 h with 5 mM KCN, ^{125}I-labeled prolactin (880,000 cpm; 4.5 ng), and 0, 5, 10, 20, 40 or 2000 ng (to assess nonspecific binding) unlabeled prolactin in 1 ml medium (without glucose). Data were corrected for nonspecific binding (13) and plotted according to the method of Scatchard. Results are the mean values for triplicate flasks. The line was fitted by least squares regression analysis (r=0.977). The average DNA content was 82 μg/flask.

difference in prolactin binding after incubation in tumor cell medium with or without 2,4 dinitrophenol (Table 3). Other experiments (not shown) indicated that 2,4 dinitrophenol did not increase prolactin binding to liver membranes directly. The stimulation of prolactin binding by 2,4 dinitrophenol was not limited to cultured tumor cells, since it also occurred in slices prepared directly from mammary tumor (Fig. 3).

DISCUSSION

The metabolic inhibitors used in this study have been examined for their effects on the binding and degradation of epidermal growth factor and human chorionic gonadotropin (10,16). In these studies, pretreatment with metabolic inhibitors did not affect initial binding although the agents prevented

Table 3: Binding of ^{125}I-labeled Prolactin After Incubation With Tumor Cells

Additions to Medium	Bound ^{125}I-labeled Prolactin (% of total)
1 mM 2,4 dinitrophenol	59 ± 1
1 mM 2,4 dinitrophenol + unlabeled prolactin (2 μg/ml)	11 ± 1
None	61 ± 2
Unlabeled prolactin (2 μg/ml)	10 ± 1

Cultured tumor cells were incubated in assay medium with ^{125}I-labeled prolactin and the indicated additions for 5 h at 37°C. The medium [0.1 ml containing 100,000 cpm (0.5 ng)] was then added to a 0.1 ml suspension of liver membranes prepared from female rats. After 16 h incubation at 22° C, the membranes were sedimented and the bound radioactivity determined. Each value is the mean ± S.D. of triplicate samples.

subsequent degradation of the ligands. Since prolactin degradation does not occur in the absence of metabolic inhibitors (Table 3), it cannot account for the low level of binding at 37°C. Further, preliminary findings indicate that the initial binding of ^{125}I-labeled epidermal growth factor to mammary tumor cells is not radically altered by 2,4 dinitrophenol. This suggests that activation of binding is specific for prolactin.

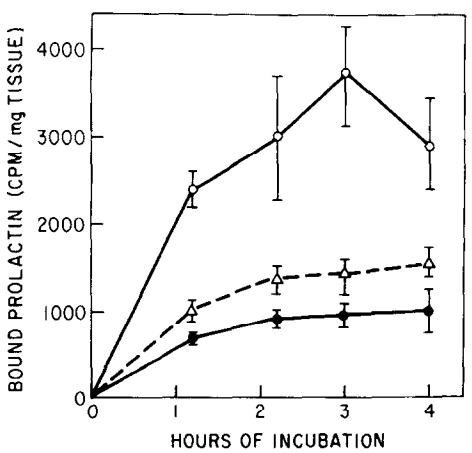

Figure 3. Effect of 2,4 dinitrophenol on prolactin binding to mammary tumor tissue slices. Tumor tissue was sliced to 0.5 mm thickness with a Stadie Riggs tissue slicer and then cut into pieces (∿ 20 mg wet weight). The pieces were incubated at 37°C in 1 ml of ^{125}I-labeled prolactin (800,000 cpm; 5.6 ng) ± unlabeled prolactin (2 μg/ml) ± 1 mM 2,4 dinitrophenol. At the indicated intervals pieces were washed four times with a total of 20 ml of wash medium. Each point is the mean (± S.D.) for 4 pieces of tissue. Nonspecific binding (x--x) was unaltered by 2,4 dinitrophenol. Specific binding with (●) and without (o) 2,4 dinitrophenol.

Although the mechanism by which these metabolic inhibitors increase prolactin binding is not known it is likely that a substantial decrease in the cellular level of ATP is involved since both uncouplers and respiratory inhibitors stimulate binding. One possibility is that energy depletion may allow the hormone to penetrate cells and bind to intracellular sites (17). Alternatively, receptors may be continuously cycled between the internal and external membrane compartments or be released from the cell surface (18) and energy depletion may lead to receptor accumulation at the cell surface. At present, these possibilities appear less likely because increased hormone binding also occurs at 4°C when membrane mobility and hormone penetration should be severely restricted. More likely are the possibilities that (a) receptors [or some regulatory component(s)] exist in both an inactive and an active form, and energy depletion shifts the equilibrium toward the active state or (b) energy depletion interferes with hormone-induced receptor "down regulation."

Studies on the mechanism of this increased binding should provide further insight into prolactin action on this cells.

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