Endocr (2007) 31:27–32
DOI 10.1007/s12020-007-0003-1

ORIGINAL PAPER

Effect of prolactin on inositol uptake in mouse mammary gland explants

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Published online: 1 June 2007

Abstract Studies were carried out to assess the role of insulin (I), cortisol (H), and prolactin (P or PRL) in regulating myoinositol (inositol) uptake in the mammary gland. Using cultured mammary gland explants from pregnant mice (12–14 days into gestation), insulin and prolactin were found to stimulate inositol uptake, while cortisol impaired inositol uptake. Optimal inositol uptake was observed when tissues were treated with all three lactogenic hormones (I, H, and PRL). Further studies were designed primarily to characterize the PRL stimulation of inositol transport. Inositol uptake in the mammary explants increased linearly for 4 h, both in IH treated tissues and those treated with IHP; distribution ratios of greater than 14 were achieved at 4 h, suggesting an active inositol transport mechanism. The PRL effect on inositol uptake is sodium-dependent, temperature-dependent, and ouabain sensitive. DIDS and furosemide did not impair inositol uptake or the PRL effect on inositol uptake. PRL stimulated inositol uptake employing PRL concentrations of 10–1000 ng/ml. The PRL effect was manifested at all PRL-treatment times of 12 h or longer, but not at earlier times. PRL thus appears to be an important and essential hormone for the stimulation of inositol accumulation in milk during lactogenesis.

Keywords Insulin · Prolactin · Cortisol · Inositol transport · Mammary gland · DIDS · Ouabain · Cytochalasin B · Furosemide

This research was supported by the Molecular Endocrinology Research Fund of the Wayne State University School of Medicine.

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Introduction

Myoinositol (inositol) is a major constituent (2 mM) of human milk, and is one important nutrient for the development of the newborn [1]. Inositol is a constituent of one of the phospholipids, phosphatidylinositol, which is essential as one of the structural elements in all cellular membranes. In addition, phosphorylated inositols are components of several important cellular signaling pathways [2].

Milk of humans, as well as other species, contains inositol at concentrations in the millimolar range, whereas the plasma concentration ranges between 10 and 50 micromolar. Inositol is thus concentrated in milk at more than an order of magnitude higher than that of the maternal plasma, which suggests that the mammary gland must have an active transport mechanism for concentrating inositol in milk. Studies were designed to investigate the characteristics of inositol uptake into mouse mammary gland explants, and to determine the role(s) of lactogenic hormones in regulating inositol uptake. Hormones studies included insulin (I), cortisol (H), and prolactin (PRL or P). These hormones have been reported in earlier studies [3–11] to be of critical importance in regulating a variety of lactogenic processes.

Materials and methods

Pregnant (12–14 days of pregnancy) Swiss-Webster mice were used in all experiments; they were purchased from Harlan Laboratories (Indianapolis, IN). Ovine prolactin (P or PRL; National Institutes of Health PO-21) was a gift from the National Institutes of Health. Other substances were purchased from the following sources: cortisol from

Charles Pfizer (New York, NY); Hanks' balanced salt solution (HBSS), medium 199-Earle's salts (EMEM), penicillin, streptomycin, furosemide, ouabain, DIDS (4,4′ diisothiocyanatolstilbene-2,2′-disulfonic acid), and cytochalasin B from Sigma-Aldrich Co. (St. Louis, MO); porcine insulin from Eli Lilly (Indianapolis, IN); [^{3}H]-inositol (18.5 Ci/mMol) from New England Nuclear Corp. (Boston, MA).

Explants of mouse mammary tissues were prepared and cultured as described earlier [4]; typically explants were pooled from 14 animals and randomly distributed for each experiment. The explants were cultured on siliconized lens paper floating on 6 ml EMEM; all incubations were carried out in 60 × 15-mm Petri dishes maintained at 37°C in an atmosphere of 95% O_2–5% CO_2. In experiments in which the effects of hormones on inositol transport were to be determined, the tissues were transferred to vessels containing [^{3}H]-inositol (0.1 μCi/ml) in 6 ml EMEM (EMEM contains inositol at 11 micromolar); incubations were carried out in a rotary water bath at 37°C (120 cycles/min). The tissues were then weighed and radioactivity was determined by scintillation spectrometry. The intracellular accumulation of radiolabeled inositol was calculated by subtracting the amount of radiolabel in the extracellular space from the total radioactivity in the tissue homogenates [10]. For these calculations the total water content (51.5%) and extracellular space (24.6%) were determined by the volume of distribution of [^{3}H]OH and [^{14}C]-inulin (1 mM), respectively. In time course studies, equilibrium was achieved with [^{3}H]OH and [^{14}C]-inulin (1 mM) by 15 min after their addition. Results of the inositol uptake studies are expressed as a distribution ratio, which represents the ratio of the intracellular specific activity divided by the extracellular specific activity of the radiolabeled inositol. All studies involving the preparation of mouse mammary gland explants were performed in compliance with the regulations of the Animal Care and Use Committee of Wayne State University. Statistical comparisons were made using ANOVA followed by Dunnett's test. All data are expressed as the mean ± standard error and means were deemed significantly different with *p*-values of less than 0.05 unless specified otherwise. All experiments in this study were repeated 2–6 times.

Results

Figure 1 shows the effect of three lactogenic hormones on inositol uptake into cultured mouse mammary tissues. The tissues were treated for 24 h with all possible combinations of insulin (1 μg/ml), cortisol (10^{-7}M) and prolactin (1 μg/ml); [^{3}H]-inositol uptake was assessed during the final hour of culture. Insulin and prolactin, both by themselves and in the presence of the other hormones, stimulated [^{3}H]-inositol uptake; cortisol, by itself, reduced the extent of [^{3}H]-inositol accumulation. All three lactogenic hormones together effected the greatest stimulation of [^{3}H]-inositol uptake. It is well established that effects of prolactin on lactogenic processes require the presence of insulin and cortisol.

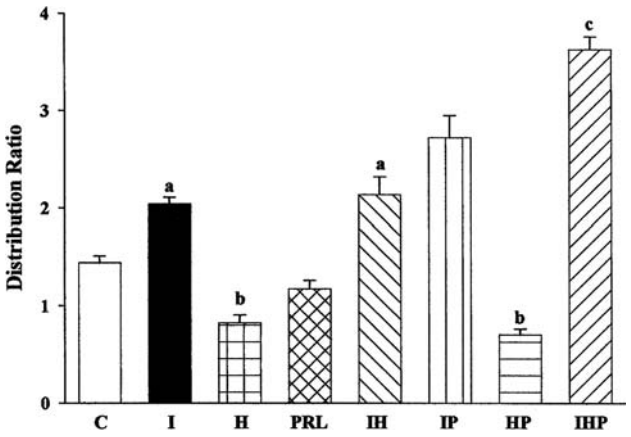

Fig. 1 Effects of hormones on inositol uptake. Mammary explants were cultured for 24 h in the absence of hormones; incubation was then continued for an additional 24 h with combinations of insulin (I, 1 μg/ml), cortisol (H, 10^{-7}M) and prolactin (PRL or P, 1 μg/ml). Tissues were then pulse-labeled for 1 h with 0.1 μCi/ml ^{3}H-inositol, after which the intracellular accumulation of [^{3}H]-inositol was calculated. (**a**) significantly greater than control; (**b**) significantly less than control; (**c**) significantly greater than I H. Numbers represent the mean ± SE of 4–8 observations

The time-course of [^{3}H]-inositol uptake in control (IH) and PRL-treated (IHP) tissues is shown in Fig. 2. In both control and PRL-treated tissues, uptake was linear for 4 h. At 4 h in the PRL-treated tissues, a distribution ratio of

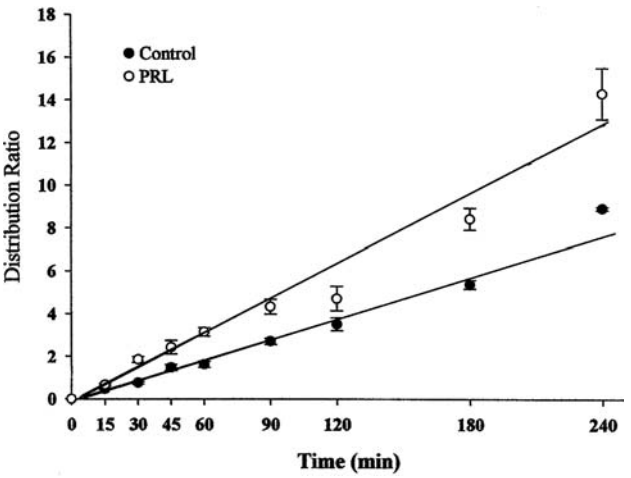

Fig. 2 Time-course of [^{3}H]-inositol uptake in control and PRL-treated tissues. Mammary explants incubated for 1 day with IH, and then an additional 1 day with IH or IHP. Tissues were then incubated for the times indicated with 0.1 μCi/ml [^{3}H]-inositol. Intracellular accumulation of [^{3}H]-inositol was then calculated. Numbers represent the mean ± SE of 4 observations

greater than 14 was observed. A mechanism in the mammary tissue for concentrating the inositol within the mammary cells is thus apparent.

The time-course for the PRL stimulation of [^{3}H]-inositol uptake in the cultured mammary explants is shown in Fig. 3. More than 8 h of PRL-treatment is required before the PRL effect becomes manifest; the PRL effect persists for at least 32 h. Figure 4 shows the effect of PRL concentration of [^{3}H]-inositol uptake after a 24 h PRL-exposure period. All PRL concentrations above 10 ng/ml elicited a significant response; this was essentially an ''all or none'' response as the magnitude of the PRL effect was as great with 10 ng/ml PRL as with 1000 ng/ml. PRL concentrations below 10 ng/ml were without effect.

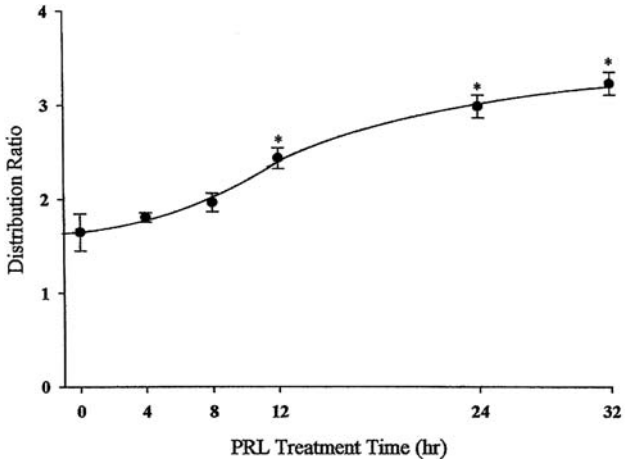

Fig. 3 Time-course for PRL stimulation of [^{3}H]-inositol uptake. Mammary explants were incubated for 24 h with IH, and then for the times indicated with IH or IH plus 1 μg/ml PRL. The tissues were pulse-labeled with 0.1 μCi/ml [^{3}H]-inositol for the final 1 h of culture. Intracellular accumulation of [^{3}H]-inositol was then calculated. Numbers represent the mean ± SE of 4 observations. * denotes greater than control with $p < .05$

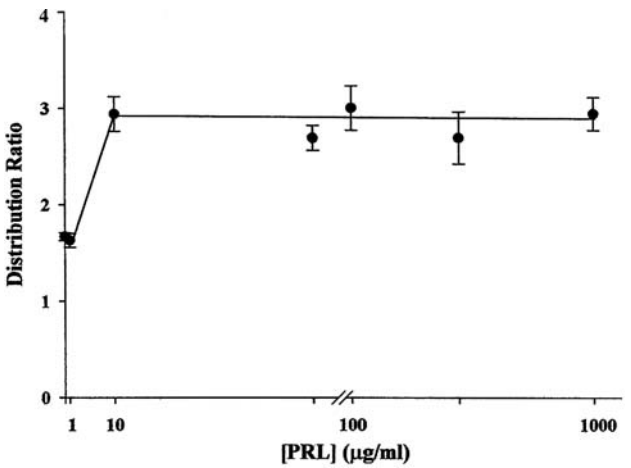

Fig. 4 Effect of PRL concentration on [^{3}H]-inositol uptake. Mammary explants were cultured for 1 day with IH, and then one additional day with PRL at the concentrations indicated. Tissues were then cultured for 1 h with [^{3}H]-inositol, and the intracellular accumulation of [^{3}H]-inositol calculated. Numbers represent the mean ± SE of 4 observations. Significant ($p < .05$) changes were noted with all PRL concentrations of 10 ng/ml and above

Figure 5 shows the effect of inositol concentration on [^{3}H]-inositol uptake in control (IH) and 24 h PRL-treated (IHP) tissues. The Lineweaver-Burke plot of the data indicates that inositol is taken up with an apparent K_m value of 0.133 mM; no difference was noted in response to PRL. The V_{max}, however, was almost doubled in the PRL-treated tissues, 0.38 vs. 0.63 mmol/l intracellular water/hour.

The results of an experiment designed to determine the sodium-dependence of inositol uptake are shown in Fig. 6. In control (IH) and 24 h, IHP-treated tissues, [^{3}H]-inositol uptake was determined for the final 2 h of culture in the following media: EMEM, HBSS, KRB, and KRB-choline (sodium is replaced with choline). Clearly, the absence of sodium impaired inositol uptake in both the control and PRL-treated tissues. Irrelevant to the sodium-dependent part of this experiment was the observation that the magnitude of the PRL stimulation of inositol uptake was greatest when [^{3}H]-inositol uptake was measured with EMEM; this is perhaps due to the high nutritional constituents in EMEM vs. HBSS or KRB.

Figure 7 shows a time-course of [^{3}H]-inositol uptake employing various uptake-temperatures. Control (IH) and 24 h, PRL-treated tissues were incubated for 1–3 h with [^{3}H]-inositol at 4, 23, 30, or 37°C. It is clearly apparent that

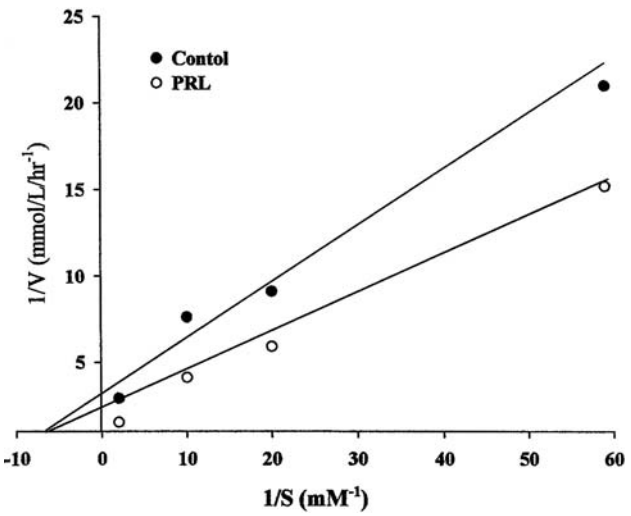

Fig. 5 Inositol concentration and PRL stimulation of inositol uptake. Mammary explants were cultured for 1 day with IH, and then 1 day with IH or IHP. Tissues were then incubated one additional hour with [^{3}H]-inositol at the concentrations indicated. The reciprocal of the substrate concentration was then plotted against the reciprocal of the intracellular accumulation of [^{3}H]-inositol. Numbers represent the mean ± SE of 4 observations

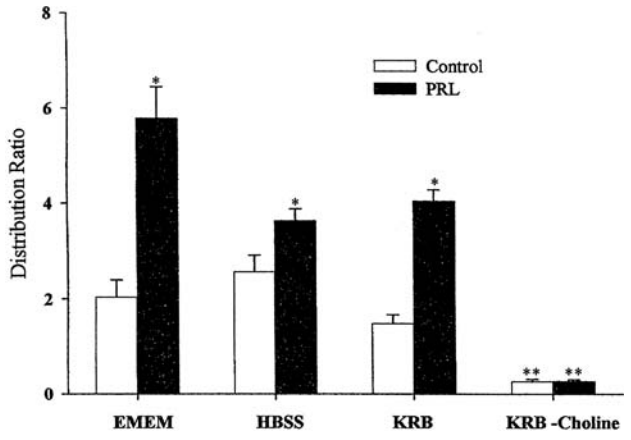

Fig. 6 Effect of sodium on inositol uptake. Mammary explants were cultured for 1 day with IH, and then 1 day with IH or IHP. Tissues were then incubated for two additional hour with 0.1 μCi/ml [^{3}H]-inositol contained in one of four media: EMEM, HBSS, KRB, or KRB with choline substituted for sodium. The intracellular uptake of [^{3}H]-inositol was then calculated. Numbers represent the mean ± SE of 4 observations. * denotes greater than control with $p < .05$. ** denotes less than EMEM control with $p < .05$

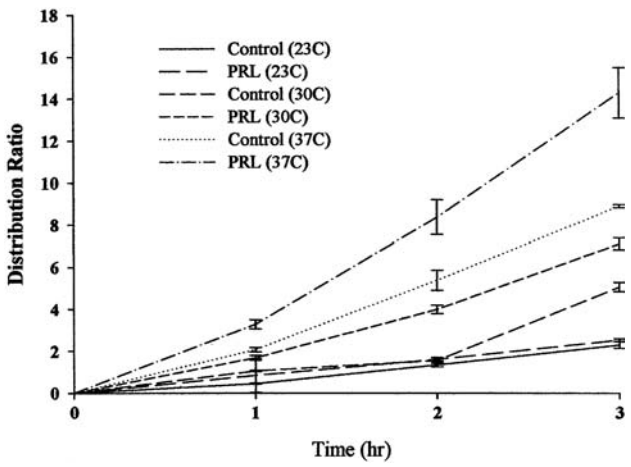

Fig. 7 Effect temperature on [^{3}H]-inositol uptake. Mammary explants were cultured for 1 day with I H, and then 1 day with IH or IHP. Tissues were then cultured for the times indicated with 0.1 μCi/ml [^{3}H]-inositol at 23, 30, or 37°C. Intracellular accumulation of [^{3}H]-inositol was then calculated. Numbers represent the mean ± SE of 4 observations

[^{3}H]-inositol uptake is temperature dependent. The PRL effect was apparent at all times with uptake temperatures of 30°C and 37°C, but not at 23°C. In addition, minimal [^{3}H]-inositol accumulated in the cells at 4°C (data not shown).

In order to investigate the specificity of the inositol transport mechanism, the effect of other sugars at 10 and 25 mM on [^{3}H]-inositol uptake in control (IH) and 24 h, IHP-treated tissues is shown in Fig. 8. Uptake in this experiment was determined with a 1-h incubation.

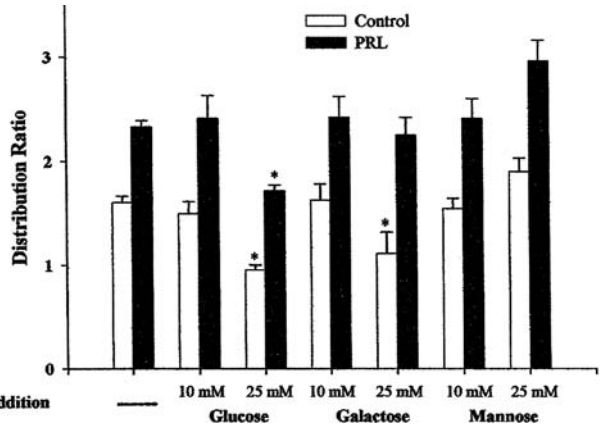

Fig. 8 Effect of Other Monosaccharides on [^{3}H]-inositol Uptake. Mammary explants were cultured for 1 day with IH, and then 1 day with IH or IHP. Tissues were then cultured for 1 h with media containing 0.1 μCi/ml [^{3}H]-inositol and 0, 10, or 25 mM glucose, galactose, or mannose; intracellular accumulation of [^{3}H]-inositol was then calculated. Numbers represent the mean ± SE of 4 observations. * denotes significantly less than appropriate control with $p < .05$

Mannose was without effect, whereas 25 mM glucose and galactose inhibited inositol uptake in the control tissues; 25 mM glucose also inhibited [^{3}H]-inositol uptake in the PRL-treated tissues.

The effects of an anion exchange inhibitor, DIDS, and a NaK2Cl cotransporter inhibitor, furosemide, on [^{3}H]-inositol uptake was determined in control (IH) and 24 h, IHP-treated tissues during a 2-h uptake period (Fig. 9). Neither of these inhibitors impaired the extent of [^{3}H]-inositol uptake in the control tissues. But, surprisingly, both

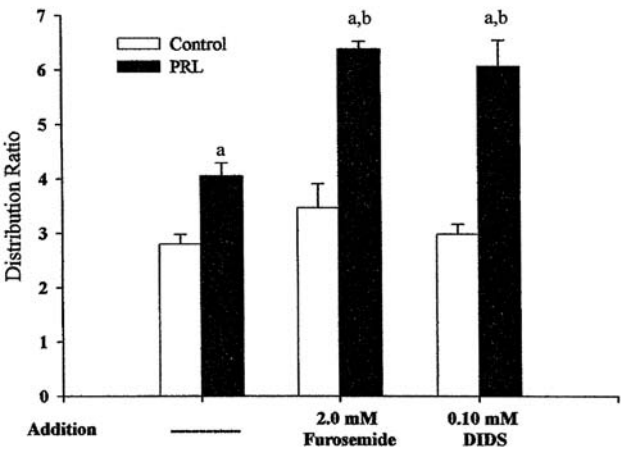

Fig. 9 Effect of Anion Exchange Inhibitors on [^{3}H]-inositol Uptake. Mammary explants were incubated 1 day with IH, and then 1 day with IH or IHP. Tissues were then incubated 2 h with media containing 0.1 μCi/ml [^{3}H]-inositol and 2 mM furosemide or 0.1 mM DIDS. Intracellular accumulation of [^{3}H]-inositol was then calculated. Numbers represent the mean ± SE of 4 observations. (**a**) greater than control with $p < .05$; (**b**) greater than PRL-treated control tissues with $p < .05$

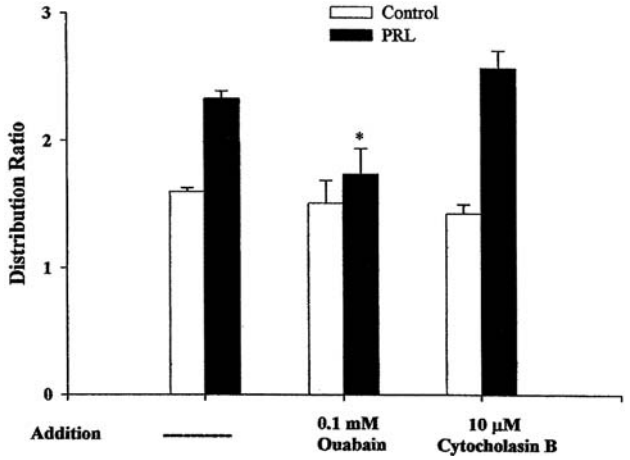

Fig. 10 Effect of Ouabain and Cytochalasin B on [^{3}H]-inositol Uptake. Mammary explants were incubated 1 day with IH, and then 1 day with IH or IHP. Tissues were then incubated 1 additional hour with media containing 0.1 μCi/ml [^{3}H]-inositol and 0.1 mM ouabain or 10 μM cytochalasin B. Intracellular accumulation of [^{3}H]-inositol was then calculated. Numbers represent the mean ± SE of 4 observations. * denotes less than PRL-treated controls with $p < .05$

inhibitors enhanced [^{3}H]-inositol uptake in the PRL-treated tissues.

In the final experiment (Fig. 10), the effect of ouabain (inhibitor of NaK-ATPase), and cytochalasin B (inhibitor of transporter insertion in plasma membrane via an actin-dependent mechanism) on [^{3}H]-inositol uptake was assessed in control (IH) and 24 h, IHP-treated tissues; uptake was assessed employing a 1-h culture period with [^{3}H]-inositol. Neither inhibitor affected the extent of [^{3}H]-inositol uptake in the control tissues; but, ouabain inhibited the PRL response, while cytochalasin B (at 10 μM) was without effect.

Discussion

Inositol is an essential nutrient in milk that is present in concentrations that are more than an order of magnitude higher than that of the maternal plasma [1, 2]. Milk is composed of a variety of substances, some of which are synthesized in the mammary alveolar epithelial cells, whereas others are taken up directly from the maternal plasma. Examples of substances taken up directly from the plasma include sodium, potassium, chloride, phosphate, iodide, calcium, citrate, choline, carnitine, glucose , amino acids, pyrimidine nucleosides, and inositol [3–5, 10–16]. A number of inositol transporters have been identified, including those that are sodium-inositol symporters [17]. A more recent article [18] identifies two inositol transporters in endothelial cells: SMIT-1—a sodium-dependent inositol cotransporter, and SMIT-2—a sodium-independent inositol transporter. The data from our studies suggests that inositol uptake in the mouse mammary gland occurs primarily via an active, sodium-dependent mechanism, likely via a sodium-inositol symporter. Inositol uptake apparently requires sodium-potassium ATPase, as ouabain impairs inositol uptake. The temperature-dependence of inositol uptake further suggests the energy dependence of this process. In addition, the achievement of distribution ratios of greater than 14 in tissues cultured for 4 h with [^{3}H]-inositol provides convincing evidence that inositol is taken up via an active mechanism. In data not presented, we found that less than one percent of the [^{3}H]-inositol taken up into the tissues was incorporated into phosphatidylinositol; incorporation into other inositol derivatives was not determined. The possible conversion of inositol into other metabolic products may be a contributing factor for interpreting the results of our experiments. The apparent K_m of 0.133 mM in mammary tissues compares favorably with that reported for other tissues including rat intestinal brush-border membrane vesicles [19].

The anion exchange inhibitor DIDS had no effect on inositol transport in control tissues. This contrasts with the inhibitory effect of DIDS on iodide uptake in cultured mammary cells [20]. An anion exchange mechanism therefore does not appear to be involved in the inositol uptake mechanism in the mammary gland in the absence of PRL. The potentiation of the magnitude of the PRL effect on inositol uptake by DIDS and furosemide was repeated in two experiments. We currently offer no speculation as to why this occurs.

At 25 mM, but not 10 mM, both glucose and galactose impaired the rate of inositol uptake; mannose at 25 mM was without effect. Since inositol uptake was determined at an inositol concentration of 10 μM, inhibition of inositol uptake by glucose or galactose required concentrations of more than three orders of magnitude higher than the inositol concentration. The uptake of inositol via its transporters thus appears to be quite specific.

The PRL stimulation of inositol uptake was elicited with physiological concentrations of prolactin (10–1000 ng/ml). PRL stimulated the apparent V_{max} of inositol uptake, which is consistent with the postulated insertion of additional inositol transporters into the plasma membranes of mammary alveolar epithelial cells. Consistent with this idea is the extended (8–10 h) PRL-treatment time required before the PRL effect was detected. The mechanism for the PRL effect on inositol transport likely involves a stimulation of mRNA production from the inositol transporter gene(s), followed by translation of the message and insertion of the transporters into the plasma membrane. This mechanism and the time-course of the PRL effect are similar to the PRL effect on several other lactogenic processes including lactose synthesis, lipid synthesis, iodide uptake, phosphate

uptake and incorporation into phospholipids and proteins, amino acid uptake, glucose uptake, nucleoside uptake, and casein synthesis [8–10, 13–16]. The stimulatory effects of insulin, by itself, and the inhibitory effects of 10^{-7}M cortisol, by itself, on lactational processes also have been observed in earlier studies [8–10, 13–16]. In addition, the cooperative effects of all three lactogenic hormones (I, H, and PRL) have been well-documented [3, 5–11, 13–16].

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