Effect of prolactin on phosphate transport and incorporation in mouse mammary gland explants

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Rillema, James A. Effect of prolactin on phosphate transport and incorporation in mouse mammary gland explants. Am J Physiol Endocrinol Metab 283: E132–E137, 2002.—Inorganic phosphate is present in milk at a concentration that is severalfold higher than in maternal plasma. In cultured mammary tissues from 12- to 14-day-pregnant mice, the intracellular concentration of $^{32}$PO$_4$ was six times higher than in the culture medium after a 4-h treatment with $^{32}$PO$_4$. Of the principal lactogenic hormones [insulin (I), cortisol (H), and prolactin (PRL)], only I and PRL (in the presence of H and I) stimulated $^{32}$PO$_4$ uptake into cultured mammary tissues; H, by itself or in the presence of I or PRL, inhibited $^{32}$PO$_4$ uptake. All three lactogenic hormones together effected the greatest stimulation of $^{32}$PO$_4$ uptake. Similar hormone effects were observed with regard to $^{32}$PO$_4$ incorporation into lipids and trichloroacetic acid-insoluble molecules. In a time course study, the onset of the PRL stimulation of $^{32}$PO$_4$ uptake and incorporation occurred 8–12 h after PRL addition; in dose-response studies, the PRL effect was manifested with PRL concentrations of 50 ng/ml and above. From kinetic studies, the apparent maximal velocity of PO$_4$ uptake was determined to be $\sim 7.7$ mM·h$^{-1}$·1·cell water$^{-1}$; the apparent Michaelis-Menten constant was $\sim 3–5$ mM. The PRL effect on $^{32}$PO$_4$ uptake was abolished when sodium was absent from the uptake medium. These studies thus demonstrate a complex interaction of three hormones (I, H, and PRL) in the regulation of $^{32}$PO$_4$ uptake and incorporation into macromolecules in cultured mouse mammary tissues. Insulin; cortisol

PHOSPHATE IS AN ESSENTIAL COMPONENT of milk for the nourishment of the neonate. In milk, it is present in several forms, including inorganic phosphate, colloidal phosphate, and casein phosphate and in phospholipids (5, 12). Because of the high concentration of phosphate in milk that meets the nutritional demands of the suckling neonate, copious amounts of phosphate are transferred from the maternal plasma into milk. One author (2) reported that all of the plasma inorganic phosphate is replaced every 10 min in lactating rats. Even when only inorganic phosphate is considered, its concentration in milk is 5- to 10-fold higher than in the maternal plasma. A highly efficient transport mechanism is therefore obviously present in the alveolar epithelial cells of the mammary gland. Shillingford et al. (13) recently characterized one phosphate transport mechanism in lactating rat mammary glands. Phosphate uptake was predominantly via a sodium-dependent transport mechanism, presumably located on the basolateral membrane of the alveolar epithelial cells; a sodium-phosphate symporter was postulated. Phosphate uptake occurred via a saturable mechanism with an apparent Michaelis-Menten constant (Km) of 1.13 mM.

The experiments reported in this study were carried out to characterize the effects of three lactogenic hormones [insulin, cortisol, and prolactin (PRL)] on phosphate uptake and incorporation in mouse mammary gland explants. The specific focus is on the prolactin regulation of phosphate uptake.

MATERIALS AND METHODS

Midpregnant (10–14 days of pregnancy) Swiss-Webster mice were used in all experiments. They were purchased from Harlan Laboratories (Indianapolis, IN). Ovine PRL (National Institutes of Health PS-19) was a gift from the National Institutes of Health. Other substances were purchased from the following sources: cortisol from Charles Pfizer (New York, NY); choline chloride, Hanks’ balanced salt solution (HBSS), and medium 199 (M-199)-Earle's salts from Sigma Chemical (St. Louis, MO); $^{32}$PO$_4$, $^3$HOOH, and [carboxy-14C]insulin (405.8 mCi/g) from New England Nuclear (Boston, MA); porcine insulin, penicillin, and streptomycin from Eli Lilly (Indianapolis, IN).

Explants of mouse mammary tissues were prepared and cultured as described earlier (8). The explants were cultured on siliconized lens paper floating on 6 ml of M-199-Earle's salts containing 1 µg/ml insulin plus 10$^{-7}$ M cortisol and/or PRL (0–1 µg/ml); 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 where the effects of PRL on phosphate uptake and incorporation were to be determined, the tissues were initially cultured for 24–36 h with insulin plus cortisol, after which PRL was added and incubations continued for the times specified for each experiment. Unless specified otherwise, for the final 2 h of culture, the tissues were transferred to vessels containing $^{32}$PO$_4$ (0.5 µCi/ml) in 4 ml of M-199; incubations were carried out in a rotary water bath at 37°C (120 cycles/min). The tissues were then weighed and homogenized in 4 ml of 5% trichloroacetic acid (TCA).

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One-milliliter samples were employed to assess the extent of $^{32}$P incorporation into the lipid fraction (3). The remaining 3 ml were centrifuged at 2,000 $g$ for 10 min. Radioactivity in 1-ml aliquots of the TCA-soluble fraction was determined by liquid scintillation techniques. After the pellet was washed with an additional 5 ml of 5% TCA, radioactivity in the TCA-insoluble fraction was determined after solubilization in 2 ml 1 N NaOH. The intracellular accumulation of radio-labeled, unincorporated PO$_4$ was calculated by subtracting the amount of radiolabel in the extracellular space from the total TCA-soluble radioactivity in the tissue homogenates (8, 11). For these calculations, the total water content (51.0%) and extracellular space (24.6%) were determined by the volume of distribution of $^{3}$HOOH and [14C]inulin (1 mM), respectively. In time course studies, equilibration was achieved with $^{3}$HOOH and [14C]insulin by 15 min after their addition. PRL had no effect on the volumes of distribution of these substances under the conditions employed by these experiments. Results of the phosphate 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 phosphate. The results of the incorporation studies are expressed as disintegrations per minute per milligram wet weight of tissues.

Statistical comparisons were made with Student’s t-test when two means were compared or by an analysis of variance followed by Dunnett’s test for multiple comparisons. Means are considered significantly different (*) when $P < 0.05$. Results are expressed as means ± SE.

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**Fig. 1.** Time course of $^{32}$PO$_4$ accumulation in cultured mammary gland explants. Tissues were cultured for 1 day with insulin (I; 1 $\mu$g/ml) plus cortisol (H; 10$^{-7}$ M). Tissues were then cultured for 1 more day with I + H or I + 1 $\mu$g/ml prolactin (PRL). $^{32}$PO$_4$ (0.5 $\mu$Ci/ml) was present during the final times indicated in the figure. Intracellular accumulation in a 5% trichloroacetic acid (TCA)-soluble tissue fraction is expressed as a distribution ratio of the means ± SE of 6 observations. *Greater than control with $P < 0.05$.

**Fig. 2.** Time course of $^{32}$PO$_4$ incorporation into lipids in cultured mammary gland explants. Experimental details are the same as in Fig. 1, except that $^{32}$PO$_4$ incorporation into lipids was determined (see MATERIALS AND METHODS). Results are expressed as means ± SE of 6 observations.

**Fig. 3.** Time course of $^{32}$PO$_4$ incorporation into a TCA-insoluble fraction from cultured mammary gland explants. Experimental details are the same as in Fig. 1, except that $^{32}$PO$_4$ incorporation into a 5% TCA-insoluble fraction was determined. Results represent means ± SE of 6 observations.

**Fig. 4.** Effects of lactogenic hormones on $^{32}$PO$_4$ accumulation in cultured mammary gland explants. Explants were cultured for 2 days with or without all combinations of 3 lactogenic hormones: 1 $\mu$g/ml insulin (I), 10$^{-7}$ M cortisol (H), and/or 1 $\mu$g/ml PRL. $^{32}$PO$_4$ (0.5 $\mu$Ci/ml) was present for the final 2 h. C, control. Numbers represent means ± SE of 6 observations of $^{32}$PO$_4$ accumulation in a 5% TCA-soluble tissue fraction. *Greater than control, $P < 0.05$; **greater than I + H, $P < 0.05$. 

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RESULTS

Figure 1 shows a time course of $^{32}$PO$_4$ uptake in mouse mammary gland explants that were cultured in the presence or absence of PRL for 24 h. Uptake increased progressively over a 4-h labeling period, and the uptake was twice as great in the PRL-treated tissues. Distribution ratios of $^{32}$PO$_4$ were achieved in the PRL-treated tissues, suggesting that an energy-requiring mechanism is involved in phosphate uptake into mammary cells. Because inorganic phosphate uptake was calculated from the $^{32}$PO$_4$ present in TCA-soluble tissue fractions, an experiment was carried out to determine the actual percentage of $^{32}$PO$_4$ in this fraction that was present as inorganic phosphate. By use of the inorganic phosphate precipitation method of Willard et al. (14), aliquots of the TCA supernatants from Fig. 1 were subjected to precipitation and subsequent quantitation; 81% of the $^{32}$PO$_4$ in the PRL-treated TCA extracts and 86% of the $^{32}$PO$_4$ in the control TCA extracts precipitated as inorganic phosphate. Most of the $^{32}$PO$_4$ in the TCA-soluble tissue extracts is thus present as inorganic phosphate.

Figures 2 and 3 show the time course of $^{32}$PO$_4$ labeling of lipids (primarily phospholipids) and TCA-insoluble molecules (primarily casein) in the tissues described in Fig. 1. The $^{32}$PO$_4$ incorporation into each of these tissue fractions increases progressively with time, and PRL causes about a twofold stimulation of incorporation in each of these fractions; this likely reflects the 50–100% stimulation of casein and phos-
pholipid synthesis that occurs in response to PRL (5, 10).

Figures 4–6 report the results of an experiment wherein explants were cultured for 2 days with all possible combinations of three lactogenic hormones (insulin, cortisol, and PRL); the tissues were then pulse labeled for 2 h with $^{32}$PO$_4$. When the hormones were tested individually (Fig. 4), insulin stimulated $^{32}$PO$_4$ uptake, PRL had no effect, and cortisol ($10^{-7}$ M) inhibited uptake. When the hormones were tested in combinations, the only combination in which PRL stimulated $^{32}$PO$_4$ uptake was when all three hormones were tested in concert. In the associated incorporation data as presented in Figs. 5 and 6, only insulin by itself stimulates $^{32}$PO$_4$ incorporation into lipids and the TCA-insoluble fraction, whereas PRL has a stimulatory effect only when combined with insulin and cortisol.

A time course for the PRL stimulation of $^{32}$PO$_4$ uptake and incorporation is presented in Fig. 7. Tissues employed in this experiment were initially cultured with insulin plus cortisol; PRL was then added for the times indicated. Effects of PRL on phosphate uptake and incorporation were first detected 8–12 h after PRL was added to the cultured tissues; the responses were maintained through 30 h. In dose-re-

![Graph](image1)

**Fig. 9.** Phosphate concentration vs. $^{32}$PO$_4$ uptake and incorporation in cultured mammary gland explants. Tissues were treated with hormones as in Fig. 1. Phosphate (0.5 Ci/ml $^{32}$PO$_4$) at the concentrations indicated was present during the final 2 h of culture. The intracellular-to-media phosphate concentration ratio was then calculated and expressed as the mean ± SE of 6 observations.

![Graph](image2)

**Fig. 10.** Effect of sodium on $^{32}$PO$_4$ uptake in cultured mammary gland explants. Tissues were treated with hormones as in Fig. 1. During the final 2 h of culture, 0.5 µCi/ml $^{32}$PO$_4$ was present in medium consisting of medium 199 (M-199), Krebs-Ringer bicarbonate buffer (KRB, containing sodium), or KRB containing choline chloride substituted for sodium chloride. In far right, the tissues were cultured for 2 h in sodium-free medium before the terminal 2-h culture with $^{32}$PO$_4$ in sodium-containing KRB. Results are expressed as means ± SE of 6 observations.

![Graph](image3)

**Fig. 11.** Effect of sodium on $^{32}$PO$_4$ incorporation into lipids in cultured mammary gland explants. Experimental details are the same as in Fig. 10, except that $^{32}$PO$_4$ incorporation into lipids was determined (see MATERIALS AND METHODS). Results are expressed as means ± SE of 6 observations.

![Graph](image4)

**Fig. 12.** Effect of sodium on $^{32}$PO$_4$ incorporation into a TCA-insoluble fraction from cultured mammary gland explants. Experimental details are the same as in Fig. 10, except that $^{32}$PO$_4$ incorporation into a 5% TCA-insoluble fraction was determined. Results are expressed as means ± SE of 6 observations.
sponse studies (Fig. 8), PRL effects were expressed with all PRL concentrations of 50 ng/ml and above; these concentrations are physiological in that they are similar to plasma concentrations in mice and other species.

With control and 24-h PRL-treated tissues, phosphate uptake was quantitated with culture medium phosphate concentrations of 1–10 mM (Fig. 9). The intracellular phosphate concentration was calculated from the TCA-soluble $^{32}$PO$_4$ that was taken up from the culture medium; the total amount of phosphate in the cells was not determined but is likely higher than the calculated concentrations presented in Fig. 9. Saturation kinetics for uptake are clearly apparent. Because a large fraction of the inorganic phosphate is rapidly incorporated into lipids and proteins, a precise estimation of transport kinetics ($K_m$ and maximum velocity, $V_{max}$) is not possible. Regardless, however, if the data in Fig. 9 are plotted as the reciprocal of the velocity vs. the phosphate concentration, an apparent $K_m$ of ~4 mM and an apparent $V_{max}$ of 7.5 mM·h$^{-1}$·1 cell water$^{-1}$ were determined. These values compare favorably with those published for rat mammary tissues (4): $K_m$ = 1.13 mM, $V_{max}$ = 13.4 mM·h$^{-1}$·1 cell water$^{-1}$.

The experiments in Figs. 10–12 were carried out to determine the sodium dependence of the PRL effects on $^{32}$PO$_4$ uptake and incorporation. In these studies, explants were cultured for 24 h in the absence (control) or presence of PRL; the tissues were then cultured for 2 additional hours with $^{32}$PO$_4$ contained in M-199, KRB, or KRB with the NaCl substituted for with choline chloride. In the final experimental combination, the tissues were cultured for 2 h with the sodium-free KRB, after which they were treated for 2 h with sodium-KRB containing $^{32}$PO$_4$. Several interesting observations evolved from these experiments. First, the PRL effects on $^{32}$PO$_4$ uptake and incorporation into lipids were abolished when these determinations were made with sodium-free medium. The PRL effect on $^{32}$PO$_4$ incorporation into TCA-precipitable molecules (primarily proteins) was attenuated but not abolished with the sodium-free medium. The 2-h exposure of the tissues to the sodium-free medium was not detrimental to the functioning of the tissues, since the magnitude of the PRL responses was fully restored in tissues that were first cultured in sodium-free medium for 2 h, after which $^{32}$PO$_4$ uptake was assessed in normal KRB. Another interesting observation was that the magnitude of $^{32}$PO$_4$ uptake and incorporation was ~50% when uptake was determined with $^{32}$PO$_4$ contained in M-199 vs. KRB. Because the phosphate concentrations in M-199 and KRB are the same (1 mM), one or more of the components of M-199 must be impairing phosphate uptake and incorporation; this component(s) has yet to be identified. A final observation in these studies is that distribution ratios greater than three were generated when $^{32}$PO$_4$ uptake was determined in the absence of sodium. This may suggest that another energy-requiring transporter for phosphate may exist in mammary tissues; this transporter is clearly not affected by PRL treatment.

**DISCUSSION**

PRL as well as insulin and cortisol is vitally important in the regulation of phosphate metabolism in the mammary gland. Because copious amounts of phosphate are secreted into milk during lactation, the hormone regulation of this process is of utmost importance. PRL at physiological concentrations (50–1,000 ng/ml) functioned in concert with insulin and cortisol to stimulate phosphate uptake as well as its incorporation into lipids and TCA-insoluble materials. The onset of these PRL responses was 8–12 h after PRL addition to the cultured tissues; this correlates well with the onset of PRL’s effect on the synthesis of a number of other milk products, including lactose, casein, and triglycerides; the PRL stimulation of iodide, amino acid, and glucose transport follows a similar time course (1, 3, 4, 6–11). Precisely how all the signaling pathways for PRL, insulin, and cortisol integrate to effect a maximum stimulation of milk product synthesis remains to be explained.

The enhanced uptake of phosphate across the basolateral surface of the alveolar epithelial cells is likely the primary mechanism accelerating the accumulation of inorganic phosphate in milk (12, 13). In addition, the increased phosphate uptake likely enhances the substrate provision for phosphate incorporation into casein, as well as combining with calcium in casein micelles. Clearly a sodium-dependent phosphate transporter is present in mammary cells; this is the only transporter that is stimulated by PRL, since the phosphate uptake effect is abolished when uptake is determined in the absence of sodium. Phosphate distribution ratios greater than three maintain, however, when phosphate uptake is determined in the absence of sodium. This suggests that a sodium-independent phosphate transporter may also exist in the mammary gland. In addition, this transporter is likely an energy-dependent, active transporter to achieve a distribution ratio greater than unity; clearly this transporter is not regulated by PRL. In the future, it remains for the phosphate transporter(s) to be isolated and their molecular structure determined.

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**REFERENCES**