Effects of Polypeptide and Protein Hormones on Lipid Monolayers

I. *Effect of insulin and parathyroid hormone on monomolecular films of monooctadecyl phosphate and stearic acid*

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ABSTRACT Insulin in low concentrations inhibits the uptake of Ca^{++} by the monooctadecyl (stearyl) phosphate monolayer (at air-water interface) and facilitates the release of Ca^{++} adsorbed to the monolayer. These effects of insulin are more pronounced at higher insulin concentrations. Evidence is presented that a relatively intact insulin molecule competes with Ca^{++} for the free phosphate group of the monolayer. Albumin has a slight inhibitory action on calcium uptake and parathyroid hormone has no observable action on calcium uptake or release.

INTRODUCTION

The essential role played by lipids in general and by phospholipids in particular in maintaining the structural and functional integrity of biological membranes has been well-established. Davson and Danielli (1) proposed that the plasma membrane is composed of a lipid bimolecular leaflet whose polar groups project inward into the aqueous cell interior and outward into the surrounding aqueous environment. A model system having the characteristics of the outer layer of the plasma membrane was employed to elucidate some of the interactions of calcium and insulin with the cell membrane. The model system consisted of a monomolecular film of monooctadecyl phosphate spread on the surface of an aqueous solution containing the reactants.

The actions of Ca^{++} and of insulin on cellular transport are well-recognized. Ca^{++} impedes the passage of Na^+ and K^+ across the nerve cell membrane (2) and reduces the influx of Na^+ into HeLa cells (3). Conversely, a lowering of the concentration of Ca^{++} in the ambient solution increases the permeability of muscle (4) and egg (5) cells to water and that of red blood cells, brain slices,

and muscle cells to Na^+ and K^+ (6). It induces loss of intracellular enzymes by liver slices (7).

In contrast to Ca^{++}, insulin increases the rate of entry of glucose and non-metabolizable sugars into skeletal and cardiac muscles, fat cells, and fibroblasts (8-12), that of amino acids into the diaphragm (13), cardiac muscle (14), and fat cells (12, 15), and that of K^+ into muscle cells (16).

Rodbell (12) and Blecher (17, 18) believe that the action of insulin on transport is similar to that of phospholipases A and C. Phospholipase C catalyzes the hydrolysis of a number of phospholipids which occur commonly in cell membranes (19). This suggests that an important aspect of the action of insulin may be its interaction with the phosphate groups of the cell membrane.

The interrelationship between the action of insulin and that of Ca^{++} on the cell membrane was explored by measuring the influence of insulin on the uptake of ^{45}Ca by the monooctadecyl phosphate monolayer (mOP) at the

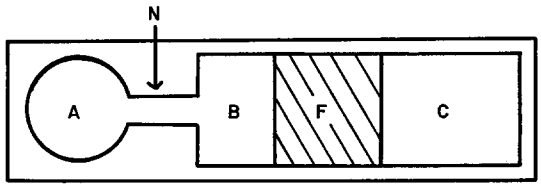

FIGURE 1. Diagram of the Teflon trough. Chamber A was 5.5 cm in diameter; chamber B-C was 5 × 21 cm; both chambers were 1.2 cm in depth. The neck measured 0.9 × 1.8 cm and was 0.3 cm deep. The Teflon float (hatched area) measured 5 × 7.5 cm and was 0.0025 cm thick. The diameter of chamber A was equal to that of the Micromil window of the G-M tube.

air-water interface. Insulin was shown to inhibit the adsorption of Ca^{++} onto the surface of a mOP monolayer; this suggests that its action may be mediated by inhibition of the effect of Ca^{++} on the cell membrane. Two other soluble reactants (albumin and parathyroid hormone) whose interactions with Ca^{++} are recognized, and another lipid monolayer (stearic acid) served as controls for the interaction.

METHODS

A modified Langmuir trough similar to that described by Pak and Arnold (20) was used in these experiments. The trough, milled from Teflon, consisted of two chambers connected by a shallow neck (Fig. 1). Suspended at a fixed height of 0.4 cm above the smaller chamber (A) was a Geiger-Mueller (G-M) gas flow tube (Nuclear Chicago D47 with a Micromil end window). A mixture of helium (98.7 %) and butane (1.3 %) (Matheson Co., Inc., East Rutherford, N. J.) under 9.5 lb of pressure flowed through the G-M tube during measurements. 1 min counts, sufficient to give fractional standard deviations of counting (20) of less than ±0.01, were measured with a scaler (Ultrascaler, Nuclear Chicago Corp., Des Plaines, Ill., Model 192A).

Reagent grade NaOH, $CaCl_2$, H_3PO_4, HCl, and methanol (absolute) were used. ^{45}Calcium chloride (S.A. 5 mc/mg Ca^{++}) was purchased from Nuclear Chicago, albumin (ovalbumin, 3 times crystalline) from Nutritional Biochemicals (Cleveland, Ohio), and ethyl laurate from Eastman Chemicals (Rochester, N.Y.). Insulin (bovine, 5 times recrystallized, Lot No. T-2482) was a gift from Eli Lilly and Co. Research Laboratories (Indianapolis, Ind.), and parathyroid hormone (porcine) a gift from Dr. John T. Potts, National Institutes of Health, Bethesda, Md.

Stearic acid (M.A., mp 70°C) and stearyl alcohol (M.A., mp 58.5°–59.0°C) were purchased from Mann Research Laboratories (New York, N.Y.), and were used without further purification. Stearic-1-^{14}C-acid (S.A. 0.1 mc/3 mg) was purchased from Tracerlab (Waltham, Mass.). Monooctadecyl phosphate (mOP) was the same as that used by Gershfeld and his associates (21). These fatty acids were dissolved in a solution of benzene:methanol (19:1). Before use, benzene (Fisher Chemicals, Fair Lawn, N.J.) was filtered through a dry florisil and silica gel column. Aqueous solutions were prepared with water triple-distilled in quartz (Amersil still, Heraeus Quartz Corp., Hanau am Main, Germany) and stored in quartz flasks to minimize the ion content. 5 M solutions of insulin, albumin, and parathyroid hormone were prepared in 5 mM hydrochloric acid and stored at 3°C. Final dilutions with water were made just prior to study.

At the beginning of each experiment the trough was filled to the brim with sodium phosphate buffer (pH 7.4) which was 3.4 mM for sodium and 1 mM for phosphate. The volume of the solution was kept constant to maintain a constant distance between the surface of the aqueous phase and the G-M tube. A Teflon float (F) was laid on the swept surface of the buffer solution at the distal end of the large chamber (B-C). Monooctadecyl phosphate was spread on the surface of chambers A and B and the solvent allowed to evaporate. A drop of ethyl laurate, a piston oil, was applied to the surface of compartment C; this compressed the monolayer with a pressure of 19.7 dyne/cm (20), which exceeds the collapse pressure of an insulin monolayer (22) and at which a liquid-solid film of mOP is formed (21). A Teflon barrier (14.0 × 1.2 × 1.2 cm) was then placed across the neck (N) connecting the two chambers to maintain constant the area of the compressed monolayer on the small chamber and to prevent leakage of the piston oil into the monolayer surface in A. The whole trough and the detector were enclosed in a 41 × 26 cm dome made of transparent methyl methacrylate. Atmospheric air, saturated with water vapor after being forced under slight positive pressure through a water reservoir, circulated within the dome. Experiments were carried out at 25°C ± 1°C.

After the monolayer had been formed and equilibrated for 3 min, a half-ml of a solution containing calcium chloride, 1×10^{-6} M, a tracer quantity of ^{45} calcium-chloride[1], and an aliquot of the substance to be studied were introduced with a Lang-Levy micropipette beneath the monolayer into the subsolution contained in the small chamber. The pipette was rinsed 15 times during 1.5 min. Adequate mixing under these conditions had been shown previously with a dye, which showed complete dispersion within the reaction chamber. Corrected for background, radioactivity was assumed to originate from emissions of ^{45}Ca at, or very near, the film interface, because of the short-range (0.65 mm) of the β-radiations of ^{45}Ca in water.

[1] $CaCl_2$ (1×10^{-6} M), labeled with a tracer of $^{45}CaCl_2$ will henceforth be called "^{45}Ca-$CaCl_2$".

RESULTS

Interaction of Ca^{++} with the mOP Monolayer

When a solution containing ^{45}Ca-$CaCl_2$ was allowed to interact with an mOP monolayer, the radioactivity of the film rose rapidly, reaching a plateau at

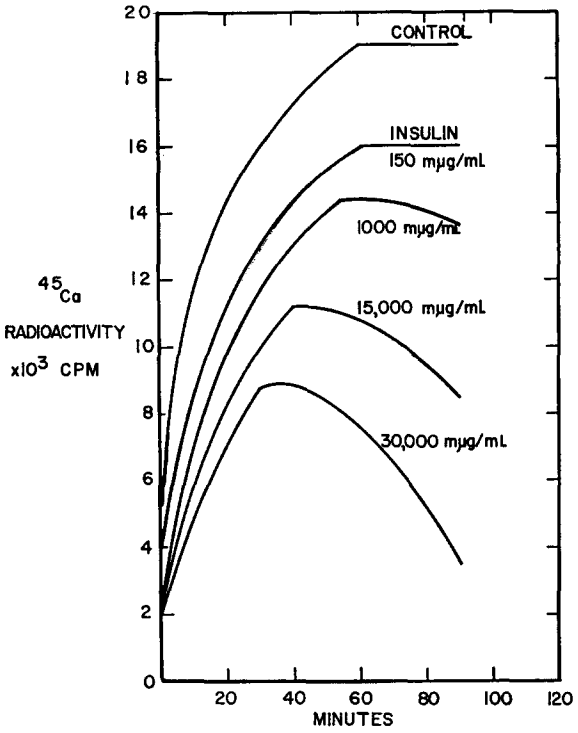

FIGURE 2. The effect of insulin on the ^{45}Ca uptake of the mOP monolayer. The subsolution, pH 7.4, contained ^{45}Ca-$CaCl_2$. Control radioactivity, with a plateau of $1.9 \times 10^4 \pm 0.02 \times 10^4$ cpm (± 1 SEM), represents nine experiments; radioactivity in the presence of insulin represents the mean of three experiments at each insulin concentration.

about 65 min and remaining at that level until the end of the study at 90 min (Fig. 2).

Influence of Insulin on the Uptake of ^{45}Ca by the mOP Monolayer

Insulin, added to the subsolution beneath the monolayer, modified the uptake of ^{45}Ca by the film (Fig. 2). In the first place, insulin reduced the magnitude of the uptake of ^{45}Ca by the monolayer. At a concentration of 150 mμg/ml, it lowered the maximum radioactivity by about 15 %. The peak ^{45}Ca radioactivity of the film was diminished further when higher concentrations of insulin were added to the subsolution, and at an insulin concentration

of 30,000 mμg/ml, it was reduced by approximately 50 %. In the second place, insulin altered the kinetics of uptake of ^{45}Ca. The kinetics of uptake of ^{45}Ca in the presence of insulin was biphasic; after an initial rapid uptake of ^{45}Ca there was a fall in radioactivity. With increasing concentrations of insulin in the solution, the fall was more pronounced, obliterating the plateau region and resulting in a more rapid decrease in count. These results suggest that the action of insulin is twofold: it inhibits the uptake of Ca^{++} and facilitates the release of adsorbed Ca^{++}.

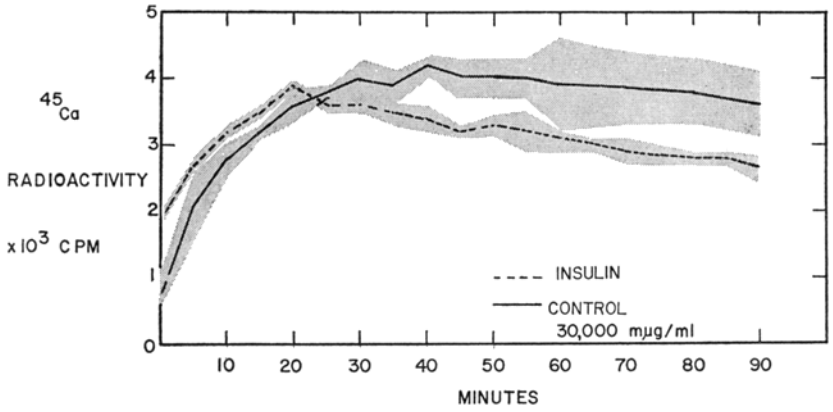

FIGURE 3. The influence of insulin on the ^{45}Ca uptake at the stearic acid monolayer. The subsolution, pH 7.4, contained ^{45}Ca-$CaCl_2$. Radioactivity represents the mean of three experiments, with the shaded areas indicating ±1 SEM.

Influence of Insulin on the Interaction between Ca^{++} and the Stearic Acid Monolayer

To examine whether the inhibitory action of insulin on Ca^{++} adsorption was specific for the phosphate monolayer, ^{45}Ca uptake studies were undertaken with a carboxyl film (stearic acid). When ^{45}Ca-$CaCl_2$ was added below a monolayer of stearic acid, radioactivity reached its peak in about 40 min, diminishing slightly by 90 min when the experiment was terminated (Fig. 3). If stearic acid were allowed to interact with the ^{45}Ca-$CaCl_2$ in the presence of insulin (30,000 mμg/ml), the maximum radioactivity was reached at a slightly lower value; this was followed by a fall in radioactivity of about 25 % by 90 min (Fig. 3).

These effects of insulin may represent facilitation either of the desorption of the ^{45}Ca-monolayer complex, or of the release of adsorbed ^{45}Ca alone. Accordingly, the following studies were carried out to distinguish between these two processes. Insulin was allowed to interact with a monolayer of stearic acid-1-^{14}C. The subsolution contained $CaCl_2$ (1×10^{-6} M) but no ^{45}Ca, so that all radioactivity was that of ^{14}C emissions. There was no change in radio-

activity, a result which indicates that desorption of the monolayer does not occur. We therefore conclude that insulin facilitates the release of Ca^{++} alone from the stearic acid monolayer, and by analogy, from the phosphate monolayer as well.

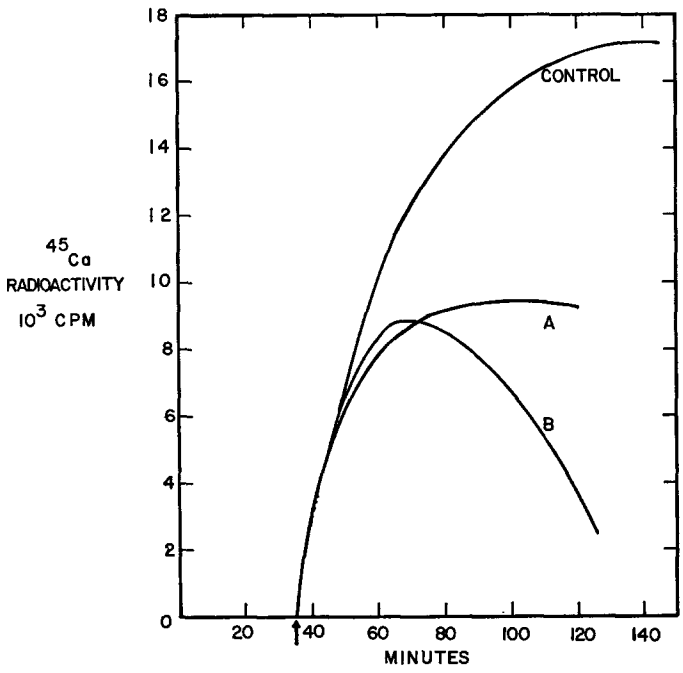

FIGURE 4. The interaction between insulin and the mOP monolayer, followed by the action of Ca^{++}. Insulin (concentration 30,000 mμg/ml) and ^{45}Ca-$CaCl_2$ were in the subsolution. Control, Ca^{++} was added 35 min after the film had formed. Curve A, insulin was added as soon as the film was formed and Ca^{++}, 35 min afterwards. Curve B, insulin and calcium were added simultaneously as soon as the film was formed.

Some Characteristics of the Interaction of Calcium and Insulin with the mOP Monolayer

(*a*) The ^{45}Ca released under the influence of insulin probably represented that not firmly bound to the monolayer, as may be appreciated from the following study. ^{45}Ca-$CaCl_2$ was allowed to interact with the mOP monolayer for 75 min before the addition of insulin (30,000 mμg/ml). Under such conditions an insoluble film of stearyl calcium phosphate should have formed. Insulin then added failed to cause any change in the ^{45}Ca radioactivity of the film. Calcium released by insulin, therefore, may represent that held to the monolayer by electrostatic forces rather than that bound to the film by covalent linkage.

(*b*) Adsorbed ^{45}Ca is not released if insulin is allowed to react with the mOP

monolayer prior to the addition of ^{45}Ca. Insulin at a concentration of 30,000 mμg/ml, sufficient to cause the release of ^{45}Ca, was allowed to interact with the monolayer for 35 min. ^{45}Ca-$CaCl_2$ was then added. The first phase of the rapid uptake of ^{45}Ca was seen as in the control experiment without insulin and in the study in which insulin (30,000 mμg/ml) and calcium were added simultaneously (Fig. 4). The second phase, the decrease in radioactivity, however, did not occur. These results suggest that the release of the adsorbed ^{45}Ca probably depends on the conformational state of the insulin molecule. During the period of adsorption of insulin onto the film, much of the secondary and tertiary bond structure of insulin may still be intact. Under these conditions, insulin facilitates the release of adsorbed Ca^{++}. When the adsorption of insulin is completed, however, many of its secondary and tertiary bonds are probably destroyed. Such a "denatured" insulin does not cause the release of adsorbed calcium. The results suggest that a relatively intact insulin molecule and Ca^{++} compete for the free carboxyl or phosphate group of the monolayer. Even though the secondary fall in ^{45}Ca radioactivity was abolished, the inhibition of the initial uptake of ^{45}Ca was still apparent when ^{45}Ca-$CaCl_2$ was added after the reaction of the monolayer with insulin (Fig. 4). This suggests that insulin neutralizes some of the negative charge of the film required for the uptake of calcium.

Influence of Insulin on the Interaction of Ca^{++} with the Stearyl Alcohol Monolayer

When ^{45}Ca-$CaCl_2$ was allowed to react with stearyl alcohol, an uncharged monolayer, the plateau of radioactivity attained was 4.9×10^3 cpm, about 25 % of that occurring at the mOP monolayer. When insulin (30,000 mμg/ml) together with ^{45}Ca-$CaCl_2$ interacted with a stearyl alcohol monolayer, the radioactivity increased to 16 % above that attained when insulin was not present. Thus, insulin slightly enhanced the uptake of $^{45}Ca^{++}$ at an electrostatically neutral surface, in contrast to its inhibition of Ca^{++} uptake by the negatively charged monolayers of mOP and stearic acid. It would appear that insulin acts to neutralize, with its positively charged groups, some of the negative charges at the monolayers (mOP and stearic acid), thereby reducing the available binding sites for Ca^{++}. In the absence of a negative charge on a monolayer (stearyl alcohol), insulin stabilizes more Ca^{++} in the region of the interface, perhaps by electrostatic attraction between the negative charges of adsorbed insulin and the positively charged Ca^{++}.

Influence of Albumin and of Parathyroid Hormone on the Interaction of Ca^{++} with the mOP Monolayer

When albumin (1,000 mμg/ml) was added with ^{45}Ca-$CaCl_2$ beneath an mOP monolayer, the peak of radioactivity was slightly lower than in the absence of albumin (Fig. 5). Further reduction by approximately 15 % was seen at a

higher concentration of albumin (30,000 mμg/ml). This inhibition by albumin of ^{45}Ca uptake is significantly less than that shown by a comparable concentration (by weight) of insulin. There was a fall in the plateau of ^{45}Ca radioactivity of 32 % at a much higher concentration of albumin (210,000 mμg/ml, comparable to 30,000 mμg/ml of insulin on a molar basis). Further, the secondary decrease in ^{45}Ca radioactivity was not observed with albumin; this suggests that albumin, unlike insulin, does not cause the release of bound ^{45}Ca.

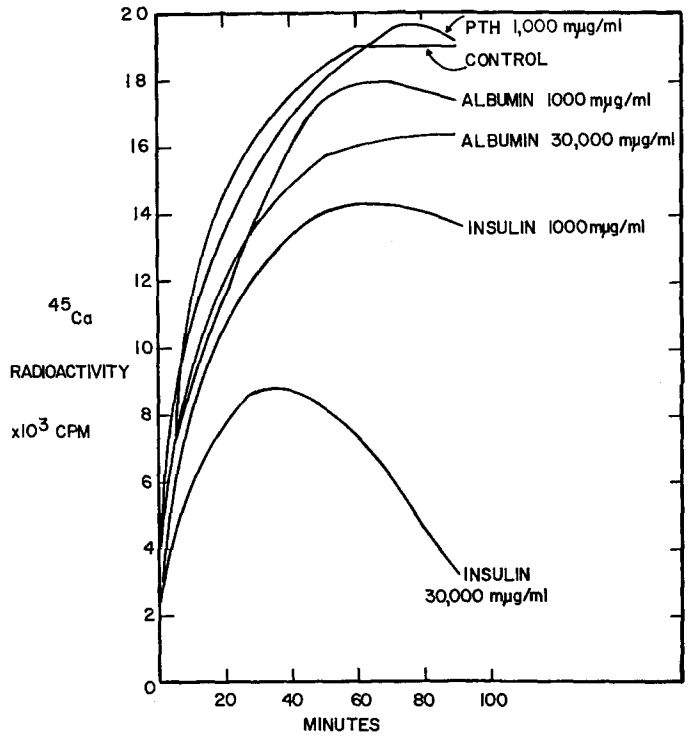

FIGURE 5. The effect of albumin and parathyroid hormone on ^{45}Ca uptake by the mOP monolayer. ^{45}Ca-$CaCl_2$ was in the subsolution.

In contrast to insulin and albumin, parathyroid hormone did not significantly affect the uptake of ^{45}Ca by the mOP monolayer.

DISCUSSION

A model system in which a highly purified monooctadecyl phosphate monolayer serves for the study of interactions similar to those taking place in phospholipid moieties of the plasma membrane has been used to observe the interactions of calcium ions in solution with phosphate-containing long-chain fatty acid molecules on the surface of the solution. In this model system the

interactions of molecules in the aqueous phase with the monolayer can be thought to represent the molecular interactions of such molecules with the phospholipids of the plasma membrane bimolecular leaflet which are oriented outward; that is, towards the interstitial fluid. These studies have shown that insulin interferes with the adsorption of Ca^{++} by the monolayer and facilitates the release of Ca^{++} already adsorbed there. The inhibition by insulin of calcium uptake is probably dependent on the net negative charge at the monolayer surface. The release by insulin of calcium from the monolayer is probably an action of undenatured insulin molecules competing with calcium for the charged groups of the monolayer. Alternatively, this release may represent desorption of a Ca^{++}-peptide complex. Such a complex may increase the ionic repulsion between the insulin molecules due to the neutralization of some of the negative charge of insulin by Ca^{++}, leading to desorption of the complex. The inhibition of adsorption of calcium at the monolayer in the presence of insulin might be thought to represent the interference with adsorption of calcium by the outer surface of the plasma membrane. Calcium is known to reduce the permeability of the membrane to a variety of substances (2, 3). If the presence of calcium at the outer surface of the plasma membrane is necessary to limit the entry of glucose and amino acids into cells, then the action of insulin might be to diminish the uptake of calcium at the surface of the plasma membrane, thereby promoting passage of such substances through the plasma membrane.

Some caution must be observed in applying conclusions based on molecular interactions studied in a model system to the behavior of living cells, as the concentrations of reactants and the significant spatial relations may differ from those observed in the whole animal.

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