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THE EFFECT OF POLYPEPTIDE HORMONES ON LIPID MONOLAYERS

II. THE EFFECT OF INSULIN ANALOGUES, VASOPRESSIN, OXYTOCIN, THYROCALCITONIN, ADRENOCORTICOTROPIN, AND 3',5'-CYCLIC AMP ON THE UPTAKE OF Ca^{2+} BY MONOMOLECULAR FILMS OF MONOOCTADECYL PHOSPHATE

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SUMMARY

1. Like insulin, some analogues of insulin inhibited the adsorption of Ca^{2+} at a monooctadecyl phosphate monolayer; the inhibition was, in all cases, less than the inhibition by intact insulin.

2. Facilitation of release of Ca^{2+} from the monolayer by insulin required that the insulin molecule be intact, as release was facilitated only very slightly when des-A^1-glycine-des-B^1-phenylalanine^1-insulin or insulin B-chain-S-sulfonate replaced insulin, and did not occur at all in the absence of alanine in position B^{30}.

3. Vasopressin and oxytocin inhibited Ca^{2+} adsorption. In addition, vasopressin facilitated Ca^{2+} release.

4. Thyrocalcitonin profoundly inhibited Ca^{2+} adsorption but facilitated only slightly its release.

5. 3',5'-cyclic AMP facilitated slightly the adsorption of Ca^{2+}.

6. It is suggested that the biphasic action of insulin and vasopressin on Ca^{2+} uptake depends upon the concurrence of a 6-membered disulfide ring at or near the N-terminus of the molecule, and a proline followed by a positively charged amino acid (arginine or lysine) in the two positions preceding the C-terminal amino acid.

INTRODUCTION

In a previous communication[1] insulin was shown to influence the interaction of Ca^{2+} with a monooctadecyl phosphate (stearyl monophosphate) monolayer at the air–water interface: insulin inhibited the uptake of Ca^{2+} by the monolayer and facilitated the release of Ca^{2+} already adsorbed* on the monolayer. In this communication we report the effects of the following polypeptide hormones and biologically active

* No underlying physicochemical mechanism for the interaction of Ca^{2+} with the monolayer is implied in the usage of the term "adsorbed". The term therefore refers to Ca^{2+} held to the monolayer by electrostatic forces as well as by hydrogen or covalent linkage.

substances (reactant) on the interaction between Ca^{2-} and the phosphate monolayer: vasopressin, oxytocin, thyrocalcitonin, analogues of insulin, and adenosine 3',5'-monophosphate (cyclic-AMP). Our studies show that whereas vasopressin, oxytocin, thyrocalcitonin, and some analogues of insulin inhibit the adsorption of Ca^{2+}, only intact insulin and vasopressin facilitate the release of adsorbed Ca^{2+}. Evidence is presented that the facilitation of Ca^{2+} release requires the integrity of the tertiary structure of the reactant molecules and may depend on the presence of the 6-membered disulfide ring near the N-terminus and a positive charge near the C-terminus.

MATERIALS AND METHODS

Materials

Reagent grade NaOH, $CaCl_2$, H_3PO_4, HCl, and methanol (absolute) were used. $^{45}Ca^{2+}$ (as $CaCl_2$, 5 mC/mg Ca^{2+}) was purchased from Nuclear Chicago, and ethyl laurate from Eastman Chemicals.

The following reactants were used: oxytocin synthetic powder (101 units/mg, Mann Research Laboratories), bovine vasopressin (dry powder, 53 pressor units and 2.6 oxytocin units/mg, Mann Research Laboratories), and cyclic-AMP (A grade, Calbiochem).

Insulin A-chain-S-sulfonate, insulin B-chain-S-sulfonate, des-B^{30}-alanine-insulin and insulin were kindly provided by Dr. William W. Bromer and Dr. J. M. McGuire of Eli Lilly and Co. Research Laboratories (Indianapolis, Ind.); des-B^{23-30}-octapeptide-insulin, des-B^{30}-alanine-des-A^{21}-asparagine-insulin, and des-A^{1}-glycine-des-B^{1}-phenylalanine-insulin by Dr. Frederick H. Carpenter of the Department of Biochemistry, University of California (Berkeley, Calif.); and porcine thyrocalcitonin (70 M.R.C. units/mg) by Dr. Robert J. Schlueter of Armour Laboratories (Kankakee, Ill.).

Monooctadecyl phosphate[2] was dissolved in a solution of benzene–methanol. Water was triple-distilled in quartz and stored in quartz flasks to minimize the ion content. 5 M solutions of peptides and other reactants were prepared in 5 mM HCl and stored at 3°. Final dilutions with water were made at the time of study. Although insulin in a concentration as low as 150 ng/ml of subsolution had been shown to inhibit the uptake of $^{45}Ca^{2+}$ by the monooctadecyl phosphate monolayer[1], test substances were used at a concentration of 30 000 ng/ml of subsolution to increase the magnitude of changes. Equal quantities by weight of each reactant were used, as the effects of the peptide were related, presumably to their amino acid composition rather than to the number of molecules present[1].

METHODS

The technique for measuring the uptake of Ca^{2+} by the monolayer was discussed in detail in a previous communication[1] and will be described here briefly. A monolayer of monooctadecyl phosphate was spread over the surface of a sodium phosphate buffer solution (pH 7.4, 1 mM phosphate) in a modified Langmuir trough. The monolayer was compressed by a lateral pressure of 19.7 dynes/cm with ethyl laurate piston oil[3]. 0.5 ml of a solution containing 1 μM $CaCl_2$ and a trace quantity of $^{45}Ca^{2+}$ (the solution to be referred to as "$^{45}Ca^{2+}$–$CaCl_2$") and an aliquot of the reactant were introduced into the subsolution beneath the monolayer. $^{45}Ca^{2+}$ radioactivity was measured with a Geiger–

Mueller gas flow counter (Nuclear Chicago D47 with a Micromil end window) suspended at a fixed distance of 1 cm above the surface of the monolayer. The radioactivity measured was assumed to originate from $^{45}Ca^{2+}$ at or near the film interface[1].

RESULTS

Influence of insulin on the uptake of $^{45}Ca^{2+}$ by the monooctadecyl phosphate monolayer

As shown previously[1], when $^{45}Ca^{2+}$–$CaCl_2$ was allowed to interact with a monooctadecyl phosphate monolayer, the film radioactivity rose swiftly, reaching a plateau at about 65 min and remaining at this level until the end of the study at 90 min (Fig. 2, Curve 1).

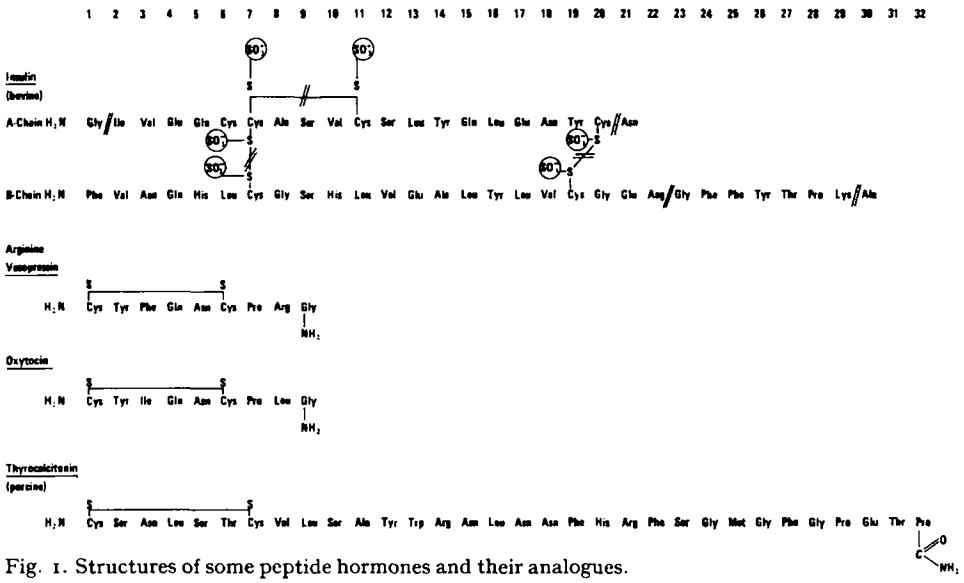


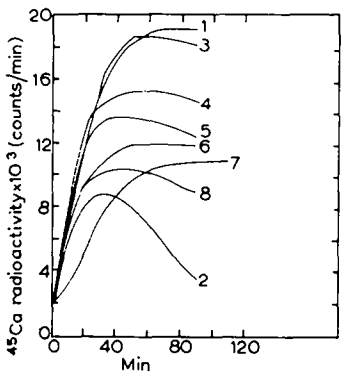
Fig. 1. Structures of some peptide hormones and their analogues.

Insulin, added to the solution beneath the monolayer, reduced the magnitude of the uptake of $^{45}Ca^{2+}$ by 54 % and facilitated the release of $^{45}Ca^{2+}$ already adsorbed (Fig. 2, Curve 2). The influence of other reactants on these two processes, that is, the initial uptake of $^{45}Ca^{2+}$ and its subsequent release, will now be considered.

Influence of some analogues of insulin on the uptake of $^{45}Ca^{2+}$ by the monooctadecyl phosphate monolayer

Insulin B-chain-S-sulfonate, added to the solution under the monolayer, reduced $^{45}Ca^{2+}$ uptake by the film (at the maximum $^{45}Ca^{2+}$ radioactivity) by 46 %; insulin A-chain-S-sulfonate, by 42 %; des-B^{30}-alanine-des-A^{21}-asparagine-insulin, by 38 %; and des-A^{1}-glycine-des-B^{1}-phenylalanine-insulin lowered $^{45}Ca^{2+}$ uptake by 28 % (Fig. 2). Des-B^{23-30}-octapeptide-insulin reduced the magnitude of $^{45}Ca^{2+}$ adsorption at the monooctadecyl phosphate monolayer by 20 %, and des-B^{30}-alanine-insulin did not interfere at all with $^{45}Ca^{2+}$ uptake (Fig. 2). Insulin A-chain-S-sulfonate in the subsolution delayed the attainment of the plateau of $^{45}Ca^{2+}$ uptake (Fig. 2).

Both insulin B-chain-S-sulfonate and des-A^1-glycine-des-B^1-phenylalanine-insulin facilitated the release of adsorbed $^{45}Ca^{2+}$ from the monolayer. The rate of $^{45}Ca^{2+}$ loss was considerably lower, however, than in the presence of insulin (33 counts/min in contrast to 120 counts/min). Other insulin analogues did not significantly alter the release of $^{45}Ca^{2+}$ from the monooctadecyl phosphate monolayer.



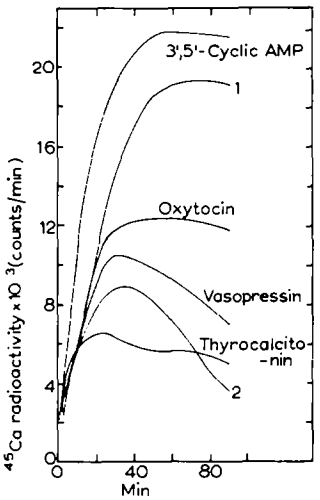


Fig. 2. The influence of some analogues of insulin on the uptake of $^{45}Ca^{2+}$ at the monooctadecyl phosphate monolayer as a function of time. 1, control: $^{45}Ca^{2+}$–$CaCl_2$ alone; 2, insulin, 30000 ng/ml subsolution + $^{45}Ca^{2+}$–$CaCl_2$. Each of Curves 3–8 represents the action of insulin analogue at a concentration of 30000 ng/ml of subsolution + $^{45}Ca^{2+}$–$CaCl_2$; 3, des-B^{30}-alanine; 4, des-B^{23-30}-octapeptide; 5, des-A^1-glycine-des-B^1-phenylalanine; 6, des-B^{30}-alanine-des-A^{21}-asparagine; 7, A-chain-S-sulfonate; 8, B-chain-S-sulfonate.

Fig. 3. The influence of vasopressin, oxytocin, thyrocalcitonin, and 3',5'-cyclic AMP on the uptake of $^{45}Ca^{2+}$ at the monooctadecyl phosphate monolayer. 1, control: $^{45}Ca^{2+}$–$CaCl_2$; 2, insulin, 30000 ng/ml of subsolution + $^{45}Ca^{2+}$–$CaCl_2$. Each of the other curves represents the interaction of the reactant indicated, at a concentration of 30000 ng/ml of subsolution + $^{45}Ca^{2+}$–$CaCl_2$.

Influence of vasopressin and oxytocin on the uptake of $^{45}Ca^{2+}$ by the monooctadecyl phosphate monolayer

Oxytocin, added to the solution beneath a monooctadecyl phosphate monolayer, reduced the magnitude of the $^{45}Ca^{2+}$ uptake by 36 %, and arginine vasopressin reduced the uptake by 45 % (Fig. 3). Vasopressin, but not oxytocin, facilitated the release of adsorbed $^{45}Ca^{2+}$ at a rate comparable to that produced by insulin.

Influence of thyrocalcitonin and cyclic-AMP on the uptake of $^{45}Ca^{2+}$ at the monooctadecyl phosphate monolayer

Thyrocalcitonin, added to the solution beneath the monolayer, inhibited $^{45}Ca^{2+}$ uptake by 66 %, and facilitated slightly (at a rate of 23 counts/min) the release of $^{45}Ca^{2+}$ already adsorbed there. Cyclic-AMP increased the $^{45}Ca^{2+}$ uptake by 14 % at the maximum radioactivity (Fig. 3); thus it facilitated slightly the adsorption of $^{45}Ca^{2+}$ at the monooctadecyl phosphate monolayer.

DISCUSSION

Inhibition by peptide reactants of the adsorption of $^{45}Ca^{2+}$ at a monooctadecyl phosphate monolayer has two phases: the inhibition of Ca^{2+} uptake at the monolayer and the release of Ca^{2+} already adsorbed on the monolayer. The studies reported here suggest that these two effects could be related to specific structural features in the peptides.

With the exception of des-B^{30}-alanine-insulin, all the peptides studied inhibited the uptake of Ca^{2+} by the monolayer. This action of peptides was greater for the intact insulin molecule than for the analogues of insulin some with disulfide bonds oxidized or with altered secondary and tertiary structure. This effect may be attributed partially to a lesser amount of insulin analogues adsorbed on the phosphate monolayer, resulting from the partial loss of surface activity. Since peptides bearing net positive charge at pH 7.4 in these studies (vasopressin, thyrocalcitonin)[4] were equally effective as those having net negative charge (insulin)[5], the effect of the peptides in inhibiting Ca^{2+} uptake is probably not entirely charge dependent. The exact structural requirement of the peptides for the inhibition of Ca^{2+} uptake is not apparent from this study.

On the other hand, the second action of peptides, the release of bound Ca^{2+}, can be related to certain structural features of the peptides.

Insulin and vasopressin facilitated markedly the release of Ca^{2+} adsorbed at the monolayer. The two hormones share the following structural features: each has the sequence proline followed by a positively charged amino acid in the two positions immediately preceding the C-terminal amino acid. Each has a six-membered disulfide ring at (vasopressin) or near the N-terminal end of the peptide, as in larger insulin molecule (Fig. 1) (Positions 6–11).

Insulin A-chain-S-sulfonate lacks the disulfide ring and the proline followed by a positively charged amino acid in the penultimate positions (Fig. 1); insulin B-chain-S-sulfonate lacks the disulfide ring but retains the proline followed by a positively charged amino acid in the penultimate position; oxytocin contains the disulfide ring and the proline, but lacks a positively charged amino acid next to the C-terminal amino acid; thyrocalcitonin, a single-chain peptide consisting of 32 amino acids, has an additional amino acid in the (7-membered) disulfide ring[6], and, in addition, has a particularly rigid C-terminal region (because of prolines in Positions 29 and 32) bearing a negatively charged glutamate in Position 30. These molecules do not affect the release of bound $^{45}Ca^{2+}$ from the phosphate monolayer (Figs. 2 and 3).

Des-A^1-glycine-des-B^1-phenylalanine-insulin, and insulin B-chain-S-sulfonate, which retain the integrity of the B-chain at the C-terminal end, facilitate the release of bound $^{45}Ca^{2+}$, though to a smaller extent than intact insulin molecules possessing both a disulfide ring and intact C-terminal end (Fig. 2).

Both oxytocin and vasopressin inhibit the uptake of $^{45}Ca^{2+}$, but only vasopressin facilitates the release of adsorbed $^{45}Ca^{2+}$. HOFMANN[7] pointed out that substitution of phenylalanine for isoleucine in Position 3, and of arginine or lysine for leucine in Position 8 converts compounds having predominantly oxytocic activity to those having predominantly vasopressor activity. Of the two kinds of substitution, the latter is the more influential. It is tempting to speculate that the positively charged arginine present in Position 8 (following proline at the C-terminal end) of vasopressin but absent in oxytocin, is important in the facilitation of Ca^{2+} release (Fig. 2).

It seems, then, that the presence of both structural features, a 6-membered disulfide ring and a penultimate positively charged amino acid preceded by proline, may be essential to the facilitation of release of bound Ca^{2+}, and, therefore, to the biphasic action on Ca^{2+} adsorption at the monolayer.

A conformational change in which negative charges are diminished—for example, micelle formation—might account for an increase in release of Ca^{2+}. Perhaps the positive charge near the C-terminal end of intact insulin or vasopressin reacts electrostatically with negative phosphate groups, binding the peptide to the monolayer, and this places the relatively less polar, more rigid disulfide ring region of the peptide in a position to form hydrogen bonds with the long-chain, non-polar portion of the monolayer substance, with resultant formation of micelles.

The biological significance of these findings may be appreciated from the following considerations.

Inhibition of adsorption of Ca^{2+} at the monooctadecyl phosphate monolayer by a peptide hormone may serve as a model for the inhibition of adsorption of Ca^{2+} at the outer surface of the plasma membrane of cells. Ca^{2+} is known to reduce the permeability of a number of different cells to a variety of substances[8-13]. Peptide hormones which diminish Ca^{2+} adsorption might therefore increase the permeability of sensitive cells to water and water-soluble substances.

If it is assumed that Ca^{2+} at the plasma membrane impedes the reabsorption of water in the renal tubule, then the action of vasopressin in inhibiting Ca^{2+} adsorption and facilitating the release of bound Ca^{2+} shown in this study could explain the antidiuretic action of the hormone *in vivo*. THORN AND SCHWARTZ[14] found that a release of Ca^{2+} accompanies both the antidiuretic action of vasopressin in the mammalian kidney and the action of vasopressin leading to an increased permeability to water by the toad bladder.

The profound inhibitory action of thyrocalcitonin on Ca^{2+} uptake at a monooctadecyl phosphate monolayer contrasts with the lack of the inhibitory activity of parathyroid hormone at a similar monolayer[1]. Structurally, parathyroid hormone is quite different from thyrocalcitonin, having no cystine[15]. A major *in vivo* action of parathyroid hormone, an enhancement of bone resorption[16], is apparently in opposition to the inhibitory action of thyrocalcitonin on bone resorption[17-19].

Cyclic-AMP, implicated as a secondary effector in some actions of insulin[20] and of vasopressin[21], was found to facilitate Ca^{2+} adsorption, in contrast to the inhibitory actions of insulin and vasopressin.

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