PROTEIN KINASE MEDIATED PHOSPHORYLATION OF THE RAT LIVER PLASMA MEMBRANE*

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Summary

Partially purified protein kinase from rat liver has been found to phosphorylate the isolated rat liver plasma membrane. The level of phosphorylation is increased two fold by 10^{-6}M cyclic AMP. Glucagon and epinephrine at 10^{-6}M to 10^{-7}M also stimulated the kinase dependent phosphorylation of the membrane, glucagon being more effective than epinephrine. The phosphorylated membrane has been found to bind more Ca^{++} than the control membrane. These studies suggest a new role for certain hormones. These hormones may influence the structure and function of the membrane, in particular the permeability of the membrane, by controlling the degree of phosphorylation and bound calcium.

Introduction

Cyclic-AMP dependent protein kinases have been found in many mammalian tissues and a variety of invertebrate phyla (1-6). The possible interrelations between calcium ions and cyclic-AMP have been discussed by Rasmussen (7). In a variety of tissues there is a connection between the stimulus (hormone or electrical), the response, Ca^{++} and cyclic AMP. It has been suggested (7) that a lack of external Ca^{++} alters the cell membrane permeability with subsequent leak of intracellular constituents into the medium. Cyclic-AMP has been shown to stimulate the phosphorylation of the isolated microsomal membranes from kidney and brain (8-10), the uptake and rate of turnover of Ca^{++} in the isolated sarcoplasmic reticulum of canine heart (11) and the calcium binding of particulate fractions of toad bladder (12).

In this paper we report the cyclic-AMP dependent phosphorylation of the isolated rat liver plasma membrane and further show that certain hormones such

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as glucagon and epinephrine also stimulate the kinase dependent membrane phosphorylation.

Methods and Materials

Rat liver plasma membranes were prepared by the method of Ray (13) and washed with EDTA and tris buffer before use. Rat liver protein kinase (through the ammonium sulfate precipitation and dialysis step) was prepared by the method of Yamamura et al (14). Aliquots were frozen at -15°. The reagents and their sources are as follows: Crystalline beef insulin and glucagon (Eli Lilly Laboratories), L-epinephrine (CalBiochem.), hydrocortisone (Nutritional Biochemicals Corp.), $^{45}CaCl_2$ and γ-^{32}P-ATP (ICN.), 3',5'-cyclic AMP (P-L Biochemicals).

The protein kinase activity was measured as follows: 1 mM γ-^{32}P-ATP. (0.1μC), 1 mM $MgCl_2$, 300μg kinase, and 500 μg of casein (Nutritional Biochem.) were incubated in 1 ml of 0.075M tris buffer pH 7.5 for 5 min. at 37° with or without 10^{-6}M cyclic-AMP. After incubation, 2.0 ml of 10% TCA were added and the mixture was passed through a Millipore filter disc (HA 0.45 μ, Millipore Corp.). The Millipore disc was washed two times with 5 ml of tris buffer and counted in 10 ml of Bray's scintillation cocktail (15).

Phosphorylation of the membrane for Ca^{++} binding: Membranes (1.5 - 1.8 mg protein) were incubated with 6 mg of kinase, 1 mM $MgCl_2$, 10^{-6}M c-AMP, and 1 mM ATP for 10 min at 37° C in a total volume of 20 ml of 0.075M tris buffer pH 7.5. The membrane pellet was obtained by centrifugation at 2000 RPM and washed two times with 5.0 ml of tris buffer. The washed pellet was resuspended in 5 ml of tris buffer. 0.2 ml aliquots (60-70 μg membrane protein) were diluted with 0.8 ml of tris buffer containing 0.5 μC of $^{45}Ca^{++}$ at a concentration range of 5×10^{-3} to 5×10^{-6}M Ca^{++}. The membranes were incubated for 10 min at 37° C, filtered through Millipore filter discs, washed two times with 5 ml of tris buffer and counted in Brays cocktail. Controls were also run in which the kinase was omitted.

Phosphorylation of the membrane was carried out by incubating 60-70 μg of membrane protein in 1 ml of 0.1 M tris buffer pH 7.5 containing 1 mM γ-^{32}P-

ATP (0.1 μC), 1 mM $MgCl_2$, 100-300 μg of kinase with and without 10^{-6} -10^{-8}M cyclic AMP. Membranes were also incubated under identical conditions without cyclic-AMP but containing 10^{-6} -10^{-7} M glucagon and epinephrine. Appropriate controls were also run in which $MgCl_2$ or the kinase were omitted. In order to distinguish phosphorylation of the membrane from ordinary binding of ATP to the membrane, the incubations were carried out with 1 mM 8 - ^{14}C-ATP (0.5 μC) (New England Nuclear Corp.) in place of γ-^{32}P-ATP. In this case the kinase or the kinase with either cyclic AMP or hormones had no effect on the binding of ^{14}C-ATP to the membrane.

Results

The results in Figure 1 show the dependency of phosphorylation of the membrane on the cyclic-AMP concentration. Cyclic AMP at 10^{-6} M gave the great-

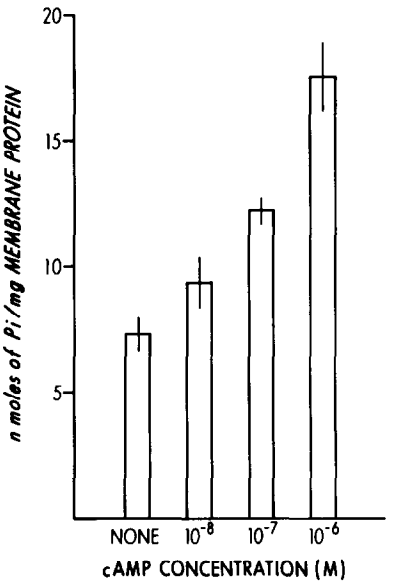

Figure 1. The effect of cyclic-AMP on the protein kinase dependent phosphorylation of the plasma membrane. Membranes (60-70 μg protein) were incubated in 0.1M tris buffer pH 7.5 in a total volume of 1.0 ml containing 300 μg of protein kinase, 1 mM $MgCl_2$, 1 mM γ-^{32}P-ATP (0.1 μC), and varying amounts of cyclic-AMP. After 10 min incubation at 37° C the membranes were collected on HA 0.45μ Millipore filter discs, washed two times with 5 ml of tris buffer. The discs were placed in scintillation vials, 10 ml of Brays cocktail were added and the radioactivity determined.

est stimulation. The stimulation was slightly over 2 fold and is in agreement with the degree of stimulation reported for rat liver protein kinase with histone as substrate (1).

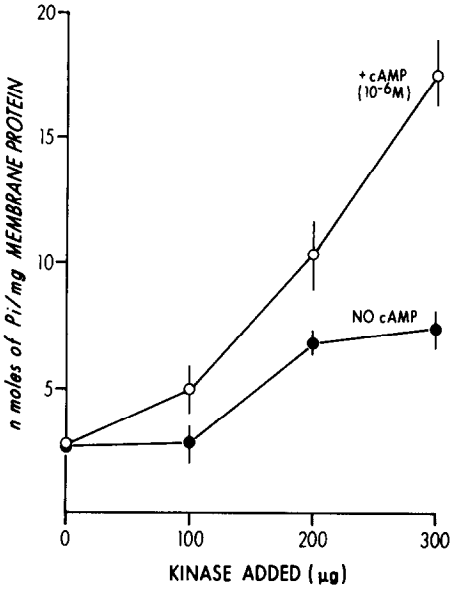

Figure 2. Phosphorylation of the plasma membrane as a function of protein kinase concentration with and without cyclic-AMP. Membranes were incubated as described in Fig. 1 except that the kinase concentration and cyclic AMP concentration were varied as shown in the Figure.

The effect of the protein kinase concentration on membrane phosphorylation is shown in Fig. 2. Between 100-300 µg of protein kinase, the degree of phosphorylation with cyclic AMP is nearly linear with respect to enzyme concentration.

Inasmuch as the plasma membrane contains a hormone responsive adenylcyclase, it was expected that the addition of hormones which stimulate adenyl cyclase would produce sufficient cyclic AMP to stimulate the protein kinase. Experiments were therefore carried out in which membranes were incubated with the protein kinase with and without glucagon and epinephrine. The results given in Figure 3 show that glucagon and epinephrine both stimulate the kinase mediated phosphorylation of the membrane. Glucagon is more effective than epinephrine. This observation is in accord with our observation (16) and that

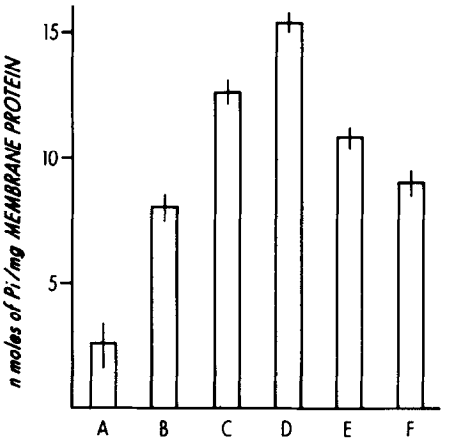

Figure 3. The effect of glucagon and epinephrine on the protein kinase dependent phosphorylation of the plasma membrane. Membranes were incubated as described in Figure 1 except that the glucagon and epinephrine concentrations were varied. A - no kinase added, B - kinase alone, C - kinase + 10^{-6} M glucagon, D - kinase + 10^{-7} M glucagon, E - kinase + 10^{-6} epinephrine, F - kinase + 10^{-7} M epinephrine.

of Pohl et al (17) that glucagon gives a greater stimulation of adenyl cyclase in rat liver plasma membranes than does epinephrine.

Since the phosphorylation of the membrane would yield polar phosphate groups which might bind Ca^{++}, the binding of Ca^{++} to control membranes and phosphorylated membranes was studied. The results in Table 1 show that the phosphorylated membrane binds more Ca^{++} than the control membrane.

These studies provide new data on hormone-Ca^{++}-protein kinase interactions with the plasma membrane and point to a system for the hormonal control of membrane permeability via Ca^{++} binding.

CALCIUM BINDING TO THE PHOSPHORYLATED AND NON-PHOSPHORYLATED PLASMA MEMBRANE*

	Binding Constants(M^{-1})		Number of Binding Sites pmoles/mg membrane protein	
	K_1	K_2	n_1	n_2
Control Membrane	4.0×10^3	3.1×10^2	22	126
Phosphorylated Membrane	3.0×10^3	3.6×10^2	30	146

* See text for experimental details.

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