

# The Glucagon-sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver

## II. COMPARISON BETWEEN GLUCAGON- AND FLUORIDE-STIMULATED ACTIVITIES\*

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### SUMMARY

Glucagon and fluoride ion stimulate the activity of a common adenyl cyclase system in plasma membranes isolated from rat liver. Their actions are noncompetitive indicating that they act at separate sites in this system. Manganous ion, above 5.0 mM, inhibited selectively the response of the enzyme to glucagon. Inorganic pyrophosphate (1.5 mM), on the other hand, inhibited the response of the enzyme to fluoride but enhanced the response to glucagon. The inhibitory effect of pyrophosphate was noncompetitive with fluoride ion. Highly purified phospholipase A caused a selective loss of the glucagon response and enhanced the stimulatory effect of fluoride ion. Treatment of liver membranes with digitonin also caused a selective loss of hormone response. Inactivation of glucagon response by digitonin was not restricted to the liver membrane adenyl cyclase system; incubation of ghosts of fat cells with digitonin resulted in loss of response of the adenyl cyclase system to glucagon, adrenocorticotropin, secretin, and epinephrine at very low concentrations of the detergent. Digitonin enhanced the response of the fat cell system to fluoride ion. Sodium dodecyl sulfate, over a narrow range of concentrations, inhibited the response of liver membrane adenyl cyclase to glucagon, and enhanced the enzyme's response to fluoride ion. Other detergents caused a parallel loss of the response to both fluoride ion and glucagon.

The present findings suggest that glucagon and fluoride ion activate adenyl cyclase by different mechanisms. It is possible that these agents react through different molecular entities in this complex enzyme system.

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It has been postulated (2) that animal adenyl cyclase systems contain distinct molecular components, termed "discriminators," which react specifically with hormones and through which the catalytic component, adenyl cyclase, is activated. This postulate is based in part from the observation that a single adenyl

cyclase enzyme in fat cells (or their "ghosts") of the rat is activated by glucagon, secretin, adrenocorticotropin, epinephrine, thyrotropin, and luteinizing hormone through sites that are specific for each of the hormones (3, 4). Fluoride ion, which activates all adenyl cyclase systems in eucaryotic cells (5, 6), also activates the hormone-sensitive adenyl cyclase system in fat cells (7). Since fluoride ion appears to act nonspecifically on adenyl cyclase systems, it is likely that the halide ion does not activate the system through the hormone-discriminators but possibly through some direct action on the catalytic component.

The purpose of this paper is to establish that glucagon and fluoride ion activate the same adenyl cyclase system in liver plasma membranes and do so by different mechanisms. Such information not only helps to establish the complexity of the adenyl cyclase system in these membranes, but also assists in efforts to isolate and characterize the fluoride-sensitive component and the discriminator for glucagon.

### EXPERIMENTAL PROCEDURE

All experiments using plasma membranes were carried out with the partially purified preparation described in the preceding report (8). Preparation of isolated fat cells and fat cell ghosts from rat adipose tissue are described elsewhere (7, 9). Highly purified phospholipase A (EC 3.1.1.4),  $\alpha$  type (10), 1400 units per mg, was generously supplied by Dr. Michael A. Wells (University of Arizona, Tucson). Digitonin, from Mann, was recrystallized twice from ethanol before it was used. EGTA<sup>1</sup> was obtained from Sigma. The sources of all other chemicals have been described previously (7, 8).

The incubation mixture for determining adenyl cyclase activity in liver membranes and fat cell ghosts contained, in 0.05-ml volume, the following: 3.2 mM ATP- $\alpha$ -<sup>32</sup>P (30 to 50 cpm per pmole), 5.0 mM MgCl<sub>2</sub>, 25 mM Tris-HCl, pH 7.6, 20 mM creatine phosphate, 1 mg per ml of creatine kinase (20 to 50 units per mg), 1 mM EDTA, and between 20 and 40  $\mu$ g of liver membranes or 50  $\mu$ g of fat cell ghost protein. The reaction was stopped as described before (7) and cyclic 3',5'-AMP formed was isolated and determined according to the method of Krishna, Weiss, and Brodie (11).

<sup>1</sup> The abbreviations used are: EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N'*-tetraacetic acid; cyclic AMP, cyclic 3',5'-monophosphate.

\* Some of the studies have been reported in preliminary form (1).

In all figures and tables, adenyl cyclase activity refers to nanomoles of cyclic 3',5'-AMP formed in 10 min per mg of protein.

### RESULTS

Glucagon and fluoride ion act on the same adenyl cyclase system in rat liver plasma membranes. This was established by the experiment shown in Table I in which maximal stimulating concentrations of fluoride ion (15 mM) and glucagon (10  $\mu$ g per ml) were added to the adenyl cyclase incubation medium either alone or in combination. Combination of the two agents failed to stimulate the activity of the enzyme more than glucagon alone. The lack of competitive interaction between the two agents suggested that fluoride ion and glucagon activate the enzyme through different sites. This finding permitted an evaluation of the possible different characteristics of the fluoride- and glucagon-responsive components contained within the same enzyme system.

**Effects of Divalent Ions**—Adenyl cyclase systems require for their activity a divalent cation, and both  $Mg^{++}$  and  $Mn^{++}$  support enzymatic activity. This was first shown for dog brain adenyl cyclase (5) and later for a variety of adenyl cyclase systems (6). The effect of varying concentrations of  $Mn^{++}$  on glucagon- and fluoride-stimulated activities in liver plasma membranes is shown in Fig. 1. At concentrations of  $Mn^{++}$  below 5.0 mM, both the response to glucagon and to fluoride ion

were stimulated. However, at concentrations higher than 5.0 mM,  $Mn^{++}$  inhibited selectively the response of the enzyme to glucagon. Selective stimulation of the fluoride response has been observed also in the fat cell ghost adenyl cyclase system (7).

$Mg^{++}$  acted somewhat differently than  $Mn^{++}$  in that it stimulated, in the presence of 1 mM EDTA, the response to glucagon over a narrow range of concentrations, and caused parallel loss of basal, fluoride-, and glucagon-stimulated activities at higher concentrations (see Fig. 6 in the previous report (8)).

**Effects of Inorganic Pyrophosphate**—Pyrophosphate, at 1.5 mM, inhibited the response of the liver enzyme to fluoride ion by 76% whereas it stimulated the response to glucagon by 30%, as shown in Fig. 2. The selective inhibitory effect of pyrophosphate on the fluoride response was noncompetitive since doubling the concentration of fluoride ion failed to reduce the inhibitory effect of pyrophosphate.

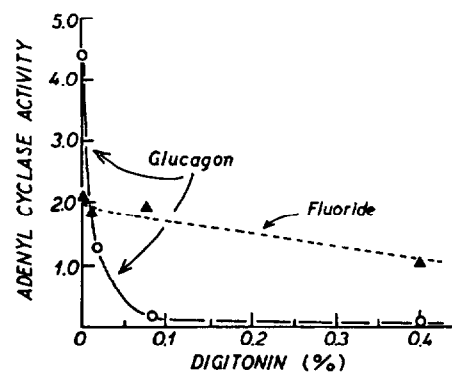


FIG. 4. Effect of digitonin on response of adenyl cyclase activity in liver plasma membranes to glucagon and fluoride ion. Liver plasma membranes (0.6 mg per ml) were incubated for adenyl cyclase activity in the presence of either 20  $\mu$ g per ml of glucagon (O) or 10 mM NaF ( $\blacktriangle$ ) and the indicated concentrations of digitonin. Incubations were carried out for 10 min at 30°.

TABLE I  
Effect of glucagon and fluoride ion on adenyl cyclase activity in liver plasma membranes

Additions	Adenyl cyclase activity <sup>a</sup>
Glucagon (10 $\mu$ g per ml).....	4.28 $\pm$ 0.23
NaF (15 mM).....	2.47 $\pm$ 0.12
Glucagon (10 $\mu$ g per ml) + NaF (15 mM)....	4.10 $\pm$ 0.15

<sup>a</sup> Values are mean  $\pm$  half the range of triplicate determinations.

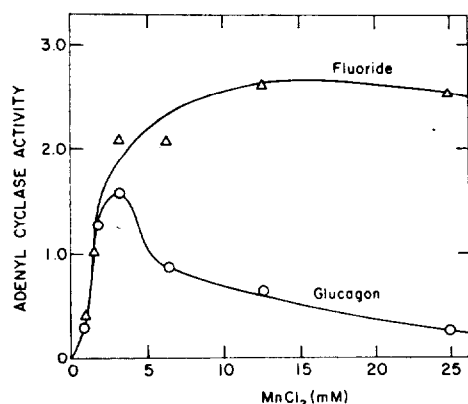


FIG. 1 (left). Effect of varying concentrations of  $MnCl_2$  on adenyl cyclase activity determined in the presence of either 10  $\mu$ g per ml of glucagon or 10 mM NaF. EDTA and  $MgCl_2$  were omitted from the standard assay medium.

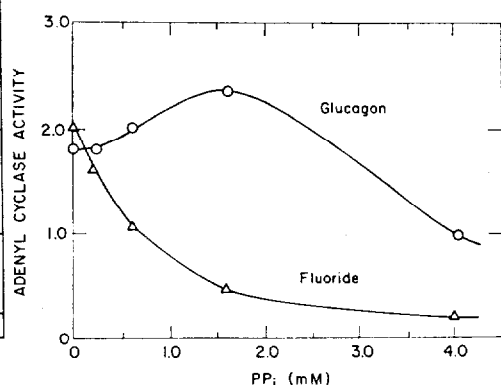


FIG. 2 (center). Effect of varying concentrations of inorganic pyrophosphate on adenyl cyclase activities determined in the presence of 10  $\mu$ g per ml of glucagon (O) or 10 mM NaF ( $\Delta$ ). EDTA was omitted from the standard assay medium.

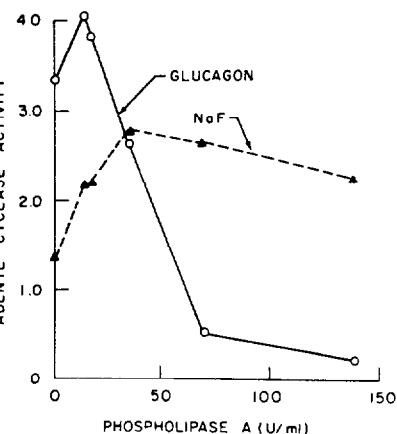


FIG. 3 (right). Effect of treatment of liver plasma membranes

with phospholipase A on the response of adenyl cyclase to glucagon and fluoride ion. Liver plasma membranes (3.5 mg per ml) were incubated in a medium containing 1 mM  $CaCl_2$ , 50 mM Tris-HCl, pH 7.6, and the indicated concentrations of phospholipase A. Incubations were carried out for 10 min at 30° and were terminated by addition of 2.0 mM EGTA. Then, 10- $\mu$ l aliquots of these mixtures were analyzed for adenyl cyclase activity in the presence of either 10  $\mu$ g per ml of glucagon (O) or 10 mM NaF ( $\blacktriangle$ ).

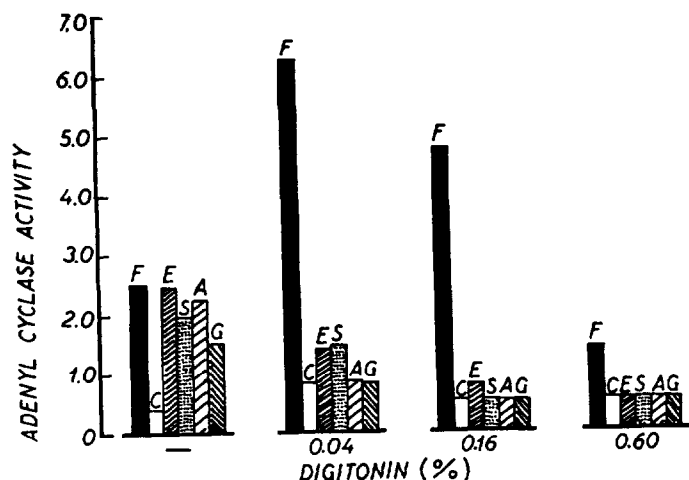


FIG. 5. Effect of digitonin on response of adenyl cyclase in fat cell ghosts to hormones and fluoride ion. Fat cell ghosts (1.0 mg per ml) were incubated in adenyl cyclase assay incubation medium (7) containing the indicated concentrations of digitonin. Activity was determined in the absence (Control, C) and the presence of either 10 mM NaF (F) or 10  $\mu$ g per ml of epinephrine (E), secretin (S), ACTH (A), and glucagon (G). Incubations were carried out for 10 min at 30°.

**Effects of Phospholipase A**—We have reported previously (12) that treatment of liver plasma membranes with heat-treated snake venom, rich in phospholipase A activity, results in selective loss of the glucagon response and stimulation of the response of the enzyme to fluoride ion. In Fig. 3, it is seen that highly purified phospholipase A has the same effects. In these experiments, membranes were treated for 5 min at 30° with phospholipase A, followed by the addition of EGTA, a calcium chelator, to stop the action of the calcium-dependent enzyme (13). Addition of the chelator prior to that of the enzyme resulted in complete inhibition of the effects of phospholipase A on the response of adenyl cyclase to glucagon and fluoride ion. This is additional evidence that the observed effects were due to the enzymatic action of phospholipase A.

**Effects of Detergents**—The effects of phospholipase A suggested that lipids play a role in the activation of adenyl cyclase by glucagon and fluoride ion. This possibility was investigated further by testing the effects of a variety of detergents. As shown in Fig. 4, digitonin (a neutral detergent) caused selective loss at concentrations less than 0.1%, of the response of liver adenyl cyclase to glucagon.

Selective inactivation by digitonin of the response of adenyl cyclase to glucagon was not restricted to the plasma membranes of rat liver. In Fig. 5, it can be seen that digitonin also inhibited the response of the fat cell ghost adenyl cyclase system to glucagon as well as to secretin, adrenocorticotropin, and epinephrine. As was observed with liver membranes treated with phospholipase A, digitonin stimulated the response of the fat cell ghost system to fluoride ion. This effect of digitonin was not observed with the liver membrane system.

In experiments not shown, sodium dodecyl sulfate, at 0.008%, stimulated the fluoride response in liver adenyl cyclase system by 2-fold and caused the loss of 40% of the response to glucagon. Higher concentrations of the detergent caused complete loss of both activities. Triton X-100 and sodium deoxycholate in-

hibited the response to both fluoride ion and glucagon in a parallel fashion.

#### DISCUSSION

The present studies show that glucagon and fluoride ion activate a common adenyl cyclase system in liver plasma membranes through processes that have markedly different characteristics. It is likely that the two activation processes represent different molecular components in this complex enzyme system, as has been suggested previously from studies with the adenyl cyclase system in fat cells (2). Understanding of the molecular basis for the selective effects of divalent cations, pyrophosphate, phospholipase A, and detergents must await isolation and characterization of the components of the adenyl cyclase system.

Selective inactivation by detergents of the glucagon-response in liver membranes or of the response of the fat cell ghost system to several hormones suggests that this is a general characteristic of the processes through which hormones activate adenyl cyclase. Other examples of selective inactivation of hormone response by detergents or agents thought to act as surfactants, such as phenothiazines, have been reported for a variety of adenyl cyclase systems (14).

The catalytic component of the adenyl cyclase system, as reflected by fluoride activation, is either unaffected or enhanced by agents that alter lipids, whereas the hormone-activated processes are inactivated by these agents. It would appear that the structures of the catalytic component and the component or components involved in hormone activation are modified differently by removal or modification of lipids. The sensitivity of the hormone-stimulated processes to detergents or phospholipase A suggests either that the specific recognition sites for the hormones, termed discriminators, are lipoproteins or that coupling between discriminator and the catalytic component requires the presence of lipids. We have reported elsewhere (1, 15) that addition of phospholipids to liver membranes depleted of lipid by digitonin-treatment results in partial restoration of the response of adenyl cyclase to glucagon. In the following report (16), evidence will be presented that the discriminator for glucagon behaves as a lipoprotein.

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