Activation of Protein Kinase and Glycogen Phosphorylase in Isolated Rat Liver Cells by Glucagon and Catecholamines

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MORRIS J. BIRNBAUM AND JOHN N. FAIN
From the Section of Physiological Chemistry, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

In liver cells isolated from fed female rats, glucagon (290 nM) increased adenosine 3':5'-monophosphate (cyclic AMP) content and decreased cyclic AMP binding 30 s after addition of hormones. Both returned to control values after 10 min. Glucagon also stimulated cyclic AMP-independent protein kinase activity at 30 s and increased protein kinase activity assayed in the presence of 2 μM cyclic AMP at 1 min. Glucagon increased the levels of glycogen phosphorylase a, but there was no change in total glycogen phosphorylase activity.

Glucagon increased glycogen phosphorylase a at concentrations considerably less than those required to affect cyclic AMP and protein kinase. The phosphodiesterase inhibitor, 1-methyl-3-isobutyl xanthine, potentiated the action of glucagon on all variables, but did not increase the maximum activation of glycogen phosphorylase.

Epinephrine (1 μM) decreased cyclic AMP binding and increased glycogen phosphorylase a after a 1-min incubation with cells. Although 0.1 μM epinephrine stimulated phosphorylase a, a concentration of 10 μM was required to increase protein kinase activity. 1 Methyl-3-isobutyl xanthine (0.1 mM) potentiated the action of epinephrine on cyclic AMP and protein kinase. (-)-Propranolol (10 μM) completely abolished the changes in cyclic AMP binding and protein kinase due to epinephrine (1 μM) in the presence of 0.1 mM 1-methyl-3-isobutyl xanthine, yet inhibited the increase in phosphorylase a by only 14%.

Phenylephrine (0.1 μM) increased glycogen phosphorylase a, although concentrations as great as 10 μM failed to affect cyclic AMP binding or protein kinase in the absence of phosphodiesterase inhibitor. Isoproterenol (0.1 μM) stimulated phosphorylase and decreased cyclic AMP binding, but only a concentration of 10 μM increased protein kinase. 1-Methyl-3-isobutyl xanthine potentiated the action of isoproterenol on cyclic AMP binding and protein kinase, and propranolol reduced the augmentation of glucose release and glycogen phosphorylase activity due to isoproterenol.

These data indicate that both α- and β-adrenergic agents are capable of stimulating glycogenolysis and glycogen phosphorylase a in isolated rat liver cells. Low concentrations of glucagon and β-adrenergic agonists stimulate glycogen phosphorylase without any detectable increase in cyclic AMP or protein kinase activity. The effects of α-adrenergic agents appear to be completely independent of changes in cyclic AMP and protein kinase activity.

Glucagon and epinephrine increase cyclic AMP accumulation and glycogen breakdown in the perfused rat liver and in isolated hepatocytes (1–4). There still exist several key problems in understanding the control of glucose mobilization by hormones. Although an activation of protein kinase, phosphorylase kinase, and glycogen phosphorylase in isolated liver cells by glucagon has been reported (5), these changes have not been systematically investigated and correlated with variations in cyclic AMP concentrations.

The nature of the adrenergic stimulation of glycogenolysis has proven even more difficult to understand. There is now substantial evidence that α-adrenergic stimulation of liver produces significant increases in glucose production, both via glycogenolysis and gluconeogenesis (4, 6). The α-adrenergic glycogenolytic response has been studied in isolated perfused liver (7–9), but this system is limited by several factors. α-Adrenergic agonists decrease blood flow in the perfused liver, and the hypoxia produces significant hepatic glycogenolysis (10). It is therefore extremely difficult to evaluate the relative roles of α- and β-adrenergic stimulation on the hepatic parenchymal cell or study the biochemical mechanisms of increased glycogen metabolism in perfused liver. The use of suspensions of isolated hepatocytes obviates these problems.

One approach to the elucidation of the biochemical mechanism of α-adrenergic increases in glycogenolysis is to determine the enzyme or enzymes that are modulated in response to stimulation. A logical place to begin would be with those enzymes postulated to be responsible for cyclic AMP-mediated augmentation of glycogenolysis, that is, the classical "cascade" or "amplification" system that ultimately results in the breakdown of hepatic glycogen to glucose 1-phosphate (11). This report describes an investigation designed to determine the nature of catecholamine effects on the first and the last enzymes in this cascade, protein kinase and glycogen phosphorylase a, respectively, and to correlate these changes with alterations in the levels of cyclic AMP.

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1 The abbreviation used is cyclic AMP, adenosine 3':5'-monophosphate.
Protein Kinase and Glycogen Phosphorylase in Liver

MATERIALS AND METHODS

Liver cells were isolated from 150- to 200-g female Sprague-Dawley rats (Charles River, CD strain) fed laboratory chow ad libitum. The isolation procedure was that of Berry and Friend (12) as previously described (13). The portal vein was cannulated with a plastic cannula and immediately connected to a perfusion apparatus equipped with a peristaltic pump (MRA Corp., Boston, Mass.). During excision and removal to the perfusion platform, the liver was continuously perfused with 80 ml of calcium-free Krebs-Ringer bicarbonate buffer (NaCl (120 mM), KCl (4.8 mM), KH₂PO₄ (1.2 mM), MgSO₄ (1.2 mM), NaHCO₃ (24 mM), pH 7.4) containing glucose (100 mg/ml), and heparin (1 to 2 units/ml) which was not recirculated. The blanched liver was perfused for 30 to 40 min with recirculating calcium-free buffer containing glucose and 12 to 15 mg of type II collagenase (Worthington, Lot 45A145) and then for 3 min with buffer without collagenase. Cells were dispersed with a metal spatula, filtered through nylon chiffon (200- to 250-µm mesh), and sedimented by centrifugation at 50 × g for 9 min. The hepatocytes were resuspended and washed twice with Krebs-Ringer bicarbonate buffer and the concentration of glucose utilized in the particular experiment. Cells were counted with a hemocytometer. The isolation procedure, as well as all subsequent incubations, were performed at 37°C in an atmosphere of 95% oxygen, 5% carbon dioxide.

The isolated hepatocytes (6 to 15 × 10⁶ cells) were incubated in plastic tubes (17 × 100 mm) containing 1 ml of ice-cold Krebs-Ringer bicarbonate buffer in a total volume of 1 ml and shaken in an orbital water bath shaker (Lab-Line Instruments, Inc.) at 150 to 200 rpm. The process of those steps becomes apparent that the reproducibility of basal values for most parameters could be greatly improved by preincubating cells in the same tubes as the final incubations and initiating the reactions by addition of the appropriate drugs. Basal glycogen phosphorylase activity was lower and more stable if the hepatocytes were incubated for at least 30 min in the presence of 100 mg/ml of glucose prior to the start of the experiments. This procedure did not affect either cyclic AMP levels or protein kinase activity. All incubations were terminated by adding 1 ml of ice cold Krebs-Ringer bicarbonate buffer to the cells and immediately placing them in an ice bath. Cells were centrifuged in the cold for 3 min. The pellet was kept briefly in an ice bath, and the supernatant either discarded or analyzed for glucose by the glucose oxidase method (14).

The cells were then suspended in 1 ml of ice-cold buffer (pH 7.0) containing 10 mM morpholinopropanesulfonic acid (Mops), 50 mM NaF, 5 mM EDTA, and 1 mM diethiothreitol, and homogenized with a Wilems Polytron (Brinkmann Instruments, Inc.) for 20 at setting 5. The homogenates were centrifuged at 10,000 × g for 15 min at 0°C, and the pellets discarded. All assays were performed in the supernatant, which contains 5 to 10 mg of protein/ml.

For assay of cyclic AMP content, an aliquot of the supernatant was removed and 2 N HCl was added to give final a concentration of 0.02 N HCl. Samples were heated in a boiling water bath for 1 min and trichloroacetic acid at 0°C, 30 min in 5% acid at room temperature, and 5 min in acetone at room temperature. The discs were dried with a heat lamp and counted in toluene with 8% 2,5-diphenyloxazole and 2% p-bis-(6- methylstyril) benzene (Pb120). Rabbit liver glycogen (Sigma) was purified by passage over an Amberlite MB-1 (Mallinckrodt) column followed by precipitation with ethanol. Caffeine was included in the incubation buffer for 5 min at 30°C before addition of the substrate to control for any possible contribution of glycogen phosphorylase activity within an experiment as well as minor unavoidable differences between assays performed on separate days. However, in all experiments presented below, increases in the protein kinase activity ratio were accompanied by increases in the activity assayed in the absence of caffeine, and the latter were often more variable and sometimes not statistically significant.

Glycogen phosphorylase was measured using a filter disc assay similar to that of Gilboe et al. (20). The total incubation volume was 0.06 ml and consisted of 60 mM morpholinoethanesulfonic acid (Mops), pH 6.1, 1% glycogen, 15 mCi [14C]glucose 1-phosphate (about 0.005 μCi/μmol, New England Nuclear), and either 0.5 mM caffeine or 0.5 mM Na₂SO₄ and 1 mM AMP. Rabbit liver glycogen (Sigma) was purified by passage over an Amberlite MB-1 (Mallinckrodt) column followed by precipitation with ethanol. Caffeine was included in the incubation buffer for 5 min at 30°C before addition of the substrate to control for any possible contribution of glycogen phosphorylase activity.

Sources of the materials used for these studies are the following: (-)-epinephrine (3,4 dihydroxy-α-(methylamine)methyll-benzyl alcohol), (-)-isoepinephrine hydrochloride (3,4 dihydroxy-α-(isopropylaminomethyl)-benzyl alcohol hydrochloride), (-)-phenylephrine hydrochloride (1-α,methylnlethyll-benzyl alcohol hydrochloride), MeS (2-N-(methylphenoil)thanesulfonic acid), Mops (morpholinopropanesulfonic acid), Tris (tris(hydroxymethyl)amino methane), glucose oxidase, α-glucose 1-phosphate (Grade V), adenosine 5'-monophosphoric acid, horseradish peroxidase, adenosine 5'-orthophosphate, and glucose 6-phosphate were purchased from Sigma Chemical Co.; 1-methyl-3-isobutyl xanthine was obtained from Aldrich Chemical Co. (–)-Propanolol (1-isopropylaminomethyl)-3-
Glucagon (290 nM) increased intracellular cyclic AMP content and decreased cyclic AMP binding in isolated liver cells from fed female rats (Fig. 1). Maximal changes were 30 s after addition of the hormone, the earliest time point measured. Content and binding varied inversely to each other, and both approached control values at 10 min. It is likely that the decrease in binding is simply a result of the increase in endogenous, competing cyclic AMP. The time course for cyclic AMP content presented in Fig. 1 is essentially consistent with one previously reported using a slightly different treatment of isolated liver cells from fasted rats for extraction of cyclic AMP (3).

Protein kinase, assayed in the absence of cyclic AMP and using a mixture of calf thymus histones as substrate, was elevated at 30 s after the addition of a large amount of glucagon. For these studies, hepatocytes were homogenized in a medium that did not contain a phosphodiesterase inhibitor, as it was determined that the inclusion of 0.1 mM 1-methyl-3-isobutyl xanthine during homogenization did not significantly improve the yield of protein kinase activity or the magnitude of hormone-induced changes. The stimulation of protein kinase activity declined gradually during the 10-min incubation of isolated cells. In other experiments, protein kinase activity returned to control levels by 20 min after addition of hormones.

Total protein kinase activity, as determined by assay in the presence of 2 μM cyclic AMP was depressed 1 min after addition of glucagon (Fig. 1). At that time, the activity decreased maximally, and then slowly returned to basal. The decrease in total protein kinase observed in several tissues has been attributed to "translocation" of kinase (24) or to the nonspecific association of free catalytic subunit with particulate matter (25), which was removed by low speed centrifugation in these studies. This decrease has not been observed before in isolated hepatocytes (5). Although the data presented here do not indicate a specific mechanism, the observed lag does suggest that the diminution in total protein kinase activity after hormonal stimulation is an event occurring in the intact cell and not an artifact generated during homogenization.

As shown in Fig. 1, the changes in cyclic AMP and protein kinase in response to 290 nM glucagon were paralleled by a rise in glucagon phosphorylase α. Phosphorylase α activity remained elevated for 10 min after the rapid activation. However, at 20 min after addition of glucagon, phosphorylase a levels had returned to control levels (data not shown). The increase in phosphorylase α activity is most likely due to the conversion of phosphorylase b to phosphorylase a by phosphorylation; the increase in activity could not be accounted for on the basis of a change in "total" phosphorylase activity (Fig. 1).

Glucagon also increased glucose release from hepatocytes isolated from fed rats and incubated in the absence of any gluconeogenic substrate (Fig. 1). It was difficult to determine changes in glucose release at early time points due to a high background, but there appeared to be an augmentation in release 4 min after the addition of hormone. This increase was more apparent after 10 min incubation, but both basal and stimulated glucose release appeared to be linear over 10 min.

Fig. 2 presents dose response curves for glucagon in the absence and presence of the phosphodiesterase inhibitor 1-methyl-3-isobutyl xanthine. Both agents were added for the last 1 min of incubation. Glucagon, at a concentration of 5 nM both decreased cyclic AMP binding and increased cyclic AMP content. 1-Methyl-3-isobutyl xanthine (0.1 mM) potentiated the action of glucagon on both variables but was without effect in the absence of hormone. 1-Methyl-3-isobutyl xanthine reduced the minimal effective concentration of glucagon which decreased cyclic AMP binding, and augmented the effect of the hormone at higher concentrations.

The dose-response curve for the effect of glucagon on protein kinase activity essentially paralleled that on cyclic AMP levels (Fig. 2). However, 29 nM glucagon maximally increased protein kinase, as expressed either as cyclic AMP-independent activity or as the activity ratio. Total protein kinase activity decreased with increasing glucagon concentration. 1-Methyl-3-isobutyl xanthine potentiated the action of glucagon on total and cyclic AMP-independent protein kinase activity, and produced a small but consistent stimulation in the absence of any hormone. In one series of experiments, 1-methyl-3-isobutyl xanthine increased the protein kinase activity ratio from 0.23 ± 0.020 to 0.25 ± 0.020 (n = 8, p < 0.001 by paired comparisons). These changes are consistent with, although considerably smaller in magnitude than those reported for activation of hepatic protein kinase in vivo by methyl xanthines (26). In isolated hepatocytes, 1-methyl-3-isobutyl xanthine lowered the minimal effective concentration of glucagon necessary for activation of the protein kinase, and increased the stimulation produced by any greater dose, including maximally active hormone concentrations. 1-Methyl-3-isobutyl xanthine potentiated the decrease in total protein kinase activity produced by
all effective concentrations of glucagon except the highest (290 nM).

The lowest concentration of glucagon tested (1 nM) increased the level of glycogen phosphorylase a (Fig. 2). Thirty times this concentration maximally activated phosphorylase, but all doses were without effect on "total" activity. The lowest concentration of glucagon that produced a detectable rise in protein kinase or decrease in cyclic AMP binding was far greater than that required for activation of glycogen phosphorylase. A similar phenomenon has been observed for the relationship between cyclic AMP content and glycogenolysis in goldfish hepatocytes after stimulation with β-adrenergic agents (27).

The effect of 1-methyl-3-isobutyl xanthine on glycogen phosphorylase a in the absence of hormone proved to be quite variable. Generally, the phosphodiesterase inhibitor alone slightly activated the enzyme, as indicated in Fig. 2. However, in any given series of experiments, this difference was quite inconsistent and sometimes not apparent (Figs. 4 to 6). It is likely that variations in the effect of the phosphodiesterase inhibitor were in fact due to fluctuation in the basal phosphorylase activity during the course of an experiment. This inconsistency was not observed for the protein kinase activity, which might contribute to the small but significant increases produced by 1-methyl-3-isobutyl xanthine on that enzyme.

1-Methyl-3-isobutyl xanthine potentiated the activation of glycogen phosphorylase by submaximal concentrations of glucagon (Fig. 2). The xanthine decreased the dose of glucagon necessary for maximal stimulation by about 10-fold, without altering the maximal activity. In the presence of 1-methyl-3-isobutyl xanthine, a half-maximal activation of protein kinase corresponds to a maximal activation of the phosphorylase. Thus, the same activity of protein kinase correlated with a maximal stimulation of phosphorylase in the absence or presence of phosphodiesterase inhibitor. It appears that while protein kinase activity is responsive to changes in cyclic AMP over a large range, a relatively small change in protein kinase is sufficient to completely activate glycogen phosphorylase.

Epinephrine, a naturally occurring catecholamine possessing both α- and β-adrenergic activity, increases glycogenolysis in isolated hepatocytes (2, 6). The time course for the effects of 1 μM epinephrine on cyclic AMP and glycogen phosphorylase are presented in Fig. 3. Cyclic AMP content also appeared to increase after exposure to catecholamine, but these differences were not statistically significant. Based on the nature of the changes in cyclic AMP binding and content, in which the two

**Fig. 2.** Dose-response curve for glucagon action on cyclic AMP binding and content, protein kinase, and glycogen phosphorylase with and without 1-methyl-3-isobutyl xanthine. Isolated liver cells (6 to 10 x 10^6 cells/ml) were incubated in Krebs-Ringer bicarbonate buffer. Glucagon was added at the indicated concentrations in the absence (circles) or presence (triangles) of 1 mM 1-methyl-3-isobutyl xanthine (1 min before the end of the incubation). Open symbols indicate protein kinase assayed in the presence of 2 μM cyclic AMP. Values are the means of two paired experiments.

**Fig. 3.** Time-course for epinephrine action on cyclic AMP binding, cyclic AMP content, and glycogen phosphorylase. Isolated liver cells (9 to 12 x 10^6 cells/ml) were incubated in Krebs-Ringer bicarbonate buffer for 20 min prior to the start of the experiment. The cells were then incubated for the time indicated in the absence (circles) or presence (squares) of 1 μM epinephrine. Data for cyclic AMP binding and content are expressed as the difference from control due to epinephrine. Values are the means ±S.E. of three paired experiments. Significant effects of epinephrine are depicted by asterisks (*p < 0.05, **p < 0.01).
were consistently inversely related to each other, it is likely that the decrease in binding simply reflects an increase in intracellular cyclic AMP content. The denaturation steps required to prepare cells for cyclic AMP assay may increase the variability of the data and account for the lack of statistical significance in these experiments. Therefore, cyclic AMP binding appears to be a better reflection of the endogenous concentration of the nucleotide than the assay of total cyclic AMP.

Epinephrine (1 μM) significantly increased glycogen phosphorylase activity 1 min after the addition of hormone. This was due to a net conversion of phosphorylase b to phosphorylase a, since there was no change in the "total" glycogen phosphorylase activity. The increased phosphorylase a levels returned to control values at 10 min. This is consistent with the effect of epinephrine on glucose release, which the hormone increased at 5 min, the earliest measurable time point (data not shown). However, at 10 min the rate of release from stimulated cells had returned to control values.

A decrease in cyclic AMP binding was seen with as little as 0.1 μM epinephrine in the presence of 1-methyl-3-isobutyl xanthine (Fig. 4). However, 10 μM epinephrine was required to significantly reduce binding in the absence of 1-methyl-3-isobutyl xanthine.

The protein kinase activity ratio correlated well with changes in cyclic AMP binding due to epinephrine (Fig. 4). Only the highest concentration of catecholamine was capable of increasing kinase activity in the absence of a phosphodiesterase inhibitor. In the presence of 0.1 mM 1-methyl-3-isobutyl xanthine, 1 μM epinephrine significantly increased protein kinase activity.

Epinephrine alone, at a concentration of 0.1 μM, significantly increased glycogen phosphorylase a without affecting cyclic AMP or protein kinase. 1-Methyl-3-isobutyl xanthine slightly potentiated epinephrine action, but it did not enhance the sensitivity of phosphorylase a to catecholamine.

We sought further clarification of the nature of epinephrine's stimulatory effects using (−)-propranolol, a relatively specific β-adrenergic blocking agent (Table I). Epinephrine was added for the last minute of incubation to cells incubated with propranolol for 15 min. 1-Methyl-3-isobutyl xanthine was added to all tubes for the last minute of incubation. Propranolol competitively inhibited the changes in cyclic AMP binding and protein kinase produced by 1 μM epinephrine. A 10-fold excess of propranolol was sufficient to completely inhibit the effects of epinephrine on these variables. However, even at these high concentrations, propranolol inhibited the activation of glycogen phosphorylase by epinephrine by only 14%.

Phenylephrine is a predominantly α-adrenergic agonist which increases glycogenolysis in both perfused liver and isolated cells (6). Fig. 5 shows the dose response curves for the effects of phenylephrine on cyclic AMP binding, protein kinase, and glycogen phosphorylase after incubation of hepatocytes for 1 min with the catecholamine. This was the earliest time point we measured and produced maximum values, as the increase in phosphorylase activity in the presence of phenylephrine rapidly decayed (data not shown). The 0.1 μM phenylephrine significantly increased the levels of glycogen phosphorylase a, but there was no significant change in cyclic AMP binding at any concentration of catecholamine. There was a tendency toward a decrease in binding at the highest dose of phenylephrine tested (10 μM). No concentration of drug affected protein kinase activity in isolated liver cells, except in the presence of 1-methyl-3-isobutyl xanthine.

We considered it possible that α-adrenergic agents produced an increase in protein kinase activity that was lost during disruption of the hepatocytes. Cells that were treated for 1 min with 10 μM phenylephrine and then homogenized in buffer containing either 150 mM KCl or 50 mM KCl did not have protein kinase activity significantly different from control cells homogenized under similar conditions. However, disruption in high salt decreased protein kinase activity assayed in the presence of cyclic AMP in both control and hormone-treated liver cells. For example, protein kinase activity assayed in the presence of 2 μM cyclic AMP decreased from 1.84 ± 0.202 nmol/mg of protein when cells were homogenized in the absence of added KCl to 1.16 ± 0.185 nmol/mg of protein when homogenized in 50 mM KCl (n = 3, p < 0.05 by paired comparisons). The increase in protein kinase activity produced by 10 μM epinephrine in the presence of 0.1 mM 1-methyl-3-isobutyl xanthine was still apparent when the hepatocytes were homogenized in the presence of KCl (data not shown).

The question of the potency of the predominantly β-adrenergic agent isoproterenol on gluconeogenesis and glycogenolysis is still unsettled (4, 6). In our studies, isoproterenol (0.1 μM) significantly decreased cyclic AMP binding in isolated hepatocytes.
Table I

**Effect of propranolol on changes in cyclic AMP binding, protein kinase, and glycogen phosphorylase due to epinephrine in presence of 1-methyl-3-isobutyl xanthine**

Isolated liver cells (8 to 10 x 10^6 cells/ml) were incubated for 40 min in Krebs-Ringer bicarbonate buffer plus 400 mg/100 ml of glucose. (-)-Propranolol, when added, was present during the last 15 min of incubation. Epinephrine (Epi) (1 μM) was added 1 min before the end of the incubation, as was 1-methyl-3-isobutyl xanthine (0.1 mM), which was added to all tubes. Values are the means ± S.E. of three paired experiments.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cyclic AMP binding</th>
<th>Protein kinase activity ratio</th>
<th>Glycogen phosphorylase a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal cpm/μg protein</td>
<td>Δ due to Epi</td>
<td>Basal μmol/mg protein</td>
</tr>
<tr>
<td>None</td>
<td>20.4 ± 3.7 ± 0.81</td>
<td>0.28 ± 0.07 ± 0.024</td>
<td>1.65 ± 0.92 ± 0.19</td>
</tr>
<tr>
<td>(-)-Propranolol</td>
<td>20.4 ± 2.7 ± 0.87</td>
<td>0.25 ± 0.03 ± 0.043</td>
<td>1.66 ± 0.92 ± 0.12</td>
</tr>
<tr>
<td>1 μM</td>
<td>19.3 ± 0.1 ± 1.34</td>
<td>0.28 ± 0 ± 0.015</td>
<td>1.73 ± 0.79 ± 0.14</td>
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<tr>
<td>10 μM</td>
<td>18.1 ± 0.1 ± 1.19</td>
<td>0.26 ± 0 ± 0.011</td>
<td>1.68 ± 0.78 ± 0.13</td>
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</table>

Fig. 5. Dose-response curve of phenylephrine effect on cyclic AMP binding, protein kinase, and glycogen phosphorylase with and without 1-methyl-3-isobutyl xanthine. Isolated liver cells (7 to 15 x 10^6 cells/ml) were incubated for 40 min in Krebs-Ringer bicarbonate buffer with 400 mg/100 ml of glucose. The indicated concentrations of phenylephrine, in the absence (○) or the presence of 0.1 mM 1-methyl-3-isobutyl xanthine (△) were added 1 min before the end of the incubation. Values are the means ± S.E. of five paired experiments for cyclic AMP binding and protein kinase and four paired experiments for glycogen phosphorylase a. Asterisks indicate statistically significant differences (p < 0.05) due to phenylephrine.

Cyclic AMP increased the levels of glycogen phosphorylase a (Fig. 6). However, a concentration of 10 μM isoproterenol was required to stimulate protein kinase. 1-Methyl-3-isobutyl xanthine (0.1 μM) potentiated the action of isoproterenol on both cyclic AMP binding and protein kinase.

We considered the possibility that the glycogenolytic effect of isoproterenol was due to something other than its β-adrenergic potential. As shown in Table II, the ability of isoproterenol to stimulate both glucose release and glycogen phosphorylase a was inhibited by the β-adrenergic blocking agent propranolol.

DISCUSSION

As evidence for β-adrenergic stimulation of rat hepatic glycogenolysis and gluconeogenesis has mounted over the past several years, the possibility has been considered that these phenomena are not related to detectable alterations in cyclic
AMP (4, 6-9). Although there have been several reports of activation of glycogen phosphorylase by catecholamines in vivo (28), in the perfused liver (6, 7, 10, 29) and in liver slices (30, 31), there has been no study of this kind on isolated liver cells. Furthermore, the control of hepatic protein kinase activity by catecholamines has never been systematically investigated.

The idea that protein kinase mediates the action of α-adrenergic agents on hepatic glycogenolysis in the rat does not appear to be tenable. Isoproterenol, a β-adrenergic agent, was considerably more potent than the predominately α-adrenergic agonist phenylephrine in increasing the activity of protein kinase, with or without the addition of a phosphodiesterase inhibitor. The activation of protein kinase by high concentrations of phenylephrine in the presence of 1-methyl-3-isobutyl xanthine is most likely due to the weak β component, which has been demonstrated in several other systems (27, 32). Furthermore, the β-blocking agent (–)-propranolol completely inhibited the increase in protein kinase activity produced by epinephrine. These data strongly support the concept that stimulation of protein kinase by α-adrenergic stimulation is a pure β-adrenergic effect. The correlation between protein kinase activity and cyclic AMP levels is consistent with the proposition that activation of adenylate cyclase is a β-adrenergic response (33), and suggests that the protein kinase measured in these studies responds to detectable alterations in total cyclic AMP. In support of this is the observation that the protein kinase inhibitor of Walsh et al. (34) completely reversed the activation of protein kinase by any of the agents reported to be effective in these studies, and inhibited basal activity by about 35%.2

One can imagine several conditions in which an activation of a hepatic protein kinase by α-adrenergic stimulation would not have been detected in these studies. Possibly increases in a small active pool of cyclic AMP or protein kinase were not seen due to a large background activity. We consider this to be unlikely in regard to protein kinase since its physiological substrate, phosphorylase kinase, is located predominately in the glycogen granule3 and this organelle is distributed throughout the cytoplasm (35). Another possibility is that the protein kinase that responds to α-adrenergic stimulation cannot utilize histone as a phosphate acceptor. Thirdly, allosteric modification of the protein kinase induced either by the α-adrenergic agent or its hypothetical second messenger, might not be preserved during disruption of the cells. However, the activation of protein kinase by cyclic AMP, which is known to act by noncovalent means (17), was preserved during the preparation and assay of the kinase in these studies. Homogenization in physiological as well as high salt concentrations did not reveal any activation of histone kinase by phenylephrine.

In contrast to the activation of protein kinase in isolated rat liver cells, the increase in glycogen phosphorylase α by hormones does not always correlate well with changes in cyclic AMP. There are two distinct conditions in which this dissociation becomes apparent: treatment of hepatocytes with low concentrations of glucagon, and stimulation by α-adrenergic agents. In regard to the action of glucagon, the differences might well be due to a very efficient biological cascade system that allows detection of only the amplified signal using current methodology. Alternatively, there could be a protein kinase-independent activation of glycogen phosphorylase by glucagon. The latter proposition is a more likely explanation for the action of α-adrenergic agents.

Isolated hepatocytes are responsive to both isoproterenol and phenylephrine at the same concentrations, and yet phenylephrine is totally without effect on protein kinase and cyclic AMP binding in the absence of a phosphodiesterase inhibitor (Figs. 5 and 6). The increase in cyclic AMP and protein kinase by all agents and the stimulation of glycogen phosphorylase by isoproterenol, but not epinephrine, is sensitive to β-adrenergic blockade (Table I and II). These data strongly support the notion that there exists in rat liver cells a cyclic AMP-independent, protein kinase-independent, activation of glycogen phosphorylase α by α-adrenergic stimulation. The use of isolated hepatocytes has completely eliminated the possibility that vascular changes alone are responsible for the α-adrenergic stimulation of gluconeogenesis (4) and glycogenolysis (6). These present studies indicate that α-adrenergic agents can increase the levels of glycogen phosphorylase α in the absence of changes in oxygenation. The data do not, however, prove or disprove the existence of a hormone-sensitive system for glycogen breakdown that is independent of alterations in the phosphorylation state of glycogen phosphorylase.

The increase in phosphorylase α in the absence of any change in protein kinase raises the intriguing question of which enzyme is responsible for α-adrenergic modulation of phosphorylase. Shimazu and Amakawa (36) have reported increased phosphorylase kinase activity in rabbit liver after injection of epinephrine into the portal vein. They did not measure protein kinase, but did find increased cyclic AMP in response to the catecholamine. Splanchnic nerve stimulation increased glycogen phosphorylase activity and decreased glycogen phosphorylase phosphatase, but epinephrine was without effect on the latter enzyme. Thus, a report of an effect of α-adrenergic stimulation on an enzyme involved with the phosphorylation or dephosphorylation of glycogen phosphorylase is still lacking. The demonstration of such a modulation would provide important information concerning the biochemical mechanism of the α-adrenergic modulation of carbohydrate metabolism, and is currently under investigation.

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### Table II

<table>
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<th>Addition</th>
<th>Glucose release</th>
<th>Glycogen phosphorylase</th>
<th>α</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Increase due to (1 μM) isoproterenol</td>
<td>Increase due to (1 μM) isoproterenol</td>
</tr>
<tr>
<td>None</td>
<td>24.8 ± 1.2</td>
<td>1.12 ± 0.16</td>
<td>0.91 ± 0.16</td>
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<tr>
<td>(–)-Propranolol</td>
<td>1 μM</td>
<td>1.19 ± 0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>20.1 ± 0.72</td>
<td>1.24 ± 0.16</td>
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</tbody>
</table>

2 Unpublished observations.
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Activation of protein kinase and glycogen phosphorylase in isolated rat liver cells
by glucagon and catecholamines.
M J Birnbaum and J N Fain


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