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*J Clin Invest.* 1976;57(2):435-443. https://doi.org/10.1172/JCI108294.

**Research Article**

Hormone-induced desensitization of hormonal regulation of cyclic AMP (cAMP) content has been described in a number of tissues. In the present study, we examined responses of rat liver to glucagon after periods of sustained exposure to the hormone in vivo and in vitro. In intact anesthetized rats infused with glucagon (50 ng/min) for 1 h or more and in liver slices incubated with the hormone (10 muM) for this period, hepatic cAMP responsiveness to glucagon was significantly blunted compared with that of tissue exposed to the hormone for shorter periods. The reduction in hepatic cAMP responsiveness to glucagon appeared to be fully expressed by 2 h. With the doses of hormone employed, the sequential alterations in hepatic responsiveness seemed to be limited to the cAMP system, since other parameters of glucagon action did not wane with time. Diminished hepatic cAMP responsiveness during sustained hormonal exposure could not be attributed to decreased glucagon availability, accelerated extracellular release of cAMP, hepatic ATP depletion, or enhanced phosphodiesterase activity. Studies in vitro suggested that modulation of the cAMP response occurred at the level of adenylate cyclase (AC). During sustained exposure of hepatic slices to glucagon, reductions in glucagon-responsive AC correlated temporally with those in cAMP and both changes were reversible. Alterations in glucagon-responsive AC were demonstrated over a wide range of ATP (10 muM-0.1 […]



# Reduced Sensitivity of the Hepatic Adenylate Cyclase-Cyclic AMP System to Glucagon during Sustained Hormonal Stimulation

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ABSTRACT Hormone-induced desensitization of hormonal regulation of cyclic AMP (cAMP) content has been described in a number of tissues. In the present study, we examined responses of rat liver to glucagon after periods of sustained exposure to the hormone in vivo and in vitro. In intact anesthetized rats infused with glucagon (50 ng/min) for 1 h or more and in liver slices incubated with the hormone (10 $\mu$M) for this period, hepatic cAMP responsiveness to glucagon was significantly blunted compared with that of tissue exposed to the hormone for shorter periods. The reduction in hepatic cAMP responsiveness to glucagon appeared to be fully expressed by 2 h. With the doses of hormone employed, the sequential alterations in hepatic responsiveness seemed to be limited to the cAMP system, since other parameters of glucagon action did not wane with time.

Diminished hepatic cAMP responsiveness during sustained hormonal exposure could not be attributed to decreased glucagon availability, accelerated extracellular release of cAMP, hepatic ATP depletion, or enhanced phosphodiesterase activity. Studies in vitro suggested that modulation of the cAMP response occurred at the level of adenylate cyclase (AC). During sustained exposure of hepatic slices to glucagon, reductions in glucagon-responsive AC correlated temporally with those in cAMP and both changes were reversible. Alterations in glucagon-responsive AC were demonstrated over a wide range of ATP (10 $\mu$M–0.1 mM) and glucagon (10 nM–5 mM) concentrations in the cyclase reaction mixture, and appeared to be a noncompetitive phenomenon relative to glucagon. Maximal NaF-responsive AC did not fall concomitantly with time. Thus, the reduction in glucagon-responsive AC was probably not related to a reduction in the catalytic unit of the enzyme, but could have been due to an alteration in glucagon binding to its receptor sites, or in the coupling mechanism involved in transmission of the hormonal signal to the catalytic unit.

Dr. DeRubertis is a Clinical Investigator of the Veterans Administration Hospital and American College of Physicians Teaching and Research Scholar.

*Received for publication 31 July 1975 and in revised form 6 October 1975.*

## INTRODUCTION

Local cellular mechanisms may exist to quench as well as to initiate hormonal signals. In liver, most, if not all, of the cellular actions of glucagon are thought to be initiated by cyclic AMP (cAMP). However, there is evidence to suggest that during periods of continuous stimulation of the liver with glucagon the hepatic cAMP response to this hormone may decrease. In man, Liljenquist and co-workers (1) observed that the sustained infusion of exogenous glucagon at a constant rate is accompanied by a peak-decline pattern in net splanchnic cAMP production. Our own previous studies (2) and those of others (3, 4) have noted a similar tendency for the hepatic cAMP content of experimental animals to decline from early peak levels during glucagon infusions. Further, in rats with persistent endogenous hyperglucagonemia induced by infection, the hepatic cAMP response to exogenous glucagon is blunted (5). Such observations suggest that the liver may become refractory to glucagon during periods of sustained stimulation with the hormone. This possibility has not been specifically evaluated with glucagon and liver. However, in several other tissues, including fat cells (6–8), brain (9–11), pineal gland (12), leukocytes (13, 14), fibroblasts (15), ovary (16), and thyroid (17), prior hormonal exposures result in a reduction in the responsiveness of the cAMP system to subsequent hormonal stimulation. It has been suggested (6, 18)

that such sequential alterations in tissue responsiveness may serve to modulate cellular cAMP generation and possibly other hormonal actions (19) during periods of sustained hormonal stimulation, and may be mediated by local cellular mechanisms (6, 18, 20, 21). Accordingly, in the present study we examined responses of rat liver to glucagon after periods of sustained exposure to the hormone in vivo and in vitro.

## METHODS

Male Sprague-Dawley rats (Zivic-Miller Laboratories Inc., Pittsburgh, Pa.) weighing 200±20 g, were studied after an 18-h fast. Rats were anesthetized with pentobarbital, 5 mg/g body weight i.p., and a femoral venous catheter inserted for infusion of test agents using a constant infusion pump (Harvard Apparatus Co., Millis, Mass., model 600-000). Rats were studied in groups of 8–10, using heating pads and heat lamps to prevent hypothermia during the prolonged infusions. In initial experiments, groups of rats were infused with either 0.85% saline (0.05 ml/min) or glucagon (50 ng/min, approximately 225–275 ng/kg body weight per min) in 0.05 ml saline for the times indicated in the Results (Table I). In subsequent experiments, rats were infused with either 5% glucose in water (11–14 mg glucose/kg per min per 0.05 ml) or glucagon (50 ng/min) in 0.05 ml 5% glucose for the times indicated in the Results (Table II). At the termination of each timed infusion, the left lobe of the liver was quick-frozen *in situ* by clamping the tissue between aluminum blocks cooled to −196°C in liquid nitrogen and the vascular pedicle to this lobe was ligated. This tissue was subsequently assayed for cAMP and ATP content, and for activities of glycogen phosphorylase, glycogen synthetase, adenylate cyclase, and cyclic phosphodiesterase, as described below. Immediately after tissue excision, a 25-gauge needle was inserted into the portal vein and advanced to the level of the liver. A 1.0-ml sample of portal blood was collected in chilled glass tubes containing 6 mg EGTA (ethylene glycol-bis(β-aminoethyl ether)*N,N,N,N'*-tetraacetate) and 500 U of Trasylol (FBA Pharmaceuticals, Inc., New York) in 50 μl, for subsequent determination of plasma glucose and immunoreactive glucagon (IRG).[1] Tissue samples were stored at −70°C and assayed within a week.

TABLE I

*Alterations in Plasma Glucose and IRG, and in Hepatic cAMP, ATP, PHOS, and GS Activities during Infusions of Glucagon and Glucose*

| | Glucose | Glucagon | |
|---|---|---|---|
| Infusion time, *min*... | 120 | 120 | Final 20 |
| Plasma | | | |
| Glucose, *mg/dl* | 196±28 | 404±51* | 365±47* |
| IRG, *ng/ml* | 0.27±0.05 | 17.3±3.6* | 15.8±3.1* |
| Hepatic | | | |
| cAMP, *nmol/g wet wt* | 0.44±0.05 | 1.86±0.31* | 6.21±1.07*‡ |
| ATP, *μmol/g wet wt* | 3.42±0.54 | 2.95±0.44 | 3.13±0.46 |
| Phosphorylase, *μmol glucose/g wet wt/min* | 9.7±1.6 | 23.4±3.8* | 20.1±3.5* |
| Synthetase-I, *% total* | 76±10 | 37±8* | 42±8* |

Groups of rats were infused with either 5% glucose (0.05 ml/min) for 120 min (glucose) or with approximately 250 ng of glucagon/kg per min in 5% glucose for the same period (glucagon 120 min). A third group was infused with glucose for the first 100 min and then with glucagon at the above rate for only the final 20 min (glucagon, final 20 min). Values are mean±SE with an *n* of 8–10 for each group.

* Indicates $P < 0.02$ vs. glucose.

‡ $P < 0.01$ glucagon-final 20 vs. −120 min.

*In vitro studies.* Fasted rats were anesthetized as described above and the liver exposed surgically. After sectioning the inferior vena cava, the liver was immediately perfused manually through the portal vein with saline at 4°C until thoroughly blanched. The liver was then quickly excised and placed in saline at 4°C. Tissue slices, 50–100 mg, were prepared with a Stadie-Riggs microtome and incubated within 30 min of excision. All slices were initially incubated for 30 min in stoppered flasks containing 2 ml of hormone-free Krebs-Ringer bicarbonate buffer (KRBG) (pH 7.4, 1 mg/ml of bovine serum albumin and glucose), with 95% $O_2$ and 5% $CO_2$ serving as the gas phase. Glucagon was then added to some flasks and the incubations continued for the times indicated in the Results (Fig. 1). Incubation media in these studies was exchanged at 15-min intervals for fresh buffer (37°C) with or without glucagon, 10 μM–5 nM to ensure a constant supply of biologically active hormone and to prevent accumulation of potential inhibitory factors in the media. Since preliminary studies indicated that peak increases in hepatic cAMP content and phosphorylase activity occurred 5 min after either initial or subsequent additions of glucagon, incubations were routinely terminated 5 min after the final addition of fresh hormone. At the termination of each timed incubation, slices were either extracted for ATP or cAMP content, or homogenized in the appropriate buffer for determination of glycogen phosphorylase, phosphodiesterase, or adenylate cyclase (AC) activities, as described below.

*Assay of hepatic parameters.* Hepatic cAMP and ATP content were extracted and assayed using, respectively, modifications of the protein-binding and luciferin-luciferase methods, as previously reported in detail (22, 23). Hepatic glycogen phosphorylase (PHOS) was prepared and assayed by the method of Gilboe et al. (23, 24). Hepatic PHOS measured by this method should reflect the physiologically active, presumably phosphorylated, form of the enzyme (23). The results are expressed as micromoles of glucose incorporated into glycogen per gram of liver per minute at 30°C. Glycogen synthetase (GS) was assayed by the method of Thomas et al. (25), with minor modifications (23). Synthetase activity was assayed in both the presence and absence of 5 mM glucose-6-phosphate (G-6-P). Activity measured in the presence of G-6-P was taken as total expressable activity (26), while activity measured in the absence of G-6-P reflects that of the dephosphorylated, physiologically active form of the enzyme (synthetase-I) and was expressed as a proportion of total activity (% GS-I).

Cyclic phosphodiesterase (PDE) activities of the 100,000-*g* soluble and particulate fractions of hepatic homogenates were prepared and assayed as previously reported (22), by the method of Thompson and Appleman (27). Activity of each fraction was determined at both a high (0.1 mM) and low (0.13 μM) concentration of cAMP. Dilutions of homogenate fractions were added which destroyed

[1] *Abbreviations used in this paper:* AC, adenylate cyclase; G-6-P, glucose-6-phosphate; GS, glycogen synthetase; IRG, immunoreactive glucagon; KRBG, Krebs-Ringer bicarbonate glucose buffer; PDE, cyclic phosphodiesterase; PHOS, glycogen phosphorylase.

TABLE II

*Effects of Prior Incubation of Hepatic Slices with Glucagon on Basal-, Glucagon-, and NaF-Responsive AC Activities*

| Addition to cyclase reaction mixture | Time of slice incubation, *min* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | | 15 | | 30 | | 60 | | 120 | |
| | C | PG | C | PG | C | PG | C | PG | C | PG |
| | *pmol cAMP formed/10 min/mg of protein* | | | | | | | | | |
| None | 42±5 | 88±10 | 37±4 | 76±9 | 39±5 | 78±9 | 48±6 | 93±11 | 44±6 | 101±14 |
| NaF, *1 mM* | 303±38 | 327±40 | 288±37 | 281±32 | 317±41 | 293±34 | 312±42 | 300±38* | 283±37 | 289±30* |
| Glucagon, *10 μM* | 340±43 | 353±46 | 313±31 | 286±35 | 324±38 | 255±34 | 339±44 | 194±29‡ | 316±40 | 181±25‡ |

Hepatic slices were incubated for the times shown in either the absence (control, C) or presence (prior glucagon, PG) of 10 μM glucagon, and washed 2,000-*g* particulate fractions were prepared at 4°C. AC activities of these fractions were then assayed in the absence (basal activity), or presence of maximal stimulatory concentrations of NaF or glucagon. AC reaction time was 10 min with 2 mM ATP present. Each value represents the mean±SE of seven duplicate AC determinations pooled from seven experiments.

* Value for NaF greater than value for glucagon at 60 and 120 min, with $P < 0.05$.

‡ $P < 0.02$ by *t* test for paired values, comparing glucagon-responsive AC of PG to C at the same incubation time.

approximately 20% of the added cAMP. Under these conditions the reaction rate was linear for the 5 min of assay, and is expressed as pmol cAMP hydrolyzed/mg protein per 5 min at 30°C.

AC activity was examined in both whole hepatic homogenates and the twice washed 2,000-*g* particulate fractions of these homogenates. Tissue preparation and assay conditions have previously been described in detail (5). Briefly, 2,000-*g* pellets were prepared by gently homogenizing approximately 400 mg of liver at 4°C in 6 ml of 10 mM Tris-HCl buffer (pH 7.4), containing 0.2 mM EDTA. The total homogenate volume, adjusted to 40 ml, was passed through gauze and centrifuged at 2,000 *g* for 30 min at 4°C. This particulate fraction was then washed twice before assay of AC with 40 ml of the same buffer. The AC reaction mixture contained 2.0 mM ATP, $2 \times 10^6$ cpm of [$^{32}$P]ATP/assay, 1.0 mM cAMP, 5.0 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.6), 20 mM caffeine, 25 mM creatine phosphate, 0.5 mg/ml of

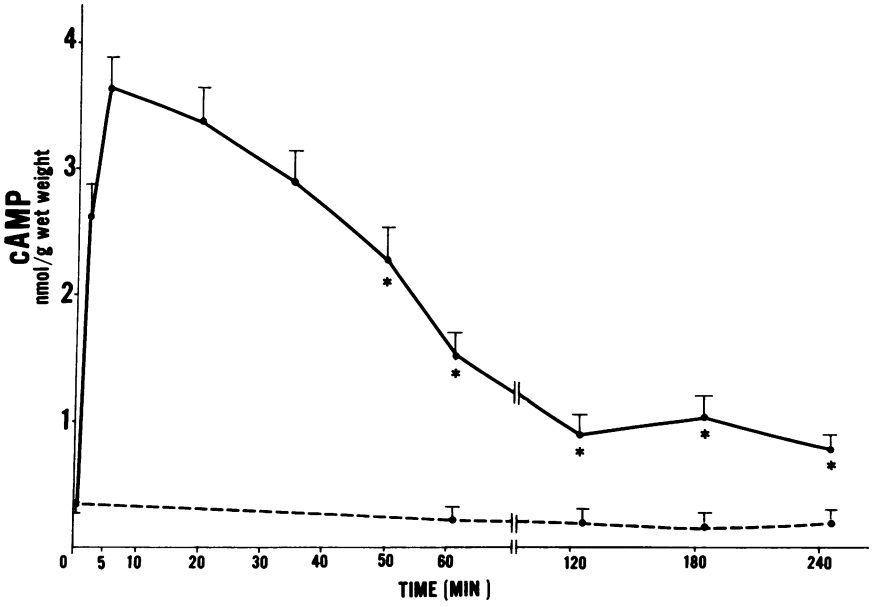


FIGURE 1 Sequential alterations in cAMP during sustained exposure of hepatic slices to glucagon in vitro. All slices were preincubated in hormone-free KRBG for 30 min. Slices were then incubated in the presence (——, glucagon) or absence (– – –, control) of 10 μM glucagon for the times shown. Incubation media was exchanged for fresh KRBG and glucagon at 15-min intervals after the initial addition of hormone, and slices subsequently extracted for cAMP 5 min after the final addition of fresh hormone. Each value represents mean±SE for nine slices pooled from three experiments. * indicates $P < 0.01$, comparing subsequent values for glucagon to the initial 5-min peak value. At all times examined, cAMP of glucagon were significantly greater than control, with $P < 0.01$.

creatine phosphokinase, and 1.3 mg bovine serum albumin and was incubated at 30°C. The reaction was initiated by adding 200–300 μg of tissue protein from the particulate fractions, or approximately 400 μg when whole hepatic homogenates were studied. The cAMP formed was isolated by the two-step column chromatographic procedure (Dowex AG 50 WX-4, followed by alumina) recently described by Salomon et al. (28). Protein was determined by the Lowry method (29).

*Determination of plasma glucose and IRG.* Glucose was measured by the glucose oxidase method (Glucose, 510, Sigma Chemical Co., St. Louis, Mo.). IRG was determined by Dr. James B. Field, University of Pittsburgh, using previously described methods (30). Antibody 30 K, purchased from the Diabetes Research Fund, University of Texas, Southwestern Medical School at Dallas, Dallas, Tex., was employed. Sources of other materials have been reported (22, 23).

Statistical differences among mean values of various parameters were assessed with Student's *t* test for unpaired values, unless otherwise indicated.

## RESULTS

*Effects of sustained glucagon infusions on hepatic responses to glucagon in vivo.* Hepatic cAMP content of rats infused with glucagon in 0.85% saline for 120 min (mean 2.01±0.37 SE nmol/g wet wt) was significantly below that of rats infused with hormone in this diluent at the same rate for only the final 20 min of the 120-min infusion period (5.94±0.99). Both these values were clearly above hepatic cAMP levels in rats receiving saline alone for the entire 120-min infusion period (0.52±0.05). Increases in plasma glucose over the saline controls were similar in rats infused with glucagon for either a 20- or 120-min period (30–40 mg/dl above saline control). However, changes in hepatic PHOS and %GS-I in response to glucagon under these conditions were small and variable, and thus prevented an evaluation of sequential changes in the action of the hormone on these parameters. Our own previous studies and those of others (3, 31, 32) have shown that infusion of glucose decreases hepatic PHOS and increases %GS-I, effects which are reversed by pharmacologic doses of glucagon (2). Accordingly, to permit a clearer assessment of the effects of brief and prolonged glucagon exposure on hepatic PHOS and %GS-I, rats were infused with either 5% glucose or glucagon in 5% glucose for 120 min, while other rats received 5% glucose for the first 100 min and glucagon in 5% glucose for only the final 20 min. (Table I). Alterations in cAMP in these three study groups were similar to those observed with saline. Levels were significantly higher in rats infused with glucagon for only the final 20 min (14-fold over glucose control) than in those infused with hormone continuously for 120 min (4.2-fold over control). Hepatic cAMP content of rats continuously infused with glucagon for 180 or 240 min was not different from that seen after a 120-min infusion (Table I). Glucose infusion depressed basal PHOS and increased %GS-I compared to values in rats given saline (data not shown). Relative to values in the glucose controls, the magnitude of the increase in hepatic PHOS, reduction in %GS-I, and the increase in plasma glucose were similar in rats infused with glucagon for either the final 20-min or the entire 120-min period (Table I). The changes in PHOS, %GS-I, and plasma glucose seen after a 120-min infusion of glucagon were not consequences of earlier and persistent hormone actions, but required sustained infusion of glucagon. Values in rats infused with glucagon for the initial 20 min of a 120-min infusion period and then given 5% glucose for the final 100 min did not differ from those receiving glucose alone for the entire 120 min (not shown). As indicated in Table I, plasma IRG were similar in rats infused with glucagon for 20 or 120 min, and levels in both these groups were markedly increased above control. Hepatic ATP content was comparable in the three study groups at the completion of 120-min infusions (Table I).

No consistent differences were demonstrated in basal-, glucagon-, or NaF-responsive AC activities or in PDE activities, when these parameters were examined in homogenates of liver from rats who had received glucagon or hormone-free infusates for 120-min periods. These comparisons were made using both frozen tissue and tissue samples homogenized, fractionated, and assayed immediately after completion of the infusions.

*Effects of sustained exposure of hepatic slices to glucagon in vitro.* As shown in Fig. 1, the peak increase in cAMP occurred 5 min after initial exposure of hepatic slices to 10 μM glucagon. With more prolonged exposure of slices to this concentration of hormone, cAMP declined from the initial peak value, reaching an apparent plateau by 2 h. Consistent with the changes observed in vivo, cAMP content of slices incubated with glucagon for prolonged periods was still significantly above control. The decline in cAMP responsiveness was not attributable to the prolonged incubation per se. The cAMP levels in slices incubated for 125 min but exposed to glucagon for the final 5 min (mean 2.64±0.39 SE pmol/g wet wt) were significantly above those of slices incubated with hormone for this entire period (1.27±0.25). ATP levels in slices without hormone exposure (1.14±0.16 μmol/g wet wt), with a terminal 5-min exposure to glucagon (0.99±0.10) and with continuous exposure to hormone for 125 min (1.10 ±0.13) were similar. Thus ATP depletion did not appear to account for reduced cAMP production in either the in vivo or in vitro models. Consistent with in vivo observations, activity of PHOS was also comparable in slices exposed to glucagon for the final 5 min (8.2

±0.8 μmol glucose per g wet wt per min) or for the entire 125 min (7.1±0.9), and both values were above PHOS of control slices (3.6±0.5). The lower absolute values for hepatic ATP and PHOS in the slice model compared to those seen in vivo have been previously described (23, 33, 34).

To assess the possibility that the apparent decline in the cAMP response of hepatic slices to glucagon with time was related to enhanced degradation or extracellular release of the nucleotide, slices which had been preincubated for 120 min in either the presence or absence of glucagon were transferred to new flasks for a terminal 10-min incubation with 10 μM glucagon and 10 mM theophylline. Both tissue and media cAMP content were reduced in slices with prior exposure to glucagon (not shown). Consistent with the failure of theophylline to restore the cAMP response to control, no differences were noted in the PDE activities of slices incubated for 120 min in the presence or absence of 10 μM glucagon. Activities in both tissues were examined in the 100,000-*g* soluble and particulate fractions, at a high (0.1 mM cAMP) and a low (0.13 μM cAMP) substrate concentration (not shown).

Since these results all indicated that neither enhanced cAMP degradation or release could account for the changes observed, the effects of sustained glucagon exposure on AC activity were examined. As shown in Table II, prior incubation of hepatic slices with 10 μM glucagon for 60 or 120 min clearly altered the glucagon-responsive AC activity of the tissue. When compared to tissue without prior hormone exposure, the formation of cAMP by washed 2,000-*g* particulate fractions from slices previously incubated with hormone for 60 or 120 min was detectably reduced after addition of 10 μM glucagon to the cyclase reaction mixture. This concentration of glucagon produced maximal increases in AC activity in control tissue. The reduction in glucagon-responsive AC occurred despite an increase in basal cAMP formation (no glucagon added to the cyclase reaction mixture) by the tissue with prior exposure to glucagon. cAMP formation increased in a linear fashion with time over the 10-min reaction period and was proportional to the concentration of tissue protein added over a 100–400-μg range (not shown). As also indicated in Table II, maximal glucagon-responsive AC activities of slices which had been previously incubated with glucagon for 30 min or less were not statistically different from those of control. However, basal AC of slices with prior glucagon exposure was increased over control at each time point studied, and glucagon-responsive AC was variably reduced at both 15 and 30 min. Activity was reduced comparably in slices preincubated with glucagon for 60 or 120 min (Table II). Qualitatively similar decreases in maximal glucagon-responsive AC activity (40–50%) were observed when enzyme activity was examined in whole hepatic homogenates rather than 2,000-*g* particulate fractions and when slices were preincubated in a single sample of KRBG with frequent additions of glucagon to the same media (not shown). In contrast to these changes in glucagon-responsive AC, no differences were noted in the AC responses of slices with and without prior glucagon exposure to a maximal dose (15 mM) of NaF (Table II). Further, maximal NaF-responsive AC, which tended to be somewhat less (10–20%) than glucagon-responsive activity in control tissue, was significantly greater than hormone-responsive activity in tissue with 1 or 2 h of prior incubation with glucagon. Reductions (30–40%) in glucagon-responsive AC in slices with prior exposure to glucagon were detectable when the ATP concentration in the cyclase reaction mixture was varied over a range of 10 μM–5 mM (data not shown). The apparent $K_m$ of the enzyme preparation from each tissue, calculated from Lineweaver-Burk plots (35), was similar (0.1 mM for control, and 0.08 mM for prior glucagon), and comparable to values previously reported by Birnbaumer and co-workers (36). As shown in Fig. 2, AC activity of slices with prior exposure to glucagon for 60 min was detectably blunted compared to control in response to doses of glucagon in the cyclase reaction mixture ranging from 10 nM to 0.1 mM. Since AC activity remained blunted in the presence of high doses of glucagon in the cyclase reaction mixture, the results

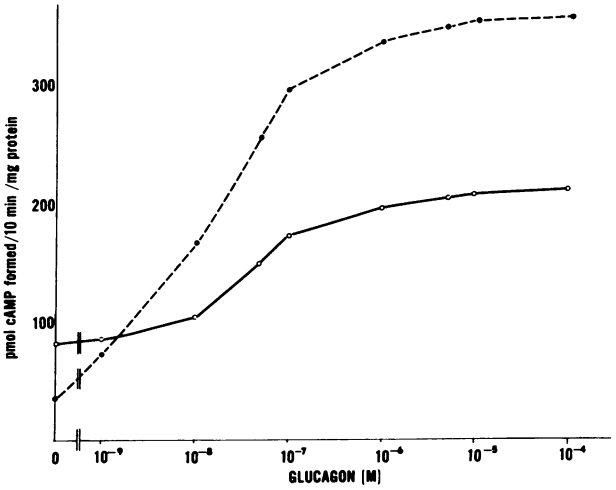


FIGURE 2 Log-concentration response to glucagon of AC activity of hepatic slices with prior exposure to glucagon. Slices were incubated for 60 min in the absence (– – –) or presence (——) of 10 μM glucagon, and 2,000-*g* particulate fractions prepared. AC activity was then assayed in the presence of 1 nM–0.1 mM glucagon in the cyclase reaction mixture (2 mM ATP, reaction time 10 min). Values shown are averages of triplicate determinations.

suggest that the inhibition of AC activity induced by prior hormone exposure may be a noncompetitive phenomenon. Prior incubation of hepatic slices for 120 min with 50 nM glucagon also significantly reduced the subsequent AC response to 10 $\mu$M glucagon in the cyclase reaction mixture, whereas preincubation of slices with lower doses of the hormone (5 or 10 nM) for this period failed to alter either basal or glucagon-responsive AC.

To assess the reversibility of the depressions in AC and cAMP responsiveness induced by prior glucagon exposures, liver slices were first incubated for 1 h at 37°C in KRBG with 10 $\mu$M glucagon, and then washed and incubated for an additional hour in hormone-free buffer. Upon reexposure to 10 $\mu$M glucagon, the increases in AC activity and cAMP content of tissue washed in this manner were both significantly greater than those seen in slices which had been continuously exposed to glucagon throughout the 1- or 2-h incubation periods (Fig. 3). Further, the AC and cAMP responses of washed slices upon reexposure to glucagon were similar to those of control tissue, which had had no prior exposure to the hormone (Fig. 3). Basal AC and cAMP of the washed tissue was also similar to control (not shown).

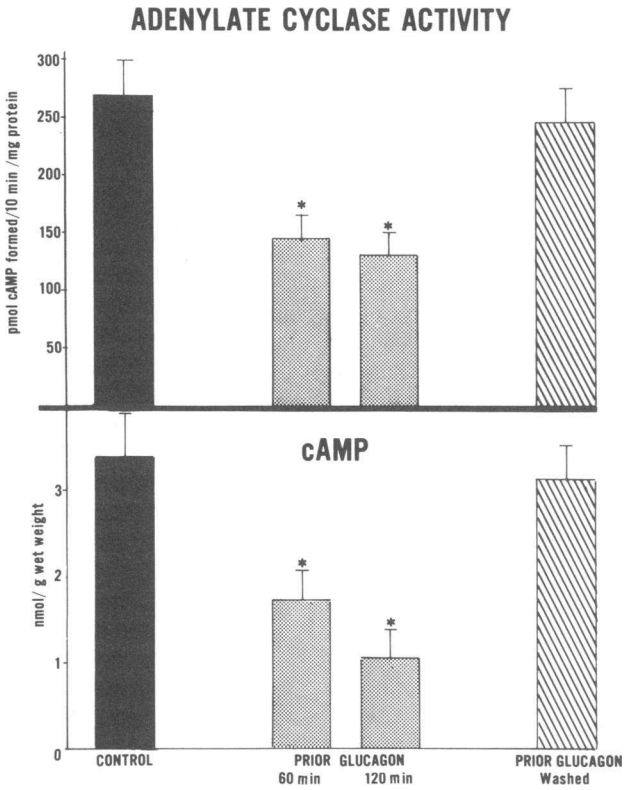


FIGURE 3 Effects of tissue washing on hepatic slices with prior glucagon exposure. Slices were initially incubated at 37°C in KRBG with 10 $\mu$M glucagon for 60 min and then washed and incubated in hormone-free buffer for an additional 60 min (Prior Glucagon-Washed). Other slices, handled identically, were continuously exposed to glucagon for 60 or 120 min (Prior Glucagon) or incubated in hormone-free buffer for 120 min (Control). The slices were then either incubated for a final 5 min with 10 $\mu$M glucagon and extracted to assess cAMP content, or alternatively were homogenized for determination of AC activity with 10 $\mu$M glucagon present in the cyclase reaction mixture. Values represent mean±SE of nine determinations pooled from three experiments; * indicates $P < 0.02$ vs. control. * $P < 0.02$ vs. control.

The possibility that reductions in glucagon-responsive AC of hepatic slices during sustained exposure to glucagon were mediated by inhibitory factors in the incubation media was also specifically evaluated. KRBG with 10 $\mu$M glucagon was incubated for 120 min in the presence and absence of liver slices. Freshly prepared liver slices were then incubated in these buffers for 15 min and glucagon-responsive AC determined. AC was comparable in slices incubated in media used for prior tissue incubation and in the media which had not previously contained tissue.

## DISCUSSION

The results of the present study indicate that sustained exposure of the liver to glucagon reduces hepatic cAMP responsiveness to the hormone. This possibility was raised by earlier observations in both man (1) and in experimental animals (2–5), but not systematically examined. With the doses of hormone employed in the present study the cAMP content of liver exposed to glucagon for 4-h periods either in vivo or in vitro remained significantly above control, despite the decline toward basal levels with time. Thus, cAMP responsiveness of the liver to the hormone was blunted but not ablated by prolonged stimulation. While this manuscript was in preparation, Plas and Nunez (37) reported the development of refractoriness to glucagon stimulation in cultured fetal hepatocytes after prior exposure to the hormone. Results of these studies also suggested that a reduction in cAMP production was responsible. However, alterations in AC or PDE activities were not specifically evaluated in their cells.

In the present study several possible mechanisms for the apparent reduction in hepatic sensitivity to glucagon were examined. These included: (*a*) decreased availability of glucagon with time; (*b*) hepatic ATP depletion; (*c*) an increase in hepatic PDE activity and cAMP degradation; (*d*) an acceleration of the rate of extracellular release of cAMP by liver; and finally, (*e*) a decrease in AC activity and cAMP formation. None of the first four mechanisms seemed to account for the changes observed. However, our observations in vitro

suggest that the decline in cAMP responsiveness with time was, at least in part, a consequence of a locally mediated reduction in glucagon-responsive AC activity. Alterations in AC responsiveness to glucagon correlated temporally with those in cAMP and both responses were restored by incubating hepatic slices with prior hormone exposure in hormone-free buffer at 37°C for 1 h. In contrast to changes in maximal glucagon-responsive AC, NaF-responsive AC activity from slices with prior exposure to glucagon was indistinguishable from that of control tissue. According to current concepts (38), NaF is thought to activate the AC system at its catalytic site. Therefore, the reduction in glucagon-responsive AC activity does not appear to be related to a reduction in the catalytic unit of the enzyme. Further, the AC obtained from each tissue had a similar apparent $K_m$ for ATP, suggesting that the change in activity induced by prior glucagon exposure was not related to a reduction in the affinity of the catalytic unit of the enzyme for ATP. The reduction in glucagon-responsiveness could reflect an alteration in glucagon binding to its receptor sites or in the coupling mechanism involved in transmission of the hormonal signal to the catalytic unit. A reduction in hormone binding induced by prior exposure of cells to hormone has been previously reported (39, 40), but the role of altered glucagon binding in the decline in hormonal responsiveness observed in the present study remains to be determined. In this regard, it is of interest that Fouchereau and co-workers have recently reported that hepatic binding of glucagon is diminished in rats after a 48-h fast, when endogenous glucagon levels are increased (41).

The increase in basal AC activity of slices with prior exposure to glucagon was most likely due to the persistent binding of biologically active hormone to tissue during the homogenization and fractionation procedures (42, 43). Accordingly, it is possible that the decrease in maximal glucagon-responsive AC of this tissue was similarly related to occupation of glucagon receptor sites by biologically inactive hormone degradation products. This possibility seems unlikely for two reasons. First, elevation of basal AC activity occurred in tissue with brief periods of prior hormone exposure but was not accompanied by a reduction in glucagon-responsive AC. Further, the diminution of AC responsiveness in tissue with more prolonged prior hormone exposure was observed over a wide range of glucagon concentrations in the cyclase reaction mixture and not overcome by high concentrations of the hormone (0.1 mM), suggesting that the inhibition of enzyme activity was noncompetitive. Glucagon refractoriness in cultured fetal hepatocytes also seemed to be a noncompetitive phenomenon (37). Persistent binding of biologically active glucagon to the liver during the homogenization and fractionation procedures may have been necessary to demonstrate the decline in maximal glucagon-responsive AC in tissue with prior hormone exposure. Neither basal nor glucagon-responsive AC were detectably altered in quick-frozen or fresh liver from rats infused with hormone for 2 h in vivo and in slices exposed to concentrations of hormone below 50 nM, implying that in these preparations any residual tissue-bound glucagon was inactivated during the fractionation procedure. However, the failure to demonstrate reductions in glucagon-responsive AC after glucagon infusion in vivo leaves open the possibility that other local or systemic factors may have mediated the decrease in hepatic cAMP responsiveness seen in intact rats. These might include the accumulation of unidentified extracellular inhibitors of hormone actions (6), formation of prostaglandins (20, 22) or adenosine (21), local generation of peptide inhibitors of AC (42, 43), an altered cellular distribution of calcium (10, 18), and enhanced local hormone degradation (44, 45). Such potential mechanisms have not been systematically evaluated in the present study. However, in agreement with the observations of Plas and Nunez (37), evidence for accumulation of an extracellular inhibitor of glucagon action was lacking in our in vitro studies.

Under conditions of the present study, hepatic cAMP responsiveness was the only biologic action of glucagon examined which waned detectably with time. Increases in plasma glucose and hepatic phosphorylase activity and decreases in synthetase activity, actions of glucagon thought to be mediated by cAMP (3, 46), were similar in liver exposed to the hormone for brief or prolonged periods. This was correlated with persistent elevation in cAMP levels during sustained glucagon stimulation. Since small increases in cAMP may be sufficient for expression of the glycogenolytic and gluconeogenic actions of glucagon (3, 46), the persistent increases in cAMP could have mediated the changes in plasma glucose, PHOS, and %GS-I. In this regard, our findings are analogous to those reported in fat cells (7, 8). However, in studies of cultured fetal hepatocytes previously exposed to glucagon, no glycogenolytic action and no further increase in cAMP was observed in response to a second addition of glucagon, even though cAMP levels in such cells remained fourfold higher than those without prior glucagon exposure (37). At present, the explanation for these apparent differences is uncertain.

Finally, since decreases in cAMP responsiveness induced by prior exposure of tissue to effector hormone have now been reported in a variety of organs, it is tempting to speculate that this change may have adaptive value. Regulation at the level of AC with an apparent reduction in responsiveness of the enzyme to

hormonal, but not to NaF stimulation, is not unique to the liver and glucagon. Similar changes occur in several other tissues (13, 14, 17, 40). Such "desensitization" of hormone-responsive AC by prior exposure of tissue to the hormone might serve to limit energetically wasteful cAMP generation during periods of prolonged stimulation. Whether such changes have physiologic significance is at present conjectural, but as noted above, reductions in both hepatic binding (41) and in hepatic cAMP responsiveness to glucagon (5) may occur during periods of sustained endogenous hyperglucagonemia.

## ACKNOWLEDGMENTS

We are greatly indebted to Charles Pfender and Nancy Oakman for their expert technical assistance, to Dr. James B. Field for determination of plasma IRG concentrations, and to Mrs. Ruth Greenlee for secretarial assistance.

This work was supported in part by a grant received from the University of Pittsburgh, School of Medicine Alumni Fund.

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