Relationship of Some Hepatic Actions of Insulin to the Intracellular Level of Cyclic Adenylate

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SUMMARY

Glucagon and/or the catecholamines elevate tissue cyclic adenylate (cAMP) and thereby change the activity of a number of metabolic processes, as studied in the isolated perfused rat liver. Insulin can lower cAMP in liver and thereby opposes these changes. In liver, in the basal state, there appears to be a substantial quantity of cAMP which is compartmentalized, unaffected by insulin and physiologically inactive. The hormone effects are exerted on a pool of "free cAMP," and changes in the level of this pool control the minute-to-minute output of glucose and presumably effect rapid regulation of potassium fluxes and other processes. The level of cAMP in the active pool in the perfused liver is reflected by the efflux of the nucleotide into the medium. Not all insulin effects in liver or other tissues are mediated by a fall in cAMP. DIABETES *21* (Suppl. 2):439-46, 1972.

Insulin has a number of direct actions on hepatic metabolism. In this report, the possibility will be discussed that several of these effects are in part or entirely the consequence of a fall in the level of cAMP within the tissue cells. In line with this proposal, we have noted that certain metabolic systems are affected detectably by insulin only when they have first been activated or inhibited, as the case may be, by a rise in tissue cAMP. On these systems, insulin acts as a direct antagonist of glucagon and catecholamine, the hormones which raise hepatic cAMP. We do not believe, however, that a fall in tissue cAMP will account for all insulin effects, as will be discussed later.

If insulin does indeed lower intracellular cAMP, it might be expected to moderate or reverse all effects caused by a rise in intracellular cAMP. In table 1, some effects of elevated cAMP (or of glucagon and epinephrine) on the liver are listed. The table shows whether or not insulin antagonizes each effect in those cases which have been studied.

The most extensively studied effect of insulin on the liver is to reduce glucose output. To show this effect,

TABLE 1

Effects of cAMP (or glucagon or catecholamine) on the isolated rat liver, and the antagonistic action of insulin. (Sources of these data are given in reference 1, except as noted).

Effect	Agent	Antagonized by insulin
Stimulation of glycogenolysis or activation of phosphorylase[1,2]	glucagon	+
	catecholamine	+
	cAMP	+
Stimulation of gluconeogenesis[1]	glucagon	+
	catecholamine	not tested
	cAMP	+
Stimulation of ureogenesis[1,2]	glucagon	+
	catecholamine	not tested
	cAMP	not tested
Stimulation of initial K uptake[1,2]	glucagon	not tested
	catecholamine	not tested
	cAMP	not tested
Stimulation of K loss[1,3]	glucagon	+
	cAMP	+
Stimulation of Ca flux[1]	glucagon	not tested
	catecholamine	not tested
	cAMP	not tested
Stimulation of histone phosphorylation[1]	glucagon	not tested
	cAMP	not tested
Stimulation of proteolysis (probably by lysosomal activation)[4]	glucagon	+
Inhibition of lipoprotein release[5]	glucagon	not tested
Induction of enzymes:		
tyrosine amino transferase[1]	glucagon	?*
	cAMP	?
phosphoenolpyruvate carboxykinase[1]	glucagon	+
	catecholamine	+
	cAMP	+

*Antagonism is difficult to test inasmuch as insulin also "induces" this enzyme, although apparently by a different mechanism.

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certain conditions are required and the numerous negative reports in the older literature often reflect the failure to meet these conditions. The essential condition in our experience is that intracellular cAMP be elevated, but not too much. To clarify this statement and to bring out some of the complexities and uncertainties in understanding the insulin response, it is useful to consider first how cAMP levels change on exposure of liver to glucagon and/or catecholamines.

The enzyme adenyl cyclase, which catalyzes the conversion of ATP to cAMP, is located in the plasma membrane.[6,7] It is activated as a result of the combination of glucagon and/or catecholamines with specific receptors,[7-9] presumably at the external surface of the cell. The nature of the signal generated by the receptor-hormone interaction is not known, nor is it known how the signal is transmitted to the cyclase. The signal must pass through the cell membrane, however, since cAMP is formed intracellularly.

The pattern of the rise in tissue cAMP on exposure to glucagon or to epinephrine is complex and very poorly understood. The response to a concentration of glucagon which gives a maximal stimulation of adenyl cyclase is shown in figure 1. The nucleotide concentration may be increased as much as fifty times. By contrast, the rise induced by a maximally effective concentration of epinephrine is relatively small, about three times (figure 2), and is biphasic. Within about sixty seconds, in the continued presence of catecholamine, the tissue cAMP declines to a stable value about two times the basal level. A current hypothesis to account for these curves is as follows. Glucagon receptors constitute a single species, distinct from the catecholamine receptors, and are exclusively stimulatory to adenyl cyclase. Epinephrine receptors, on the other hand, are of two kinds: One is the so-called beta type, which activates adenyl cyclase and raises cAMP, and the other is the alpha type, which brings about a lowering of cAMP by mechanisms that are not yet clear.* It is possible that opposing influences exerted by activation of these receptors result in the biphasic response. A similar pattern of response to cAMP has also been seen in heart[11,12] and adipose cells in the presence of caffeine (see below). In these tissues there is other evidence for two receptor types.

Recent observations by Ho and Sutherland[13] and Manganiello et al.[14] are of great interest and suggest other possibilities in regard to the above response. Working with isolated fat cell suspensions, Ho has found that the cells become refractory to epinephrine stimulation within a minute or less. As shown in figure 3, addition of more catecholamine at any time after the intracellular level of cAMP begins to fall following the initial catecholamine stimulation fails to elicit any elevation of the nucleotide concentration. It is suggested

*Attempts to characterize receptors as alpha or beta in rat liver by use of the usual activators and blocking agents has been very unsatisfactory.[10] It is attractive to follow the suggestion of Robison[20] that alpha receptors be considered, by definition, those which lead to lower levels of cAMP and that beta receptors are those which raise cAMP.

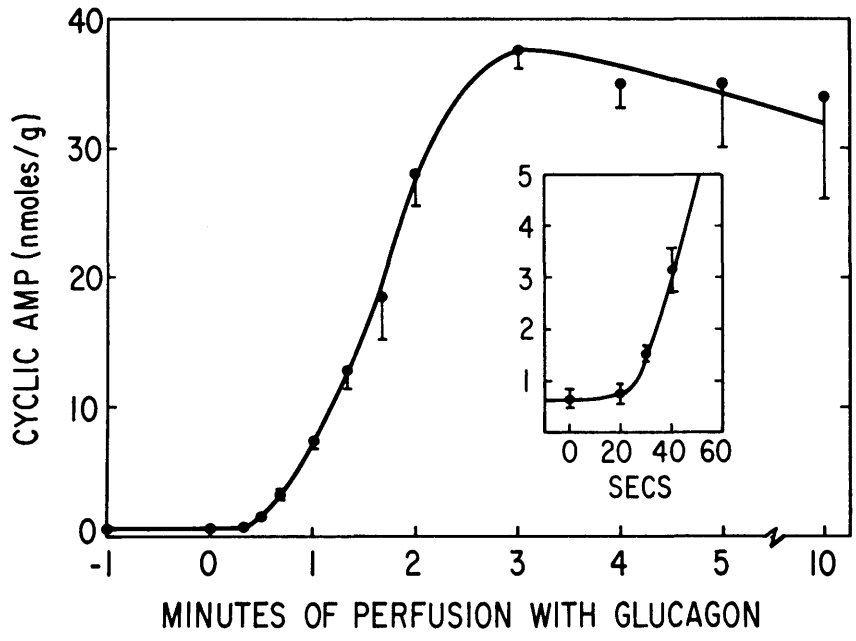

FIGURE 1

Time course of cyclic AMP accumulation in livers perfused with glucagon. Livers from fed male rats were perfused without recirculation. After a twenty-minute control period, glucagon was infused at 0.1 nmole per min. into the influent medium. Tissue samples were rapidly frozen at the times indicated and analyzed for cyclic AMP. Mean values of at least four members are plotted. The vertical lines show standard errors of the means.

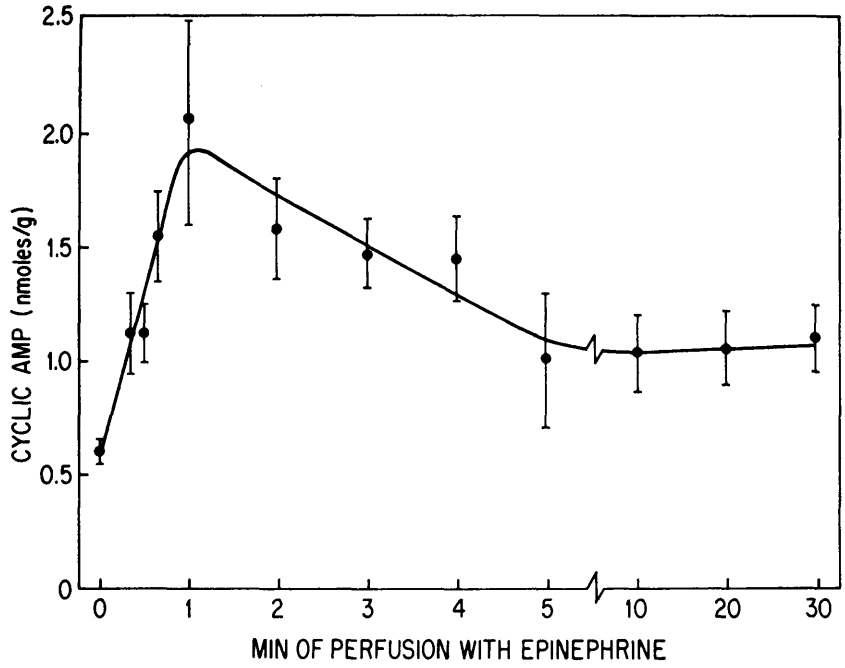

FIGURE 2

Time course of cyclic AMP accumulation in livers perfused with epinephrine. Experimental details are the same as for figure 1 except that epinephrine was infused at 14 nmoles per min.

that this refractoriness is due to the formation and/or release of an inhibitory substance. This is evidenced by the finding that the incubation medium from cells exposed to epinephrine suppresses the response of fresh cells to catecholamine (table 2). One action of the inhibitory material is to suppress adenyl cyclase activation directly or indirectly. Whether or not it also stimulates cAMP phosphodiesterase has not been determined. The inhibitor, as it appears in the medium, has not yet been characterized chemically except to note that it is largely albumin bound but does not seem to be a common fatty acid. It is heat stable and partially extracted by lipid solvents. Its effect is not reproduced by a number of prostaglandins.

The formation of the inhibitor is not peculiar to catecholamine action but is also observed on addition of other hormones which elevate cAMP in fat cells, such as ACTH and glucagon.[13] In fact, exposure of

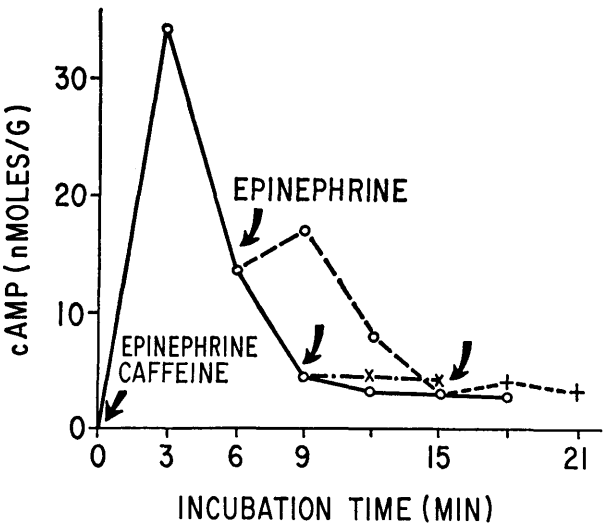

FIG. 3. Levels of cAMP following single and repeated exposure to epinephrine. Fat cell suspension, 4.6 ml., was incubated at 37° in a Krebs-Ringer bicarbonate-albumin medium. To each vial, epinephrine (6.1 x 10^{-7} M) and caffeine (1.1 mM) were added at zero time. To some vials, a second dose of epinephrine was added at 6, 9, or 15 min. (O - - - O, X — • — X, and + — — — +, respectively). Samples were fixed at the times indicated and the results are expressed as nanomoles of cAMP per gm. of fat cell lipid. Each point is a mean of two or three cell incubations.

TABLE 2

Suppression of epinephrine-induced accumulation of cAMP in fat cells by a factor released into the medium.

Fresh fat cells were incubated in various proportions of "incubated" and fresh medium and cAMP accumulation determined. "Incubated medium" was prepared by a separate incubation of fat cells with epinephrine and caffeine for fifteen minutes followed by removal of the cells. Details are given in reference 13.

Incubated medium (ml.)	Control medium (ml.)	cAMP in cells (pmoles)	Inhibition (%)
0	4	76	0
1	3	56	25
2	2	42	45
3	1	30	60
4	0	25	67

cells to dibutyryl-cAMP or cAMP itself causes production of the substance, which suggests that cAMP itself is the common factor in all conditions giving rise to the inhibitor.[13] It is possible, therefore, that Ho and Sutherland are observing the operation of an interesting feedback loop in which cAMP production is to some extent self-limiting.

The question of whether the above system is present in other tissues is under investigation. We do not yet know, for example, whether or not exposure of liver to suboptimal (physiological) levels of glucagon will cause, like epinephrine, a biphasic cAMP response. Preliminary suggestions, however, are that this may be the case and that the liver may produce a similar inhibitory substance. In any case, Ho and Sutherland's observations suggest the presence of a previously unrecognized mechanism by which cAMP is controlled and may bear on the question of how insulin can lower tissue cAMP. It will also be interesting to see if the inhibitor substance, when characterized, can be legitimately classified as another "second messenger," analogous to cAMP itself.

The relationship of the tissue level of cAMP to activation of metabolic process is not a simple one. This point can be illustrated by consideration of the effects of the nucleotide on glycogenolysis. In the first place, a substantial "basal" amount of cAMP is present in livers perfused under conditions in which glycogenolysis is inactive. The concentration of the nucleotide, if it were evenly distributed in the intracellular water, would be about 1×10^{-6} M, which would be expected to activate substantially the cAMP-sensitive histone kinase in liver described recently by Langan[15] and Kumon et al.[16] However, this kinase, which may be identical to the kinase which activates the phosphorylase system, is not activated in liver in the basal state (see below). While studies have not yet been carried out with liver enzyme, Corbin and Soderling in unpublished work from this laboratory have found that the protein kinase in extracts of fat cells is fully dissociated, i.e. activated[17] on exposure to 1×10^{-6} M cAMP. It appears probable, therefore, that the basal cAMP is bound or sequestered out of contact with this kinase in the cytosol. Alternatively, the basal cAMP may be inactive because of the presence of inhibitors. Preliminary experiments favor the idea of sequestration since a substantial portion of cAMP can be centrifuged down in particulate fractions of sucrose homogenates of liver tissue (J.D. Corbin, unpublished results, this laboratory). It may also be noted at this point that the basal level of cyclic nucleotide is not lowered by insulin.

If the above considerations are correct, it would follow that very small elevations of "free" or "active" cAMP might cause a large activation of glucose production but might be difficult to detect analytically because of the large background of inert cAMP. This seems, in fact, to be a problem in studies of the relation of cAMP level to enzyme activity in all tissues examined, since they all have a high background level of cAMP (see reference 1). In the liver, a rise in tissue cAMP to two times the background is sufficient to cause full activation of glycogenolysis. This degree of stimulation is not unreasonable since, according to the above discussion, the level of free cAMP may have risen very many fold. This argument could also explain why epinephrine brings about as high a rate of glucose production as glucagon although the latter can cause a much higher elevation of cAMP.

It is possible that various metabolic processes require different levels of cAMP for activation. One does not know, for example, by what mechanism most processes are affected by cAMP, although it is attractive to consider that activation of protein kinase could be the common factor. If sensitivities to cAMP do indeed differ, epinephrine or glucagon could have different metabolic effects although the same "second messenger," as cAMP has been termed by Sutherland, were involved in all cases. Glucagon could activate the less sensitive processes which epinephrine could not affect. Rough estimates of the relative sensitivity of four processes to glucagon, epinephrine and exogenous cAMP are shown in table 3. The values suggest that glycogenolysis and gluconeogenesis are about equally sensitive to stimulation and are much more so than ureogenesis and ketogenesis. Thus, glucagon should be more effective than epinephrine for activation of the latter processes. This suggestion ap-

TABLE 3

Estimates of the concentration of glucagon or epinephrine for half-maximal activation of several metabolic processes.

Livers of fed rats were perfused without recirculation of the medium. Glycogenolysis was estimated from the glucose output in perfusions without added substrates; gluconeogenesis was estimated in other experiments from the conversion of [^{14}C]lactate (20mM) to [^{14}C]glucose. Ureogenesis and ketogenesis were determined in perfusions which contained no added amino or fatty acids.

Hormone	Glycogenolysis	Gluconeogenesis	Ureogenesis	Ketogenesis
	M			
Glucagon	5×10^{-11}	1×10^{-10}	5×10^{-8}	5×10^{-8}
Epinephrine	1×10^{-7}	1×10^{-7}	*	†
cAMP	4×10^{-5}			

* Beginning stimulation at 5×10^{-5} M.
† No significant stimulation at 5×10^{-5} M.

pears to be supported by the data of table 4 which show substantial stimulation of ureogenesis and ketogenesis by glucagon but little or none by epinephrine. Whether or not these differences have physiological significance is uncertain since secretion of catecholamines or glucagon may not reach high enough levels in the intact animal to result in a separation of functions. It should be emphasized further that the above experiments have examined ketogenesis and ureogenesis from endogenous liver substrates, whereas the extrahepatic tissues are usually the major sources of these substrates. Activation of these four processes is suppressed by insulin, consistent with an effect of the hormone to lower tissue cAMP.

The minute-to-minute control of glycogenolysis, gluconeogenesis and other processes which are very sensitive to cAMP probably involves small changes in level of "free" cAMP in a concentration range near the limits of detection by direct analysis of hepatic tissue for the reasons mentioned earlier. Recently, however, a new approach has been developed which may provide a means of monitoring these changes. The liver is exceptional among tissues in its relatively high sensitivity to extracellular cAMP, which suggests that the hepatic cells are unusually permeable to the nucleotide. This suggestion has been supported by the recent findings[18] that glucagon administration in man can cause a large rise in plasma cAMP, presumably reflecting hepatic efflux. Our studies with the isolated rat liver show that glucagon brings about a net efflux of the nucleotide into the perfusion medium. It is reasonable to suppose that this efflux reflects a rise in the fraction of cAMP that is free in the cytosol and is little affected by sequestered or bound nucleotide.

TABLE 4

Differential effects of optimal concentrations of glucagon or epinephrine on metabolic processes in perfused liver. Hormones were infused continuously to give inflow concentrations of about 1×10^{-7} M for glucagon and 1×10^{-5} M for epinephrine.

Additions	Glycogenolysis	Gluconeogenesis	Ureogenesis	Ketogenesis*
	μmoles. 100 gm. rat^{-1} • hr.$^{-1}$			
None	180 ± 8	122† ± 9	43 ± 2	11 ± 1
Glucagon	665 ± 25	257† ± 36	90 ± 8	25 ± 2
None	205 ± 9	105† ± 6	45 ± 2	11 ± 1
Epinephrine	530 ± 30	182† ± 4	60 ± 2	14 ± 1

*Acetoacetate plus betahydroxybutyrate.
†c.p.m. × 10^{-3} of [^{14}C]glucose from [^{14}C]lactate in medium.

The correlation between cAMP levels and activation of glucose production is shown in figure 4. Without glucagon addition, almost no cAMP appears in the medium despite substantial amounts in the tissue, as noted earlier (see also table 5). With increasing, but suboptimal, concentrations of glucagon, cAMP in the medium rises very markedly in a manner parallel with the increase in glucose production. The rise in cAMP in the medium is suppressed by insulin with a concomitant suppression of glucose output. With higher concentrations of glu-

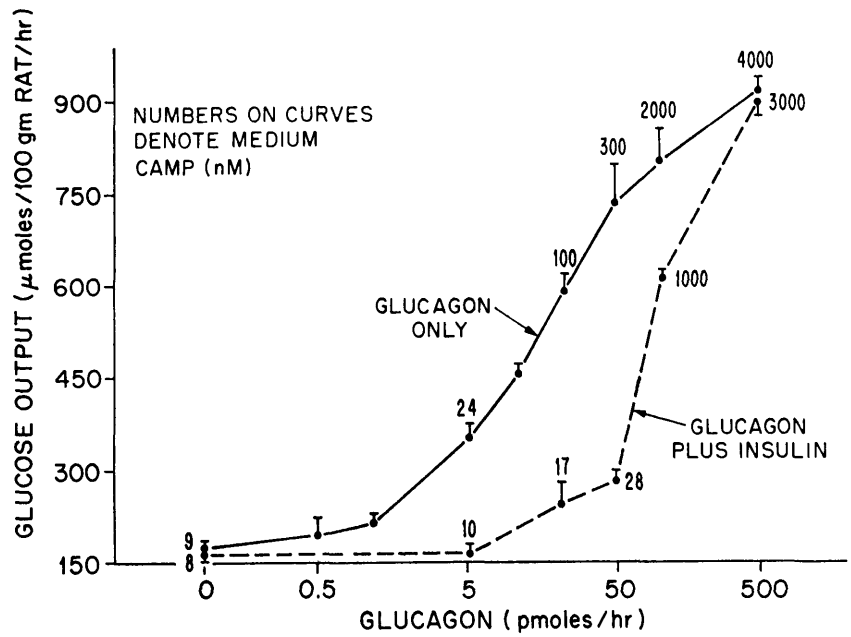

FIGURE 4

Effects of glucagon and insulin on effluxes of glucose and cAMP from the perfused rat liver. Livers from fed rats were perfused with recirculation for sixty minutes with a constant infusion of glucagon to give the concentration shown. Insulin in addition to glucagon was administered as a constant infusion in the lower curve. The effluent medium was collected for estimation of cAMP. The values for cAMP in nanomoles are shown by the numbers beside the points on the curves.

TABLE 5

Effect of alloxan diabetes, insulin, fasting and feeding on level of cAMP in liver. Livers were rapidly removed from anesthetized rats and fixed by freeze clamping for estimation of cAMP. Livers from similarly treated rats were perfused for one hour with [^{14}C]lactate (20 mM) to estimate gluconeogenesis by formation of [^{14}C]glucose.

Conditions	Hepatic cAMP	Gluconeogenesis
	mμmole/gm. liver	c.p.m. × 10^{-3} • 100 gm. rat^{-1} • $hr.^{-1}$
Normal fed	0.49 ± 0.06	83
Alloxan diabetic	0.96 ± 0.07	246
Alloxan diabetic treated 30 min. with insulin in vivo	0.49 ± 0.07	
Normal fasted, 24 hr.	0.85 ± 0.18	164

cagon, glucose production and cAMP levels in medium rise to very high values and the suppressive effect of insulin is no longer observable. It is possible that insulin still lowers cyclic nucleotide levels under these circumstances, but the effect becomes lost in the variability of the measurements. In any case, the insulin effect, if it occurs, probably does not lower the cAMP enough to bring it into the concentration range in which it is rate-limiting for glucose production. It is to be noted that the effects of insulin on glucose production and efflux of cAMP occur in the physiological range of glucagon concentrations. Estimates of the levels of cAMP in the tissue in these same studies (not shown) show a rise with increasing glucagon concentrations, but the suppressive effect of insulin is small relative to the changes in the medium. The insulin effect on the tissue is detectable only at the lower, physiological concentration of glucagon.

Since insulin is always present in the portal blood, it would seem probable that it exerts a variable but ever-present depressant effect in vivo on the levels of hepatic free cAMP. This concept is supported by the observation that injection of insulin antiserum into the normal rat brings about an immediate rise in hepatic cAMP.[19] The sole action of the antiserum, as far as is known, is to inactivate insulin, thus leaving unopposed the influence of glucagon and catecholamines.* The resultant rise in cAMP is associated with increased glycogenolysis and gluconeogenesis. Severe acute alloxan diabetes also causes a rise in liver cyclic nucleotide which is restored to normal by insulin administration (table 5). Fasting is associated with low insulin and elevated glucagon secretion, and, in this condition also, hepatic cAMP is elevated and gluconeogenesis activated (table 5). Furthermore, hepatic cAMP rises to higher levels than it does in livers from fed rats upon exposure to glucagon,[21] which suggests an altered balance between the adenylate cyclase and phosphodiesterase systems. Recent studies by Exton et al.,[22] however, suggest that a chronic elevation of cAMP in liver differs functionally in certain regards from an acute elevation. In livers of alloxan diabetic rats, cAMP does not leak into the medium as it does when cAMP is raised acutely to the same level by glucagon in liver from a normal rat. Furthermore, the high rate of glucose output by the diabetic liver responds very poorly to insulin in vitro over a period of three hours, whereas substantial insulin effects are seen in vivo within sixty minutes.

At the present time, it would seem likely that the balance between insulin and glucagon secretion, as it affects hepatic cAMP, may be the most important regulatory mechanism for the minute-to-minute control of glucose output. An example of how such a regulation might occur is illustrated in the experiment of figure 5. The output of cAMP is taken as an index of the level of functional cAMP in the tissue. Changes in cAMP efflux with glucagon and insulin precede, but are in the same direction as, those in glucose output. This figure also illustrates the time course of cAMP and glucose changes in response to low levels of the hormones. The response of cAMP is considerably faster than that of glucose and is fast enough to suggest that enzyme activation or deactivation is involved rather than enzyme synthesis.

Another system illustrating the possible mediation of certain insulin effects by a reduction in intracellular cAMP is that which controls cellular potassium.[3,23] Some studies[23] bearing on this point are shown in table 6. The isolated liver from a normal fed rat was first perfused for one hour, during which time the endogenous cAMP fell to a minimal level. This fall presumably reflects the subsidence of adenyl cyclase activation[10] induced by sympathetic discharge during the operative procedures. The fall is accompanied by a decline in the high initial glucose output to a very low value. During the second hour of perfusion, the net changes in potassium content of the recirculating perfusate were measured in the absence or presence of the hormonal agents. It can be seen that K^+ release was very low in the absence of cAMP and rose substantially as the cAMP level was raised. Insulin had little effect in the absence of cAMP but suppressed the effects of the low level of cAMP almost completely. At high rates of cAMP infusion, however, insulin was

*It is not likely that circulating catecholamines reach high enough concentrations to be effective, but catecholamine released at nerve endings in the liver may be very important.

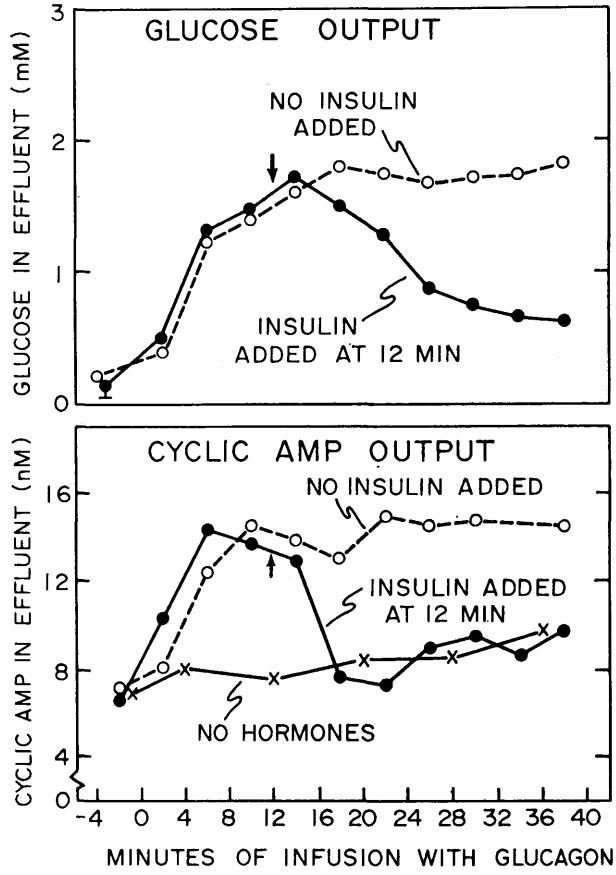

FIG. 5. Interaction of glucagon and insulin in the control of glucose output by the perfused rat liver. Livers of fed rats were perfused with recirculating media for a one-hour control period and then perfusion without recirculation was begun at −4 min. Glucagon infusion was maintained from 0 time (except for the curve shown by x's) at a steady rate well below that giving a maximal effect. Insulin infusion was superimposed on the glucagon infusion where indicated.

again ineffective. This pattern of cAMP-insulin antagonism is exactly as seen in the case of glucose output, and the interpretation is the same. Insulin is effective only when "free" cAMP is slightly or moderately elevated. If cAMP is too high, however, insulin is ineffective. It is possible that the hormone may still lower cAMP levels but not enough to make it rate-limiting. The mechanism by which cAMP affects K fluxes is unknown.

The history of research into the mechanism of insulin action is notable for an abundance of errors, oversimplifications and misinterpretations. With this in mind, we would like to emphasize some of the uncertainties and questions with regard to the present study: (1) A dose-response curve suggests that the effects of insulin on

TABLE 6

Effects of insulin and cyclic cAMP on net potassium efflux from the perfused rat liver. Livers were first perfused for one hour with recirculating media; then 1 ml. insulin solution or 1 ml. saline was added to give a perfusate insulin concentration of approximately 25 mU./ml. Very slow infusions of cAMP were then begun into the tube leading to the liver at concentrations to give the indicated concentrations in the perfusate fluid. Insulin infusion was begun, where indicated, at the same time as the infusion of cAMP. These infusions were continued throughout the second hour, with continued recirculation.

Cyclic AMP concentration entering liver	Change in perfusate K^+ μmoles/100 gm. rat/second hr. Insulin	No insulin	p
0	+ 6.2 ± 3.8	+18.2 ± 2.3	.05
3×10^{-6} M	+ 3.9 ± 3.9	+30.6 ± 4.4	.005
6×10^{-6} M	− 6.9 ± 4.0	+42.2 ± 4.3	.005
1.2×10^{-5} M	+ 4.2 ± 3.6	+35.6 ± 4.3	.005
2.4×10^{-5} M	+17.2 ± 3.2	+41.2 ± 3.8	.005
4.8×10^{-5} M	+22.9 ± 2.6	+41.5 ± 3.2	.005
9.6×10^{-5} M	+40.2 ± 6.8	+47.4 ± 7.2	n.s.
1.2×10^{-4} M	+40.3 ± 4.9	+44.4 ± 4.5	n.s.

cAMP-mediated processes in liver require substantially higher concentrations of hormone than do the antilipolytic or glucose transport effects in adipose and muscle tissue. On the other hand, the liver is exposed physiologically to much higher insulin loads (concentration X plasma flow) than muscle and fat. We have not yet tested monocomponent insulin (see Schlichtkrull et al., this symposium, page 649) in our system, but insulin antibody blocks the effects of the insulin preparations tested (Novo insulin, ten times recrystallized). (2) The lowering of cAMP levels by insulin may be an associative rather than causative factor in the effects we have seen. For example, insulin could lead to the formation of its own particular "second messenger" which could act to oppose certain actions of cAMP as well as to lower cAMP. Studies of Soderling and Corbin[24] have shown that insulin brings about recombination of the catalytic and regulatory subunits of cAMP-dependent protein kinase in adipose tissue exposed to epinephrine. This is the expected consequence of a lowering of cAMP in that tissue but does not exclude a more direct effect of another second messenger on the protein kinase. We feel that a number of effects of insulin on the liver (e.g. induction of certain enzymes) and in other tissues (e.g. stimulation of glucose transport) are probably not due to a lowering of cAMP. These cAMP independent effects may reflect the presence of an unidentified second messenger system and may be closer to the primary action of the hormone than the effects mediated through lowered cAMP.

ACKNOWLEDGMENT

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