Insulin inhibition of hepatic cAMP-dependent protein kinase: Decreased affinity of protein kinase for cAMP and possible differential regulation of intrachain sites 1 and 2

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ABSTRACT In hepatocytes stimulated with 8-bromo-cAMP, insulin decreases the affinity of the cAMP-dependent protein kinase for cAMP, shifting the $K_a$ without affecting the $V_{max}$ activity. This occurs under conditions where cyclic adenine nucleotide concentrations are unchanged. We report here that glycolysis stimulated by 8-(4-chlorophenylthio)-cAMP, an analog with 100 times tighter affinity than cAMP for the protein kinase regulatory subunit, was only slightly antagonized by insulin. The tight binding of this analog appears to overcome the protein kinase affinity change induced by insulin. The relative importance of the two intrachain cAMP binding sites of the cAMP-dependent protein kinase regulatory subunit was investigated by using analogs with relative selectivity for each site. Analogs exhibiting preferential binding to site 2 were far less sensitive to insulin antagonism than were analogs binding preferentially at site 1 and less well at site 2. No other property of these analogs, including the rate of hydrolysis by phosphodiesterase, the $IC_{50}$ for phosphodiesterase, the $K_a$ for protein kinase, or the type I versus type II kinase specificity, could account for the ability of insulin to antagonize glycolysis stimulated by these analogs. These data indicate that insulin may act to decrease the affinity of protein kinases for cAMP through a possible regulation of intrachain site 2 binding.

Glucagon, by increasing intracellular cAMP levels, activates cAMP-dependent protein kinases. Its counterregulatory hormone, insulin, can antagonize these responses through several mechanisms (for a review, see ref. 1). Insulin stimulates hepatic cAMP phosphodiesterase to decrease cAMP concentrations (2, 3); however, cAMP destruction is not required to terminate the glucagon signal (4). Insulin inhibits hepatic cAMP-dependent protein kinase in the absence of any changes in cyclic adenine nucleotide concentrations (4–6). A similar inhibition of protein kinase activity has been observed in diaphragm (7) and skeletal muscle (8); however, the mechanism of this response has remained obscure.

In recent years, many analogs of cAMP have been developed with differing specificities towards protein kinase (9, 10). Corbin and coworkers (11, 12) have discovered that the regulatory subunit of the cAMP-dependent protein kinase contains two intrachain cAMP binding domains, termed site 1 and site 2, based on their sensitivity to various cAMP analogs. Both binding sites appear to be involved in protein kinase activation (13, 14). The differing specificities of these cAMP analogs permit the examination of protein kinase regulation in intact cells. In perfused heart, insulin decreases $[^3H]cAMP$ binding, which is specific for intrachain site 2, suggesting the possible regulation of this binding site on protein kinase (15).

Here we demonstrate that, in liver, insulin can decrease the affinity of cAMP-dependent protein kinase for cAMP and indicate a possible role for intrachain site 2 binding based on the specificity of insulin antagonism of several cAMP analogs. A preliminary report of these findings has appeared (16).

MATERIALS AND METHODS

Materials. 8-Thiopropyl-cAMP was from ICN; 8-(4-chlorophenylthio)-cAMP (CIPhs-cAMP) and $\alpha_2$-macroglobulin, Boehringer Mannheim; insulin, Eli Lilly; [$\gamma$-32P]ATP, Amersham; and all other reagents, Sigma.

Hepatocyte Incubations. Hepatocytes were isolated from fed rats and incubated as described (4) in Krebs-Henseleit buffer containing 2.4 mM CaCl$_2$, 1.5% defatted bovine serum albumin, and 0.8 mg of bacitracin per ml for 5 and 20 min for protein kinase and glycolysis measurements, respectively. Glucose was measured in KOH-neutralized perchloric acid extracts (17).

Protein Kinase Measurements. Cell extracts were prepared and assayed for cAMP-dependent protein kinase as described (4, 18) with the following modifications. Sedimented hepatocytes were washed twice in the homogenization buffer (100 mM KCl containing 0.5 mM 1-methyl-3-isobutylxanthine, 45 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 50 μg of $\alpha_2$-macroglobulin per ml, 10 mM EDTA, and 10 mM potassium phosphate at pH 6.8) to remove exogenous cyclic nucleotides. After homogenization, extracts were centrifuged (100 × g for 2 min) through 1-ml syringe columns of Sephadex G-50 and equilibrated in homogenization buffer to eliminate low molecular weight substances that might interfere with the cAMP titrations of protein kinase activity. The final concentrations of the assay components were 5 mM Mg(OAc)$_2$, 10 mM NaF, 0.23 mM [$\gamma$-32P]ATP (100 cpm/μmol), 30 mM potassium phosphate (pH 6.8), and 105 μM kemptide, as the phosphorylatable substrate. Protein kinase activity was measured within 45 min of the termination of hepatocyte incubations. This was necessary to preserve the effect of insulin on cAMP-dependent protein kinase (see below). Total protein kinase activity in homogenates was inhibited >90% by muscle cAMP protein kinase inhibitor. The cAMP-independent protein kinase activity was, in all cases, subtracted from the activities measured above.

Stability of the Insulin Effect. The inhibitory effect of insulin on cAMP-dependent protein kinase was reversed following incubation of cell extracts for 1 hr at 4°C, as manifested by an increase in the activity of the insulin-treated sample to levels approaching those of the control samples (which remained relatively constant). Therefore, the previous isolation procedure (4) was modified to include protease inhibitors and NaF (as indicated above) to stabilize the insulin response. This permitted the consistent observation of an effect of insulin on protein kinase, providing extracts were assayed

Abbreviation: CIPhs-cAMP, 8-(4-chlorophenylthio)-cAMP.

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within 45 min of hepatocyte homogenization (Fig. 1). Larner and colleagues (7) have reported a similar transient inhibition of protein kinase in rat diaphragm. In agreement with their observations, when more concentrated extracts of cells were prepared (20–30 μl of buffer per mg wet weight of cells), the insulin response was abolished. However, when our standard homogenization dilution was used (50 μl of buffer per mg wet weight of cells) and extracts were assayed at a higher concentration in the assay cocktail (so that the amount of cellular protein was equal to that used in the concentrated homogenization conditions), the response to insulin was retained. This indicates that the loss of protein kinase inhibition by insulin in more concentrated homogenates is the result of factors affecting the enzyme between homogenization and initiation of the assay and not the consequence of factors influencing expression of enzyme activity.

Expression of Results. The effect of insulin on glucose release is expressed as the percent inhibition by insulin of the increment of glucose release (75%) induced by cAMP or its analogs. The percent insulin effect is calculated from the basal rate of glucose release in the absence of any effector additions to the hepatocyte incubation (B), the rate in the presence of the analog stimulator (S), and the rate in the presence of both S and insulin (I) as follows:

\[
\% \text{ I effect} = \frac{[S - I]}{[S - B]} \times 100.
\]

Because insulin had no effect on the basal rate of glucose release, this expression serves as an indicator of the ability of insulin to antagonize the stimulatory effects of the agents used in this study.

RESULTS

Insulin responses in hepatocytes have been demonstrated for the most part only in antagonism of hormone stimulation (19) and may be mediated in part by inhibition of adenylyl cyclase and enhancement of phosphodiesterase activity. Insulin was found (4) to antagonize the enhancement of the cAMP-dependent protein kinase activity by 8-bromo-cAMP, an analog not appreciably hydrolyzed by phosphodiesterase (20). This inhibition of protein kinase activity is not mediated by any change in cyclic adenosine nucleotide concentration (4).

To investigate the mechanism of protein kinase inhibition, cell extracts were passed through a Sephadex G-50 column to remove any interfering small molecular weight compounds; this treatment did not alter the response to insulin (data not shown). In hepatocytes incubated with 8-bromo-cAMP, insulin decreased the affinity of the cAMP-dependent protein kinase for cAMP (Fig. 1). The apparent Ka for cAMP was increased 41% (from 5.6 to 7.9 × 10^−5 M), while no change in Vmax activity was observed. The effect of insulin on the Ka for cAMP is consistent with the 47% inhibition of hepatic 8-bromo-cAMP-activated protein kinase activity (Fig. 1) and the 57% inhibition by insulin of the rate of 8-bromo-cAMP-stimulated glycogenolysis (4). In cells not stimulated with 8-bromo-cAMP, insulin did not influence hepatic protein kinase activity.

CIPhS-cAMP is an analog that, like 8-bromo-cAMP, is an extremely poor substrate for phosphodiesterase but has 100 times higher affinity for the regulatory subunit of cAMP-dependent protein kinase than either 8-bromo-cAMP or cAMP (20). In isolated hepatocytes, insulin antagonized cAMP-stimulation of glycogenolysis by shifting the dose–response curve for cAMP to the right (Fig. 2). Insulin was only weakly effective, however, against the stimulation of glucose release by CIPhS-cAMP. This supports the hypothesis that, under conditions where the hydrolysis of cNMP is prevented (as is the case with CIPhS-cAMP), alterations in protein kinase affinity for cNMP play an important role in insulin antagonism of cAMP-mediated phenomena. It appears that the tight binding of CIPhS-cAMP to protein kinase can overcome the small shift in affinity induced by insulin.

Two distinct intrachain cAMP binding domains on the regulatory subunit of cAMP-dependent protein kinase have been discovered based on their differential sensitivity to several cAMP analogs (11, 12). The ability of insulin to antagonize the actions of various analogs that differ in site specificity was examined in the glycogenolysis assay. The degree of insulin inhibition of analog stimulation decreased at higher levels of activation just as in the cases shown in Figs. 1 and 2. Consequently, analog concentrations were titrated in each experiment to stimulate the basal rate of glucose release by 75%.

The ability of insulin to antagonize glycogenolysis stimulated by the analogs tested varied from an 82% inhibition of the increment of analog stimulation (for cAMP) to 18% or less for six of the analogs examined (Table 1). Two groups of analogs could be identified, those antagonized by insulin (≥ 35% antagonism) and those termed “relatively insensitive to insulin” (≤ 18% of the 75% stimulation by the analog). For the latter group, the dose–response curves for analog stim-

![Fig. 1. The effect of insulin on the cAMP sensitivity of protein kinase activity. Hepatocytes were incubated for 5 min with 2 μM 8-bromo-cAMP in the absence (○) and presence (●) of 10 nM insulin. The cells were homogenized and assayed for cAMP-dependent protein kinase activity within 45 min as described. Total protein kinase activity, measured in the presence of 5 μM cAMP, was 39 units per mg wet weight of cells. cAMP concentrations were determined spectrophotometrically by using A500 = 15.4 μM⁻¹. Results are expressed as the means ± SEM for duplicate incubations from three different hepatocyte preparations. The stars denote P < 0.01 statistical difference between 8-bromo-cAMP with and without insulin.](image-url)
ulation of glycogenolysis in the presence and absence of insulin were similar to that for CIPHS-cAMP (Fig. 2).

In Table 1 the analogs are listed in the order of decreasing effectiveness of insulin action. Analogs preferentially selective for intrachain site 2 binding were relatively insensitive to insulin action, as compared with analogs preferentially selective for site 1. Although 8-amino-cAMP appeared to be an exception, this analog was hydrolyzed by phosphodiesterase at 90% of the rate of cAMP. Therefore, insulin can antagonize this analog by stimulating its destruction by phosphodiesterase. The relative inability of insulin to antagonize glycogen breakdown stimulated by CIPHS-cAMP is due to the tight binding of this analog to both intrachain cAMP binding sites. 8-Thioisopropyl-cAMP was not antagonized by insulin in liver cells despite its relative nonspecificity for either binding site. This is in marked contrast to the ability of insulin to inhibit lipolysis stimulated by this analog in isolated rat adipocytes under conditions where neither adenylyl cyclase nor phosphodiesterase are involved in insulin action (27). This indicates that insulin may mediate its effects on protein kinase in a tissue-specific manner.

The relative potency of insulin antagonism toward these analogs does not correlate with any of the other parameters compiled from the literature in Table 1. The rate of phosphodiesterase hydrolysis is similar in both groups of analogs. It seems surprising that insulin did not decrease glycogenolysis activated by N\(^6\)-benzoyl-cAMP, since it has been reported that this analog is hydrolyzed by phosphodiesterase at 55% of the rate of cAMP (20). However, this analog may inhibit hepatic phosphodiesterase and not be hydrolyzed in

### Table 1. Percent inhibition by insulin of hepatic glycogenolysis stimulated 75% by various cAMP analogs and comparison with several properties of the analogs

<table>
<thead>
<tr>
<th>cAMP analog</th>
<th>% inhibition by insulin</th>
<th>Site specificity</th>
<th>Rate of hepatic PDE hydrolysis relative to cAMP*</th>
<th>(K_a \times 10^8) of liver protein kinase*</th>
<th>PDE IC(_{50}), (\mu)M</th>
<th>Ratio (K_a) types I/II</th>
<th>Ratio Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antagonized by insulin</td>
<td></td>
<td>Site</td>
<td>Ref.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>82</td>
<td>None</td>
<td></td>
<td>1.0</td>
<td>2.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8-Amino</td>
<td>62</td>
<td>2 (18×)</td>
<td>14</td>
<td>0.90</td>
<td>1.2</td>
<td>83</td>
<td>40</td>
</tr>
<tr>
<td>8-Aminomethyl</td>
<td>58</td>
<td>1 (17×)</td>
<td>24</td>
<td>0.07</td>
<td>1.1</td>
<td>—</td>
<td>33</td>
</tr>
<tr>
<td>8-Bromo</td>
<td>57</td>
<td>1</td>
<td>11</td>
<td>0.11</td>
<td>0.7</td>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td>8-Thiomethyl</td>
<td>49</td>
<td>1</td>
<td>26</td>
<td>0.08</td>
<td>1.0</td>
<td>69</td>
<td>46</td>
</tr>
<tr>
<td>8-Methoxy</td>
<td>35</td>
<td>1</td>
<td>10</td>
<td>0.06</td>
<td>8.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Relatively insensitive to insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(^6)-Aminohexyl-carbamoylmethyl</td>
<td>18</td>
<td>2</td>
<td>26</td>
<td>0.06</td>
<td>1.7</td>
<td>33</td>
<td>597</td>
</tr>
<tr>
<td>N(^6)-Butyryl</td>
<td>15</td>
<td>2</td>
<td>26</td>
<td>&lt;0.05</td>
<td>0.7</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td>N(^6)-Benzoyl</td>
<td>12</td>
<td>2</td>
<td>26</td>
<td>0.55</td>
<td>1.7</td>
<td>—</td>
<td>90</td>
</tr>
<tr>
<td>8-Thioisopropyl</td>
<td>12</td>
<td>2 (15×)</td>
<td>24</td>
<td>0.06</td>
<td>24.0</td>
<td>—</td>
<td>202</td>
</tr>
<tr>
<td>CIPHS-cAMP</td>
<td>13</td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.22</td>
<td>200</td>
<td>27</td>
</tr>
</tbody>
</table>

The effect by insulin is expressed as the percent inhibition of the increment of stimulation (in this case, 75% stimulation) as described. Results are the means of duplicate incubations from three separate hepatocyte preparations. Glycogenolysis was determined by measuring the rate of glucose release during a 20-min incubation as described (the average basal rate was 11.9 \(\mu\)mol/20 min per g wet weight of cells). The concentration of insulin was 10 nM. Analogs are listed in decreasing order of insulin effectiveness. IC\(_{50}\) values for phosphodiesterase (PDE) represent the concentration of analog required to inhibit by 50% the hydrolysis of cAMP by isolated low-\(K_m\) cAMP phosphodiesterase from the sources indicated. All other experimental details are given in the cited references.

*Ref. 20.
†Ref. 21 and 22.
‡Ref. 23.
the intact cell because the addition of a phosphodiesterase inhibitor (Ro 20-1724) only slightly stimulated the glycolytic actions of this analog. The $K_i$ for hepatic kinase and the relative selectivity for type I and type II protein kinases (both present in approximately equal proportion in liver) are comparable for both insulin-sensitive and -insensitive analogs. By titrating analog levels to attain identical degrees of glycolysis stimulation, the potential influence of differences in analog permeability can be avoided; no effect of insulin on hepatocyte permeability to $cAMP$ has been found (28).

For certain analogs large variations have been reported for the IC$_{50}$ values for the low-$K_m$ phosphodiesterase in different tissues (29). Nevertheless, no correlation between the IC$_{50}$ values for lung, adipose, or heart and the susceptibility to insulin antagonism was apparent for these analogs. In many cases, small differences in IC$_{50}$ values corresponded to widely disparate degrees of insulin inhibition. This is consistent with the stability of these analogs to phosphodiesterase and the nonobligatory nature of phosphodiesterase activation in the mechanism of insulin antagonism of cAMP-mediated events (4). The parameter that best correlates with the ability of insulin to inhibit glycolysis stimulated by these analogs is the intrachain cAMP binding site specificity.

**DISCUSSION**

We have demonstrated (4) that insulin inhibits hepatic glycolysis stimulated by 8-bromo-$cAMP$ through a mechanism independent of adenylate cyclase and phosphodiesterase action. The effects of insulin under these conditions can be accounted for by a decrease in the activity ratio for cAMP-dependent protein kinase in the absence of any changes in cyclic adenine nucleotide concentrations (4). Here we report that insulin decreased the affinity of protein kinase for $cAMP$, raising the $K_i$ without influencing the $V_{max}$ activity.

Mor et al. (6) have reported that insulin, acting as the sole hormone, caused a small shift in affinity of protein kinase for cAMP in isolated hepatocytes. Since insulin effects in hepatocytes have been demonstrated for the most part only in antagonism of hormone stimulation (19), the physiological significance of this observation is unclear. No metabolic response of hepatocytes to insulin was demonstrated in their studies (6). Here we report that insulin decreases the protein kinase affinity for cAMP in hepatocytes stimulated with 8-bromo-$cAMP$. The observed increase in the $K_i$ appears sufficient to account for the inhibition of glycogen breakdown observed under these conditions. In contrast to our findings, Mor et al. (6) did not observe any response to insulin when protein kinase was assayed in the absence of added cAMP (i.e., the conventional activity ratio was unaffected). Kinase activity measured without added cAMP in the assay is the best indicator of enzyme activity in vivo because cells are homogenized under conditions where the dissociation/reassociation state of the enzyme remains unchanged (18, 30). In rat diaphragm, insulin also raised the $K_i$ for cAMP without affecting $V_{max}$ activity (7); however, no effect was detected in the absence of added cAMP.

Evidence favoring an important role for protein kinase regulation in the mechanism of insulin antagonism of cAMP-mediated phenomena was obtained by using CIPhs-$cAMP$, an analog with 100 times higher affinity than $cAMP$ for the regulatory subunit of protein kinase (20). Insulin only slightly decreased glycolysis stimulated by this analog (Fig. 2), but antagonized some other analogs that also were relatively resistant to phosphodiesterase hydrolysis (Table 1). This suggests that the weak ability of insulin to antagonize the effects of this analog (31) (Fig. 1) is the result of the analog's high affinity for protein kinase and not its nonsusceptibility to phosphodiesterase hydrolysis, as has been proposed (31). Comparison of the properties of the various analogs and the ability of insulin to antagonize their effects indicates that those that have higher affinity for intrachain site 2 of the regulatory subunit of protein kinase were far less sensitive to insulin regulation. Cyclic nucleotides interact with both types of intrachain binding sites in the process of protein kinase activation (13, 14). Just as the tight-binding affinity of CIPhs-$cAMP$ prevailed over insulin-induced protein kinase affinity changes to render this analog insensitive to insulin, the tighter binding of site 2-selective analogs may have overcome a possible effect of insulin on intrachain site 2 binding affinity.

Recently, Beebe et al. (32) have reported that when several cAMP analogs were tested in hepatocytes and adipocytes only those with a low IC$_{50}$ for phosphodiesterase could be antagonized by insulin; analogs with high IC$_{50}$ values were insensitive to insulin. Several of the analogs with low IC$_{50}$ values for phosphodiesterase were found to be more susceptible to phosphodiesterase hydrolysis than were analogs with high IC$_{50}$ values. Based on these observations, the authors concluded that analog hydrolysis by phosphodiesterase was necessary for insulin antagonism.

In the cases where the same analogs were tested for insulin sensitivity, our results are in general agreement with these authors. However, we differ greatly in our interpretations. By using a new, sensitive method for the detection of hydrolysis of cAMP analogs by phosphodiesterase, hydrolysis of 8-bromo-$cAMP$ and CIPhs-$cAMP$ by adipocyte low-$K_m$ phosphodiesterase could be observed (32). However, the concentrations of 8-bromo-$cAMP$ and CIPhs-$cAMP$ chosen to measure the degree of hydrolysis by phosphodiesterase (17 and 22 $\mu$M, respectively) was far greater than the analog concentrations used in this study (2 and 0.5 $\mu$M, respectively). Under conditions where insulin antagonized the glycolytic actions of 8-bromo-$cAMP$, no change in cyclic adenine nucleotide concentrations (8-bromo-$cAMP$ + cAMP) was observed (4). The addition of maximally effective concentrations of phosphodiesterase inhibitors (1-methyl-3-isobutylxanthine or Ro 20-1724) did not influence 8-bromo-$cAMP$ or insulin action (4). Furthermore, since both 8-bromo-$cAMP$ and CIPhs-$cAMP$ were hydrolyzed at approximately the same rate (32), we would expect equivalent sensitivity to insulin antagonism. This was not observed by us (Table 1) nor by Beebe et al. (32).

The holoenzyme of the cAMP-dependent protein kinase is dissociated by cAMP according to the following equation:

$$R_2C_2 + 4cAMP \rightarrow R_2cAMP_4 + 2C.$$  

Based on our data, it appears that insulin shifts the equilibrium of this reaction to the left by decreasing the affinity of the regulatory subunit for cAMP. The two intrachain binding sites for cAMP on the regulatory subunit are required for activation (13, 14), although their respective roles are unclear. We provide evidence in this paper that insulin inhibition of protein kinase activity in liver may involve an effect on intrachain site 2 binding. In perfused heart, insulin decreases protein kinase binding of $[^{3}H]cIMP$ (15), a site 2-selective analog, supporting a similar mechanism in this tissue. In both heart (33) and liver (under the conditions of this study), insulin does not affect cyclic adenine nucleotide concentrations. However, insulin could regulate the cAMP affinity of protein kinase through changes in the phosphorylation state of the type II kinase (34–37), as has been suggested for steroid hormones in liver (38) and epinephrine action in muscle (39).
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