Cyclic AMP-dependent and Cyclic AMP-independent Antagonism of Insulin Activation of Cardiac Glycogen Synthase*

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The hormonal regulation of glycogen synthase has been studied with isolated perfused hearts that were depleted of 85% of their endogenous glycogen. Glycogen depletion alone promoted a 3-fold activation of glycogen synthase and magnified by 3-fold the response to insulin. Glycogen depletion also facilitated the detection of epinephrine-promoted glycogen synthase inactivation. Hormonal effects on glycogen synthase have been correlated with changes in phosphorylase, phosphorylase kinase, and tissue cAMP levels. Insulin activation of glycogen synthase was observed within 90 s of hormone addition and was maximal by 4 min. A half-maximum effect was obtained at an insulin concentration of 100 micromolar/\text{ml}. Insulin-dependent activation is reversed by \beta-adrenergic agonists, \alpha-adrenergic agonists, and glucagon. Each promote the same degree of inactivation and the maximum extent of inactivation produced by each is independent of whether or not the tissue has been stimulated with insulin. \beta-adrenergic agonists and glucagon act via cAMP, \alpha-agonists most likely act via intracellular Ca^{2+} translocation, and insulin action would appear to be independent of either cAMP or Ca^{2+}. The action of epinephrine on cardiac glycogen synthase is mediated by interaction with both \alpha- and \beta-receptors. As indicated by dose-response curves, receptor occupancy of each occurs to an almost equal extent at suboptimal epinephrine concentrations. Regulation of cardiac glycogen synthase by epinephrine thus is mediated by two second messenger systems which converge to produce the end physiological response.

Glycogen synthase (UDP-glucose:glycogen 4\alpha-d-glucosyltransferase, EC 2.4.1.11) is the rate-limiting step in mammalian glycogenesis and a focal point of hormonal control. Observations, initiated by the work of Larner's laboratory (see Ref. 1 for review), have shown: (i) that the enzyme is regulated by phosphorylation, (ii) that this covalent modification results in the conversion of the active I form to a form (D) whose activity is dependent on glucose 6-phosphate, and (iii) that this inactivation is catalyzed by the cAMP-dependent protein kinase (2, 9). The subsequent work of many investigators (see Ref. 4 for detailed literature citation) has demonstrated that the inactivation of glycogen synthase is a complex reaction. Regulation occurs via multisite phosphorylation and what was initially denoted as the D form in fact refers to one or a mixture of forms that are phosphorylated in distinct peptide sites. Maximum phosphorylation of glycogen synthase would appear to be in the range of 6 to 12 phosphates per enzyme subunit (5-7). Protein kinases that catalyze the phosphorylation of glycogen synthase include not only the cAMP-dependent protein kinase, but also phosphorylase kinase, a Ca^{2+}-calmodulin-dependent protein kinase, and at least one other glycogen synthase kinase that is both cyclic nucleotide- and Ca^{2+}-independent.

We have recently established conditions for studying the phosphorylation of glycogen synthase in the isolated perfused heart (8). This system will permit a comparison to be made between which peptide sites on glycogen synthase are phosphorylated or dephosphorylated in response to different hormones, and the site specificities currently being identified for the various protein kinases that catalyze the phosphorylation of the purified cardiac enzyme (9-11). The hormonal regulation of cardiac glycogen synthase remains to be fully clarified. As has been observed for a wide range of tissues (1, 7, 12-22) insulin activates cardiac glycogen synthase although, in comparison to other tissues, the increases in the I/D ratio have been in general modest (23-26). In addition, in comparison to the well documented effects of catecholamines and/or glucagon on the inactivation of skeletal muscle, liver, and adipose tissue glycogen synthases (18-22, 27-34), epinephrine has been variably reported to activate, inhibit, or have no effect on the cardiac muscle enzyme (8, 26, 35, 36). This current report documents the effects of both catecholamines and glucagon as antagonists of the activation of cardiac glycogen synthase by insulin. It defines what role cAMP has in these processes. It also compares the effects of \alpha- and \beta-adrenergic agonists in the control of cardiac glycogen synthase with their respective effects in the regulation of this enzyme as shown for other tissues (28-34).

EXPERIMENTAL PROCEDURES

Heart Perfusion

Hearts from male Sprague-Dawley rats (230 to 240 g) were perfused essentially as described previously (8, 37, 38). Briefly, hearts were removed under heparin Nembutal-induced anesthesia and perfused by the Langendorff technique with Krebs-Henselet bicarbonate-buffered media (39) either with (experiment of Table I) or without (all other experiments) 11 mM glucose. Perfusion temperature was 37 °C. The hearts were perfused by dripproof for a 3- to 4-min equilibration period and then for 30 min with 26 ml of recirculating media either with or without 11 mM glucose as noted above. At the times indicated in each experiment, the various hormones were added to the recirculating reservoir (see Fig. 2 in Ref. 38) so that the final concentration in the total recirculating perfusate medium was as indicated in each figure legend. Due to the time of circulation through the bubble trap, pump, solenoids, and aortic cannula, the interval between addition of hormone and delivery to the tissue was 20 s. The method of hormone addition employed eliminated the delivery of a bolus of effector to the tissue that would have been in substantial excess of the circulating concentration, and permitted the simple addition of several antagonists and agonists at specific concentrations.
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and times. Under all circumstances, the perfusion with recycling media was for 30 min, independent of which, and in which order, agonists and antagonists were added. Contractile force was measured at approximately 80% of the length tension curve with a force displacement transducer attached by the thread to the apex of the heart. Any heart that did not maintain a rate of greater than 240 beats/min throughout the experiment was rejected. The parameters of the hypertrophic response to a-adrenergic agonists were within the normal ranges we have previously reported (37). At the termination of the perfusion, hearts were freeze-clamped by the method of Wollenberger et al. (40), powdered in a percussion mortar precooled in dry ice, and the powders stored at –80°C.

Tissue Extraction and Enzyme Assays

Glycogen Synthase—The procedure for tissue extraction and determination of I/D activity ratio and other kinetic parameters of glycogen synthase is a modification of that described previously (8). The frozen heart powder (approximately 100 mg) was suspended in 1 ml of 30 mM Tris/chloride (pH 7.5) containing 30 mM KCl, 5 mM EDTA, 100 mM NaF, 45 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 16,000 × g for 15 min and the supernatant decanted through glass wool. The filtrate was diluted 5-fold in extraction buffer containing 1 mg/ml bovine serum albumin. Glycogen synthase activity was assayed by a modification of the method of Thomas et al. (41). The reaction was initiated by the addition of 0.02 ml of diluted heart extract to 0.1 ml of a solution containing 60 mM Tris/chloride (pH 7.8), 40 mM NaF, 12 mg/ml of glycogen, 5 mM EDTA, 5 mM [14C] UDP-glucose, in the presence or absence of 10 mM glucose-1-phosphate. The extent of 14C incorporation into glycogen was determined as previously described (8). Glycogen synthase activity was linear for at least 40 min and was routinely determined from both the 15- and 30-min time points. The per cent I glycogen synthase refers to the ratio of activity observed in the absence and presence of glucose 6-phosphate.

Phosphorylase Kinase—Cardiac phosphorylase kinase was assayed by a modification of the procedure reported previously (37). Between 150 and 200 mg of frozen heart powder were homogenized in 0.75 ml of 30 mM Tris/chloride (pH 7.6), 30 mM KCl, 5 mM EDTA, 100 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. The extract was centrifuged at 39,000 × g and the supernatant decanted through glass wool. Prior to assay the filtrate was diluted with an equal volume of 10 mM glyceral phosphate, pH 6.8, containing 5 mM EDTA, 125 mM NaF, and 45 mM 2-mercaptoethanol. Phosphorylase kinase activity was determined from the extent of incorporation of 32P into skeletal muscle phosphorylase b. For assay, 10 µl of diluted heart extract at 0°C was added to 50 µl of a solution which had been preincubated at 30°C and which contained 50 mM glyceral phosphate, 50 mM Tris/chloride, at either pH 6.8 or 8.2, plus 3.6 mM [γ-32P]ATP, 12 mM magnesium chloride, 18 mM 2-mercaptoethanol, 20 mM CaCl2, 6 mg/ml of phosphorylase b, and 12 mg/ml of glycogen. The extent of 32P incorporation into phosphorylase b was determined for the initial 30-min period by the procedure described previously (37). Data are presented as activity at pH 6.8/6.2 activity ratio that has been employed more routinely (42). This activity ratio was determined for all experimental protocols reported here and, with the exception of a higher degree of variability in the values, always mimicked the data based upon specific activity (see Table II for a comparison). For measurement of phosphorylase kinase activity, phosphorylase b was prepared from skeletal muscle by the procedure of Fischer and Krebs (43) and stored as the lyophilized powder (44). For a sequence of assays the powder was dissolved as we have described (44) and the solution stored at 1°C. For each assay, the phosphorylase b solution was incubated for 20 h at 15°C and only added to the preincubation mixture after the latter had reached a final glucose-1-phosphate concentration of 10 mM and a final temperature of 20°C. The solution was then preincubated for a 10-to 15-min period at 30°C prior to addition of the heart extract (see above). This protocol negated problems of cold-promoted conformational changes in phosphorylase b which affect its efficacy as a substrate.

Phosphorylase—Cardiac phosphorylase was assayed by a modification of the procedure of Cori et al. (45). Heart powder (100 mg) was extracted in 1 ml of 50 mM MES1 (pH 6.1) containing 100 mM NaF, 5 mM EDTA, 45 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The extract was centrifuged at 12,000 × g for 10 min, decanted through glass wool, and the filtrate diluted 5-fold in extraction buffer containing 1 mg/ml of bovine serum albumin. For assay the incubation media contained diluted extract, 100 mM NaF, 50 mM MES (pH 6.1), 5 mM EDTA, 20 mg/ml of glycogen, and 30 mM glucose-1-phosphate in the presence or absence of 2 mM 5'-AMP. Phosphate release was determined as described by Cori et al. (46). The rate of reaction was linear for at least 40 min and phosphorylase activity was routinely determined from the extent of reaction between 0 and 22.5 min. The addition of Nort (10 mg/ml) to the initial heart extract, with its subsequent removal by centrifugation, did not modify the value of per cent of phosphorylase a as determined from the ratio of activities in the absence and presence of 5'-AMP.

Other Analytical Determinations

Glycogen was determined in 100 mg of frozen heart powder as described by Lo et al. (46). 5'-AMP was extracted as in Ref. 8 and assayed by the hydroxyapatite adsorption procedure of Prostrann and Kon (47) using beef heart 5'-AMP-dependent protein kinase. Protein was determined by the method of Lowry et al. (48).

Materials

Insulin (Iletin regular) and glucagon were from Eli Lilly and Co. Epinephrine was from Elkins-Sinn, Inc. Phenoxbenzamine hydrochloride was from Smith, Kline and French. L-Phenylephrine hydrochloride, D(-)-isoproterenol hydrochloride, D(-)-propranolol, phenylmethylsulfonyl fluoride, and hydroxyapatite were from Sigma. The source and/or preparation of all other compounds have been described previously (3, 8, 37).

RESULTS

Comparison of the Hormonal Response in Glycogenesis and Glycogenolysis for Hearts Perfused in the Presence or Absence of Glucose—As has been routinely observed (8, 23–25, 34–36) glycogen synthase in rat hearts perfused with glucose exists primarily in the inactive phosphorylated form as indicated by the low I/D activity ratio (Table I). As others have reported (23–26), the enzyme may be activated by addition of physiological concentrations of insulin. The increase, however, while statistically significant, is only modest (Table I). In all probability, the restriction on glycogen synthase activation is due to the presence of high tissue concentrations of glycogen. In both skeletal muscle (49) and heart (26, 50), an inverse relationship has been shown between glycogen concentration in the cell and the percentage of glycogen synthase in the I form. Thus, the level of glycogen is a predominating factor in control of the extent of its own synthesis. To relieve this restriction on the potential for insulin-dependent activation of glycogen synthase, hearts were perfused in the absence of glucose for 30 min thus reducing by 85% the tissue concentra-

Table I

<table>
<thead>
<tr>
<th>Glucose present</th>
<th>Glucose absent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percent I Glycogen synthase</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td>8.36 ± 2.0</td>
<td>12.86 ± 2.17</td>
</tr>
<tr>
<td>(n = 15)</td>
<td>(n = 6)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycogen (mg</th>
<th><strong>Control</strong></th>
<th><strong>Insulin</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>(extract 50 mg heart)</td>
<td>146.1 ± 10.6</td>
<td>ND</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

1 ND, not determined.
stimulated tissue, the level of phosphorylase a is slightly presence or absence of glucose. In control, nonhormonally genolytic response to epinephrine for hearts perfused in the presented). In Table I is presented a comparison of the glyco- increase is probably insufficient to account for the elevated decrease in ATP, and a consequential allosteric activation of phosphorylase; this is as described by Morgan and Parmeg- ian (51) for the enhanced cardiac glycogenolysis that occurs in response to epinephrine (1 PM) was added 1 min before freeze-clamping of the tissue. Assays as described under "Experimental Procedures." Table II

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glycogen synthase</th>
<th>Phosphorylase activity at pH 6.8</th>
<th>Specific activity pH 6.8</th>
<th>cAMP pmol/mg min</th>
<th>pmol/mg heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + Glucose</td>
<td>12.1</td>
<td>15.1</td>
<td>0.12</td>
<td>105</td>
<td>0.24</td>
</tr>
<tr>
<td>− Glucose</td>
<td>27.5</td>
<td>18.4</td>
<td>0.13</td>
<td>122</td>
<td>0.22</td>
</tr>
<tr>
<td>Epinephrine + Glucose</td>
<td>9.2</td>
<td>87.9</td>
<td>0.25</td>
<td>304</td>
<td>1.21</td>
</tr>
<tr>
<td>− Glucose</td>
<td>18.6</td>
<td>82.1</td>
<td>0.23</td>
<td>368</td>
<td>1.11</td>
</tr>
</tbody>
</table>

The system is clearly responsive to insulin at concentrations that are physiological.

Adrenergic Antagonism of Insulin Activation of Cardiac Glycogen Synthase—We have examined the effects of cate-

![Fig. 1](http://www.jbc.org/)
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Phosphorylase perfused hearts. The protocols were as follows: (1) and (2) glycogen synthase; (3) and (4) tissue cAMP levels; (5) and (6) phosphorylase and phosphorylase kinase activities. The graphs indicate: (a) cAMP levels, (b) glycogen synthase activity, (c) phosphorylase activity, and (d) phosphorylase kinase activity. The effects of epinephrine on insulin-stimulated activation of glycogen synthase are presented in Fig. 2. Dose-response curves are indicated in the top three panels and time course of response in the lower three. Glycogen synthase activities are on the left-hand side, cAMP determinations in the center panels, and phosphorylase and phosphorylase kinase activities on the right-hand side. (An identical format of presentation is used in subsequent figures.) As is indicated in Fig. 2, epinephrine reversed the insulin-dependent activation of glycogen synthase. There was no significant difference in either the dose-response or time-response for insulin-stimulated or control tissues. Epinephrine inactivated glycogen synthase to the same extent (as measured by the I/D ratio) independent of whether or not the tissue had been pretreated with insulin (Fig. 2a and d). Insulin alone had no effect on basal cAMP concentrations (see also Fig. 1a) nor did it effect the level of cAMP produced by either a suboptimal or maximal dose of epinephrine (Fig. 2b and e). (In this figure, as appropriate in subsequent figures, a single line has been used to denote data for control and insulin-stimulated tissue when there is no significant difference between each.) As has been extensively documented elsewhere (53-55), epinephrine activates both phosphorylase and phosphorylase kinase (Fig. 2c and f). In contrast to what is observed with glycogen synthase, insulin alone does not change the basal activation state of either phosphorylase or phosphorylase kinase, and, consonant with what is observed with cAMP levels, there was no effect of insulin on either the dose-response or time-response of epinephrine-stimulated activation of either enzyme. The dose-response and time-response for the effects of catecholamines on glycogen synthase (plus or minus insulin pretreatment), cAMP levels, and phosphorylase and phosphorylase kinase activation, are quite similar. It would appear that a low dose of epinephrine (10^{-8} M) was possibly slightly more effective in activating phosphorylase and depressing insulin-stimulated glycogen synthase activation, than it was in activating phosphorylase kinase or increasing cAMP concentrations above the basal threshold levels. This possibility is explored in more detail below.

There is growing evidence that the major action of epinephrine in rat liver is mediated by α-adrenergic mechanisms and that one such effect is upon glycogen synthase inactivation (30-32, 56-59). Hence, we have investigated the effect of a relatively pure α-agonist, L-phenylephrine; and a β-agonist, DL-isoproterenol, on cardiac glycogen synthase. Data obtained for DL-isoproterenol action are fully compatible with a conclusion that it reverses the insulin-dependent activation of glycogen synthase via stimulation of the cAMP second messenger system, even though the insulin action itself is not mediated by the cyclic nucleotide (Fig. 3). Thus there is a clear dose-dependent and time-dependent correlation between isoproterenol-stimulated increase in tissue cAMP concentration, phosphorylase kinase activation, phosphorylase activation, and glycogen synthase inactivation, with phosphorylase kinase activation appearing to slightly precede either of the latter two. As with epinephrine, the dose-response and time-response of isoproterenol on glycogen synthase inactivation, and the maximal effect of isoproterenol on this inactivation, were independent of whether or not the enzyme had been activated by insulin (Fig. 3a and d). As also with epinephrine, insulin did not effect either the dose-response or time-response of recirculating perfusate at 5 min and 1 min, respectively, prior to freeze-clamping of the tissue; solid symbols (d to f), insulin (10 milliunits/ml) and epinephrine (2 μM) were added at 5.5 min, and at the indicated times, respectively, prior to freeze-clamping the tissue; open symbols (all graphs) identical protocol as for the respective solid symbols but without addition of insulin. For all, the total time of perfusion with recirculating media, irrespective of hormone addition, was 30 min.
isoproterenol-stimulated increases in cAMP levels (Fig. 3, b and e), or the activation of phosphorylase or phosphorylase kinase (Fig. 3, c and f). Dose-response curves for isoproterenol in these hearts perfused without glucose are fully consistent with an extensive literature that has documented the concentration range in which cardiac function is modulated.

In addition to the cAMP-dependent inactivation of glycogen synthase that is evidenced by the data presented in Fig. 3, there is also clearly a CAMP-independent mechanism for catecholamine-promoted inactivation. Presented in Fig. 4 are the dose-response and time-response curves for phenylephrine effects on cardiac glycogen synthase, phosphorylase, phosphorylase kinase, and tissue cAMP concentrations. At concentrations of phenylephrine below 10^{-8} M, there is no activation of phosphorylase kinase (Fig. 4c) and essentially no change in tissue cAMP levels (Fig. 4b). In contrast, low concentrations of phenylephrine inactivate both control and insulin-stimulated glycogen synthase and activate phosphorylase (Fig. 4, a and c). The dose-response curve for each are identical. At higher concentrations of phenylephrine (10^{-7} to 10^{-5}) tissue concentrations of cAMP are increased, phosphorylase kinase is activated, and there is an additional activation of phosphorylase. Two distinct phases of phosphorylase activation are evident, one, at higher concentrations of phenylephrine, is correlated with phosphorylase kinase activation and increased tissue cAMP levels; the other, at lower concentrations, is independent of each of these (Fig. 4, b and c). Presumably, these effects of phenylephrine at very high concentrations are due to a weak, β-adrenergic agonist action that has been reported by others (60, 61). To clarify these observations, the time dependency of phenylephrine effects (at 1 μM) were examined in the presence of the β-antagonist, dl-propranolol. With such conditions (Fig. 4, d to f), it is clear that phenylephrine inactivates glycogen synthase in either control or insulin-stimulated tissue and activates phosphorylase. These occur under conditions in which tissue cAMP concentration remains at a basal level and phosphorylase kinase, which serves as an intracellular indicator of effective cAMP concentration, remains inactive. The time of response for glycogen synthase inactivation in control and in insulin-stimulated tissue, and of phosphorylase activation, are identical. The maximal effect of phenylephrine on glycogen synthase inactivation is the same whether or not the tissue has been pretreated with insulin (Fig. 4, a and d). Phenylephrine activation of phosphorylase was independent of whether or not the tissue had been treated with insulin (Fig. 4f). It is also to be noted that, whereas epinephrine and isoproterenol maximally activated phospho- 

rlyase to a level of 80% α (Figs. 2 and 3), phenylephrine, in the absence of elevated tissue concentrations of cAMP, only causes a conversion to 30 to 35% α (Fig. 4, c and f). As shown in Fig. 4, phenylephrine action on either glycogen synthase or phosphorylase is blocked by the α-antagonist phenoxybenzamine; note that phenoxybenzamine did not block the insulin-stimulated activation of glycogen synthase.

Since glycogen synthase inactivation and the reversal of insulin-stimulated activation can each be promoted by either α-adrenergic or β-adrenergic action, we have examined the efficacy of the natural catecholamine, epinephrine, to act via each of these receptors in the regulation of glycogen synthase. To do so we have examined the dose-response curves for epinephrine in the presence of either the α-antagonist phenoxybenzamine (Fig. 5, a to c) or the β-antagonist, dl-propranolol (Fig. 5, d to f), each added to the perfusate 0.5 min prior to the addition of epinephrine. The results are fully complementary to, and in agreement with, what has been observed with α- and β-agonists (Figs. 3 and 4). Epinephrine-dependent inactivation of glycogen synthase in the presence of an α-blockade (Fig. 5, a to c) is correlated with increases in tissue cAMP levels, activation of phosphorylase kinase, and extensive activation of phosphorylase. In contrast, in the presence of a β-blocking agent (Fig. 5, d to f) epinephrine-stimulated glycogen synthase inactivation occurs with a concomitant restricted activation of phosphorylase and no changes in either tissue concentrations of cAMP or the activation of phosphorylase kinase. From the dose-response curves for activation of insulin-stimulated glycogen synthase, isoproterenol (Fig. 3a) is about 2- to 4-fold more potent than epinephrine (in the presence of an α-blocking agent, Fig. 5a), and epinephrine (in the presence of a β-blocking agent, Fig. 5d) is about 20- to 80-fold more potent than phenylephrine (Fig. 4). These data are consistent with the observations of Lefkowitz's laboratory (62, 63) that isoproterenol binds to the β-receptor of cardiac muscle with a 4-fold higher affinity than does epinephrine, but that epinephrine binds with a 100- fold higher affinity to the α-receptor of cardiac muscle than does phenylephrine. Of special note is the observation that epinephrine exhibits an essentially identical dose-response curve for inactivation of insulin-stimulated glycogen synthase when mediated by either the α- or β-receptors (cf. Fig. 5, a and d). Williams and Lefkowitz (62) have reported that myocardial membranes contain almost equal numbers of α- and β-receptors. Presumably under physiological conditions, the effect of epinephrine on cardiac glycogenesis is the result of the co-ordination of its interaction with both types of recep-
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Fig. 4. Dose-response curves and time-response curves for the effects of phenylephrine on insulin-stimulated and control perfused hearts. The experiment was performed as described for Fig. 2 except with the following protocols: solid symbols (a to c), insulin (10 milliunits/ml), and phenylephrine (at indicated concentrations) were added to the recirculating perfusate at 5.5 min and 1.5 min, respectively, prior to freeze-clamping the tissue. Solid symbols (d to f) except for +, insulin (10 milliunits/ml), DL-propranolol (10 μM), and phenylephrine (1 μM) were added at 6 min, 2 min, and at the times indicated, respectively, prior to freeze-clamping. +, identical protocol as for other solid symbols except for replacement of DL-propranolol by phenoxybenzamine (10 μM) in d for glycogen synthase and in f for phosphorylase. Open symbols (all graphs, identical protocol) as for the respective solid symbols without addition of insulin. For all, the total time of perfusion with recirculating media, irrespective of hormone addition, was 30 min.

Fig. 5. Effect of phenoxybenzamine and DL-propranolol on the response to epinephrine of insulin-stimulated and control perfused hearts. The experiments were performed as described in Fig. 2 with the following protocols: solid symbols (a to c), insulin (10 milliunits/ml), phenoxybenzamine (10 μM) and epinephrine (at indicated concentrations) were added at 5.5 min, 1.5 min, and 1.0 min, respectively, prior to freeze-clamping of the tissue; solid symbols (d to f), insulin (10 milliunits/ml), DL-propranolol (10 μM), and epinephrine (at indicated concentrations) were added at 5.5 min, 1.5 min, and 1.0 min, respectively, prior to freeze-clamping of the tissue; solid symbols (a to c), insulin (10 milliunits/ml), DL-propranolol (10 μM), and epinephrine (at indicated concentrations) were added at 5.5 min, 1.5 min, respectively, prior to freeze-clamping of the tissue; solid symbols (a to c), insulin (10 milliunits/ml), DL-propranolol (10 μM), and epinephrine (at indicated concentrations) were added at 5.5 min, 1.5 min, respectively, prior to freeze-clamping of the tissue; open symbols (all graphs), identical protocol as for the respective solid symbols but without addition of insulin. For all the total time of perfusion with recirculating media, irrespective of hormone addition, was 30 min.

Hormonal Effects on the Kinetic Parameters of Glycogen Synthase—As has been emphasized elsewhere (7, 22, 28, 67, 68) covalent modification of glycogen synthase affects not only the I/D activity ratio but also other kinetic parameters such as the apparent $V_{0.5}$ for glucose 6-phosphate, the $S_{0.5}$ inactivation was independent of whether or not the enzyme had been activated by insulin. In contrast to such tissues as liver (66), insulin not only did not affect either the basal or maximally stimulated level of cAMP, it also did not depress the level of cAMP produced at suboptimal levels of glucagon. Therefore, the magnitude of response of both phosphorylase activation and phosphorylase kinase activation were essentially identical with those observed with either isoproterenol (Fig. 3), or epinephrine in the presence of an α-antagonist (Fig. 5c); each of these is also acting extensively through the cAMP second messenger system.
Effect of insulin, catecholamines, and glucagon on the kinetic parameters of cardiac glycogen synthase

The kinetic constants indicated were determined for cardiac glycogen synthase for tissue perfused under the conditions presented in Figs. 1 to 6 and under "Experimental Procedures." Assays were performed under the conditions described under "Experimental Procedures," except for the necessary changes in variable parameters. The degree of significance was determined by Student's t test. NS indicates no significant difference, a dash (---) indicates an inconsequential comparison. ND indicates no determination.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glycogen Synthase Activity Ratio</th>
<th>a</th>
<th>Glucose-6-P Activation</th>
<th>b</th>
<th>Kinetic Constants in Absence of Glucose-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% V Value vs Control</td>
<td>% V Value vs Insulin</td>
<td>[Glucagon] μM</td>
<td>Value vs Control</td>
<td>Hill Coeff</td>
</tr>
<tr>
<td>Control</td>
<td>27.4 ± 2.2 (36)</td>
<td>---</td>
<td>173 ± 45 (6)</td>
<td>1.29</td>
<td>5.8 ± 1.8</td>
</tr>
<tr>
<td>Insulin</td>
<td>47.0 ± 5.3 (40)</td>
<td>&lt;0.001</td>
<td>86 ± 42 (6)</td>
<td>&lt;0.01</td>
<td>3.5 ± 1.0</td>
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<tr>
<td>Insulin plus Epinephrine</td>
<td>19.5 ± 4.8 (15)</td>
<td>&lt;0.001</td>
<td>304 ± 59 (5)</td>
<td>&lt;0.01</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>Insulin plus Glucagon</td>
<td>21.1 ± 2.7 (6)</td>
<td>&lt;0.001</td>
<td>311 (2)</td>
<td>---</td>
<td>5.8 ± 2.7</td>
</tr>
<tr>
<td>Insulin plus Isoproterenol</td>
<td>21.2 ± 1.5 (6)</td>
<td>&lt;0.001</td>
<td>ND</td>
<td>---</td>
<td>ND</td>
</tr>
<tr>
<td>Insulin plus Phenylephrine</td>
<td>20.8 ± 3.2 (6)</td>
<td>&lt;0.001</td>
<td>330 (1)</td>
<td>---</td>
<td>4.6 ± 2.1</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>17.6 ± 4.5 (11)</td>
<td>&lt;0.001</td>
<td>328 ± 73 (3)</td>
<td>&lt;0.01</td>
<td>4.6 ± 1.2</td>
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<tr>
<td>Glucagon</td>
<td>19.9 ± 1.4 (7)</td>
<td>&lt;0.001</td>
<td>244 (1)</td>
<td>---</td>
<td>ND</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>19.0 ± 2.6 (9)</td>
<td>&lt;0.001</td>
<td>394</td>
<td>---</td>
<td>ND</td>
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<tr>
<td>Isoproterenol</td>
<td>20.6 ± 1.8 (9)</td>
<td>&lt;0.001</td>
<td>ND</td>
<td>---</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Data obtained at a maximal level of hormonal effect under the conditions presented in Figs. 1 to 6.

*Data obtained for hearts perfused in the absence of glucose. Insulin (10 milliunits/ml) was added 5 min before freeze-clamping, except in the experiment with L-phenylephrine where it was added 5.5 min before termination. Epinephrine (2 μM), glucagon (0.1 μM), and L-phenylephrine (1 μM) were added at 1 min, 1.5 min, and 1.5 min, respectively, before freeze-clamping.

*The number of determinations is indicated in parentheses.

In single experiment for direct comparison, the values for control, insulin-treated, epinephrine-treated, and phenylephrine-treated tissue were 197, 75, 410, and 394 μM, respectively.
UDP-glucose constant and the $V_{\text{max}}$ at saturating UDP-glucose in the absence of glucose 6-phosphate. Under some conditions, especially with high levels of phosphorylation, these values can be a more sensitive index of change than the I/D activity ratio (see Ref. 69 for discussion). Accordingly, we have determined these kinetic constants for glycogen synthase from hearts perfused under a number of the conditions which gave a maximal hormonal response (see Figs 1 to 6). These values are presented in Table III. Also included in Table III is a summation of the effects of maximal hormonal stimulation on the I/D ratio, the standard deviation for these values, and the p value for the degree of significance between each condition. These values are in full confirmation of all the data presented in the preceding sections (Figs. 1 to 6). Insulin activation, as measured by the percent I value is clearly significantly increased above the control ($p < 0.001$), but is reversed to an equivalent degree by epinephrine, glucagon, isoproterenol, or phylephrine. There is no significant difference between the effect of any of these four on the I/D ratio with either control or insulin-preincubated tissue.

Coomassite with the increase in the I/D ratio, insulin promotes a decrease in the apparent $K_{\text{m}}$ glucose 6-phosphate value. Insulin also causes a small decrease in the $S_{\text{0.5}}$ UDP-glucose value but no change in the $V_{\text{max}}$ as measured in the absence of glucose 6-phosphate. In none of the experimental conditions examined was any change in the $V_{\text{max}}$ in the presence of glucose 6-phosphate observed (data not shown). All of the agents that reverse insulin-dependent activation of glycogen synthase do so with equivalent resultant effects on its kinetic parameters. Inactivation in control tissue and reversal of insulin stimulation are characterized by an increase in the apparent $K_{\text{m}}$ glucose 6-phosphate value, no change in the $S_{\text{0.5}}$ UDP-glucose value (as compared to control tissue), but a depression in the $V_{\text{max}}$ of the enzyme as assayed in the absence of glucose 6-phosphate. Within the limitations of the procedures to determine these kinetic constants, the changes observed were quantitatively identical for glycogen synthase as inactivated by a CAMP-dependent process (as exemplified by glucagon), a totally CAMP-independent process (as exemplified by phylephrine), or a combination of both (as exemplified by epinephrine). We have previously presented consideration of the limitations of determinations of the $A_{\text{max}}$ value for an enzyme solution containing a mixture of enzyme forms (69). Within these restrictions, no distinction can be made between the various agents that promote glycogen synthase inactivation.

**DISCUSSION**

This report describes a procedure whereby cardiac glycogenesis becomes sensitized to hormonal regulation. The physiological function of glycogen in cardiac muscle is to serve as a reserve energy source. A consequence of this is that a high cellular level of glycogen is a predominant force that suppresses its own rate of synthesis. High tissue concentrations of glycogen alone result in inactivation of glycogen synthase and when glycogen synthase is already extensively inactivated (i.e. by high glycogen), the effect of hormones, which may act to decrease glycogenesis, cannot be readily detected. This accounts for the variable reports that epinephrine had little or no effect in the regulation of cardiac glycogen synthase (8, 26, 35, 36). In this current study, cardiac glycogen levels were depressed and as a consequence catecholamine-dependent inactivation of glycogen synthase was readily observed. The magnitude of the response has permitted its detailed characterization. A second consequence of the predominating effect of glycogen in regulating its own synthesis is that in tissues with high glycogen, despite the fact that glycogen synthase is primarily in an inactive form, the response to insulin-stimulated activation is severely muted. Thus, the glycogen-depleted heart is also a good system to study hormonal activation of glycogen synthase even though the basal activation state of the enzyme has been increased.

In this characterization of the hormonal regulation of glycogen synthase in the glycogen-depleted heart we have shown: (a) that insulin activates cardiac glycogen synthase as rapidly as, or more rapidly than, any other intracellular event (i.e. excluding membrane transport). Both the magnitude of the response, and the on-going characterization of ex vivo (8) and in vitro (9–11) phosphorylation of glycogen synthase should permit a substantial delineation of the mechanism by which insulin regulates glycogen synthase. (b) that in cardiac muscle, insulin activation of cardiac glycogen synthase, an event that is clearly not CAMP-mediated, is antagonized by activation of the CAMP system. This is evidenced by the effects of isoproterenol (Fig. 3), glucagon (Fig. 6), and epinephrine in the presence of an α-antagonist (Fig. 5, a to c). (c) that in cardiac muscle, insulin activation of cardiac glycogen synthase is likewise antagonized by a CAMP-independent mechanism. This is supported by the observations with both phylephrine (Fig. 4), and epinephrine in the presence of a β-antagonist (Fig. 5, d to f). In addition to direct measurement of tissue CAMP levels, the absence of significant changes in CAMP were confirmed by measurement of the activation status of phosphorylase kinase; the latter serves as an intracellular marker of effective intracellular CAMP concentration.

The regulation of glycogen synthase by hormones has become extensively studied for liver, adipose tissue, and skeletal and cardiac muscle. Each of these exhibit distinct features. Skeletal muscle glycogen synthase is primarily regulated by β-adrenergic effectors, which promote inactivation and are mediated by CAMP (28), and by insulin, which activates by a mechanism totally independent of changes in CAMP concentrations (27). Neither glucagon nor α-adrenergic agonists regulate skeletal muscle glycogenesis. In contrast, catecholamines regulate rat hepatic glycogenesis primarily via α-adrenergic stimulation and this action is mediated not by CAMP but by intracellular Ca++ translocation (30, 31, 59). CAMP is an important regulator of hepatic glycogenesis. It mediates glucagon-induced inactivation of glycogen synthase. Probably also, the depression of CAMP by insulin, especially as it occurs in counteracting suboptimal concentrations of glucagon, accounts, at least partially, for insulin-stimulated activation of hepatic glycogen synthase. In adipocytes, catecholamines promote inactivation of glycogen synthase both as β- and α-agonists (33, 34). The β-agonist effect predominates and is mediated by elevation of tissue levels of CAMP. α-Adrenergic effectors are considerably less potent than β-agonists in modulating adipocyte glycogen (22, 33) but, like with the hepatocyte, are probably mediated by intracellular Ca++ translocation (34). Insulin action in adipocytes is complex. Clearly it acts by depressing CAMP levels, but in all probability, this is not the only mechanism by which insulin activates adipocyte glycogen synthase (17, 20).

The hormonal regulation of cardiac muscle glycogen synthase is distinct from that of the other three tissues. It is the only system in which α-adrenergic and β-adrenergic stimulation are equally effective in promoting glycogen synthase inactivation (Fig. 5). Furthermore, there is no ambiguity as to insulin’s mechanism of action. Measurements of both tissue levels of CAMP and the activation state of phosphorylase kinase confirm that insulin has no effect on either basal levels of cardiac cAMP or levels of CAMP promoted by either suboptimal or optimal concentrations of either β-agonists (Figs. 3 and 5, a to c) or glucagon (Fig. 6). These unique
features of regulation of cardiac muscle glycogen synthesis may facilitate a delineation of exactly how each hormone acts to regulate glycogen synthase.

It would appear most likely that in cardiac muscle the β-adrenergic effects are mediated by the well-established cAMP second messenger system and that α-agonists, as has been shown with liver (30, 31, 59), are mediated by intracellular translocation of Ca++. Although the role of Ca++ in controlling cardiac glycogen synthesis has not been directly addressed experimentally, the observed activation of phosphorylase in the absence of changes in phosphorylase kinase activation state (Figs. 4 and 5f) is explained logically as a Ca++-dependent allosteric activation of phosphorylase kinase. Insulin action on cardiac glycogen synthesis would not appear to be simply mediated by either cAMP or Ca++. If the latter were occurring, it would presumably have been detected as a change in allosteric activation of phosphorylase kinase. Insulin action on cardiac glycogen synthase have illustrated another complexity in this pathway for an effector to modulate a response certainly extends the gamut of potential control.

REFERENCES

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REFERENCES

51. Morgan, H. E., and Parmeggiani, A. (1964) J. Biol. Chem. 239,
Hormonal Regulation of Glycogen Synthase in the Perfused Rat Heart

2435-2439

Cyclic AMP-dependent and cyclic AMP-independent antagonism of insulin activation of cardiac glycogen synthase.
C Ramachandran, K L Angelos and D A Walsh


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