0026-895X/86/060624-07$02.00/0

MOLECULAR PHARMACOLOGY, 30:624-630

Hexose-Independent Activation of Glycogen Synthase and Pyruvate Dehydrogenase by Insulin Is Dissociated in the Mouse BC_3H-1 Cell Line

LOUIS M. LUTTRELL and ALAN D. ROGOL

Departments of Pediatrics and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received May 1, 1986; Accepted October 1, 1986

SUMMARY

We have studied the effects of insulin on several aspects of cell metabolism in the insulin-sensitive, nonfusing muscle cell line BC_3H-1. In the absence of exogenous hexose, insulin did not alter basal glycogen synthase percentage *I* activity, or attenuate the increase in intracellular cAMP content, the activation of glycogen phosphorylase *a*, or the decrease in glycogen synthase *I* brought about by β-adrenergic receptor activation with epinephrine. In contrast, both insulin and the tumor-promoting phorbol ester, tetradecanoylyl phorbol acetate markedly increased mitochondrial pyruvate dehydrogenase activity in the absence of hexose. Both glycogen synthase phosphatase and glycogen synthase kinase activities were present in BC_3H-1 cell extracts and were regulated in the expected manner by glucose 6-phosphate and cAMP, respectively. Since the pattern of partial insulin resistance seen in BC_3H-1 myocytes would require that several potentially insulin-sensitive enzymes be insensitive to insulin-generated signals, the most likely explanation for these data is that the myocytes are defective in some mechanism of insulin signaling which is independent of the mechanism for pyruvate dehydrogenase activation.

BC_3H-1 is a vascular smooth muscle-like tumor cell line derived from the C_3H mouse strain (1). Upon differentiation in culture, the myocytes express high affinity receptors for insulin (2) and exhibit several insulin-stimulated metabolic actions, including increased hexose transport (2), activation of mitochondrial PDH (3), and stimulation of phospholipid synthesis (4). We have previously shown that insulin stimulates glycogen synthesis and increases GS *I* activity in these cells, but only through a hexose-6-phosphate-dependent mechanism (5). In the absence of exogenous hexose, insulin has no significant effect on GS *I* activity and the insulin-dependent activation of GS *I* in the presence of 6-phosphorylatable hexose is blocked by inhibitors of hexose transport.

In the intact cell, GS *I* activity is determined by the opposing actions of GS phosphatases, which convert the enzyme from the *D* to the *I* form by dephosphorylation, and GS kinases, among them cAMP-dependent protein kinase, which reverse the conversion by phosphorylation (6). The activity of these enzymes is normally subject to insulin regulation by hexose-dependent and hexose-independent mechanisms (7–9). Thus, the failure of insulin to activate GS in BC_3H-1 myocytes in the absence of hexose might represent either an insensitivity of the synthase to regulatory signals or a lack of signal transmission.

To determine whether the resistance of BC_3H-1 myocyte GS to hexose-independent activation by insulin is part of a broader defect of insulin action in the cells, we have assayed for other hexose-independent effects of insulin, including attenuation of β-adrenergic receptor-mediated increases in cAMP content and GP *a* activity, and activation of mitochondrial PDH. We have also assayed for GS phosphatase(s) and cAMP-activated GS kinase activity in myocyte extracts to determine whether the interconversion of GS *D* and *I* by phosphorylation/dephosphorylation can be detected *in vitro*. We present evidence that the hexose-independent effects of insulin on GS *I* activity and responses to β-adrenergic agonist are dissociated from its effect on PDH activity in BC_3H-1 myocytes and may represent a specific defect in one pathway of insulin signal transduction.

Experimental Procedures

Materials

Crystalline bovine insulin (lot 28C-0136, specific activity: 26.8 IU/mg), adenosine 5′-monophosphate, adenosine 3′, 5′-cyclic monophos-

These studies were supported in part by the Medical Scientist Training Program at the University of Virginia (L. M. L.) and by a Pilot and Feasibility grant from the Diabetes Research and Training Center (A. D. R.), Grant AM 22125.

ABBREVIATIONS: PDH, pyruvate dehydrogenase; 2-DOG, 2-deoxy-D-glucose; EGF, epidermal growth factor; G-6-P, D-glucose 6-phosphate; GS, glycogen synthase; GS *I*, glycogen synthase activity independent of glucose 6-phosphate; GP *a*, glycogen phosphorylase activity independent of AMP; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; TPA, tetradecanoylyl phorbol acetate; IBMX, isobutylmethylxanthine.

phate, adenosine 5′-triphosphate, rabbit liver glycogen (grade III), dithiothreitol, bovine serum albumin (Cohn fraction V), 2-DOG, G-6-P, glucose 1-phosphate, epinephrine, isoproterenol, phentolamine, phenylephrine, propranolol, and IBMX were purchased from Sigma Chemical Co. Forskolin was obtained from Calbiochem. UDP-[U-^{14}C] glucose was a gift from Dr. Joseph Larner and was synthesized in his laboratory by published methods (10). 2,5-Diphenyloxazol and D-[U-^{14}C]glucose 1-phosphate were obtained from New England Nuclear. Tissue culture media and supplies were from Grand Island Biological Co. All other materials were of reagent grade. The glycogen was purified before use by passing a 5% solution over a mixed bed ion exchange resin (Amberlite MB-3) as described (11). The bovine serum albumin was tested for low insulin-like activity prior to use (7).

Methods

Cell culture. BC_3H-1 myocytes were cultured at 37° in Dulbecco's modified Eagle's medium plus 20% fetal bovine serum in a humidified 10% CO_2, 90% air atmosphere as described (5) and were used during the period of full differentiation after 10–12 days in culture. C3H/10T1/2 (10T1/2) cells, a fibroblast line of C3H mouse origin (12), were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and were used when confluent after 4–5 days in culture.

Determination of enzyme activities. Stimulation of confluent cultures with insulin, epinephrine, or phorbol ester in serum- and hexose-free incubation medium (Dulbecco's phosphate-buffered saline supplemented with 0.1% bovine serum albumin, 1% basal minimum essential vitamins, 1% basal minimal essential amino acids, and 2 mM L-glutamine, pH 7.4) was carried out as described (5). GS *I* activity was measured according to the method of Thomas *et al.* (10) as modified by Guinovart *et al.* (13). GP *a* activity was determined by the method of Gilboe *et al.* (14). GS phosphatase activity was determined by the method of Lawrence and Larner (8) using endogenous GS as substrate.

For the determination of GS kinase activity, aliquots of unstimulated cell homogenates (500 μl) in buffer containing 50 mM Tris-HCl, 0.5 mM EDTA, 0.3 mM EGTA, pH 7.0, were incubated at 30° for 1 hr to permit endogenous phosphatases to elevate GS *I* activity in the extracts to 70–80%. KF was then added to a concentration of 10 mM to inhibit phosphatase activity, $MgCl_2$ (1 mM) and ATP (25 μM) plus or minus cAMP (5 μM) were added to initiate kinase activity, and the incubation continued at 20°. At intervals, the kinase reaction was stopped by the addition of EDTA (10 mM) and 2 volumes of saturated, neutralized ammonium sulfate. Precipitation, resuspension, and desalting of the extracts was then carried out as in the GS phosphatase assay, and GS kinase activity was determined as the rate of inactivation of endogenous GS.

For cAMP determinations, myocytes grown in 12-well tissue cultures plates were stimulated as described and cAMP samples were prepared by extracting each cell monolayer for 1 hr with 1 ml of 0.1 N HCl. The cAMP content of acetylated, appropriately diluted extracts was determined by radioimmunoassay (15). Protein content of each well was determined after solubilizing the monolayer in 1 ml of 0.2 N NaOH by the method Lowry *et al.* (16). PDH activity was determined on the 700 × *g* supernatant of extracts from stimulated or nonstimulated cultures (duplicate determinations per 100-mm culture dish) by the method of Taylor *et al.* (17).

Liquid scintillation counting. Counting of dried filter papers bearing precipitated ^{14}C-glycogen was done in a Beckman LSC-7000 liquid scintillation counter in a scintillation cocktail consisting of 0.5% 2,5-diphenyloxazole in toluene.

Data presented are representative results from experiments performed at least in triplicate. Student's paired and unpaired *t* tests were used to determine the statistical significance of results.

Results

Effects of insulin on GS *I* activity in the presence and absence of exogenous hexose. Table 1 summarizes the effects of insulin on GS *I* activity in differentiated BC_3H-1 myocytes and 10T1/2 cells, another cell line of C_3H mouse origin (12). In the 10T1/2 fibroblast cell line, insulin induces a marked increase in GS *I* activity in the absence of exogenous hexose. 2-DOG alone also causes an elevation of GS *I* activity that is enhanced in the presence of insulin. EGF does not activate GS in the absence of hexose but does enhance the effect of 2-DOG, probably secondary to increased hexose transport (18). In contrast, the effects of insulin on GS *I* activity in BC_3H-1 myocytes qualitatively resemble those of EGF in the 10T1/2 cells. Insulin does not significantly increase GS *I* activity in the absence of hexose but markedly augments the effects of hexose alone. The hexose-dependent activation of GS in BC_3H-1 myocytes requires a 6-phosphorylatable hexose and is abolished in the presence of cytochalasin B (5).

TABLE 1

Comparison of hexose-dependent and -independent activation of GS *I* in BC_3H-1 and 10T1/2 cells

Hexose-dependent and -independent activation of GS *I* in BC_3H-1 and 10T1/2 cells by insulin and EGF is compared. Confluent 10–12-day cultures of BC_3H-1 myocytes and 4–5-day cultures of 10T1/2 fibroblasts were incubated for 30 min at 37° in incubation medium with or without insulin (100 nM) or EGF (30 ng/ml). 2-DOG (2 mM) was then added if appropriate, and the incubation continued for an additional 30 min before GS *I* activity was determined as described. Data shown represent mean ± standard error values for four separate determinations.

Cell line	Additions	GS %*I* activity
BC_3H-1	None	8.44 ± 0.72
	Insulin, 100 nM	10.07 ± 1.08[a]
	2-DOG, 2 mM	20.82 ± 2.22[b]
	Insulin plus 2-DOG	33.07 ± 1.81[c]
10T1/2	None	18.03 ± 1.90
	Insulin, 100 nM	52.65 ± 7.81[b]
	EGF, 30 ng/ml	19.56 ± 2.13[a]
	2-DOG, 2 mM	34.03 ± 1.02[b]
	Insulin plus 2-DOG	67.36 ± 4.03[c]
	EGF plus 2-DOG	43.86 ± 2.73[c]

[a] Not significantly different from nonstimulated.
[b] Greater than nonstimulated, $p < 0.02$ ($n = 4$).
[c] Greater than 2-DOG alone, $p < 0.05$ ($n = 4$).

Effects of insulin on basal and epinephrine-stimulated cAMP, GP *a*, and GS *I* levels. Fig. 1 depicts the effects of epinephrine on intracellular cAMP level in BC_3H-1 myocytes in the presence and absence of insulin. In hexose-free medium, basal cAMP levels were 6.89 ± 1.47 pmol/mg of cell protein and were increased 3- to 4-fold to 21.95 ± 3.45 pmol/mg after 15 min incubation with 10 μM epinephrine, [epinephrine-stimulated greater than control, $p < 0.01$ ($n = 5$)]. This effect of epinephrine was maximal from 2 to 15 min after addition and declined gradually over 60 min (see Fig. 3A), with half-maximal activation occurring at approximately 3×10^{-8} M. In contrast to its effects in rat adipocytes (19), the presence of insulin at concentrations up to 1 μM in the incubation medium during the stimulation had no effect on either the time course or dose dependence of the epinephrine effect. Basal cAMP levels in the presence of insulin were 6.93 ± 1.26 pmol/mg [not significantly different from control, $p > 0.9$ ($n = 5$)] and after 15 min of epinephrine stimulation rose to 22.05 ± 2.29 [not significantly different from epinephrine alone, $p > 0.9$ ($n = 5$)]. When 2 mM 2-DOG was included in the medium, the basal and stimulated cAMP levels were unchanged and no effect of insulin was detectable.

As shown in Fig. 2A, incubation with epinephrine significantly increased GP *a* activity with no change in total phosphorylase activity. Without hexose, basal GP *a* activity was

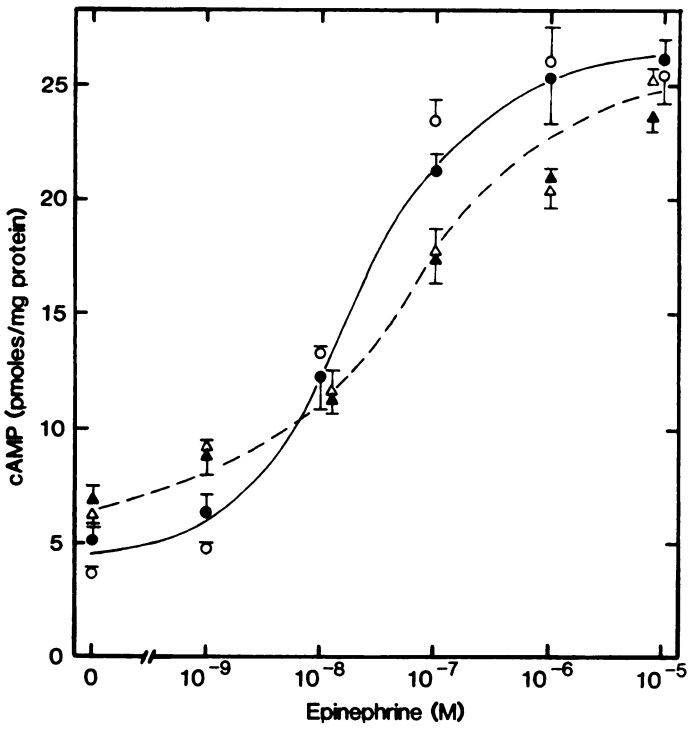

Fig. 1. Dose response of epinephrine effects on intracellular cAMP content in the presence and absence of insulin and 2-DOG. Ten- to 12-day cultures of BC_3H-1 myocytes were incubated for 30 min in serum-free incubation medium with and without insulin or hexose prior to the addition of epinephrine at the indicated concentration for 15 in. Intracellular cAMP content was then determined as described. Data shown are from one of three similar experiments. *Error bars* represent the mean ± standard error of triplicate determinations. —, cells in hexose-free medium incubated with (●) and without (○) insulin (1 μM). – – –, cells incubated with 2-DOG (2 mM), with (▲) and without (△) insulin (1 μM).

61.35 ± 4.16% and was increased to 78.33 ± 4.30% after 15 min exposure to epinephrine [epinephrine-stimulated greater than control, $p < 0.01$ ($n = 6$)]. The effect was half-maximal at approximately 1×10^{-7} M epinephrine and the time course roughly paralleled that of the epinephrine-induced increase in cAMP content (Fig. 3B). The presence of insulin had no significant effect on the response. Basal GP *a* activity with insulin was 61.20 ± 4.69% [not significantly different from control, $p > 0.5$ ($n = 6$)]. Epinephrine-stimulated GP *a* activity in the presence of insulin was 80.4 ± 4.09% [not significantly different from epinephrine alone, $p > 0.5$ ($n = 6$)]. In the presence of 2 mM 2-DOG, GP *a* activity was consistently lowered, but the activation by epinephrine was unchanged. Insulin enhanced the effect of the hexose (5) but still did not attenuate the effect of epinephrine.

Insulin was similarly ineffective in attenuating the epinephrine-induced decrease in GS *I* activity (Fig. 2B). Basal GS *I* activity was 8.60 ± 0.89% in the absence of insulin and 8.81 ± 0.77% when insulin was present [insulin-treated not significantly different from control, $p > 0.5$ ($n = 6$)]. Incubation for 15 min with epinephrine lowered the value to 5.11 ± 0.67% or 5.81 ± 0.49% in the absence or presence of insulin, respectively [not significantly different from epinephrine alone, $p > 0.15$ ($n = 6$)]. The effect of epinephrine on GS *I* activity was half-maximal at approximately 3×10^{-8} M and the time course of the effect paralleled that of the increase in cAMP content. Dose response curves to insulin (10^{-11}–10^{-6} M) in the presence

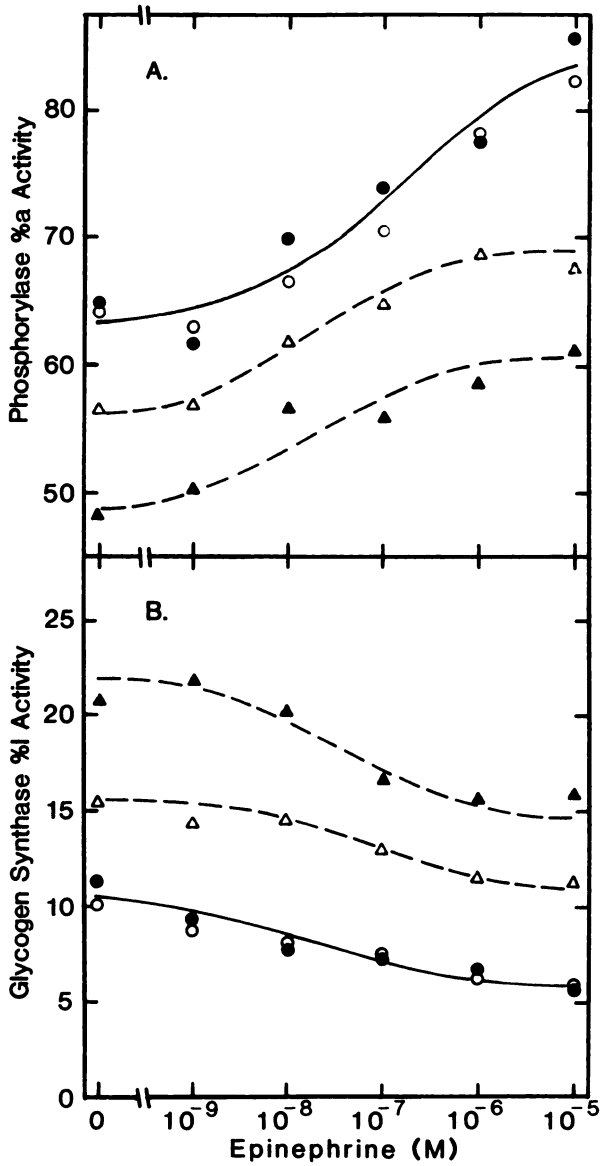

Fig. 2. Dose response of epinephrine effects on GP *a* and GS *I* in the presence or absence of insulin and 2-DOG. Ten- to 12-day cultures of BC_3H-1 myocytes were incubated for 30 min in serum- and hexose-free incubation medium with and without insulin prior to the addition of epinephrine at the indicated concentration for 15 min. GP *a* and GS *I* activities were then determined as described. Data shown are from one of three similar experiments. A. Dose response curved for GP *a* activity. —, cells in hexose-free medium incubated with (●) and without (○) insulin (1 μM). – – –, cells incubated with 2-DOG (2 mM), with (▲) and without (△) insulin (1 μM). B. Dose response curves for GS *I* activity. —, cells in hexose-free medium incubated with (●) and without (○) insulin (1 μM). – – –, cells incubated with 2-DOG (2 mM), with (▲) and without (△) insulin (1 μM).

and absence of 1 μM epinephrine were parallel to the abscissa for cAMP, GS *I*, and GP *a*, indicating that insulin was without effect over the entire concentration range at which it affects hexose transport (3–5). Two mM 2-DOG and insulin in the presence of 2-DOG produced successive upward shifts in the epinephrine dose response curves for GS *I*, but the presence of the hexose did not affect the inability of insulin to attenuate the action of epinephrine.

In rat hepatocytes, the α-adrenergic agonist phenylephrine activates glycogen phosphorylase and inhibits GS in a Ca^{2+}-dependent manner (20). These effects, like the β-adrenergic

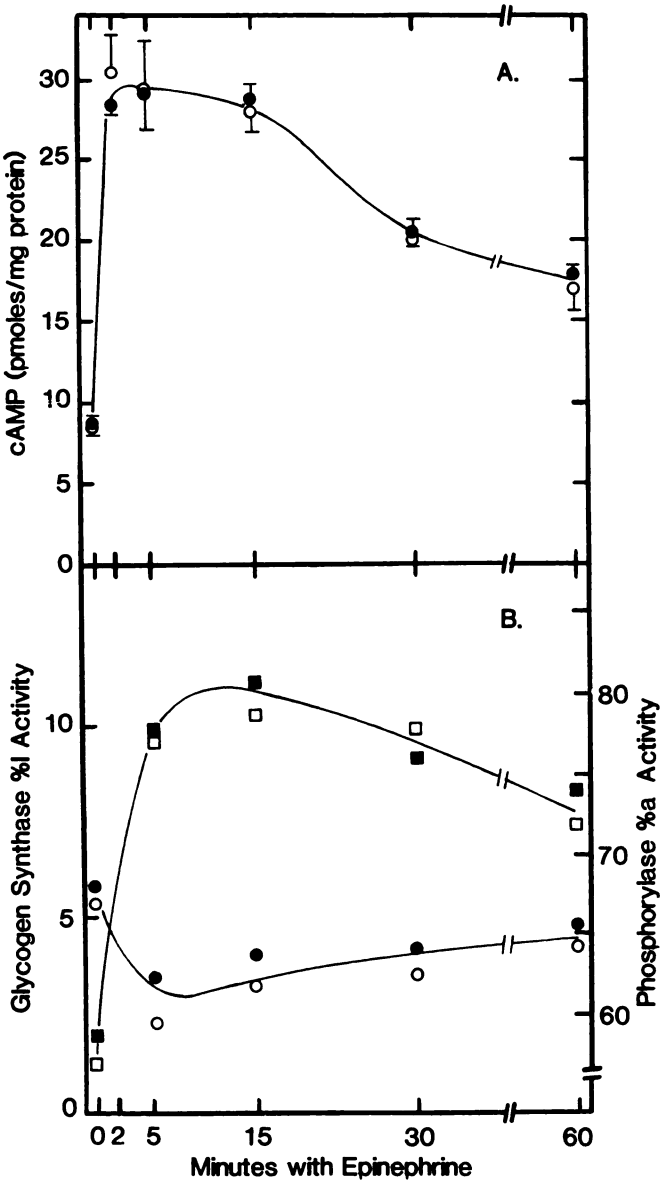

Fig. 3. Time course of epinephrine effects on intracellular cAMP content, GP *a*, and GS *I* in the presence and absence of insulin. Myocyte cultures were incubated for 30 min in serum- and hexose-free incubation medium with and without insulin prior to the addition of epinephrine (1 μM) for the indicated times. Intracellular cAMP content, and GP *a* and GS *I* activities were then determined as described. Data shown are from one of three similar experiments. A. Time course of epinephrine-stimulated increase in intracellular cAMP content in the presence (●) and absence (○) of insulin (1 μM). *Error bars* represent the mean ± standard error of triplicate determinations. B. Time course of epinephrine-induced activation of GP *a* (■, □) and inhibition of GS *I* (●, ○) activities in the presence (■, ●) and absence (□, ○) of insulin (1 μM).

receptor-mediated effects seen in adipocytes, are attenuated by insulin (21). To determine whether the effects of epinephrine in BC_3H-1 were mediated via a β-adrenergic receptor and were dependent upon altered cAMP concentrations, the pharmacologic specificity of the epinephrine effects were determined as shown in Table 2. The ability of epinephrine to increase cAMP content and GP *a* activity and to reduce GS *I* activity was specifically antagonized by the β-receptor blocker, propranolol, but was not affected by the α-antagonist, phentolamine. The β-agonist isoproterenol qualitatively mimicked epinephrine,

TABLE 2

β-Adrenergic regulation of cAMP level, GS *I*, and GP *a*

A pharmacologic characterization of epinephrine's effects on intracellular cAMP content, and GS *I* and GP *a* activities is shown. Ten- to 12-day cultures of BC_3H-1 myocytes in 12-well tissue culture plates (for cAMP determinations) or 100-mm dishes (for enzyme activity determinations) were incubated for 30 min in serum- and hexose-free incubation medium in the presence or absence of receptor antagonists as indicated, followed by 15 min further incubation in the presence of the indicated agonists. Incubations with IBMX and forskolin were for 30 min only. Following incubations, intracellular cAMP content, and GS *I* and GP *a* activities were determined as described. Data represent mean ± standard error values for triplicate determinations in representative experiments.

Agent	cAMP	GS activity %*I*	GP activity %*a*
	pmol/mg protein		
None	12.66 ± 0.61	8.90 ± 0.02	70.8 ± 0.4
Epinephrine, 1 μM	47.91 ± 1.33	4.14 ± 0.03	87.0 ± 2.5
Epinephrine, 1 μM, plus propranolol, 10 μM	13.01 ± 0.69	8.06 ± 0.34	71.9 ± 2.2
Propranolol, 10 μM	14.36 ± 0.14	ND*	ND
Epinephrine, 1 μM, plus phentolamine, 10 μM	46.28 ± 3.38	5.62 ± 0.02	84.4 ± 2.1
Phentolamine, 10 μM	13.76 ± 1.52	ND	ND
Isoproterenol, 1 μM	37.13 ± 2.73	4.98 ± 0.20	86.3 ± 0.8
Phenylephrine, 1 μM	12.45 ± 0.63	10.04 ± 1.00	74.2 ± 1.8
None	11.35 ± 0.88	9.19 ± 0.06	67.2 ± 3.2
IBMX, 5×10^{-4} M	37.65 ± 0.85	5.05 ± 0.20	89.7 ± 0.8
Forskolin, 10 μM	46.07 ± 1.88	5.55 ± 0.22	85.1 ± 0.7

* ND, not determined.

whereas 1 μM phenylephrine had no effect on cAMP content or enzyme activities. Higher concentrations of phenylephrine (10 μM) elevated cAMP level and GP *a* activity; however, this effect was inhibited by propranolol and was not affected by phentolamine. Thus, these results may represent a weak β-adrenergic effect of high concentrations of phenylephrine (data not shown). Forskolin and the phosphodiesterase inhibitor, IBMX, which elevate cAMP levels without interacting directly with the adrenergic receptor, mimicked the effects of epinephrine on GP *a* and GS *I* activity, confirming the cAMP dependence of the epinephrine effect.

In contrast to its failure to activate GS *I* in the absence of hexose and attenuate the epinephrine-induced activation of GP *a* and inhibition of GS *I*, insulin has been reported to markedly stimulate PDH activity in BC_3H-1 myocytes in the absence of hexose (3, 4). Thus, the effects of insulin and the phorbol ester, TPA, on GS *I* and PDH activity of extracts prepared from the same myocyte culture were compared. A 20-min incubation with insulin (100 nM) or TPA (1×10^{-6} M) resulted in an increase in PDH activity from 1.41 ± 0.20 nmol of CO_2/min/mg of cell protein to 2.49 ± 0.14 or 2.83 ± 0.22 nmol/min/mg, respectively [insulin- and TPA-treated values greater than control, $p < 0.05$ ($n = 3$)]. GS *I* activity was 6.57 ± 0.15% in control cultures and 6.99 ± 0.44% or 6.94 ± 0.21% in the presence of insulin or TPA, respectively [insulin and TPA treated values not significantly different from control, $p > 0.20$ ($n = 3$)]. As shown in Table 3, incubation with insulin or TPA significantly increased PDH activity in both BC_3H-1 myocytes and 10T1/2 cells with no change in total PDH activity.

GS phosphatase and GS kinase activity in BC_3H-1 myocyte extracts. To determine whether GS from BC_3H-1 myocytes can be regulated by phosphorylation/dephosphorylation, assays were performed *in vitro* for GS phosphatase and GS kinase (cAMP-dependent and -independent activity). Cytosolic extracts of differentiated BC_3H-1 myocytes were pre-

TABLE 3

Effects of insulin and TPA on cellular PDH activity

Effects of insulin and phorbol ester on active and total PDH activities in BC_3H-1 and 10T1/2 cells are shown. Ten- to 12-day myocyte cultures were incubated for 30 min in serum- and hexose-free incubation medium in the presence or absence of insulin or TPA. Each culture was then homogenized in 1 ml of ice-cold buffer (potassium phosphate, 10 mM; dithiothreitol, 1 mM, pH 7.4). One aliquot of each homogenate received EDTA (1 mM) and was maintained on ice until assayed for PDH activity. Another aliquot received 1 mM $CaCl_2$ and 5 mM $MgCl_2$ and was incubated at 37° for 5 min before the determination of total PDH activity (22). Data shown are mean ± standard error values of five or six separate determinations.

	PDH activity		
	Active	Total	% Active
	nmol CO_2/min/mg protein		
BC_3H-1			
Basal	1.64 ± 0.06	9.77 ± 0.99	16.8
Insulin, 100 nM	3.11 ± 0.25[a]	9.31 ± 0.79[b]	33.4
TPA, 1×10^{-6} M	2.72 ± 0.17[a]	9.65 ± 0.62[b]	28.2
10T1/2			
Basal	0.12 ± 0.03	4.19 ± 0.32	2.8
Insulin, 100 nM	0.31 ± 0.04[c]	3.86 ± 0.12[b]	8.0
TPA, 1×10^{-6} M	0.18 ± 0.03[c]	3.93 ± 0.37[b]	4.6

[a] Greater than basal, $p < 0.02$ ($n = 6$).
[b] Not significantly different from basal, $p > 0.50$ ($n = 5$).
[c] Greater than basal, $p < 0.05$ ($n = 5$).

pared and the activity of the enzymes was determined as a function of alterations in endogenous GS *I* activity under conditions favoring either phosphatase or kinase activity.

Fig. 4A shows G-6-P-sensitive, KF-inhibitible, GS phosphatase activity in the cytosolic extracts, measured as the rate of increase in GS *I* activity during incubation at 30° in the absence of phosphatase inhibitor. The initial rate of phosphatase activity was increased 2-fold in the presence of maximally activating concentrations of G-6-P. Fig. 3B depicts the results for cAMP-dependent and -independent GS kinase activities in the extracts measured as the rate of inactivation of GS *I* during incubation at 20° in the presence of ATP and KF, with and without cAMP. The initial rate of GS *I* inactivation in the absence of added cAMP was approximately doubled in the presence of a maximally effective concentration of cAMP. These data suggest that BC_3H-1 GS is sensitive to regulation by phosphorylation/dephosphorylation, and that a G-6-P-activatable phosphatase and cAMP-dependent protein kinase are present in the cell. However, the complexity of the phosphorylation/dephosphorylation reactions that regulate GS activity *in vivo* do not allow us to conclude from these data that the enzymes normally responsible for the hexose-independent regulation by insulin of GS are active in BC_3H-1 cells.

Discussion

Differentiated BC_3H-1 myocytes exhibit several insulin-regulated metabolic activities including enhanced hexose and amino acid transport (2), activation of mitochondrial PDH (3, 4), stimulation of glycogen synthesis, and hexose-dependent activation of GS *I* and inhibition of GP *a* (5). Unlike other systems (8, 23, 24), however, insulin does not detectably activate GS *I* by a direct hexose-independent mechanism. We have carried out the present series of experiments to determine the extent to which BC_3H-1 myocytes are insensitive to the hexose-independent effects of insulin and to ascertain whether this represents an insensitivity of target enzymes or a more proximal failure of insulin signaling.

We assayed for several of the hexose-independent effects of insulin reported in other systems. Although insulin has no effect on basal cAMP levels (9), it markedly attenuates β-adrenergic receptor-stimulated increases in cAMP level and the resultant activation of GP *a* and inhibition of GS *I* in adipocytes

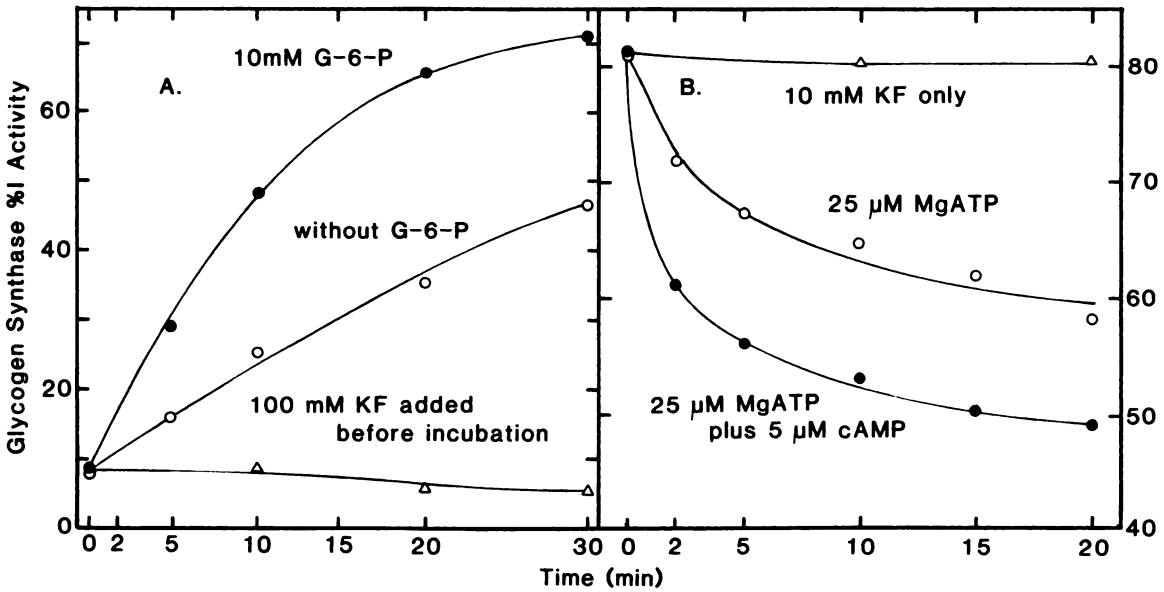

Fig. 4. Presence of G-6-P-activatable GS phosphatase and cAMP-stimulatable GS kinase activities in BC_3H-1 myocyte extracts using endogenous GS as substrate. Data shown are from one of four similar experiments. A. Extracts of unstimulated BC_3H-1 myocytes were incubated at 30° in the presence (●) or absence (○) of 10 mM G-6-P for the indicated times, after which phosphatase activity was inhibited by the addition of KF (100 mM), a two-step procedure of ammonium sulfate precipitation and Sephadex G-25 chromatography was used to remove the G-6-P, and the GS *I* activity of the extracts was determined. KF (100 mM) added prior to incubation at 30° prevented the increase in GS *I* activity (8). B. Extracts of unstimulated BC_3H-1 myocytes were incubated at 30° for 1 hr to increase endogenous GS *I* activity, after which KF (10 mM) was added to inhibit GS phosphatase activity, along with $MgCl_2$ (1 mM) and ATP (25 μM) with (●) or without (○) cAMP (5 μM) to activate endogenase GS kinase activity. Incubations were then continued at 20° for the times indicated, after which ammonium sulfate precipitation, Sephadex G-25 chromatography, and GS *I* activity determinations were carried out as in the GS phosphatase assay.

(19). In BC_3H-1 myocytes we have found that epinephrine induces a β-adrenergic receptor-dependent elevation of intracellular cAMP concentrations with concomitant activation of GP *a* and inhibition of GS *I*, but that even supramaximal concentrations of insulin do not affect the dose dependence or time course of the response. In the rat diaphragm, insulin does not alter basal or epinephrine-stimulated cAMP levels and GS *a* activity (25). However, diaphragm muscle exhibits both hexose-dependent and -independent activation of GS *I* by insulin (23).

A striking observation is that the absence of hexose-independent actions of insulin in the BC_3H-1 myocyte does not extend to the activation of mitochondrial PDH. Both insulin and the tumor-promoting phorbol ester, TPA, markedly activate PDH in the myocytes, whereas neither significantly affects GS *I* activity in the absence of hexose. PDH is phosphorylated and inactivated by an Mg-ATP-dependent, cAMP-independent protein kinase and is dephosphorylated and activated by an Mg^{2+}-dependent, Ca^{2+}-stimulated phosphatase (26). The activation of PDH by insulin is accompanied by dephosphorylation of the α-subunit of the enzyme (27), apparently without increasing the intramitochondrial Ca^{2+} concentrations (28). Farese *et al.* (4) have reported that insulin and TPA produce nonadditive activation of PDH and that insulin stimulates inositol phospholipid synthesis and elevates the intracellular DAG concentration in BC_3H-1 myocytes. Koepfer-Hobelsberger and Wieland (29) have found that inositol triphosphate activates PDH in permeabilized adipocytes, and indomethacin has been shown to block the effects of insulin on PDH in a manner reversible by prostaglandin E_2 (30). Collectively, these data suggest that some product of inositol phospholipid metabolism may be required for insulin-dependent activation of PDH, but the mechanism is as yet unclear.

Assays for GS phosphatase and cAMP-dependent and -independent GS kinase activities using native GS as substrate indicated that these activities were present in BC_3H-1 cell extracts and regulated by G-6-P and cAMP in the expected manner. Since these activities were measured as a function of changes in the level of native GS *I*, these data suggest that BC_3H-1 GS can be regulated appropriately by phosphorylation/dephosphorylation. However, since the sites of insulin-induced dephosphorylation of adipocyte GS differ in the presence and absence of hexose (31), it is not clear from these *in vitro* assays that the phosphatase(s) involved in the hexose-independent activation of GS is (are) functional in the myocytes.

Huang and Tao (32) have reported the presence of phosphatase inhibitors in L6 myocytes which prevent the dephosphorylation of GS and GP. In glucose-deprived L-6 mycoytes, refeeding results in an increase in glycogen content without a concomitant increase in GS *I* activity. The failure of insulin to activate GS in the absence of hexose in BC_3H-1 myocytes, however, is unlikely to be due to similar inhibitors, since GS and GP activities are both modulated by hexose in an insulin-stimulatable manner, and the altered activity in the presence of hexose is stable to Sephadex G-25 desalting and, thus, represents dephosphorylation (8).

The hexose-independent activation of GS by insulin in other systems may involve activation of specific GS phosphatase(s) (33) and inhibition of cAMP-dependent protein kinase (34). Attenuation of the effects of epinephrine on cAMP levels and GP activity involves inhibition of cAMP-dependent protein kinase (34–36) and activation of a low K_m phosphodiesterase (37). Since none of these effects of insulin is detectable in BC_3H-1 myocytes, it seems unlikely that insensitivity of any single target enzyme could account for the observed pattern of insulin resistance. Nor does there appear to be an abnormality of insulin binding or of the immediate sequelae of binding, i.e., hexose transport (2, 5). We have also examined the ability of insulin to stimulate BC_3H-1 insulin receptor β-subunit tyrosine kinase activity in wheat germ agglutinin-purified receptor preparations and found that insulin enhances β-subunit tyrosine phosphorylation 4- to 5-fold with a K_D of about 5 nM,[1] values which are consistent with those reported for the insulin receptors of other cultured cell types (38, 39). Thus, the lack of hexose-independent insulin effects in BC_3H-1 myocytes probably arises at some point between insulin binding to its cell surface receptor and the target metabolic enzymes themselves. This implies either a failure to generate sufficient metabolically active quantities of a second messenger, or the coincident insensitivity of several insulin-regulated enzymes to the appropriate second messenger.

The hexose-independent effects of insulin in adipocytes and skeletal muscle have been attributed to the insulin-dependent generation of low molecular weight "mediators" whose actions include the stimulation of GS phosphatase activity (33), inhibition of GS kinase(s), including cAMP-dependent protein kinase, through a mediator-induced reassociation of the R- and C-subunits (34–36), stimulation of a low K_m phosphodiesterase, and activation of mitochondrial PDH (37). Data from the purification of insulin "mediators" have shown that the cAMP-dependent protein kinase and PDH mediators are resolved into separate fractions by gel filtration (33, 40). Metabolic studies of the effects of partially purified "mediators" on intact adipocytes have emphasized that different functional classes of "mediators" exist (41, 42). The partial absence of hexose-independent effects of insulin in BC_3H-1 myocytes is consistent with the hypothesis that some, but not all, insulin "mediators" are not functional.

The nature of insulin-induced intracellular signals and the sequence of post-binding events which leads to their generation remains unclear. However, the ability of insulin to activate PDH in BC_3H-1 myocytes in the absence of several other effects underscores the distinctiveness of the mechanism whereby this enzyme is activated from the effects of insulin on GS and its regulatory enzymes. Our data strongly suggest that the second messenger necessary for PDH activation can be generated independently of that responsible for the inhibition of GS kinases and activation of GS phosphatase, although it is not clear whether the two classes of signals are produced at different steps of a single insulin-stimulated cascade or are the products of distinct signal-transducing mechanisms.

Acknowledgments

We thank the Core Analytical Laboratory (Dr. George VandenHoff) and the Tissue Culture Core Laboratory (Ms. Hannah Anderson) of the Diabetes Research and Training Center for their assistance. We thank Leslie Powell for skillful editorial assistance.

References

1. Shubert, D., J. A. Harris, C. E. Devine, and S. Heineman. Characterization of a unique muscle cell line. *J. Cell Biol.* **61**:398–413 (1974).
2. Standaert, M. L., S. D. Schimmel, and R. J. Pollet. The development of insulin receptors and responses in the differentiating nonfusing muscle cell line BC_3H-1. *J. Biol. Chem.* **259**:2337–2345 (1984).

[1] L. M. Luttrell and A. D. Rogol, unpublished observations.

3. Farese, R. V., M. L. Standaert, D. E. Barnes, J. S. Davis, and R. J. Pollet. Phorbol ester provokes insulin-like effects on glucose transport, amino acid uptake, and pyruvate dehydrogenase activity in BC_3H-1 cultured myocytes. *Endocrinology* **116**:2650–2655 (1985).
4. Farese, R. V., D. E. Barnes, J. S. Davis, M. L. Standaert, and R. J. Pollet. Effects of insulin and protein synthesis inhibitors on phospholipid metabolism, diacylglycerol levels, and pyruvate dehydrogenase activity in BC_3H-1 cultured myocytes. *J. Biol. Chem.* **259**:7094–7100 (1984).
5. Luttrell, L. M., and A. D. Rogol. Partial insulin resistance in the mouse BC_3H-1 cell line: absent hexose-independent actions of insulin. *Endocrinology* **119**:331–342 (1986).
6. Larner, J., J. C. Lawrence, R. J. Walkenbach, P. J. Roach, R. J. Hazen, and L. C. Huang. Insulin control of glycogen synthesis, in *Endocrine Pancreas and Diabetes*. International Congress Series 459 (José Pierluissi, ed.). Elsevier/North Holland, New York, 147–171 (1977).
7. Lawrence, J. C., J. J. Guinovart, and J. Larner. Activation of rat adipocyte glycogen synthase by insulin. *J. Biol. Chem.* **252**:44–450 (1977).
8. Lawerence, J. C., and J. Larner. Activation of glycogen synthase in rat adipocytes by insulin and glucose involves increased glucose transport and phosphorylation. *J. Biol. Chem.* **253**:2104–2113 (1978).
9. Larner, J., K. Cheng, C. Schwartz, K. Kikuchi, S. Tamura, S. Creasy, R. Dubler, G. Galasko, C. Pullin, and M. Katz. Insulin mediators and their control of metabolism through protein phosphorylation. *Recent Prog. Horm. Res.* **38**:511–556 (1982).
10. Thomas, J. A., K. K. Schendler, and J. Larner. A rapid filter paper assay for UDP glucose-glycogen glucosyltransferase including an improved biosynthesis of UDP-^{14}C-glucose. *Anal. Biochem.* **25**:486–499 (1968).
11. Larner, J., C. Villar-Palasi, and N. E. Brown. Uridine diphosphate glucose: α-1,4-glucan α-4-glucosyltransferase in heart. Two forms of the enzyme. Interconversion reactions and properties. *Biochim. Biophys. Acta* **178**:470–479 (1969).
12. Reznikoff, C. A., D. W. Brankow, and C. Heidelberger. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* **33**:3231–3238 (1973).
13. Guinovart, J. J., A. Salavert, J. Massague, C. J. Cuidad, and E. Harte. Glycogen synthase: a new activity ratio assay expressing a high sensitivity to the phosphorylation state. *FEBS Lett.* **106**: 284–288 (1979).
14. Gilboe, D. P., K. L. Larson, and F. Q. Nuttall. Radioactive method for the assay of glycogen phosphorylases. *Anal. Biochem.* **47**:20–27 (1972).
15. Harper, J. F., and G. Brooker. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′*O* acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**:207–218 (1975).
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
17. Taylor, S. I., C. Mukhurjee, and R. Jungas. Studies on the mechanism of activation of adipose tissue pyruvate dehydrogenase by insulin. *J. Biol. Chem.* **248**:73–81 (1973).
18. Chan, C. P., and E. F. Krebs. Epidermal growth factor stimulates glycogen synthase activity in cultured cells. *Proc. Natl. Acad. Sci. USA* **82**:4563–4567 (1985).
19. Guinovart, J. J., J. C. Lawrence, and J. Larner. Hormonal effects on fat cell adenosine 3′,5′-monophosphate dependent protein kinase. *Biochim. Biophys. Acta* **539**:181–194 (1978).
20. Hutson, N. J., F. T. Brumley, F. D. Assimacopoulos, S. C. Harper, and J. H. Exton. Studies on the α-adrenergic activation of hepatic glucose output. *J. Biol. Chem.* **251**:5200–5208 (1977).
21. Massaque, J., and J. J. Guinovart. Insulin counteraction of α-adrenergic effect on liver glycogen metabolism. *Biochim. Biophys. Acta* **543**:269–272 (1978).
22. Cheng, K., and J. Larner. Unidirectional actions of insulin and Ca^{2+}-dependent hormones on adipocyte pyruvate dehydrogenase. *J. Biol. Chem.* **260**:5279–5285 (1985).
23. Oron, Y., and J. Larner. Insulin action in intact mouse diaphragm. I. Activation of glycogen synthase through stimulation of sugar transport and phosphorylation. *Mol. Cell. Biochem.* **32**:153–160 (1980).
24. Hidaka, H., B. V. Howard, F. C. Kosmakos, R. M. Fields, J. W. Craig, P. H. Bennett, and J. Larner. Insulin stimulation of glycogen synthase in cultured human diploid fibroblasts. *Diabetes* **29**:806–810 (1980).
25. Craig, J. W., T. W. Rall, and J. Larner. The influence of insulin and epinephrine on adenosine 3′,5′-phosphate and glycogen transferase in muscle. *Biochim. Biophys. Acta* **177**:213–219 (1969).
26. Reed, L. J. Regulation of mammalian pyruvate dehydrogenase complex by a phosphorylation-dephosphorylation cycle. *Curr. Top. Cell. Regul.* **18**:95–106 (1981).
27. Hughes, W. A., R. W. Brownsey, and R. M. Denton. Studies on the incorporation of [^{32}P] phosphate into pyruvate dehydrogenase in intact rat fat-cells. *Biochem. J.* **192**:469–481 (1980).
28. Marshall, S. E., J. G. McCormack, and R. M. Denton. Role of Ca^{2+} ions in the regulation of intramitochondrial metabolism in rat epididymal adipose tissue. *Biochim. J.* **218**:249–260 (1984).
29. Koepfer-Hobelsberger, B., and O. H. Wieland. Inositol triphosphate activates pyruvate dehydrogenase in isolated fat cells. *FEBS Lett.* **176**:411–413 (1984).
30. Begum, N., H. M. Tepperman, and J. Tepperman. Studies on the possible involvement of prostaglandins in insulin generation of pyruvate dehydrogenase activator. *Diabetes* **34**:29–37 (1985).
31. Lawrence, J. C., and C. James. Activation of glycogen synthase by insulin in rat adipocytes. Evidence of hormonal stimulation of multisite dephosphorylation by glucose transport-dependent and -independent pathways. *J. Biol. Chem.* **259**:7975–7982 (1984).
32. Huang, F. L., and S. H. Tao. Glycogen metabolism of myogenic cells in culture. Presence of inhibitors for dephosphorylation of glycogen synthase and glycogen phosphorylase. *Arch. Biochem. Biophys.* **199**:123–132 (1980).
33. Cheng, K., G. Galasko, L. Huang, J. Kellogg, and J. Larner. Studies on the insulin mediator. II. Separation of two antagonistic biologically active materials from fraction II. *Diabetes* **29**:659–661 (1980).
34. Larner, J., G. Galasko, K. Cheng, A. A. Depaoli-Roach, L. Huang, P. Daggy, and J. Kellogg. Generation by insulin of a chemical mediator that controls protein phosphorylation and dephosphorylation. *Science (Wash. D. C.)* **206**:1408–1410 (1979).
35. Walkenbach, R. J., R. Hazen, and J. Larner. Reversible inhibition of cyclic AMP dependent protein kinase by insulin. *Mol. Cell. Biochem.* **19**:31–41 (1978).
36. Walkenbach, R. J., R. Hazen, and J. Larner. Hormonal regulation of glycogen synthase. Insulin decreases protein kinase sensitivity to cyclic AMP. *Biochim. Biophys. Acta* **629**:421–430 (1980).
37. Jarett, L., F. L. Kiechle, and J. L. Parker. Chemical mediator or mediators of insulin action: response to insulin and mode of action. *Fed. Proc.* **41**:2736–2741 (1982).
38. Kasuga, M., F. A. Karlssor, and C. R. Kahn. Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. *Science (Wash. D. C.)* **215**:185–187 (1982).
39. White, M. F., H. U. Haring, M. Kasuga, and C. R. Kahn. Kinetic properties and sites of autophosphorylation of the partially purified insulin receptor from hepatoma cells. *J. Biol. Chem.* **259**:255–264 (1984).
40. Kiechle, F. L., L. Jarett, N. Kotagal, and D. A. Popp. Partial purification from fat adipocyte plasma membranes of a chemical mediator which stimulates the action of insulin on pyruvate dehydrogenase. *J. Biol. Chem.* **256**:2945–2951 (1981).
41. Jarett, L., E. H. A. Wong, S. L. Macaulay, and J. A. Smith. Insulin mediators from rat skeletal muscle have differential effects on insulin-sensitive pathways of intact adipocytes. *Science (Wash. D. C.)* **227**:533–535 (1985).
42. Jarett, L., E. H. A. Wong, J. A. Smith, and S. L. Macaulay. Stimulation of pyruvate dehydrogenase activity in intact rat adipocytes by insulin mediator from rat skeletal muscle. *Endocrinology* **116**:1011–1016 (1985).

Send reprint requests to: Dr. Alan D. Rogol, Department of Pediatrics, Box 386, University of Virginia Medical Center, Charlottesville, VA 22908.