**Effects of CGS 9343B (a Putative Calmodulin Antagonist) on Isolated Skeletal Muscle**

**DISSOCIATION OF SIGNALING PATHWAYS FOR INSULIN-MEDIATED ACTIVATION OF GLYCOGEN SYNTHASE AND HEXOSE TRANSPORT**

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The role of calmodulin in control of carbohydrate metabolism in the absence and presence of insulin in isolated mouse soleus muscle was investigated. The calmodulin antagonist CGS 9343B had no effect on basal glycogen synthase activity, the contents of high energy phosphates, glucose-6-P, or glycogen synthesis. However, CGS 9343B inhibited the basal rates of 2-deoxyglucose uptake and 3-O-methylglucose transport by 30% (p < 0.05) and 40% (p < 0.001), respectively. Insulin activated glycogen synthase by almost 40% (p < 0.01) and this increase was not altered in the presence of CGS 9343B. Insulin increased the muscle content of glucose-6-P (~2-fold), as well as glycogen synthesis (~8-fold), 2-deoxyglucose uptake (~3-fold), and 3-O-methylglucose transport (~2-fold), and these increases were inhibited by CGS 9343B. In additional experiments on isolated rat epitrochlearis muscle, it was found that the hypoxia-mediated activation of 3-O-methylglucose transport was also inhibited by CGS 9343B. These data demonstrate that: 1) hexose transport, both in the absence and presence of external stimuli (insulin and hypoxia), requires functional calmodulin; and 2) insulin-mediated activation of glycogen synthase does not require functional calmodulin, nor can it be accounted for by increases in glucose transport or glucose-6-P.

Skeletal muscle is the major site of insulin-stimulated glucose uptake (1). Insulin stimulates glucose transport (2), GS1 and glycogen synthesis in skeletal muscle (3, 4). A defect in insulin-mediated glycogen synthesis, i.e. insulin resistance, is considered to underlie and contribute to the development of noninsulin-dependent diabetes mellitus (5, 6). It is therefore important to elucidate the signaling mechanisms whereby insulin stimulates glycogenesis in skeletal muscle.

Recent studies by Beitner and associates (7) indicate that insulin-mediated increases in binding of hexokinase to mitochondrial membrane and binding of phosphofructokinase and aldolase to cytoskeletal proteins in skeletal muscle are blocked by calmodulin antagonists. These results suggest that calmodulin, generally considered to be a Ca2+-transducing protein, is involved in insulin-mediated activation of glycogenesis (8). Using nonspecific calmodulin antagonists (TFP and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), a number of investigators have suggested that calmodulin also plays a role in insulin-mediated activation of hexose transport/uptake in adipocytes and skeletal muscle (9–12). Whether calmodulin plays a role in insulin-mediated activation of GS has, to our knowledge, not been investigated. It has been demonstrated that calmodulin can be phosphorylated by the insulin-receptor tyrosine kinase in vitro, and that insulin stimulates calmodulin phosphorylation in intact hepatocytes (13). Since activation of tyrosine kinase activity is a relatively early event in insulin action, we reasoned that other insulin-sensitive pathways, in addition to glycogenesis, may also require calmodulin. We have therefore assessed the effects of CGS 9343B, a calmodulin antagonist (14), on insulin-mediated activation of GS, glycogen synthesis and hexose transport in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

Materials—2-Deoxy-O-[1,2-3H]glucose, 3-O-[1H]methylglucose, [14C]mannitol, and carbonyl-[14C]insulin were purchased from DuPont NEN, and O-[U-14C]glucose, O-[U-14C]glucose-1-P, and UDP-[U-14C]glucose were purchased from Amersham. TFP was purchased from Sigma, and CGS 9343B was a generous gift from Dr. Peter Fels (Zyma SA, Switzerland). Human insulin (Actrapid) was purchased from Novo Nordisk (Denmark). All reagents and enzymes were of analytical grade and purchased from either Sigma or Boehringer Mannheim.

Muscle Preparation—Male Swiss albino mice, fed ad libitum, weighing ~35g and overnight fasted Wistar rats, weighing ~120g were housed at room temperature. Mice were killed by cervical dislocation and both solei were removed. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (8 mg/100 g body weight) and both epitrochlearis muscles were removed. Muscles were incubated in glass vials containing KBB continuously gassed with 95% O2, 5% CO2 in a shaking water bath (110 oscillations/min) (15). In some experiments hypoxia was induced by gassing with 95% N2, 5% CO2. When insulin was added it was always at a final concentration of 20 milliunits/ml.

Glycogen Synthase and Phosphorylase—Paired solei were incubated at 35°C in 1.5 ml of KBB containing 5 mM glucose for 90 min, followed by immediate freezing in liquid N2. Insulin was added during the last 30 min of incubation. Stock solutions of the calmodulin antagonists (TFP and CGS 9343B) were made in doubly distilled water on the morning of the experiment and then diluted in KBB. Whenever a compound was added it remained present throughout the incubation (i.e. if the medium was changed, the original compound was also present in the new medium). Muscles were processed and...
analyzed for GS activity using the filter paper technique following the incorporation of UDP-[14C]glucose into glycogen (16) exactly as described elsewhere (15). GS fractional activity is the activity assayed at 0.17 mM glucose-6-P divided by the activity measured at 7.2 mM glucose-6-P.

Measurements of phosphorylase, when performed, were on the same extracts used for GS. Briefly, an aliquot of the 10,000 × g supernatant (see Ref. 15) was diluted (2:1) with ice-cold buffer consisting of 100 mM NaOH. The muscle weight was recorded and the muscles were then digested in a hot water bath (70°C) for 5 min. Phosphorylase a was assayed at 15 mM glucose-1-P (0.14 mM/mmol) and 3.3 mg/ml glycogen at pH 6.3. Phosphorylase a + b was assayed in the same manner except that 3.3 mM AMP was included and the glucose-1-P (0.003 mM/mmol) and glycogen concentrations were increased to 66 mM and 6.7 mg/ml, respectively. All enzyme activities were linear with time and extract volume used (data not shown).

**Glycogen Synthesis—** Paired mouse solei were incubated as described above. At 60 min, [14C]glucose (0.05 mM/mmol) was added to all the vials. Insulin, when present, was added together with the isotope. At the end of incubation (90 min) the muscles were quickly blotted, frozen, and processed for measurement of label incorporation into glycogen as described elsewhere (18). Briefly, frozen muscles were added to preweighed Eppendorf tubes containing 120 μl of 5 N KOH, and the muscle weight was recorded. The muscles were then digested in a hot water bath (100°C) for 5 min, cooled on ice, and centrifuged at 3,000 × g for 5 min. 100 μl of supernatant was spotted onto filter paper (as for GS and phosphorylase) and washed in 67% ethanol (3 × 20 min). The papers were dried, immersed in scintillation mixture, and counted. Glycogen synthesis rates (in the presence and absence of insulin) were linear under the conditions studied (data not shown).

**Muscle Metabolism—** Paired solei were incubated as for GS and phosphorylase (see above). Frozen muscles were added to chilled ground glass homogenizing tubes followed by immediate addition of 250 μl of ice-cold 0.5 M perchloric acid. The muscles were homogenized on ice and kept in an ice bath for 10 min with occasional vortexing. The homogenate was then centrifuged at 15,000 × g for 3 min, and the supernatant was assayed for glycogen synthase, phosphorylase, and total creatine with enzymatic techniques adapted for fluorometry (19). In separate experiments, solei were dissected free and immediately frozen in liquid N2. The sum of phosphocreatine and creatine (i.e. total creatine) in these muscles averaged 18.9 ± 0.4 μmol/g wet wt (n = 5). Metabolites in the incubated muscles were adjusted to this total creatine content to allow for expression in μmol/g wet wt (metabolite, μmol/total creatine, μmol × 18.9 μmol/g wet wt).

**Hexose Transport—** Measurement of 2-DG uptake was essentially as described elsewhere (20). Briefly, paired solei were incubated at 35°C in 1.5 ml of KBB containing 2 mM pyruvate (no glucose) for a total of 80 min. After the initial 30-min incubation, insulin was added to the designated vials, and 30 min later 2-DG (final concentration = 1 mM; 1 mM/mmol) and insulin (0.2 μCi/ml medium, for assessment of extracellular space) were added to the incubation medium. 20 min after addition of 2-DG the muscles were blotted and frozen. The frozen muscles were added to preweighed Eppendorf tubes containing 0.5 ml of 1 N NaOH. The muscle weight was recorded and the muscles were then digested in a hot water bath (70°C) for 5 min. The tubes were cooled on ice and centrifuged at 3,000 × g for 5 min. Aliquots of the supernatant were added to scintillation mixture and counted for 14C and 3H. Isotope spillover between channels was corrected and intracellular concentrations of 2-DG were calculated as described elsewhere (21). 2-DG uptake rates were linear under the conditions studied (data not shown).

**Tetanic Force Measurements—** The tendons of soleus muscles were gripped with aluminum clips. One clip was mounted on a force transducer and another on a moveable hook, so that the muscle length could be adjusted to that giving maximum tetanic force. The muscle was immersed in oxygenated KBB (5 mM glucose) and stimulated at room temperature (22°C) by passing brief (0.5 ms) current pulses between two plate electrodes lying parallel to the muscle. Tetanic contractions (70 Hz, 500 ms duration) were produced at 1-min intervals. After a stabilization period, wherein 3 similar consecutive tetani were produced, the bath medium was exchanged for one containing CGS 9343B (40 μM). 10 min later, the medium containing CGS 9343B was washed out and fresh KBB was added. The signal from the force transducer was digitized and stored in a personal computer. The relaxation speed was assessed from stored data as the half-relaxation time: the time from the end of the stimulus train until the force had fallen to 50% peak tetanic force and half- relaxation times before drug exposure and 3 min after drug washout were not significantly different (n = 4).

**Statistics—** Significant differences (p < 0.05) between means were determined with the paired or unpaired t test. Values are reported as means ± S.E. unless otherwise indicated.

**RESULTS**

**Calmodulin Antagonists Do Not Affect GS and Phosphorylase Activity, nor High Energy Phosphates—** We first studied the effect of TFP, a known calmodulin antagonist, on the insulin-mediated activation of GS. A TFP concentration of 15 μM was chosen with the intent of maximizing the specific effect on calmodulin while minimizing nonspecific effects (14). TFP did not affect the insulin-mediated activation of GS (Table I). We then studied the effect of CGS 9343B, a more specific calmodulin antagonist (IC50 for inhibition of calmodulin stimulated phosphodiesterase activity = 3 μM, with no effect on protein kinase C) (14), on the insulin-mediated activation of GS. Again, there was no effect on insulin-mediated activation of GS (Table I). Furthermore, CGS 9343B had no significant effect on phosphorylase fractional activity (control = 0.03 ± 0.00; CGS 9343B = 0.04 ± 0.01, n = 6, p > 0.05). Insulin does not affect phosphorylase fractional activity in isolated mouse soleus (24). CGS 9343B (40 μM) had no significant effects on the muscle contents of high energy phosphates (ATP; control = 4.3 ± 0.1

| Treatment | GSF | n  |
|-----------|-----|----|
| None      | 0.55 ± 0.05 | 6  |
| Insulin   | 0.75 ± 0.05a | 6  |
| TFP (15 μM) | 0.55 ± 0.05 | 6  |
| TFP (15 μM) + insulin | 0.52 ± 0.02 | 5  |
| TFP (15 μM) + insulin | 0.74 ± 0.02a | 5  |
| None      | 0.56 ± 0.03 | 6  |
| CGS (40 μM) | 0.62 ± 0.04 | 6  |
| CGS (10 μM) | 0.58 ± 0.04 | 6  |
| CGS (10 μM) + insulin | 0.72 ± 0.05a | 6  |
| CGS (40 μM) | 0.58 ± 0.02 | 6  |
| CGS (40 μM) + insulin | 0.77 ± 0.06b | 6  |

* a = p < 0.01 (paired two-tailed t test).
| b = p < 0.05 (paired two-tailed t test).
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Glucose-6-P concentrations in isolated mouse soleus muscle. Muscles were incubated at 35°C for 90 min in KBB containing 5 mM glucose ± 40 μM CGS 9343B and frozen in liquid N2. Muscles were processed and analyzed for glucose-6-P as described under “Experimental Procedures.” When insulin was present, it was during the last 30 min of incubation at 20 milliunits/ml. Values are means ± S.E. for 5–6 muscles per group. **, p < 0.01 (paired two-tailed t test).

Glycogen synthesis in isolated mouse soleus muscle. Muscles were incubated for 90 min as described in legend to Fig. 1. After 60 min [14C]glucose (0.05 mCi/mmol) was added to all vials. When insulin was present (20 milliunits/ml), it was added together with the isotope. Muscles were frozen, processed, and analyzed for label incorporation into glycogen as described under “Experimental Procedures.” Values are means ± S.E. for 6 muscles per group. ***, p < 0.001 (paired two-tailed t test).

Concentration dependence of CGS 9343B mediated inhibition of glycogen synthesis in isolated mouse soleus muscle. Incubations were as described in the legend to Fig. 2. 100% glycogen synthesis = 2.34 ± 0.21 nmol/mg wet wt/30 min. Values are means of 4–8 muscles per group.

We therefore studied the effect of CGS 9343B on the transport of 3-OMG, a glucose analogue that is transported across the cell membrane but not phosphorylated to any appreciable extent. Insulin stimulated 3-OMG transport 2-fold, and this effect was blocked by CGS 9343B (Fig. 5). Additionally, CGS 9343B decreased the basal rate of 3-OMG transport. These results are similar to those observed with 2-DG.

Because the basal rate of 3-OMG transport in the mouse soleus was so high, it was difficult to detect a large increase in insulin-mediated transport. Therefore to study the concentration dependence of the CGS 9343B effect on insulin-mediated transport.

2 We have attempted a number of different protocols, including various concentrations of nonradioactive 3-OMG (0.1–10 mM), various incubation temperatures (25–35°C), and various incubation durations (2–15 min), but the protocol described under “Experimental Procedures” gave the largest and most reproducible insulin effect.
activation of 3-OMG transport, we used the rat epitrochlearis muscle, in which transport has previously been shown to increase 6-fold in the presence of insulin (27). Insulin activated 3-OMG transport 5-fold and this activation was inhibited by CGS 9343B in a concentration-dependent manner (Fig. 6). As was the case in mouse soleus muscle, CGS 9343B also inhibited the basal rate of 3-OMG transport in the epitrochlearis muscle by 45% (n = 10, p < 0.05 by paired two-tailed t test).

Since CGS 9343B inhibited basal and insulin-mediated activation of 3-OMG transport, we reasoned that the drug may also interfere with the actions of other activators of hexose transport. Hypoxia potently stimulates 3-OMG transport in skeletal muscle by a pathway separate to that of insulin (23). We therefore studied the effect of CGS 9343B on hypoxia-mediated activation of 3-OMG transport in the epitrochlearis muscle. Hypoxia increased 3-OMG transport 7-fold (cf. Figs. 7 and 6, wherein basal transport averages 0.14 μmol/ml/10 min), and CGS 9343B inhibited this increase by 65%.

CGS 9343B Does Not Affect Tetanic Force—Peak tetanic force was not significantly affected by 40 μM CGS 9343B; after 10 min exposure to the drug, force was 99 ± 3% (n = 4) of that prior to drug exposure. Relaxation, on the other hand, was significantly slowed in the presence of the drug (Fig. 8). Half-relaxation time was 145 ± 11 ms in the absence and 168 ± 15 ms in the presence of CGS 9343B (p < 0.05, n = 4). Since intracellular calmodulin is involved in the relaxation process (see "Discussion"), these results indicate that CGS 9343B permeated the muscle cells and presumably bound to calmodulin (14).

DISCUSSION

These findings suggest that calmodulin is involved in insulin-mediated activation of glucose transport and glycogen synthesis, but not in insulin-mediated activation of GS. This interpretation is valid only to the extent that CGS 9343B, under the conditions studied, specifically antagonized calmodulin function. Others have suggested that CGS 9343B is specific for calmodulin, and in contrast to other antagonists (e.g. TFP), has no effect on protein kinase C activity (at least up to a concentration of 100 μM) (14). On the other hand, it was recently demonstrated that CGS 9343B inhibits membrane currents and prevents increases in intracellular Ca2+ in rat pheochromocytoma cells. These results served as a basis for the conclusion that CGS 9343B affects ion fluxes in a manner unrelated to calmodulin inhibition (28).

These results warranted further testing on potential sites of CGS 9343B action in mouse soleus muscle. Because CGS 9343B did not affect GS and phosphorylase fractional activities under basal conditions, it is unlikely that it had any major...
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3-O-Methylglucose transport

![CGS 9343B inhibits hypoxia-mediated activation of 3-OMG transport in isolated rat epitrochlearis muscle.](image)

**Fig. 7.** CGS 9343B inhibits hypoxia-mediated activation of 3-OMG transport in isolated rat epitrochlearis muscle. Muscles were incubated for 90 min in KBB continuously gassed with 95% N₂, 5% CO₂ (40 μM CGS 9343B). Thereafter, muscles were rinsed and transport was measured as described in the legend to Fig. 6. Values are means ± S.E. for 6 muscles per group. *, p < 0.05 (paired two-tailed test).

![Force vs. Time Graph](image)

**Fig. 8.** CGS 9343B slows relaxation in isolated mouse soleus muscle. Original force records showing the slowing of relaxation after tetanic stimulation in one muscle. Similar results were obtained in 3 additional muscles. Stimulation protocol is described under "Experimental Procedures." The superimposed records were obtained after 10 min exposure to 40 μM CGS 9343B and 3 min after washout of the drug. Period of stimulation is shown below the force records (the elevated bar on the left side of abscissa). Observe that while relaxation was slower in the presence of CGS 9343B, the peak tetanic force (which generally occurred at the end of each tetanus) was unaffected by the drug.

The precise step where calmodulin modulates glucose transport is not known. Recently, a photoaffinity labeling technique has been used to demonstrate that insulin-mediated activation of glucose transport in isolated adipocytes and rat soleus muscle is accounted for by an increase in surface accessible Glut-4 transporter proteins (32, 33). And studies on isolated adipocytes indicate that the surface accessible affinity labeled Glut 4 transporters translocate to the plasma membrane from an intracellular pool upon insulin stimulation (32). By using Western blotting it has also been shown that insulin and hypoxia stimulate 3-OMG transport by separate pathways (23), it is likely that calmodulin is involved in control of hexose transport at a site beyond where the two pathways converge.

It is reasonable to assume that CGS 9343B specifically antagonized calmodulin activity under the conditions reported herein.

CGS 9343B inhibited insulin-mediated activation of hexose transport/uptake by ≥75%, but insulin-mediated glycerogen synthesis by only 40%. Presumably, there was a redistribution of the glucose entering the muscle such that in the presence of CGS 9343B relatively more glucose was diverted toward glycogen synthesis and less toward glycolysis. This can be explained by a CGS 9343B-mediated inhibition of glycolysis in the presence of insulin (7, 8), coupled with a maintained high activity of GS (present results). These findings indicate that insulin does not control glucose metabolism by simply accelerating glucose transport. The high glucose transport rate in the presence of insulin must be coordinated with activation of glucose metabolizing enzymes (e.g. hexokinase, GS, phosphofructokinase, etc.) to achieve the glucose metabolizing rates required by the muscle. In this context it is noteworthy that CGS 9343B inhibited the basal rate of hexose transport/uptake but had no effect on glycogen synthesis, which may also be due to a drug-dependent inhibition of glycolysis.

The observation that CGS 9343B inhibited both the basal and insulin-stimulated rate of glucose transport raised the possibility that calmodulin may be involved in other modes of glucose transport activation (e.g. hypoxia and exercise) (23). This possibility was verified by the finding that CGS 9343B also inhibited hypoxia mediated activation of 3-OMG transport. Because insulin and hypoxia stimulate 3-OMG transport by separate pathways (23), it is likely that calmodulin is involved in control of hexose transport at a site beyond where the two pathways converge.

It may be argued that because CGS 9343B (40 μM) inhibited basal 3-OMG transport, the drug-dependent inhibition of insulin- and hypoxia-mediated activation of 3-OMG transport was not due to inhibition of an insulin (or hypoxia)-dependent process. However, the fact that the CGS 9343B (40 μM) dependent inhibition of 3-OMG transport in the presence of insulin (~0.3 μmol/ml/10 min) and after hypoxia (~0.5) were greater than the inhibition of basal transport (~0.1) indicates that CGS 9343B exerted an additional effect on transport in the presence of insulin or after hypoxia. The same reasoning can also be applied to the results in the soleus muscle, wherein the inhibition of insulin-mediated hexose transport/uptake by CGS 9343B was markedly greater than the inhibition of basal transport/uptake by CGS 9343B (see Figs. 4 and 5).

The next question arises as to how calmodulin modulates activation of glucose transport. It is generally believed that an increase in intracellular Ca²⁺ will result in an increased binding of Ca²⁺ to calmodulin, thereby inducing a conformational
change in the latter, which will alter the affinity of calmodulin for its site of action (enzyme activity or structural component) (34). For this to be the mechanism of action in the present study would require that hypoxia and insulin should increase myoplasmic Ca\(^{2+}\). The finding that dantrolene, which inhibits sarcoplasmic reticulum Ca\(^{2+}\) release, inhibits the hypoxia-mediated activation of 3-OMG transport in skeletal muscle (23) provides indirect support for the idea that hypoxia causes an increase in myoplasmic Ca\(^{2+}\). In this context it is noteworthy that 10 min of cyanide incubation, which inhibits mitochondrial oxygen utilization, significantly increased myoplasmic free Ca\(^{2+}\) by 10 ± 1 nm (basal value = 28 nm) in isolated single intact mouse muscle fibers.\(^3\) Whether insulin increases intracellular Ca\(^{2+}\) has been debated (see Ref. 12 and references therein). The finding that dantrolene abolished the insulin-mediated activation of 3-OMG in isolated rat muscle (12) suggests that insulin also increases myoplasmic Ca\(^{2+}\). However, direct measurements of myoplasmic Ca\(^{2+}\) in intact mammalian skeletal muscle after exposure to insulin have, to our knowledge, not been performed.

Alternatively, insulin may not alter myoplasmic Ca\(^{2+}\), but rather stimulate the phosphorylation of calmodulin. It has recently been demonstrated that insulin increases the phosphorylation state of calmodulin in isolated hepatocytes, and that phosphocalmodulin exhibits altered biologic activity in a manner that can be Ca\(^{2+}\)-independent (13, 35). Which, if any of these explanations is applicable to the present study, as well as which calmodulin-dependent enzyme/structure is altered by insulin in skeletal muscle, remains to be determined.

Although CGS 9343B had no effect on GS (presence and absence of insulin), the fact that it abolished the insulin-mediated increase in glucose-6-P allows for assessment of the role of glucose-6-P in insulin-mediated activation of GS. Glucose-6-P stimulates the dephosphorylation of GS by GS phosphatases by allosterically altering the configuration of GS, rendering GS a more suitable substrate for these phosphatases (36). It has been suggested that an increase in glucose-6-P by insulin, subsequent to activation of glucose transport, stimulates GS activation (i.e. dephosphorylation) (18, 37). The present findings indicate that neither increases in glucose transport nor glucose-6-P are required for the insulin-mediated activation of GS in mouse soleus. Indeed close examination of the relationship between exogenously added glucose-6-P and GS phosphatase activity in skeletal muscle homogenates (Ref. 37, see Fig. 9) indicates that the insulin-mediated increase in glucose-6-P observed in the present study would be expected to have only a minor effect on GS phosphatase.

In conclusion, these data demonstrate that in isolated skeletal muscle: 1) hexose transport both in the absence and presence of external stimuli (insulin and hypoxia) requires functional calmodulin; and 2) insulin-mediated activation of GS does not require functional calmodulin, nor can it be accounted for by increases in glucose transport or glucose-6-P.

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\(^3\) H. Westerblad and D. G. Allen, unpublished observations.