MOLECULAR PHARMACOLOGY, 14, 1079-1091

# Effects of Insulin, Methoxamine, and Calcium on Glycogen Synthase in Rat Adipocytes

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(Received March 16, 1978)
(Accepted June 21, 1978)

SUMMARY

LAWRENCE, JOHN C., JR. & LARNER, JOSEPH (1978) Effects of insulin, methoxamine, and calcium on glycogen synthase in rat adipocytes. *Mol. Pharmacol.*, **14**, 1079-1091.

Inactivation of glycogen synthase by methoxamine was not associated with changes in cyclic AMP and was abolished by the *alpha* adrenergic antagonists phentolamine, phenoxybenzamine, and dihydroergotamine. The effect of methoxamine was not reversed by atropine or the *beta* adrenergic antagonists propranolol or practolol. Methoxamine decreased the percentage of glycogen synthase *I* activity that had been increased by incubating cells with 100 microunits/ml insulin; however, an effect of insulin was observed at the highest concentration of methoxamine tested (100 $\mu$M). The effect of 10 $\mu$M methoxamine was not overcome by 25 milliunits/ml of insulin. The inactivation of glycogen synthase by methoxamine was abolished by incubating cells in calcium-free medium containing 1 mM EGTA, and the effect of methoxamine was restored by adding calcium. Incubation of cells with the divalent cation ionophore A23187 decreased the percentage of glycogen synthase *I* activity in the presence of, but not the absence of calcium. When A23187 and methoxamine were added together, no further decrease in the percentage of glycogen synthase *I* activity was observed below that produced by either agent alone. The activation of glycogen synthase by insulin was not diminished by incubating cells in calcium-free medium plus 1 mM EGTA. An effect of insulin on glycogen synthase was observed even in the presence of A23187. The results indicate that the increase in the percentage of glycogen synthase *I* activity due to insulin is independent of extracellular calcium. *Alpha* adrenergic receptor stimulation may lead to a decrease in the percentage of glycogen synthase *I* activity by increasing the concentration of cytosolic calcium. If the activation of glycogen synthase involves calcium, presumably insulin would act by decreasing cytosolic calcium since the effects of insulin on glycogen synthase are opposite to those of methoxamine and A23187.

## INTRODUCTION

We have previously shown that stimulation of either *alpha* or *beta* adrenergic receptors in rat adipocytes leads to inactivation of glycogen synthase (1). While it is likely that the decreases in the percentage of glycogen synthase *I* activity produced by *beta* adrenergic agonists result from elevations of cellular cyclic AMP, the mechanisms mediating the effects of *alpha* adrenergic agonists on glycogen synthase are less certain. Assimacopoulos-Jeannet *et al.* (2) suggested that stimulation of *alpha* adrenergic receptors in rat hepatocytes leads to an increase in the level of cytosolic $Ca^{++}$ which results in increased phosphorylase kinase activity and phosphorylase activation. This is an attractive hypothesis since it accomodates both the requirement of

This work was partially supported by USPHS grant #AM 14334 and the University of Virginia Diabetes Center grant #AM 17042.



0026-895X/78/0146-1079$02.00/0

extracellular $Ca^{++}$ for activation of phosphorylase by *alpha* adrenergic agonists (2) and the data of Khoo and Steinberg (3) which show that the activity of hepatic phosphorylase kinase can be stimulated by micromolar concentrations of $Ca^{++}$. However, the role of $Ca^{++}$ in the *alpha* adrenergic receptor-mediated decrease in glycogen synthase *I* activity is unknown.

It has been proposed that insulin action in adipose tissue involves an increase in cytoplasmic free calcium (4–10). However, insulin increases the percentage of glycogen synthase *I* activity and *alpha* adrenergic agonists decrease this percentage in fat cells (1, 11). Clearly, both activation and inactivation of glycogen synthase cannot be easily explained by an increase in cytoplasmic calcium.

The purpose of the present study was to investigate the interactions between insulin and the relatively pure *alpha* adrenergic agonist, methoxamine. In particular, we wanted to clarify the role of calcium in mediating the effects of these two agents on glycogen synthase. The results presented demonstrate that the inactivation of glycogen synthase by methoxamine is dependent upon calcium, while the activation of glycogen synthase by insulin is not dependent on extracellular calcium. We propose that *alpha* adrenergic receptor activation results in a decrease in the percentage of glycogen synthase *I* activity by increasing the cytosolic concentration of calcium. Therefore, if the effects of insulin involve calcium, the hormone must act by decreasing the concentration of calcium in the cytosol.

## MATERIALS AND METHODS

Adipocytes were prepared from the epididymal adipose tissue of 120 to 180 g rats (Wistar strain) fed *ad libitum* (12). All experiments with adipocytes were conducted in plastic bottles or tubes. Two media were used in the experiments presented. Regular medium was prepared by dissolving bovine serum albumin (30 mg/ml) in Krebs-Ringer phosphate buffer (128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, and 10 mM $Na_2HPO_4$, pH 7.4). The pH was adjusted to 7.4 with 10 N NaOH after the albumin had dissolved. Calcium-free medium was prepared from Krebs-Ringer phosphate buffer to which no calcium chloride was added.[1] EGTA[2] was added to this medium from a concentrated solution. The pH of this solution was adjusted so that the pH of the medium was maintained at 7.4. Fat pads were cut into small pieces, and 1 to 3 g of adipose tissue were incubated in 3 to 8 ml regular medium containing 1 mg/ml crude collagenase (*Clostridium histolyticum*, Worthington Biochemical Co.) for 60 min at 37°. The dispersed cells were strained through two layers of cheesecloth, washed twice at 37° with 10 ml of the appropriate medium, and suspended in 10 ml of medium/g of original tissue. Cell number was estimated by direct counting using a hemocytometer, and 0.5–0.8 × $10^6$ cells/ml were used.

Incubations of cells for enzyme assays were performed at 37° in 5 ml medium with air as the gas phase. When appropriate, a 1 ml sample of cells was removed for ATP and cyclic AMP determination and processed as described later. After centrifugation of the remaining cells for 15 sec in a clinical centrifuge, the medium was aspirated and the incubation terminated by adding 0.5 ml of 100 mM KF and 10 mM EDTA (pH 7.0) at 0° and immediately homogenizing the cells in a chilled glass homogenizer (ten strokes with a Teflon pestle driven at 1,000 rpm). The homogenates were centrifuged at 10,000 × *g* for 15 min before the supernatants were collected for assay of glycogen synthase and phosphorylase activities. Glycogen synthase activity was assayed essentially as described by Thomas *et al.* (13). Glycogen synthase *I* activity is expressed as a percentage of the total synthase activity (assayed in the presence of 7.2 mM glucose-6-P).[3] Phosphorylase activ-

[1] This medium is of course not truly calcium free since calcium is present as an impurity in the reagents used in its preparation.

[2] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethylether)-N,N′-tetraacetic acid.

[3] Although the total glycogen synthase activity varied somewhat among different experiments (50–70 nmol of [$^{14}$C]glucose incorporated into glycogen from UDP-[U-$^{14}$C]glucose/min/$10^7$ cells), the total activi-

ity was assayed essentially by the method of Gilboe *et al.* (14). The percentage of phosphorylase *a* activity was obtained by dividing the activity found in the absence of AMP by that observed in the presence of 2.0 mM AMP and multiplying by 100. A more detailed description of the experimental conditions used in the glycogen synthase and phosphorylase assays can be found in a previous report (11).

For measurements of ATP and cyclic AMP, the incubation of the 1 ml cell sample was terminated by adding 1 ml of 10% trichloroacetic acid and homogenizing at 0° for 15 sec with a Polytron homogenizer. After centrifugation at 10,000 × *g* for 10 min, the supernatants were removed and extracted 4 times with 6 ml of water-saturated ether. ATP was assayed in the samples essentially by the method of Williamson and Corkey (15). The concentration of cyclic AMP in the samples was determined by radioimmunoassay, using the procedures described by Harper and Brooker (16).

Dihydroergotamine tartrate, atropine, bovine serum albumin (Fraction V), and rabbit liver glycogen were obtained from Sigma Chemical Company. The glycogen was purified before use by passing a 5% solution over a mixed-bed ion exchange resin (Amberlite (MB-3) as described by Larner *et al.* (17). Phentolamine/HCl was from Ciba-Geigy. Phenoxybenzamine/HCl was from Smith, Kline & French. (−)-Propranolol was a gift from Ayerst Laboratories. Methoxamine was from Burroughs Wellcome. A23187 was a gift from Dr. R. L. Hamill of Lilly Research Laboratories. In experiments with A23187, the ionophore was dissolved in dimethylsulfoxide and added to cells in 5 μl aliquots. Cells not incubated with A23187 were incubated with 5 μl of dimethylsulfoxide. Radioactive compounds were purchased from New England Nuclear.

## RESULTS

We previously reported that the *alpha* adrenergic agonist methoxamine decreased the percentage of glycogen synthase *I* activity in isolated fat cells (1). As shown in Table 1, the effects of 10 μM methoxamine were abolished by 10 μM phentolamine, 10 μM phenoxybenzamine or 1 μM dihydroergotamine, but not by 10 μM atropine, 10 μM propranolol, or 10 μM practolol. These adrenergic antagonists and atropine were without effect on the percentage of glycogen synthase *I* activity when added alone. Therefore, it seems likely that methoxamine inactivates glycogen synthase via an *alpha* adrenergic receptor-mediated mechanism.

In adipocytes, insulin has been shown to oppose the actions of epinephrine to increase phosphorylase *a* activity and decrease glycogen synthase *I* activity (11, 18, 19). Because these actions of epinephrine involve activation of both *alpha* and *beta* adrenergic receptors (1), it is not known if insulin opposes the effects of *alpha* adrenergic receptor activation. Therefore, the effects of insulin in the presence of the specific *alpha* adrenergic agonist methoxamine were investigated (Figs. 1–3).

The effects of incubating fat cells for increasing times with methoxamine were investigated (Fig. 1). In these experiments,

TABLE 1

*Inhibition of the effect of methoxamine on glycogen synthase by alpha adrenergic antagonists*

Adipocytes were incubated at 37° for 10 min without or with different adrenergic antagonists or atropine. Fat cells were next incubated at 37° with or without methoxamine. After 4.5 min, the incubations were terminated and glycogen synthase was assayed. The results presented represent the mean values ± S.E. of 4 experiments performed on different days.

| Antagonists | Without Methoxamine | 10 μM Methoxamine |
|---|---|---|
| | *Glycogen synthase % I activity* | |
| None | 9.3 ± 0.6 | 5.8 ± 0.4 |
| Phentolamine, 10 μM | 8.9 ± 0.9 | 9.1 ± 0.8 |
| Phenoxybenzamine, 10 μM | 9.5 ± 0.7 | 9.2 ± 0.5 |
| Dihydroergotamine, 1 μM | 8.9 ± 0.7 | 9.2 ± 0.7 |
| Atropine, 10 μM | 9.1 ± 0.7 | 6.5 ± 0.4 |
| Propranolol, 10 μM | 8.7 ± 0.5 | 6.5 ± 0.5 |
| Practolol, 10 μM | 9.0 ± 0.6 | 6.1 ± 0.5 |

ties were unchanged by the conditions used in the experiments presented.

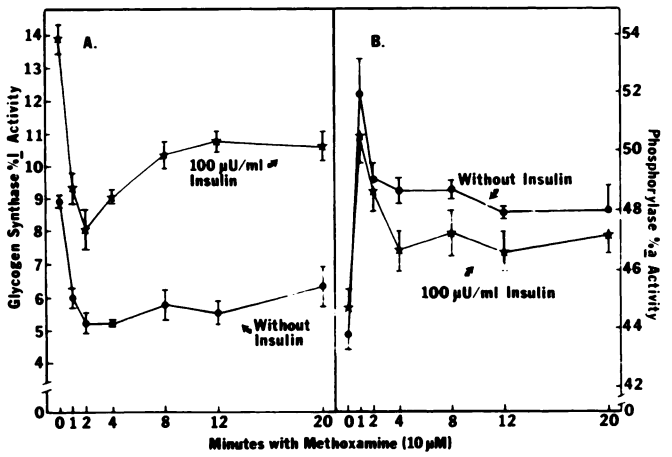


FIG. 1. *Time course of adipocyte glycogen synthase inactivation (A) and phosphorylase activation (B) by methoxamine in the absence and presence of insulin*

Adipocytes were incubated at 37° for 10 min in the absence and presence of 100 microunits/ml insulin. Methoxamine (10 μM) was then added and the incubations terminated after the appropriate times. The results presented represent the mean values ± S.E. of 3 experiments performed on different days.

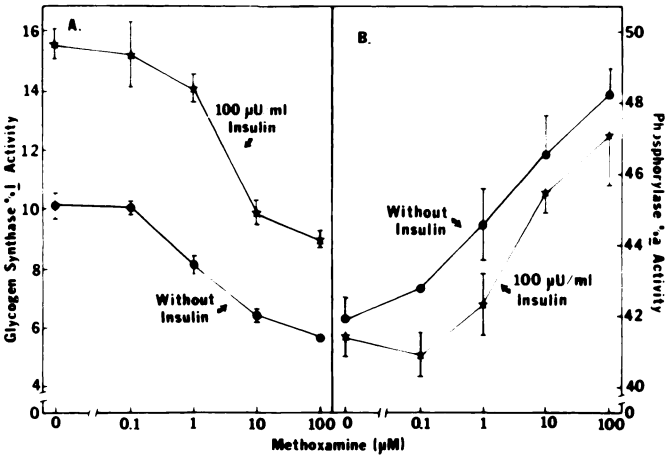


FIG. 2. *Inactivation of adipocyte glycogen synthase (A) and activation of phosphorylase (B) by increasing concentrations of methoxamine in the absence and presence of insulin*

Adipocytes were incubated with and without insulin for 10 min at 37° before the indicated concentrations of methoxamine were added. After 4 min the incubations were terminated and glycogen synthase and phosphorylase activities were assayed. The results presented represent the mean values ± S.E. from 3 experiments performed on different days.

cells were incubated for 10 min in either the presence or absence of 100 microunits/ml of insulin. Under these conditions we have found that insulin activates glycogen synthase without decreasing the concentration of cyclic AMP.[4]

[4] J. Lawrence, and J. Larner, manuscript submitted for publication.

Cells were next incubated with 10 μM methoxamine for the times shown. Methoxamine decreased the percentage of glycogen synthase *I* activity in both the presence and absence of insulin (Fig. 1A). In the absence of insulin inactivation of glycogen synthase by methoxamine was maximal after 2 min and the decreased percentage of glycogen synthase *I* activity was

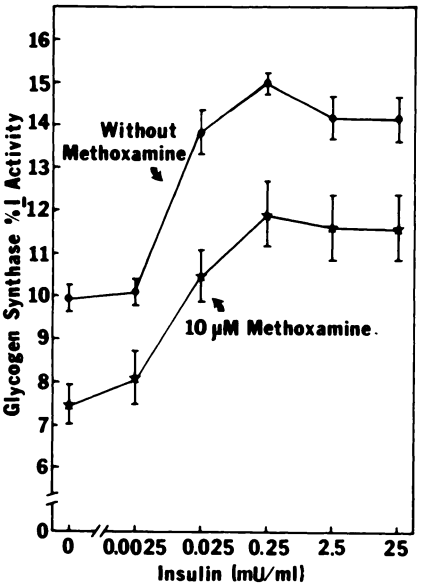


FIG. 3. *The effect of increasing concentrations of insulin on adipocyte glycogen synthase in the absence and presence of methoxamine*

Adipocytes were incubated at 37° with or without 10 μM methoxamine and the indicated concentrations of insulin. After 4 min the incubations were terminated and glycogen synthase activities were assayed. The results presented represent the mean values ± S.E. from 3 experiments performed on different days.

unchanged for at least 12 min. In the presence of insulin, the maximal effect of methoxamine was also observed after 2 min; however, with longer incubation periods, the maximal effect of methoxamine was not maintained.

The percentage of phosphorylase *a* activity was increased by methoxamine (Fig. 1B) in both the presence and absence of insulin. The effect of methoxamine was greatest after 1 min and was smaller after longer periods of incubation. Little, if any, effect of insulin was observed on phosphorylase in either the presence or absence of methoxamine.

Adipocytes were next incubated for 10 min in the presence or absence of 100 microunits/ml insulin and then for 4.5 min with increasing concentrations of methoxamine (Fig. 2). No action of insulin to oppose the activation of phosphorylase by methoxamine was observed in these experiments (Fig. 2B). Methoxamine decreased the percentage of glycogen synthase *I* activity that had been elevated by insulin, but even at concentrations of 100 μM, methoxamine did not overcome the effect of insulin (Fig. 2A).

In the experiments presented in Fig. 3, cells were incubated for 4.5 min with or without 10 μM methoxamine and increasing concentrations of insulin. Although insulin opposed the action of methoxamine to decrease glycogen synthase *I* activity, the hormone was not able to overcome completely the effects of methoxamine.

It has been suggested that an increase in cytosolic calcium is involved in both the action of insulin in adipocytes (4–10) and the action of *alpha* adrenergic agonists in rat hepatocytes (2). Because the effects of insulin and the *alpha* adrenergic agonist methoxamine on glycogen synthase are opposite (Figs. 1–3), it is highly unlikely that an increase in cytosolic calcium is responsible for the effects of both agents. The remaining experiments presented were therefore designed to investigate the role of calcium in methoxamine and insulin action in fat cells.

When cells were incubated in regular medium (represented by the bar graphs) for 4.5 min with 10 μM methoxamine, a decrease in the percentage of glycogen synthase *I* activity was observed (Fig. 4A), with no change in the concentration of cyclic AMP (Fig. 4C) or the percentage of phosphorylase *a* activity (Fig. 4B). In calcium-free medium with increasing concentrations of EGTA, the effect of methoxamine on glycogen synthase was not observed (Fig. 4A). The inactivation of glycogen synthase (Fig. 4A) and activation of phosphorylase (Fig. 4B) by 1 μM isoproterenol were not reversed by EGTA. Concentrations of EGTA up to 10 μM were without effect on the concentration of cellular ATP (Fig. 4D).

In calcium-free medium, 10 μM EGTA facilitated the ability of isoproterenol to increase the accumulation of cyclic AMP (Fig. 4C). No effect of incubating cells in calcium-free medium containing EGTA on the basal levels of cyclic AMP was observed in these experiments.

In order to determine if the effect of methoxamine could be restored by adding

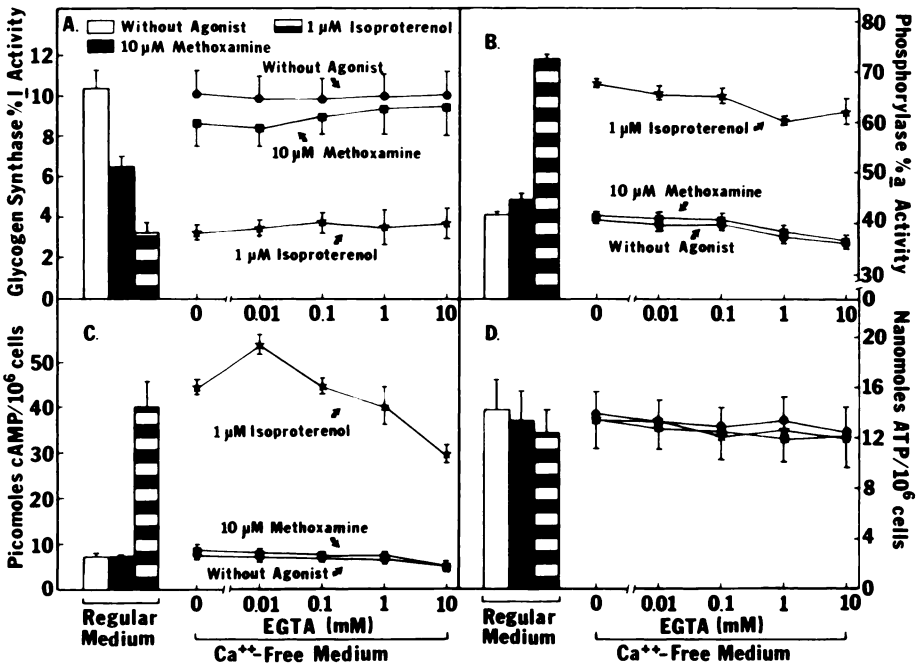


FIG. 4. *Effect of increasing concentrations of EGTA on glycogen synthase I activity (A), phosphorylase a activity (B), and the concentrations of cyclic AMP (C) and ATP (D) in adipocytes incubated with methoxamine and isoproterenol*

Fat cells were washed and suspended in either regular medium or calcium-free medium. Adipocytes were then incubated at 37° for 10 min in regular medium (bar graphs) or in calcium-free medium with increasing concentrations of EGTA before methoxamine (10 μM) or (-)-isoproterenol (1 μM) was added. The incubations were terminated 4.5 min after the addition of the agonists. The results presented represent the mean values ± S.E. from 3 experiments performed on different days.

calcium to cells first incubated in calcium-free medium containing EGTA, the experiments presented in Fig. 5 were performed. The percentage of glycogen synthase *I* activity was higher in adipocytes incubated in calcium-free medium containing 1 mM EGTA than in cells incubated in regular medium (Fig. 5A). Incubation of cells in regular medium (but not calcium-free medium) with increasing concentrations of methoxamine resulted in inactivation of glycogen synthase. The action of methoxamine to decrease glycogen synthase *I* activity was observed in cells first incubated in calcium-free medium with 1 mM EGTA when 2.4 mM calcium chloride was added together with the methoxamine.

Incubation of fat cells with increasing concentrations of methoxamine in regular medium did not change the concentration of cyclic AMP (Fig. 5B). In these experiments, higher concentrations of cyclic AMP were observed in cells incubated in calcium-free medium and 1 mM EGTA than in cells incubated in regular medium. Addition of calcium did not decrease these levels of cyclic AMP.

If *alpha* adrenergic agonists produce their effects by increasing calcium entry into the cell, then, in the presence of calcium, the ionophore A23187 should produce qualitatively similar effects. As shown in Fig. 6A, incubation of cells for 14.5 min with increasing concentrations of A23187 did not decrease the percentage of glycogen synthase *I* activity in cells incubated in calcium-free medium containing 1 mM EGTA. In cells incubated in regular medium, A23187 inactivated glycogen synthase. The ionophore also decreased the percentage of glycogen synthase *I* activity, when 2.4 mM calcium chloride was present during the

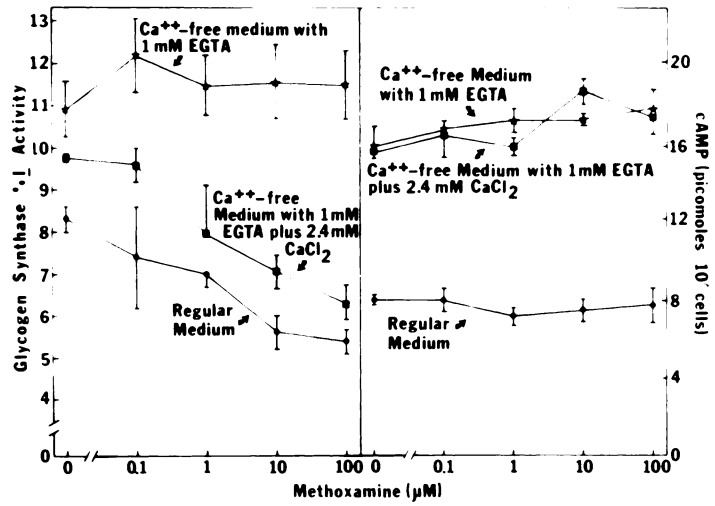


FIG. 5. *Dependence of the inactivation of glycogen synthase by methoxamine on medium calcium*

Adipocytes were incubated at 37° in calcium-free medium containing 1 mM EGTA or regular medium for 10 min. Cells were then incubated with increasing concentrations of methoxamine without or with 2.4 mM $CaCl_2$ as indicated. After 1 min the incubations were terminated and the concentrations of cyclic AMP and activities of glycogen synthase were determined. The results presented represent the mean values ± S.E. from 3 experiments performed on different days.

final 4.5 minutes of incubation, in cells first incubated in calcium-free medium. Under these conditions, and in regular medium, the maximal effect was observed with 1 μM A23187. A23187 did not activate phosphorylase in cells incubated in regular medium (Fig. 6B). When cells were incubated in calcium-free medium containing 1 mM EGTA, the percentage of phosphorylase *a* activity was decreased by the ionophore. Addition of calcium increased the percentage of phosphorylase *a* activity to approximately the same level as observed in cells incubated in regular medium without the ionophore. The changes in glycogen synthase and phosphorylase activities produced by A23187 were not associated with changes in cellular ATP (Fig. 6D). Also, A23187 did not increase the concentration of cyclic AMP (Fig. 6C). Again, in these experiments, incubation of cells in calcium-free medium with 1 mM EGTA resulted in elevated levels of cyclic AMP (Fig. 6C) when compared to the levels in cells incubated in regular medium. With calcium, A23187 decreased these elevated levels of cyclic AMP to those observed in regular medium.

In the experiments presented in Fig. 7, cells were incubated for 10 min in calcium-free medium with and without 10 μM A23187. Adipocytes were then incubated with and without 2.4 mM calcium chloride for increasing periods of time. The maximal inactivation of glycogen synthase in the presence of A23187 was observed when cells were incubated with calcium chloride for 4 min (Fig. 7A). Incubation of cells in calcium-free buffer containing 1 mM EGTA and 10 μM A23187 for 10 min resulted in a lower level of phosphorylase *a* activity compared with cells incubated without the ionophore. Addition of calcium to cells incubated with the ionophore increased the percentage of phosphorylase *a* activity to the level produced by adding calcium to cells incubated without the ionophore (Fig. 7B). This increase was maximal after 1 min.

In the experiments presented in Fig. 8, the effects of 100 μM methoxamine and 2.5 milliunits/ml insulin on glycogen synthase were investigated in cells incubated with A23187. Adipocytes were incubated in calcium-free medium containing 1 mM EGTA for 10 min in the presence and absence of 10 μM A23187. This concentration of the ionophore is ten times greater than that which produces a maximal decrease in gly-

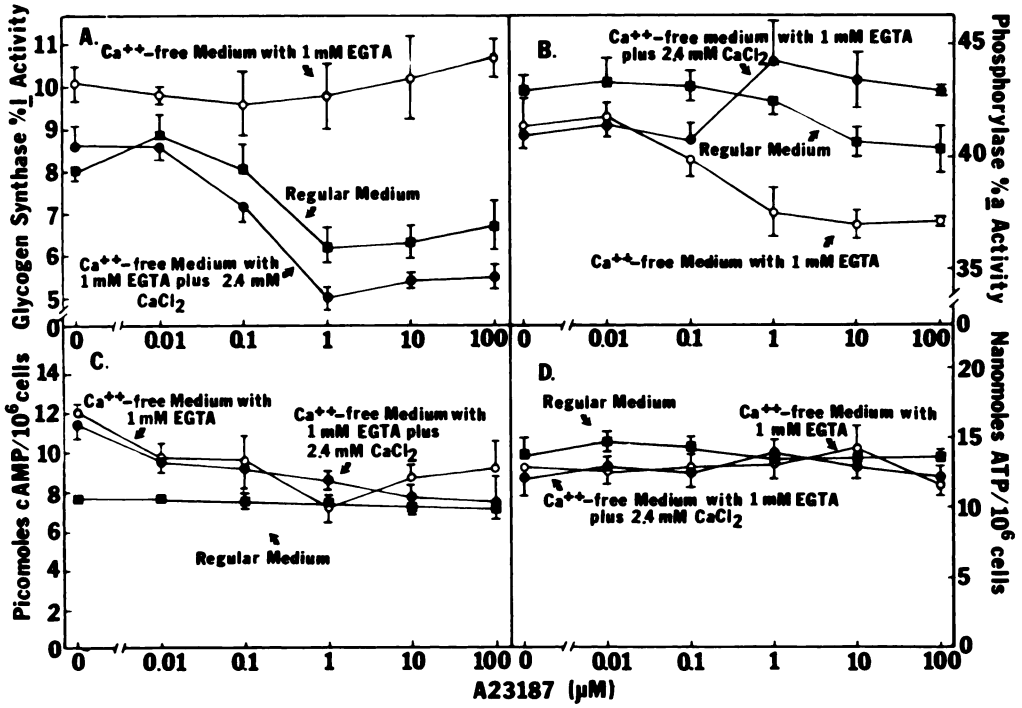


FIG. 6. *Effect of increasing concentrations of A23187 on glycogen synthase I activity (A), phosphorylase a activity (B) and the concentrations of cellular cyclic AMP (C) and ATP (D)*

Adipocytes were washed and suspended in either regular medium or calcium-free medium. Adipocytes in regular medium were incubated at 37° for 14.5 min with increasing concentrations of A23187. Cells suspended in calcium-free medium were incubated at 37° for 10 min with 1 mM EGTA and increasing concentrations of A23187, and then for 4.5 min in the presence and absence of 2.4 mM $CaCl_2$. The results presented represent the mean values ± S.E. from 3 experiments performed on different days.

cogen synthase *I* activity under the conditions used in this experiment (Fig. 6A). Cells were next incubated for 4.5 min in the presence or absence of 2.4 mM calcium chloride with and without insulin or methoxamine. In the absence of calcium, insulin activated glycogen synthase both in the presence and absence of A23187. Even in the presence of calcium and A23187, an effect of insulin was observed ($p < 0.05$).

No effect of methoxamine was observed in the absence of calcium (Fig. 8). Incubation of cells with calcium produced a decrease in the percentage of glycogen synthase *I* activity. When cells were incubated with both A23187 and methoxamine in the presence of calcium, no further decrease in the percentage of glycogen synthase *I* activity below that produced by methoxamine or A23187 alone was observed. This was not due to glycogen synthase *I* activity being too low to detect an additional decrease in activity. In other experiments, addition of 1 μM isoproterenol further decreased the percentage of glycogen synthase *I* activity in cells incubated with methoxamine or A23187.[5] This finding suggests that the ionophore and methoxamine inactivate glycogen synthase through a common pathway.

## DISCUSSION

We have investigated the mechanism of *alpha* adrenergic receptor-mediated inactivation of glycogen synthase using methoxamine. The interpretation of the results of this study is based largely on the premise that methoxamine acts through mechanisms involving *alpha* adrenergic receptors.

[5] J. Lawrence, and J. Larner, unpublished experiments.

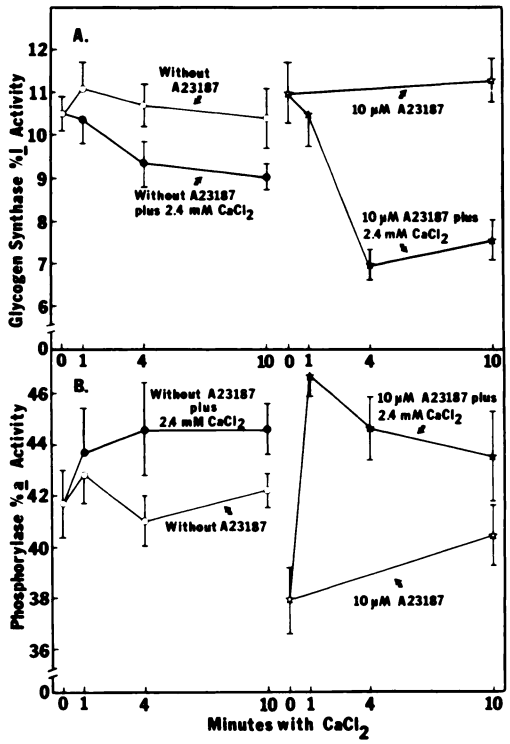


FIG. 7. *Dependence of the inactivation of glycogen synthase (A) and activation of phosphorylase (B) by A23187 on the time of incubation with calcium*

Fat cells were incubated at 37° in calcium-free medium containing 1 mM EGTA in the absence and presence of 10 μM A23187. After 10 min 2.4 mM $CaCl_2$ was added as indicated and the incubations terminated after the appropriate period of time. The results presented represent the mean values ± S.E. from 3 experiments performed on different days.

This is substantiated by the finding that the *alpha* adrenergic antagonists phentolamine, phenoxybenzamine, and dihydroergotamine completely abolished the effect of methoxamine on glycogen synthase (Table 1). The cholinergic muscarinic antagonist atropine was without effect. Furthermore, it seems unlikely that methoxamine inactivates glycogen synthase by activating *beta* adrenergic receptors, since the *beta* adrenergic antagonists propranolol and practolol did not reverse the effects of methoxamine (Table 1), and the inactivation of glycogen synthase by methoxamine occurred without changes in cyclic AMP (Figs. 4 and 5).

It is probable that calcium is involved in mediating the inactivation of glycogen synthase by methoxamine. In medium containing 1 mM EGTA without added calcium, no effect of methoxamine on glycogen synthase was observed (Figs. 4, 5, 8). The chelator was routinely added in experiments in which the calcium dependency of methoxamine and other agents was investigated. This precaution was taken because substantial amounts of calcium are present in commercial preparations of bovine serum albumin, which is present in the medium; however, the inclusion of 1 mM EGTA in the calcium-free medium should reduce the free $Ca^{++}$ to below $10^{-7}$ molar (20). The inactivation of glycogen synthase by 1 μM isoproterenol was not diminished by EGTA, and levels of ATP were unchanged

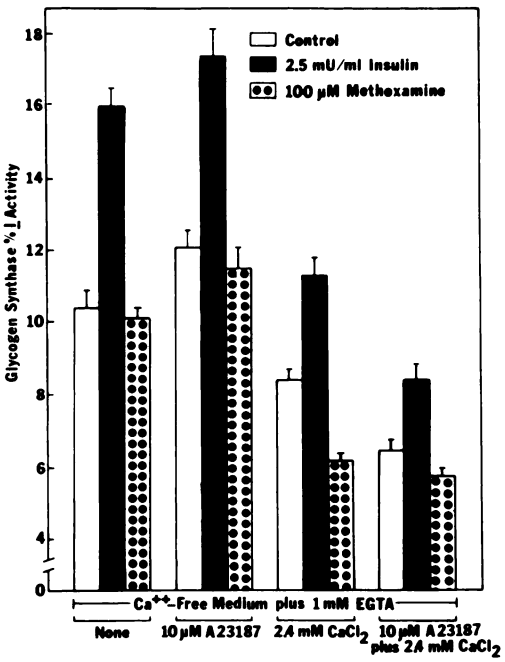


FIG. 8. *The effect of A23187 on the response of adipocyte glycogen synthase to insulin and methoxamine*

Fat cells were incubated at 37° in calcium-free medium containing 1 mM EGTA in the presence or absence of 10 μM A23187. After 10 min, cells were incubated with insulin or methoxamine in both the presence and absence of 2.4 mM calcium chloride. The incubations were terminated after 4 min and glycogen synthase activities were assayed. The results presented represent the mean values ± S.E. of 4 experiments performed on different days.

by incubation of cells with the chelator (Fig. 4). It is therefore unlikely that EGTA reversed the effect of methoxamine by nonspecifically inhibiting the glycogen synthase inactivation system or by depleting cellular ATP. That chelation of calcium by EGTA was responsible for the failure of methoxamine to inactivate glycogen synthase is suggested by the observation that the effect of methoxamine on glycogen synthase was rapidly restored by the addition of calcium (Fig. 5).

Chan and Exton (21) have reported that *alpha* adrenergic receptor stimulation in hepatocytes depleted of calcium with EGTA results in increased cyclic AMP accumulation. However, methoxamine did not significantly increase cyclic AMP in adipocytes incubated in calcium-free buffer containing EGTA (Figs. 4, 5). Perhaps a more extensive depletion of calcium is required to observe this effect of *alpha* adrenergic receptor stimulation in adipocytes.

Assimocopoulos-Jeannet *et al.* (2) have shown that phenylephrine increased the rate of both $^{45}Ca$ uptake and exchange in rat hepatocytes, and on the basis of this and other evidence, they proposed that *alpha* adrenergic agonists activate phosphorylase in hepatocytes by stimulating the entry of extracellular calcium. Perhaps a similar mechanism is responsible for the effects of methoxamine in adipocytes. Clausen (22) showed that epinephrine increased the efflux of $^{45}Ca$ from whole adipose tissue. Kissebah *et al.* (6) found that epinephrine doubled the rate of $^{45}Ca$ efflux from adipocytes. However, it is not possible to determine from the data presented whether *alpha* or *beta* adrenergic receptor stimulation was responsible. Nevertheless, the finding that the divalent cation ionophore A23187 mimicked the effects of methoxamine (Figs. 6, 8) is consistent with the hypothesis that an increase in intracellular calcium is involved in methoxamine action. When methoxamine and A23187 were added together at concentrations that produce maximal effects when the agents were added alone, no further decrease in the percentage of synthase *I* activity was observed (Fig. 8). This finding suggests that inactivation of glycogen synthase by methoxamine and A23187 involves a common pathway.

In most of the experiments presented in the present report, the effects of methoxamine on phosphorylase were relatively small compared to the effects on glycogen synthase. In part this was caused by the incubation period of 4.5 min that was routinely used. Phosphorylase activation by methoxamine is maximal after 1 min of incubation, and declines with more prolonged incubation (Fig. 1). The effect of methoxamine on glycogen synthase was maximal after 4 min and maintained through 12 min (Fig. 1).

A23187 had no effect on phosphorylase unless cells were first incubated in calcium-free medium containing EGTA. Under these conditions phosphorylase *a* activity was decreased (Fig. 6). Addition of calcium served only to reverse the effect of the ionophore with no significant net activation over the level of phosphorylase *a* observed in cells incubated in regular medium (Fig. 6), or the level produced by adding calcium to cells incubated in calcium-free medium without the ionophore (Fig. 7). The effects of A23187 on glycogen synthase were more pronounced (Figs. 6, 7).

Conversion of glycogen synthase *I* to *D* can be achieved by inhibiting glycogen synthase phosphatase activity or by increasing synthase kinase activity. In general, the effect of $Ca^{++}$ on glycogen synthase phosphatase is to stimulate its activity. For example, Kato and Bishop (23), using a partially purified rabbit skeletal muscle phosphatase, and Thomas and Nakai (24), using a partially purified enzyme from rat heart, found that calcium, as well as a number of other divalent cations, stimulated glycogen synthase phosphatase activity. Similarly, Hope-Gill *et al.* (8) reported that calcium increased glycogen synthase phosphatase activity in adipose tissue homogenates. It seems clear that stimulation of glycogen synthase phosphatase activity is not involved in the calcium-mediated inactivation of glycogen synthase.

*Alpha* adrenergic receptor-mediated activation of phosphorylase in hepatocytes may be mediated by an activation of phosphorylase kinase by increasing concentrations of cytosolic calcium (2). A similar

mechanism for phosphorylase activation by *alpha* adrenergic agonists is possible in fat cells, since adipose phosphorylase kinase has been shown to be stimulated by calcium (25). However, whether this is a possible mechanism for glycogen synthase inactivation by methoxamine is not known. Perhaps adipose phosphorylase kinase can utilize glycogen synthase as substrate. In fact, recent studies in this laborotory have shown that muscle phosphorylase kinase can indeed phosphorylate glycogen synthase in a reaction stimulated by $Ca^{++}$.[6] Even so, the present results might not be explained solely by an effect of calcium on phosphorylase kinase because both the magnitude and duration of the effects of methoxamine (Fig. 1) and A23187 (Figs. 6, 7) on glycogen synthase are greater than on phosphorylase. These results might be better explained by an effect of calcium to increase the activity of a glycogen synthase kinase. To our knowledge, no glycogen synthase kinase that is directly stimulated by calcium has been described. Takai *et al.* (26) and Inoue *et al.* (27) have provided evidence for a cyclic nucleotide-independent protein kinase that is activated by the action of a calcium-dependent protease on an inactive proenzyme. Preliminary studies indicated that both protease and proenzyme are present in adipose tissue (27). Unfortunately, it is not known whether this kinase can catalyse the phosphorylation of glycogen synthase. Regardless of the mechanism involved, the present results are consistent with the hypothesis that an increase in cytosolic calcium mediates the effect of *alpha* adrenergic receptor stimulation on glycogen synthase.

It has been proposed that an increase in cytoplasmic calcium mediates the effects of insulin on glycogen synthase, lipolysis, and other cellular events (4–10). This hypothesis is based to a large extent on measurements of $^{45}Ca$ fluxes in adipocytes incubated with the hormone. Clausen and Martin (10) showed that insulin increased $^{45}Ca$ efflux from adipocytes and argued that this was probably due to an action of the hormone to increase free calcium in the cytoplasm on the basis that the efflux of calcium is mainly a function of the concentration of free calcium in the cytosol (28, 29). Other possible explanations for this effect of insulin exist. As discussed by Clausen and Martin (10), the increased rate of efflux could be due to increased permeability of the plasma membrane to calcium, an increased rate of exchange between extracellular and intracellular calcium, or the release of calcium from membranes. The first two possibilities seem unlikely in light of the failure of insulin to increase the initial rate of $^{45}Ca$ uptake in studies with adipocytes by Clausen *et al.* (9). Hope-Gill *et al.* (8) also observed an effect of insulin to promote a rapid release of $^{45}Ca$ from fat cells preloaded with $^{45}Ca$. It was argued that because the rate of efflux was several times faster than the rate for transmembrane fluxes of $^{45}Ca$ in other cells (8, 30), the source of the $^{45}Ca$ was probably on the external surface of the plasma membrane. The calcium released from the membrane could then enter the cell and cause an increased concentration of cytoplasmic calcium. Experiments using fat cell ghosts in which insulin decreased $^{45}Ca$ binding might be taken as evidence in support of this hypothesis (6). However, fat cell ghosts contain other cellular constituents in addition to plasma membranes (notably mitochondria and endoplasmic reticulum) that probably contributed to the observed calcium binding (31). McDonald *et al.* (32, 33) have characterized the binding of $^{45}Ca$ to purified plasma membranes from adipocytes. Membranes prepared from cells incubated with insulin have an increased binding capacity (but the same binding constants) for calcium when compared to plasma membranes prepared from control cells (33). These data do not support the hypothesis that insulin promotes an increase in cytosolic calcium by decreasing calcium binding to plasma membranes.

In studies of Hope-Gill *et al.* (8) insulin was found to decrease the rate of $^{45}Ca$ release at later points (after 10 minutes) in the time course of calcium efflux. It has been argued on the basis of kinetic analysis that $^{45}Ca$ efflux monitored under these con-

[6] P. Roach, A. DePaoli-Roach, and J. Larner, manuscript submitted for publication.

ditions represents calcium derived from intracellular pools (6). Epinephrine increased $^{45}Ca$ efflux under these conditions, and insulin opposed this effect of epinephrine (6). It is suggested that, by blocking the exit of calcium from the cell, insulin would increase cytoplasmic calcium (6, 8). However, these results would also be consistent with an action of insulin to lower cytosolic calcium. For example, incubation of cells with insulin could increase the sequestration of calcium by cellular organelles, thus decreasing the availability of calcium for efflux. There is some evidence supporting this hypothesis. Hales *et al.* (34) have shown by electron probe microscopy that the endoplasmic reticulum in fat cells contains relatively high concentrations of calcium, and thus may serve as an important storage site for intracellular calcium. Bruns *et al.* (35, 36) have shown that endoplasmic reticulum isolated from fat cells possesses high-affinity binding sites for calcium and actively accumulates the ion. Furthermore, McDonald *et al.* (37) demonstrated using atomic absorption that endoplasmic reticulum from fat cells incubated with insulin contained more calcium than membranes isolated from control cells. In a more recent study, McDonald *et al.* (38) found that the endoplasmic reticula from cells incubated with insulin accumulated calcium at a faster rate than such membranes from control cells. These data suggest that insulin might lower the cytosolic concentration of calcium by increasing calcium uptake by endoplasmic reticulum. Such an interpretation would be consistent with the results of the present report since the actions of insulin on glycogen synthase are opposite to those produced by incubating cells with A23187 and calcium which probably increases the calcium concentration in the cytosol.

Certainly the results of the present report do not support an action of insulin to increase cytoplasmic calcium. Because the effect of insulin on glycogen synthase is not diminished in calcium-free medium containing 1 mM EGTA, it is unlikely that extracellular calcium is involved in activation of glycogen synthase by the hormone. However, the present results do not necessarily mean that insulin decreases cytoplasmic calcium. An action of insulin independent of calcium might also be involved. In fact, this is suggested by the ability of insulin to act in the presence of A23187, which tends to disrupt calcium gradients (Fig. 8).

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