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Effects of Lithium Ions on Glycogen Synthase and Phosphorylase in Rat Hepatocytes*

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Incubation of hepatocytes from fasted rats with LiCl provoked a concentration- and time-dependent activation of glycogen synthase. This effect was observed in the absence of glucose in the incubation medium. No changes in the intracellular concentrations of ATP or glucose-6-phosphate were detected. Lithium was also able to activate glycogen synthase in the absence of extracellular calcium. If hepatocytes were incubated with lithium and insulin, an additive effect of both agents on glycogen synthase activity was observed. LiCl was also effective in activating the enzyme in hepatocytes obtained from fed rats. When hepatocytes were incubated with [^{33}P]phosphate and then treated with LiCl, a decrease in the amount of [^{32}P]phosphate incorporated in the enzyme was observed. This dephosphorylation affected two CNBr fragments of the enzyme (CB-2 and CB-1), suggesting that several phosphorylation sites were involved. Lithium was also able to activate glycogen phosphorylase from both fasted and fed rats. Phosphorylase activation was concentration- and time-dependent, either in the presence or absence of calcium in the incubation medium. These findings demonstrate that although lithium appears to mimic the effects of insulin on glycogen synthase activity, its mechanism of action must be different from that of the hormone.

Glycogen synthase and glycogen phosphorylase are the rate-limiting enzymes in glycogen metabolism. Both enzymes are regulated by phosphorylation and dephosphorylation. Phosphorylation provokes the inactivation of glycogen synthase and the activation of glycogen phosphorylase while dephosphorylation causes the opposite effects (1, 2). Glycogen synthase may be phosphorylated *in vitro* at multiple sites by several protein kinases and all phosphorylation sites are located in two CNBr fragments of the enzyme (CB-2 and CB-1) (3). In rat liver these phosphopeptides show molecular masses of 28 (CB-2) and 14 kDa (CB-1) (4, 5).

Rat liver glycogen synthase activity in the cell is subject to several controls. Glycogenolytic hormones such as glucagon, epinephrine, or vasopressin provoke the phosphorylation and the inactivation of the enzyme (4–6). The phosphorylation caused by these hormones occurs in the same two CNBr fragments (CB-2 and CB-1) which are phosphorylated *in vitro* by protein kinases (4, 5). Insulin, in contrast, is able to activate glycogen synthase (7–10). The mechanism by which insulin is able to elicit this metabolic response remains unknown.

It has been reported that in several tissues lithium ions are able to exert insulin-like effects. Lithium stimulates glucose utilization, glycogen synthesis, and glycogen synthase activity in rat diaphragms (11). In rat adipocytes, lithium presents a wide range of insulin-like effects. Incubation of these cells with LiCl leads to an increased appearance of ^{14}C from [^{14}C] glucose into CO_2, glycogen, or lipids (12). Furthermore, the lithium ion activates adipocyte glycogen synthase with or without glucose in the medium (13). As far as hepatic glycogen metabolism is concerned, lithium stimulates the production of glycogen from glucose in rat hepatocytes, and the ion is also able to potentiate the activation of glycogen synthase by glucose (14, 15). In contrast, no effects of LiCl on glycogen phosphorylase activity have been observed (16). In general these results agree with an insulin-like action of lithium ions.

Since lithium is able to mimic some of the effects of insulin, it has been proposed as a tool in the study of the mechanism of action of the hormone. In the present work we have studied the effects of lithium on rat hepatocyte glycogen synthase and phosphorylase. We show that although lithium shares some of the effects of insulin on glycogen metabolism its mechanism of action cannot be identical to that of the insulin.

EXPERIMENTAL PROCEDURES

Incubation of the Cells—Suspensions of isolated parenchymal liver cells were prepared from starved (24 h) or, when stated, fed male Wistar rats (180–250 g) as in (Ref. 17). Cells were finally resuspended in Krebs bicarbonate buffer (pH 7.4) free of glucose, as described in Ref. 17 and aliquots (3.5 ml, 4–5 × 10^6 cells/ml) were incubated at 37 °C with continuous shaking. At the end of the incubations, cells were centrifuged and cell pellets immediately homogenized with a Polytron homogenizer (setting 6) in 300 μl ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 150 mM potassium fluoride, 15 mM EDTA, 0.6 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 25 μg/ml leupeptin, and 50 mM β-mercaptoethanol.

Cell homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C, and the supernatants were tested for both glycogen synthase and phosphorylase activity. Aliquots of the cells were treated with perchloric acid and processed for metabolite determination as described below.

Isolation of ^{32}P-Labeled Glycogen Synthase—Isotopic labeling of rat hepatocyte glycogen synthase was carried out by incubation of the cells (5 ml/vial, 4–6 × 10^6 cells/ml) with [^{32}P]phosphate (0.1 mCi/ml) in a low phosphate (0.1 mM) Krebs bicarbonate buffer (pH 7.4).

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After a 45-min exposure to [^{32}P]phosphate, LiCl was added as required and cells treated as described before in order to obtain the 10,000 × *g* supernatants. Cytosolic supernatants containing glycogen synthase activity were prepared from the 10,000 × *g* fraction by ultracentrifugation at 100,000 × *g* for 60 min at 4 °C.

Isolation of ^{32}P-labeled glycogen synthase was carried out essentially as described in Ref. 4 using specific antibodies developed in rabbits against rat liver glycogen synthase. For CNBr cleavage of glycogen synthase, immunopellets were treated as described in Ref. 18.

Analytical Procedures—Samples for SDS-polyacrylamide gel electrophoresis were evaporated to dryness and resuspended in 30 μl of sample buffer containing 40 mM Tris (pH 6.5), 5% (w/v) SDS, 0.6 M β-mercaptoethanol, 20% (w/v) sucrose, and 0.0125% (w/v), bromphenol blue as marker. Electrophoresis in polyacrylamide gels was performed as described in Ref. 19 using 6-20% gradient acrylamide slab gels.

Autoradiograms were obtained by placing the dried gels at −80 °C in x-ray cassettes containing films (Kodak X-Omat S) and intensifying screens. The amount of radioactivity was quantified by scanning the developed films at 546 nm.

Assay Methods—Glycogen synthase activity ratio was measured using the low glucose 6-P/high glucose 6-P method as described in Ref. 20. Glycogen phosphorylase activity was measured as in Ref. 21. Protein was determined by the Biuret method (22) as described in Ref. 23.

For glucose 6-phosphate and ATP determination, pellets from 2 ml of cell suspension were homogenized with a Polytron homogenizer (setting 5) for 10 s in 250 μl of ice-cold 10% perchloric acid solution. Glucose 6-phosphate and ATP were measured enzymatically in neutralized perchloric extracts as described in Ref. 24.

Suppliers—[^{33}P]Phosphate was obtained from Amersham Corp. Lithium chloride (Suprapur) was from Merck. All the other reagents were analytical grade.

RESULTS

Effects of Lithium Ions on Basal Glycogen Synthase Activity—Incubation of hepatocytes from 24-h fasted rats in the absence of glucose with 10 or 20 mM LiCl resulted in a time-dependent activation of glycogen synthase. The maximal activation was achieved after 20 min of incubation (Fig. 1*A*) and was maintained at least for 2 h (data not shown). This effect was also dependent on the concentration of the ion (Fig. 1*B*). A significant increase in the glycogen synthase activity ratio was observed at a concentration as low as 1 mM. Maximal effect was reached at about 20 mM LiCl. The enzyme activity was unaffected when NaCl substituted for LiCl.

The effect of lithium in the presence or absence of insulin on glycogen synthase activity is shown in Fig. 2. Cells were incubated for 20 min with different concentrations of LiCl and then 1 μM insulin was added and the incubation was continued for 10 more min. At the end, glycogen synthase activity ratio was measured. As can be observed, an additive effect of the hormone was obtained even at the highest concentration of LiCl tested. These results suggest that lithium and insulin activate glycogen synthase through different mechanisms of action.

Glycogen synthase activity can be regulated by covalent modification through phosphorylation-dephosphorylation reactions or by allosteric interactions with low molecular weight effectors, such as glucose 6-phosphate. In order to determine whether lithium modified the intracellular concentration of glucose 6-phosphate, the hepatic cells were incubated with 20 mM LiCl for periods up to 30 min. The incubation was terminated by the addition of perchloric acid, and the level of glucose 6-phosphate in the extracts was determined. The results in Table I indicate that glucose 6-phosphate levels were unchanged after the exposure of the cells to this ion. Experiments performed at different concentrations of LiCl or times of incubation did not result in a modification of the glucose 6-phosphate concentrations (data not shown). Another way the lithium ion could increase the activation state of glycogen synthase would be to decrease ATP concentration in the hepatocyte. The data in Table I indicate that ATP concentrations were not significantly changed by lithium.

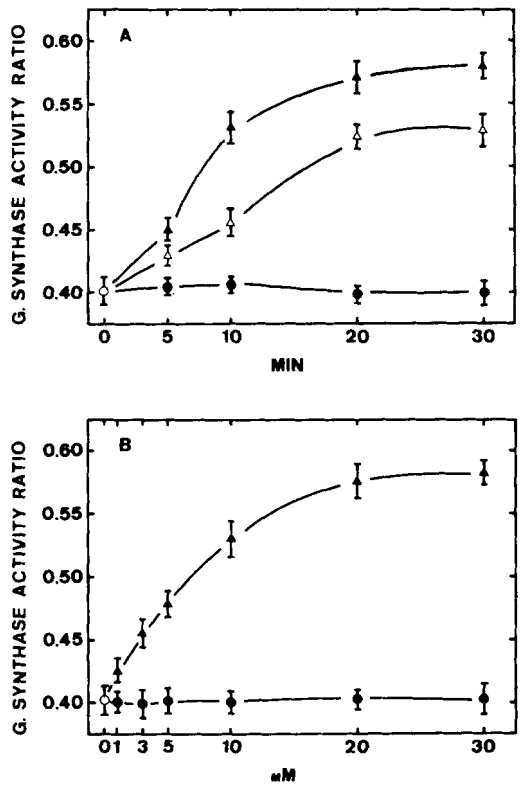

FIG. 1. *A*, time course of glycogen synthase activation by lithium ions. Cells were incubated with LiCl 10 mM (△), 20 mM LiCl (▲), or 20 mM NaCl (●) for the indicated times. At the end of the incubations, the cells were homogenized and glycogen synthase low (0.25 mM)/high (10 mM) glucose 6-P activity ratio was measured. Results are mean ± S.E. of at least six independent experiments. *B*, concentration-dependent effects of lithium on glycogen synthase activity ratio. Cells were incubated with different concentrations of LiCl (▲) or NaCl (●) for 30 min and then glycogen synthase activity ratio determined as indicated in legend to Fig. 1*A*.

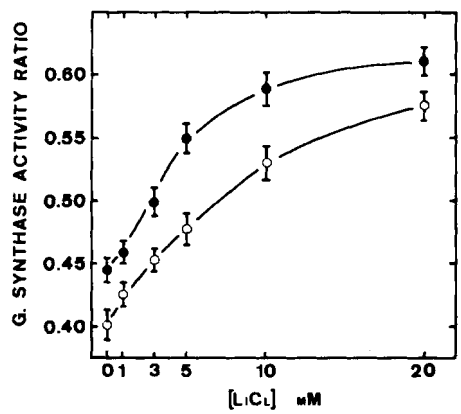

FIG. 2. **Effect of lithium ions on glycogen synthase activity ratio in the presence or absence of insulin.** Cells were incubated for 20 min with different concentrations of LiCl and then 10 more min with (●) or without (○) 1 μM insulin. Glycogen synthase low glucose 6-P/high glucose 6-P activity ratio was measured. Results are mean ± S.E. of at least four independent experiments.

In order to determine whether the effects of LiCl on glycogen synthase were permanent, extracts were filtered on Sephadex G-50 columns. The activity ratio of the enzyme

TABLE I

Effects of LiCl on ATP and glucose 6-P intracellular levels

Metabolites were measured as described under "Experimental Procedures" section. Results are mean ± S.E. of at least seven independent experiments.

	ATP	Glucose 6-P	Glycogen synthase activity ratio
	μmol/g (w/w)	*nmol/g (w/w)*	
Control	2.6 ± 0.4	75 ± 1	40 ± 2
LiCl 20 mM			
5 min	2.6 ± 0.1	74 ± 2	45 ± 1
10 min	2.7 ± 0.2	77 ± 1	53 ± 1
20 min	2.5 ± 0.2	77 ± 3	57 ± 2
30 min	2.4 ± 0.1	76 ± 2	59 ± 3

TABLE II

Stability of lithium-activated glycogen synthase to gel filtration

Cells were incubated with LiCl 20 mM for 30 min. Cells were homogenized and 0.5 ml of the extracts were filtered through Sephadex G-50 columns. Glycogen synthase low glucose 6-P/high glucose 6-P activity ratio was measured. Results are mean ± S.E. of three experiments.

	Glycogen synthase activity ratio	
	Before filtration	After filtration
Control	40 ± 2	41 ± 2
LiCl	60 ± 2	66 ± 3

TABLE III

Effects of LiCl on the phosphorylation state of rat hepatocyte glycogen synthase

^{32}P-Labeled glycogen synthase from control or lithium-treated cells was immunoprecipitated. Electrophoresis was performed before or after CNBr treatment. Autoradiograms of gels were scanned, and the peaks were integrated. Results are expressed as a percentage of the values of the peaks from control cells and are mean ± S.E. of at least five independent experiments.

	Glycogen synthase activity ratio	^{32}P-Subunit	^{32}P-CNBr Fragments		
			CB-2	CB-1	CB-2/CB-1
Control	43 ± 1	100	100	100	3.2 ± 0.3
LiCl 20 mM	68 ± 2[a]	78 ± 2[b]	75 ± 6[c]	74 ± 4[b]	3.2 ± 0.2

[a] $p \leq 0.001$.
[b] $p \leq 0.01$.
[c] $p \leq 0.05$.

remained unchanged after the treatment, supporting the idea that lithium ions activate glycogen synthase through a covalent modification of the enzyme (Table II).

Effects of Lithium on the Phosphorylation State of Glycogen Synthase—Because glycogen synthase activity is regulated through phosphorylation and dephosphorylation mechanisms, experiments were carried out in order to determine the effect of lithium on the phosphorylation state of glycogen synthase.

Glycogen synthase was rapidly isolated from ^{32}P-labeled cells by immunoprecipitation using specific antibodies raised in rabbits against homogeneous rat liver enzyme. Immunopellets from control and LiCl-treated cells were submitted to electrophoresis in the presence of SDS.[1] As shown in Table III, exposure of rat hepatocytes to 20 mM LiCl for 15 min provoked a significant dephosphorylation of the 88-kDa glycogen synthase subunit (approximately 25% with respect to the control cells).

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

In previous reports (4, 18, 25) we have shown that, in rat hepatocytes, glycogen synthase is phosphorylated in two regions of the enzyme that can be separated by CNBr cleavage (CB-2, 28 kDa and CB-1, 14 kDa). In order to examine the effect of lithium ions on these two regions, immunoprecipitated ^{32}P-labeled glycogen synthase was subjected to CNBr cleavage, and fragments were resolved by electrophoresis. The ^{32}P content of both CNBr fragments decreased approximately in the same proportion (Table III). These findings suggest that the lithium ion acts on glycogen synthase through a mechanism that involves the dephosphorylation of the enzyme at several sites.

Effects of Lithium on Glycogen Synthase from Calcium-depleted Cells—In order to investigate whether the extracellular calcium is required for the action of lithium on glycogen synthase, hepatocytes were incubated in either a normal or a calcium-depleted medium containing 1 mM EGTA with different concentrations of LiCl for 30 min. In the absence of calcium, cells showed a higher glycogen synthase activity ratio. The data of Fig. 3*A* show that lithium provoked a dose-dependent activation of glycogen synthase in both cell preparations. This effect was also time-dependent (Fig. 3*B*). These results indicate that lithium, like insulin, exerts its effects on glycogen synthase through a mechanism independent of the availability of extracellular calcium.

Effects of Lithium Ion on Hepatic Glycogen Phosphorylase—Insulin does not affect basal phosphorylase activity (17). In order to investigate whether lithium exerts an action on this enzyme, hepatocytes were incubated with LiCl in the absence

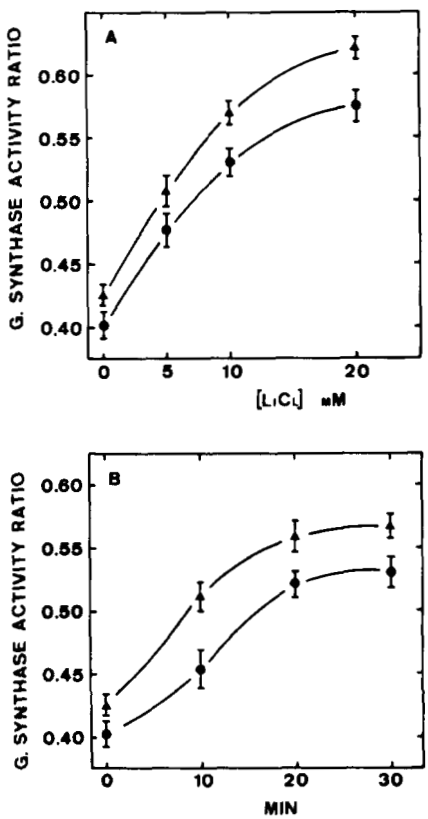

FIG. 3. **Effects of lithium ions on glycogen synthase activity ratio in calcium-free medium.** Cells incubated in Krebs-Ringer medium with (●) or without $CaCl_2$ (plus 1 mM EGTA) (▲) were incubated with LiCl. Then glycogen synthase low glucose 6-P/high glucose 6-P activity ratio was measured. *A*, hepatocytes incubated for 30 min with different concentrations of LiCl. *B*, cells incubated with 10 mM LiCl for the indicated times. Results are mean ± S.E. of at least four independent experiments.

of glucose, and glycogen phosphorylase activity was measured.

Short-time incubation of the cells with 20 mM LiCl had no effect on glycogen phosphorylase activity. However, exposure of the cells to LiCl for longer periods (more than 10 min) resulted in a clear activation of glycogen phosphorylase. At the longest time studied (60 min), enzyme activity was 185% of the control values (Fig. 4). A significant activation of phosphorylase was also observed at lower (5 and 10 mM) concentrations of LiCl (data not shown). When LiCl was replaced by NaCl, no effects on phosphorylase activity were observed. Removal of calcium from the incubation medium did not abolish the glycogen phosphorylase activation provoked by lithium (Fig. 4), although in the absence of calcium the absolute values of glycogen phosphorylase activity were lower than in its presence.

Lithium Effects on Glycogen Synthase and Glycogen Phosphorylase in Isolated Hepatocytes from Fed Rats—In order to determine whether the effects of lithium on glycogen synthase and phosphorylase activities could be influenced by the nutritional state of the animals, experiments were performed using cells prepared from fed rats. When these hepatocytes were incubated with 20 mM LiCl for 20 and 50 min, a clear increase on the activation state of both, glycogen synthase and glycogen phosphorylase, was observed (Fig. 5), although the response was slightly less marked than in fasted animals. In any case, the effects of lithium were qualitatively maintained in cells from fed rats.

DISCUSSION

The results presented in this paper demonstrate that in rat hepatocytes lithium ions are powerful activators of glycogen synthase and that this effect does not require the presence of glucose in the incubation medium. This is an important point since in a previous paper (14) it was reported that the effect of lithium was to potentiate the effect of glucose on glycogen synthase activation. Here we have shown that lithium is also able to activate glycogen synthase under basal conditions and that the presence of glucose is not required for this action.

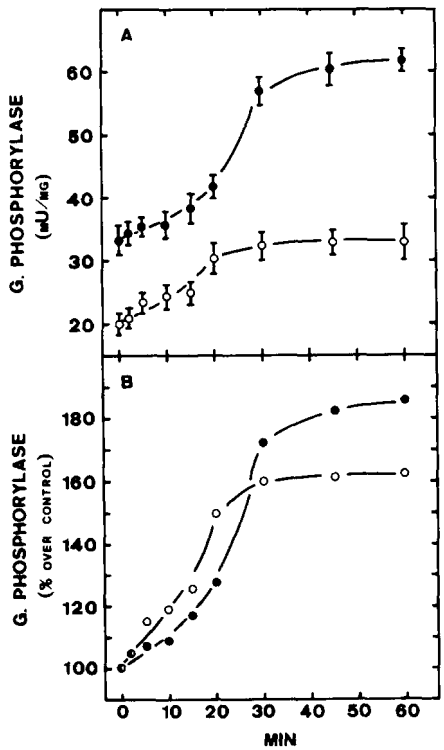

FIG. 4. **Time-dependent effect of LiCl on glycogen phosphorylase activity.** Cells incubated in a Krebs-Ringer medium with (●) or without (○) calcium were incubated with 20 mM LiCl for different times. At the end of the incubations, the cells were homogenized and glycogen phosphorylase activity was measured. Results are expressed in munits/mg (*A*) or in percentage over the control (*B*). Results are mean ± S.E. of at least four independent experiments.

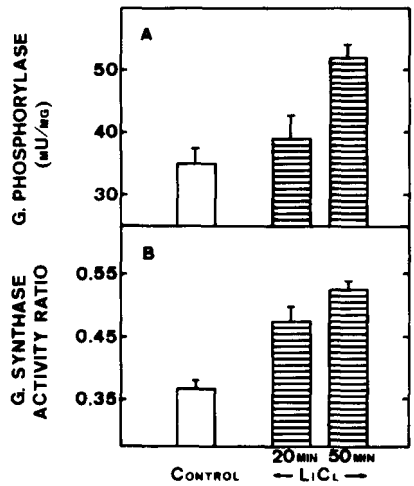

FIG. 5. **Effects of lithium ions on glycogen synthase and glycogen phosphorylase activities of hepatocytes from fed rats.** Hepatocytes were prepared from fed rats and incubated with 20 mM LiCl for 20 and 50 min. Glycogen phosphorylase activity (*A*) and glycogen synthase activity ratio (*B*) were measured. Results are mean ± S.E. of at least three independent experiments.

The effects of lithium on glycogen synthase persist after gel filtration of the extracts, indicating that they are not due to changes in a low molecular weight substance able to allosterically activate glycogen synthase. Furthermore, metabolite analysis showed that neither glucose 6-phosphate nor ATP, the two most important effectors capable of interacting with glycogen synthase and glycogen synthase interconverting enzymes, were modified by incubation of the cells with the cation.

Definitive proof of the covalent nature of the modification of glycogen synthase caused by lithium was obtained in experiments using ^{32}P-labeled cells. This approach, which has been very useful in the study of phosphorylation of glycogen synthase in rat heart (26, 27), rat diaphragms (28, 29), and rat hepatocytes (4–6) has also proven to be useful in dephosphorylation studies (30). After incubation of the cells with [^{33}P]phosphate, ^{32}P-labeled glycogen synthase was rapidly purified from extracts of these cells by immunoprecipitation using antibodies raised in rabbits against rat liver enzyme. These antibodies were capable of completely removing glycogen synthase activity from extracts (4). In addition, the immunoprecipitation reaction was highly specific since a single radioactive band corresponding to the 88-kDa glycogen synthase subunit was observed after polyacrylamide gel electrophoresis of the immunoprecipitate.

Glycogen synthase isolated by this method from control cells has a significant amount of ^{32}P bound to it, indicating that at least some of the phosphorylation sites of the enzyme undergo significant turnover. The amount of ^{32}P bound to glycogen synthase was reduced in cells that had been incubated with LiCl, thus proving that the activation provoked by the cation is a consequence of the dephosphorylation of the enzyme subunit. This dephosphorylation is about 25% of the control value, indicating that the removal of only one-fourth of the interchangeable phosphate content can result in a very noticeable increase in the activity of the enzyme. In this

regard it may be pointed out that the degree of the dephosphorylation observed is on the same order as that reported in rat heart after insulin treatment (27). In contrast, it is known that glycogenolytic hormones such as glucagon, epinephrine, or vasopressin (4–6) or other agents such as phorbol esters (18) provoke the inactivation of glycogen synthase by increasing its [^{32}P]phosphate content 2 to 3-fold.

Analysis of the relative amount of the [^{32}P]phosphate present in the two ^{33}P-containing CNBr fragments of the enzyme showed that both were equally affected. An important conclusion of this finding is that multiple phosphorylation sites are affected by lithium treatment.

A paradoxical effect of the lithium ion is that it provokes the simultaneous activation of glycogen synthase and phosphorylase. A similar situation has been described when hepatocytes are incubated with fructose (31, 32) or ATP-depleting agents (33). However, the effect of lithium cannot be attributed to changes in ATP levels, since they remain stable after incubation of the cells with the cation. This effect of lithium provides further evidence that inactivation of glycogen phosphorylase is not necessarily a prerequisite for the activation of liver glycogen synthase.

Since no glycogen synthase phosphatase or kinase modified by lithium has been described so far, it appears necessary to postulate the existence of an intermediary step between the initial action of lithium and the final modifications of phosphatase or kinase activity (or both). In any case, the lithium ion does not need extracellular calcium to carry out its effect on glycogen synthase activity since the activation of the enzyme is also observed in calcium-depleted cells. The same is true for insulin (34).

Since the effects of lithium are additive to those of a saturating concentration of insulin, the mechanism of action of both effectors cannot be identical. In addition, our results showing that basal glycogen phosphorylase is activated by LiCl are consistent with the idea that lithium and insulin actions on glycogen metabolism are not mediated by the same mechanism(s), since no effect of insulin on rat hepatocyte glycogen phosphorylase has been described thus far. It is evident that more experimental work is needed to elucidate the mechanism of action of lithium and to relate its effects to those of insulin and other hormones acting on glycogen metabolism.

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