Glucose transport rate and glycogen synthase activity both limit skeletal muscle glycogen accumulation

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Fisher, Jonathan S., Lorraine A. Nolte, Kentaro Kawanaka, Dong-Ho Han, Terry E. Jones, and John O. Holloszy. Glucose transport rate and glycogen synthase activity both limit skeletal muscle glycogen accumulation. Am J Physiol Endocrinol Metab 282: E1214–E1221, 2002.—We varied rates of glucose transport and glycogen synthase I (GS-I) activity (%GS-I) in isolated rat epitrochlearis muscle to examine the role of each process in determining the rate of glycogen accumulation. %GS-I was maintained at or above the fasting basal range during 3 h of incubation with 36 mM glucose and 60 μU/ml insulin. Lithium (2 mM LiCl) added to insulin increased glucose transport rate and muscle glycogen content compared with insulin alone. The glycogen synthase kinase-3β inhibitor GF-109203x (GF; 10 μM) maintained %GS-I about twofold higher than insulin with or without lithium but did not increase glycogen accumulation. When %GS-I was lowered below the fasting basal range by prolonged incubation with 36 mM glucose and 2 mM LiCl insulin, raising rates of glucose transport with bpV(phen) or of %GS-I with GF produced additive increases in glycogen concentration. Phosphorylase activity was unaffected by GF or bpV(phen). In muscles of fed animals, %GS-I was ~30% lower than in those of fasted rats, and insulin-stimulated glycogen accumulation did not occur unless %GS-I was raised with GF. We conclude that the rate of glucose transport is rate limiting for glycogen accumulation unless %GS-I is below the fasting range, in which case both glucose transport rate and GS activity can limit glycogen accumulation.

glucose synthase kinase-3β; lithium; insulin; fasting state; fed state

GLUCOSE TRANSPORT and glycogen synthase activity are considered the key regulatory factors for glycogen synthesis in skeletal muscle (for reviews see Refs. 19 and 29). Under varying conditions, either the rate of glucose transport or glycogen synthase activity has been shown to control the extent of glycogen accumulation. For example, mice that overexpress the glucose transporter GLUT1 have extremely high insulin-independent glucose uptake rates and enormous glycogen stores despite low glycogen synthase activity levels (26). Similarly, glycogen accumulates in exercise-trained muscle at about double the rate for untrained muscle, even though glycogen synthase activities are unaffected by training, and postexercise glycogen accumulation is directly related to muscle content of GLUT4 (the exercise- and insulin-responsive glucose transporter) and glucose transport capacity (21, 27).

On the other hand, transgenic mice that overexpress glycogen synthase in muscle have a fivefold increase in glycogen compared with wild-type mice with no changes in glucose transport (1, 20). Moreover, decreased glycogen synthase activity after physiological hyperinsulinemia is associated with impaired nonoxidative glucose disposal (13, 28).

Skeletal muscle glycogen synthesis accounts for ~90% of whole body glucose metabolism and nearly all of the insulin-stimulated nonoxidative glucose disposal in resting normal and diabetic subjects (14, 30). Phosphorylation of glycogen is limited in resting skeletal muscle by the availability of inorganic phosphate (25). For example, a sixfold stimulation of phosphorylase activity by epinephrine does not increase net glycogenolysis in resting, oxygenated muscle (25). Likewise, flux of glucosyl units through phosphorylase is not different in control and glycogen-loaded muscle (18). Glucose oxidation and nonoxidative disposal of glucose other than by storage as glycogen in muscle do not account for a significant amount of glucose transported into resting muscle in response to insulin unless glycogen synthase activity is low, and glucose is thereby shunted away from glycogen synthesis (13, 18, 28). Thus factors other than glucose transport and glycogen synthase activity play relatively minor roles in determining the extent of glycogen accumulation in skeletal muscle.

The purpose of the present study was to examine the conditions under which glucose transport rate and glycogen synthase activity are individually important to glycogen accumulation. Glucose transport rate was increased with lithium, which increases the sensitivity of skeletal muscle and adipocyte glucose transport to stimulation by insulin (3, 32). The tyrosine phospho-
tase inhibitor bpV(phen) (2), which stimulates the insulin-signaling pathway to an extent greater than a maximally effective concentration of insulin (22), was used as a second means of increasing glucose transport. Glycogen synthase activity was increased with the use of lithium and GF-109203x (GF; bisindolylmaleimide I), which both inhibit glycogen synthase kinase (GSK3β) (10, 15). Glycogen synthase activity was reduced by means of prolonged incubation of isolated skeletal muscle with high glucose and insulin concentrations or by overnight feeding.

MATERIALS AND METHODS

Animals. Male Wistar rats (~125 g) were fed a diet of Purina rat chow and water ad libitum. Food was removed from the cages of fasted animals ~16 h before experiments. Fed animals ate ad libitum the night before experiments. This research was approved by the Washington University Animal Studies Committee.

Chemicals. GF and bpV(phen) were obtained from Alexis Biochemicals (San Diego, CA). Purified porcine insulin was purchased from Eli Lilly (Indianapolis, IN). 3-O-methyl-d-[3H]glucose (3-MG) was obtained from American Radiolabeled Chemicals (St. Louis, MO), and d-[1-14C]mannitol was obtained from NEN Life Science Products (Boston, MA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

In vitro muscle incubations. Animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt). Thereafter, muscles were incubated in Dubnoff shakers in 25-ml Erlenmeyer flasks containing 2 ml of incubation media consisting of 0.1% radioimmunooassay grade bovine serum albumin (BSA) in Krebs-Henseleit bicarbonate buffer (KHB) (17) with various agents (described in following sections) and sufficient mannitol to maintain constant osmolarity. Flasks were gassed with 5% CO2-95% O2. Muscles were allowed to recover for 30 min after dissection at 35°C with 2 mM sodium pyruvate. All media, except for the recovery solution, contained 0.2% dimethyl sulfoxide. When the medium contained light-sensitive compounds, the flasks were covered with aluminum foil.

Experiment 1. Experiment 1 (time line in Fig. 1A) was undertaken to determine whether raising the rate of glucose transport and/or glycogen synthase activity above the levels normally induced by physiological insulin concentrations would increase the rate of insulin-stimulated glycogen accumulation. We used lithium (2 mM LiCl) to potentiate insulin-stimulated glucose uptake (32). Lithium also increases glycogen synthase activity in skeletal muscle (12) through inhibition (~40% inhibition with 2 mM Li+) of GSK3β (15). We also raised glycogen synthase activity with GF, which has been reported to inhibit 100% of GSK3β activity at a concentration of 10 μM (10) but does not increase insulin-stimulated glucose transport in skeletal muscle from healthy subjects (5).

Muscles were preincubated at 35°C for 1 h with 2 mM pyruvate with or without 60 μU/ml insulin (physiological concentration) in the presence or absence of 10 μM GF or 2 mM LiCl (as shown in Fig. 1). Muscles were then incubated with the same agents and the addition of 36 mM glucose instead of pyruvate for 3 h. Although 36 mM glucose is supraphysiological, we used this glucose concentration to produce high glucose transport rates while using a physiological insulin concentration and to bring about measurable changes in glycogen concentration. After the 3-h incubation, muscle samples were either clamp-frozen for future analysis of glycogen synthase activity and glycogen content or assayed for glucose transport. Muscle samples were also clamp-frozen directly after the initial 30-min recovery period for determination of basal glycogen level and glycogen synthase activity. Additional samples were incubated with 60 μU/ml insulin for 1 h for determination of glycogen synthase activity.

Experiment 2. Experiment 2 (time line in Fig. 1B) was performed to determine whether increasing glycogen synthase activity or glucose transport rate would increase glycogen accumulation when glycogen synthase activity was low. We incubated muscles with high insulin (2 μU/ml) and glucose (36 mM) levels to promote a large increase in glycogen and evoke the characteristic decline in glycogen synthase activity that occurs as glycogen concentration increases (6, 21). We found in preliminary experiments that glycogen synthase activity fell below the fastest basal level and that the rate of glycogen accumulation declined at some time between 3 and 5 h of incubation under these conditions.

To prevent the decline in glycogen synthase activity, we used 10 μM GF. To increase the rate of glucose transport after 3 h of incubation, we added 0.1 mM bpV(phen), a tyrosine phosphatase inhibitor (2) that produces rates of glucose uptake that are higher than are induced by a maximally effective insulin concentration (22). Muscles were incubated for 3 h with 36 mM glucose and 2 μU/ml insulin with or without 10 μM GF. After 3 h, incubations continued with the same agents with or without 0.1 mM bpV(phen). After a total of 4 h, muscles were rinsed, and 3-MG uptake assays were performed (g).
were performed. Other muscles were clamp-frozen after 5 h and stored at −80°C for determination of glycogen synthase activity and glycogen content.

**Experiment 3.** In Experiment 3 (time line in Fig. 1C), rats were fed ad libitum the night before experiments to reduce muscle glycogen synthase activity. Muscles were then incubated with physiological insulin and glucose concentrations to determine whether raising the depressed glycogen synthase activity with GF would increase insulin-stimulated glycogen accumulation. Muscles from fed animals were incubated for 1 h with 2 mM sodium pyruvate with or without 100 μU/ml insulin (high physiological concentration) in the absence or presence of 10 μM GF. Incubations were then terminated for some samples without insulin (fed basal), and the remaining muscles were then incubated with 8 mM glucose and either no insulin for 3 h, 100 μU/ml insulin for 1 or 3 h, or 100 μU/ml insulin with 10 μM GF for 3 h. After incubations, muscles were assayed for 3-MG uptake rate or frozen for measurement of glycogen synthase activity and glycogen content.

**Measurement of glucose transport activity.** Glucose transport activity was measured using the nonmetabolizable glucose analog 3-MG as described previously (33). To remove glucose from the interstitial space, muscles were washed for 10 min twice in KHB containing 36–40 mM mannitol, 0.1% BSA, and insulin or other agents that were present in previous incubations. After the wash, muscles were incubated at 30°C for 10 min in 1.5 ml of KHB containing 8 mM [3-3H]MG (2 μCi/ml), 28–32 mM [14C]mannitol (0.2 μCi/ml), and the other agents that were present in previous incubations. Extracellular space and intracellular 3-MG concentration (μmol·ml intracellular water−1·10 min−1) were determined as previously described (33).

**Muscle glycogen.** Percoll dense extracts of muscle were assayed for glycogen by the amyloglucosidase method (23).

**Glycogen synthase and phosphorylase assays.** Muscle samples were homogenized at 4°C in buffer containing (in mM) 50 Tris-HCl, pH 7.5, 1 EGTA, 1 EDTA, 10 β-glycerophosphate, 50 NaF, 5 sodium pyrophosphate, 1 benzamidine, 1 Na2VO4, 1 phenylmethylsulfonyl fluoride, and 1% Triton X-100, 100 mM okadaic acid, 0.1% β-mercaptoethanol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 10 μg/ml pepstatin. Samples were assayed for glycogen synthase activity at 38°C in the presence or absence of 5 mM glucose 6-phosphate (24).

Glycogen synthase I (GS-I) activity was expressed as the percentage of the total activity (measured in the presence of 5 mM glucose 6-phosphate) that was independent of glucose 6-phosphate. The activity of the active form of phosphorylase, phosphorylase α, was assayed at 30°C in the absence of AMP in the direction of glycogen degradation, as described by Young et al. (34).

**Statistics.** Data were examined with one-way analyses of variance, with the level of statistical significance set at α = 0.05. Post hoc comparisons were performed with Fisher least significant difference tests.

**RESULTS**

**Experiment 1: glycogen synthase activity at fasting basal level.** This experiment was designed to provide information regarding whether or not glycogen synthase activity in the normal, fasting range is rate limiting for glycogen synthesis. After 1 h of incubation with physiological insulin concentration (60 μU/ml), glycogen synthase activity was increased by ~50% above the basal fasting level (Fig. 2A). However, after 4 h of incubation, glycogen synthase activity was significantly, but not dramatically (~20%), reduced compared with the fasting basal level but was markedly lower than the 1-h insulin-stimulated value. This decrease in glycogen synthase activity was completely prevented by GF (Fig. 2A). However, despite maintenance of high glycogen synthase activity in the presence of GF, glycogen accumulation was not different...
from that in the muscles incubated for 4 h with insulin alone (Fig. 2C). On the other hand, lithium treatment, which did not prevent return of glycogen synthase activity to the fasting level after 4 h, resulted in ~50% higher accumulation of glycogen (Fig. 2C; glycogen increase from basal: insulin + GF 5.6 μmol glucosyl units/g, insulin + lithium 8.3 μmol/g). Lithium also resulted in a large increase in insulin sensitivity of the glucose transport process, as evidenced by a large increase (>70%) in glucose transport compared with the value obtained with insulin alone (Fig. 2B). Thus it appears that glucose uptake, rather than glycogen synthase activity, limits glycogen accumulation as long as glycogen synthase activity does not decrease much below the fasting activity range.

Experiment 2: effects of maintaining high glycogen synthase activity or increasing glucose uptake when glycogen synthase activity is below fasting basal level. The purpose of this experiment was to determine whether glycogen synthase activity becomes rate limiting for glycogen synthesis when glycogen synthase activity falls to the low levels that are seen in the presence of high muscle glycogen concentrations (6, 21). We therefore exposed muscles to high concentrations of glucose and insulin to maximize glycogen synthesis. Five hours of exposure to high insulin and glucose concentrations led to a dramatic (~70%) decrease in glycogen synthase activity from the fasting basal level (Figs. 3A and 4A), presumably as a result of the fivefold increase in glycogen concentration (Figs. 3B and 4C). As shown in Fig. 3, the decline in glycogen synthase activity occurred between 3 and 5 h of incubation with insulin (Fig. 2C). On the other hand, lithium treatment, which did not prevent return of glycogen synthase activity to the fasting level after 4 h, resulted in ~50% higher accumulation of glycogen (Fig. 2C; glycogen increase from basal: insulin + GF 5.6 μmol glucosyl units/g, insulin + lithium 8.3 μmol/g). Lithium also resulted in a large increase in insulin sensitivity of the glucose transport process, as evidenced by a large increase (>70%) in glucose transport compared with the value obtained with insulin alone (Fig. 2B). Thus it appears that glucose uptake, rather than glycogen synthase activity, limits glycogen accumulation as long as glycogen synthase activity does not decrease much below the fasting activity range.

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limiting for glycogen synthesis. However, despite the low glycogen synthase activity, increasing the rate of glucose entry by ≥45% by the addition of bpV(phen) (Fig. 4B) also resulted in a 20% greater accumulation (P < 0.05) of glycogen than occurred in the presence of insulin alone. When muscles were treated with both GF and bpV(phen) to increase both glycogen synthase activity and glucose uptake rate, their effects on glycogen accumulation were additive. These findings provide evidence that when glycogen synthase activity falls far below the basal, fasting level, the rate of glycogen accumulation is regulated by both glycogen synthase activity and glucose uptake rate.

Experiment 3: effects of low glycogen synthase activity on glycogen accumulation under physiological conditions. In the previous experiment, we used unphysiologically high concentrations of glucose and insulin to reproduce in vitro the changes in glycogen synthase activity and glycogen that occur in vivo over 24 h in response to carbohydrate feeding after exercise. The purpose of this experiment was to further evaluate the role of glycogen synthase activity in regulating glycogen accumulation under more physiological conditions (100 μU/ml insulin, 8 mM glucose). To this end, we used muscles from fed, instead of fasted, rats, which, probably because of higher glycogen, have low levels of glycogen synthase activity (6, 21). As shown in Fig. 5A, basal glycogen synthase activity was considerably lower in muscles from fed compared with fasting animals. In contrast to the stimulation of glycogen synthase activity that occurred in response to 60 μU/ml insulin in muscles from fasted animals, a higher physiological insulin concentration (100 μU/ml) did not significantly increase glycogen synthase activity in muscles from fed animals. Thus insulin-stimulated glycogen synthase activity (at the 1-h time point) in fed animals was only ~50% of the insulin-stimulated glycogen synthase activity in fasted animals. Under these conditions, the rate of glucose transport was also low (Fig. 5B). As a consequence, glycogen concentration did not change significantly over the 4-h incubation period (Fig. 5C). Stimulation of glycogen synthase activity with GF in muscles of fed rats resulted in a significant increase in glycogen accumulation. These results show that, under physiological conditions, low glycogen synthase activity can limit the rate of glycogen accumulation.

Phosphorylase activity. As shown in Table 1, phosphorylase α activity was unaffected by either GF or bpV(phen).

DISCUSSION

The new information provided by this study is that raising glycogen synthase activity when it is below the fasting basal level results in increased glycogen accumulation. By experimental manipulation of glucose transport rate and glycogen synthase activity, we have provided evidence that both factors can be rate limiting for glycogen accumulation. Raising either glucose transport or glycogen synthase activity when glycogen synthase activity is low increases the rate of glycogen accumulation. An important new finding is that, under conditions of physiologically induced low glycogen synthase activity, raising glycogen synthase activity increases glycogen accumulation in muscles exposed to physiological concentrations of insulin and glucose.

In a series of studies of postexercise glycogen supercompensation, it was previously shown that glycogen accumulates faster in exercise-trained than in un-
trained muscle (8, 11, 21, 27). Higher glycogen accumulation was related to higher muscle content of GLUT4, which resulted in a higher glucose transport capacity, whereas glycogen synthase activities were not different between trained and untrained groups. However, in both trained and untrained muscle, the plateau in glycogen repletion coincided with a decrease of glycogen synthase activity to ~20% in the I-form (21). Data from the present study support the hypothesis that the decline in glycogen synthase activity during glycogen supercompensation contributes to the cessation of glycogen accumulation (13, 28) and is consistent with in vivo nuclear magnetic resonance spectroscopy data showing that the rate of glycogen accumulation declines before the rate of glucose transport decreases in glycogen-supercompensated human muscle (18). Increased glycogen synthase activity, such as is present in transgenic mice that overexpress glycogen synthase (1, 20), would probably cause a further rise in glycogen levels in glycogen-supercompensated muscle.

Like virtually all of the enzyme inhibitors that are used in biological research, the inhibitors used in the study are nonspecific. For example, in addition to GSK3β, GF has been reported to inhibit p70 ribosomal S6 kinase, phosphorylase kinase, protein kinase C (PKC), and a handful of other kinases (7, 10). Lithium inhibits GSK3β, three kinases in the p38 pathway, casein kinase 2, IMPase, and a few other enzymes (3, 7, 15), whereas bpV(phen) is a nonspecific tyrosine phosphatase inhibitor (2). However, none of these inhibitors could exert specific effects on glycogen accumulation other than through modulation of glucose transport rate and/or glycogen synthase activity, the two driving forces for net glycogen synthesis (19, 29). Under normal conditions, virtually all glucose entering muscle in response to insulin (i.e., the insulin-stimulated increase in glucose transported above basal uptake) is stored as glycogen (30). Approximately 90% of whole body glucose disposal under these conditions can be attributed to muscle glycogen deposition (14, 30). Therefore, even if the inhibitors used in this study were to completely block all forms of glucose disposal other than glycogen storage, there would be little effect on glycogen. Only when glycogen synthase activity is extremely low is a significant amount of glucose shunted away from storage as glycogen toward oxidation or lactate production (18). Our data show that maintenance of high glycogen synthase activity by the use of GF increases glycogen accumulation. This ability of GF to stimulate glycogen accumulation is not due to an inhibitory effect on alternative glucose disposal pathways, because GF had no effect on glycogen accumulation when glycogen synthase activity was not limiting, i.e., above the fasting basal level. We have measured the effects of the inhibitors we used on the two major factors that limit glycogen accumulation, glucose transport rate, and glycogen synthase activity (19, 29), and changes in these two functions can explain the differences in glycogen accumulation that we found. We have also shown that GF and bpV(phen) have no effect on phosphorylase activity. These findings support the conclusion that the inhibitors used in this study mediated their effects by modulating glucose transport activity or glycogen synthase activity, rather than by nonspecific effects.

Lithium increases glycogen synthase activity, insulin-stimulated glucose uptake, and glycogen accumulation in skeletal muscle (4, 12, 16, 32). The increase in glycogen synthase activity appears to be mediated by inhibition of GSK3β (15). Henriksen et al. (9) have shown in pilot studies that inhibition of GSK3β by CT-98014 reverses insulin resistance of glucose transport in muscle from diabetic rats, although the compound had no effect on insulin-stimulated glucose transport in healthy muscle. Similarly, Cortright et al. (5) found that GF and another GSK3β inhibitor, Rottlerin (7), both of which also inhibit PKC, increased glucose uptake in adipocytes and muscle from insulin-resistant subjects but not in muscles from insulin-sensitive subjects (5). It seems likely that the effects of GF and Rottlerin were mediated by inhibition of GSK3β, because two other PKC inhibitors, staurosporine and calphostin C, did not mimic the effects of GF and Rottlerin on glucose uptake (5).

Using GF and lithium, we have shown a dissociation between inhibition of GSK3β activity and increased sensitivity to insulin-stimulated glucose uptake in healthy muscle. GF had no influence on glucose transport, whereas lithium exerted its insulinsensitizing effect on glucose transport (32). Our data are consistent with those of Summers et al. (31), who found little effect of a constitutively active form of GSK3β on glucose uptake in adipocytes and no effect on GLUT4 translocation during insulin stimulation. Although findings in adipocytes are not necessarily generalizable to skeletal muscle, they may be in this case, because 1) lithium has been shown to potentiate insulin-stimulated glucose transport in both adipocytes and muscle (3, 32), and 2) in preliminary studies, the specific GSK3β inhibitor CT-98014 (which is not commercially available) had no effect on insulin-stimulated glucose transport in normal skeletal muscle (9). Thus it appears that inhibition of

Table 1. GF and bpV(phen) do not alter phosphorylase activity

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<tr>
<th>Experiment 1: 60 μU/ml insulin, 4 h</th>
<th>Phosphorylase a activity</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>4.7 ± 0.5</td>
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<tr>
<td>Insulin + GF</td>
<td>3.7 ± 0.5</td>
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<tr>
<th>Experiment 2: 2 mU/ml insulin, 5 h (bpV(phen) added in final 2 h)</th>
<th>Phosphorylase a activity</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>4.8 ± 0.8</td>
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<tr>
<td>Insulin + GF</td>
<td>4.6 ± 1.0</td>
</tr>
<tr>
<td>Insulin + bpV(phen)</td>
<td>5.9 ± 0.9</td>
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Data are means ± SE (experiment 1, n = 6–7; experiment 2, n = 5–6) expressed as μmol·g⁻¹·min⁻¹. GF, bisindolylmaleimide I (GF-109203x); bpV(phen), a tyrosine phosphatase inhibitor. There are no statistically significant differences between groups.
GSK\(3\beta\) does not increase insulin sensitivity of glucose transport and that the increase in insulin sensitivity of glucose transport induced by lithium is mediated by a separate mechanism. Lithium inhibits a number of kinases and phosphatases, including GSK\(3\beta\) and IMPase (3, 15). Chen et al. (3) demonstrated that IMPase inhibition does not mimic the lithium effect on potentiation of insulin-stimulated glucose transport, and none of the other known targets for inhibition by lithium stands out as a potential mediator of insulin sensitivity.

In conclusion, our results show that both the rate of glucose transport and glycogen synthase activity can be limiting for glycogen accumulation. When glycogen synthase I activity is at or above the fasting basal level, further increasing glycogen synthase I activity does not lead to an increase in insulin-stimulated glycogen accumulation. Under these conditions, the rate of glucose transport determines the extent of glycogen accumulation. When glycogen synthase I activity is below the fasting basal level, i.e., near the fed basal level, there is little or no glycogen accumulation. Under these conditions raising either glucose transport rate or glycogen synthase activity allows glycogen accumulation.

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