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Evidence for Differential Roles of the Rho Subfamily of GTP-Binding Proteins in Glucose- and Calcium-Induced Insulin Secretion from Pancreatic β Cells*

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ABSTRACT. We utilized clostridial toxins (with known specificities for inhibition of GTPases) to ascertain the contribution of candidate GTPases in physiologic insulin secretion from β cells. Exposure of normal rat islets or isolated β (HIT-T15) cells to *Clostridium difficile* toxins A and B catalyzed the glucosylation (and thereby the inactivation) of Rac, Cdc42, and Rho endogenous to β cells; concomitantly, either toxin reduced glucose- or potassium-induced insulin secretion from rat islets and HIT cells. Treatment of β cells with *Clostridium sordellii* lethal toxin (LT; which modified only Ras, Rap, and Rac) also reduced glucose- or potassium-induced secretion. However, clostridial toxin C3-exoenzyme (which ADP-ribosylates and inactivates only Rho) was without any effect on either glucose- or potassium-induced insulin secretion. These data suggest that Cdc42, Rac, Ras, and/or Rap (but not Rho) may be needed for glucose- or potassium-mediated secretion. The effects of these toxins appear to be specific on stimulus–secretion coupling, since no difference in metabolic viability (assessed colorimetrically by quantitating the conversion of the tetrazolium salt into a formazan in a reduction reaction driven by nutrient metabolism) was demonstrable between control and toxin (A or LT)-treated β cells. Toxin (A or LT) treatment also did not alter glucose- or potassium-mediated rises in cytosolic free calcium concentrations ($[Ca^{2+}]_i$), suggesting that these GTPases are involved in steps distal to elevations in $[Ca^{2+}]_i$. Recent findings indicate that the carboxyl methylation of Cdc42 is stimulated by only glucose, whereas that of Rap (Kowluru *et al.*, *J Clin Invest* **98:** 540–555, 1996) and Rac (present study) are regulated by glucose or potassium. Together, these findings provide direct evidence, for the first time, that the Rho subfamily of GTPases plays a key regulatory role(s) in insulin secretion, and they suggest that Cdc42 may be required for early steps in glucose stimulation of insulin release, whereas Rap and/or Rac may be required for a later step(s) in the stimulus–secretion coupling cascade (i.e. Ca^{2+}-induced exocytosis of insulin). BIOCHEM PHARMACOL **54**;10: 1097–1108, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Cdc42; Rho; Rap; Rac; C3 exoenzyme; insulin secretion; pancreatic β cell; clostridial toxins

Glucose-induced insulin secretion from pancreatic β cells is largely mediated via generation of soluble second messengers such as ions, cyclic nucleotides, hydrolytic products of phospholipases (A_2, C and D), and adenine nucleotides [1, 2]. Recently, using specific inhibitors of GTP synthesis (e.g. mycophenolic acid), we identified a permissive role for GTP as one of the modulators of physiologic insulin secretion from normal rat islets and isolated β (HIT-T15) cells [3, 4]. While the exact mechanism(s) underlying GTP-mediated regulation of insulin secretion remains unidentified, one of the possible loci was identified as the activation of phospholipase C. The latter, in turn, appeared to reflect the activation of one (or more) unidentified low molecular mass or heterotrimeric GTP-binding proteins (GTPases¶), several of which have been identified in pancreatic β cells [5–9].

In the pancreatic β cell (as in many other cell types), low molecular mass GTPases and γ subunits of trimeric G-proteins undergo post-translational modifications at their C-termini; these include isoprenylation and carboxyl methylation [6, 10, 11]. Selective inhibitors of these reactions (e.g. lovastatin, an inhibitor of isoprenylation, or acetyl

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¶ *Abbreviations:* $[Ca^{2+}]_i$, cytosolic free calcium concentration; GTPase, guanosine triphosphatase; LT, lethal toxin; PMSF, phenylmethylsulfonyl fluoride; SAM, *S*-adenosyl methionine; and UDPG, uridine diphosphate glucose.

farnesyl cysteine, an inhibitor of the carboxyl methylation) have been shown to interfere with nutrient-induced insulin secretion, suggesting a functional role for certain GTPases in insulin exocytosis induced by fuels [6, 10, 11]. We observed that Cdc42, a member of the Rho subfamily of small GTPases [6], undergoes GTP-dependent translocation from cytosol to the membrane fraction, and is subsequently carboxyl methylated by a membrane-bound methyl transferase [6, 12]. Stimulatory concentrations of glucose augment the carboxyl methylation of Cdc42 in intact rat islets and HIT cells [6]; conversely, inhibition of carboxyl methylation of Cdc42 using either acetyl farnesyl cysteine or the pharmacologic depletion of GTP markedly attenuates glucose-induced phospholipase C activation in, and insulin secretion from, β cells [6]. Based on these findings, we concluded that Cdc42 may represent (one of the) GTPases essential for glucose-induced insulin secretion; however, lacking was *direct* evidence linking the carboxyl methylation of Cdc42 specifically to insulin release.

The present study was undertaken to seek further support for our formulation that Rho proteins (e.g. Cdc42) are involved in β cell signal transduction. Recent studies [13–16] have utilized clostridial toxins that specifically, but differentially, monoglucosylate (and thereby inactivate) the Rho subfamily of GTPases. For example, *Clostridium difficile* toxins (A or B) monoglucosylate (at threonine residues) Rho, Rac, and Cdc42 (but not Ras, Rab, or ARF proteins); this modification impairs the function of these small GTPases. *Clostridium sordellii* LT also has been shown to monoglucosylate Rac, Rap, and Ras specifically (but not Cdc42, Rho, or Rab). More recently, Selzer *et al.* [17] reported that *Clostridium novyi* α-toxin catalyzed the *N*-acetyl glucosaminylation (and subsequent inactivation) of Rho, Rac, and Cdc42. *Clostridium* exoenzyme C3 specifically inactivates Rho proteins (e.g. Rho A, B, and C) through ADP-ribosylation at the Asn-41 residue. Therefore, we examined the effects of three of these toxins on glucose- or potassium-induced insulin secretion from normal rat islets as well as from isolated β (HIT-T15) cells in order to identify the candidate GTPases involved in the cascade of events leading to insulin secretion. Our studies demonstrated that either toxin A (or B) or LT, but not C3 exoenzyme, markedly reduces glucose as well as potassium-induced insulin secretion from β cells, apparently via glucosylation of low molecular mass GTPases. These data support a selective role for islet Cdc42, Rac, and/or Rap, but not Rho, in insulin secretion from the pancreatic β cell elicited by glucose or potassium.

MATERIALS AND METHODS

Materials

[^{125}I]Insulin (80–120 μCi/mmol), [^{32}P-*adenylate*]NAD (10–50 Ci/mmol), [^{14}C]UDPG (287 mCi/mmol), [^{3}H-*methyl*]methionine (70 Ci/mmol), and *S*-adenosyl [^{3}H-*methyl*]methionine (SAM; 73 Ci/mmol) were purchased from NEN-Dupont (Boston, MA). Unlabelled NAD, UDPG, GTP, GTPγS, and GDP were obtained from the Sigma Chemical Co. (St. Louis, MO). All reagents were of analytical grade and of the highest purity available. Toxins A, B, and C3 exoenzyme were purified as described in Refs. 18–20. Some experiments utilized C3 exoenzyme purchased from Upstate Biotechnology (Lake Placid, NY) or Calbiochem Novabiochem (La Jolla, CA). LT, purified from the *C. sordellii* IP82 strain, was provided by Dr. Michel Popoff, Pasteur Institute, Paris, France [16]. Protein A-agarose was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Pansorbin (binding capacity of 2 mg/mL of human IgG/mL suspension) was purchased from the Calbiochem Novabiochem Corp. (La Jolla, CA).

Antisera directed against low molecular mass GTPases were purchased from Santa Cruz Biotechnology, Inc. Anti-Rho A (Cat. No. SC-179; epitope corresponding to amino acid residues 119–132 of Rho A p21) is specific for Rho A 21 and non-cross-reactive with Rho B, Rho C, or other GTPases belonging to the Ras superfamily. Anti-Rac2 (Cat. No. SC-96; epitope corresponding to amino acid residues 178–188 mapping with the C-terminal domain of Rac2) is specific for Rac2 p21 and non-cross-reactive with Rac1 and other GTPases belonging to the Ras superfamily. Anti-Cdc42Hs (Cat. No. SC-087; epitope corresponding to amino acid residues 166–182 near the C-terminus of Cdc42) is specific for Cdc42 and non-cross-reactive with Rac1, Rac2, and other Ras family GTPases. Anti-Rap 1/Krev-1 (Cat. No. SC-065; epitope corresponding to amino acid residues 121–136 of Rap1A) is specific for Rap1A and non-reactive with Rap2. Anti-Ras was purchased from Oncogene Science (New York, NY).

Pancreatic Islets and Isolated β Cells

Intact islets were isolated from male Sprague–Dawley rats as previously described [3–6]. Islets were hand-picked under a stereo-microscope twice to avoid contamination by acinar cells. HIT cells (passages 72–81) and INS-1 cells (passages 45–75) were provided by Dr. Paul Robertson (University of Minnesota Medical School, Minneapolis, MN) and Dr. C. B. Wollheim (Geneva, Switzerland), respectively. Human islets were provided by Dr. T. Mohanakumar, Islet Isolation Core, Washington University Medical School, MO. Particulate and cytosolic fractions were isolated from homogenates of β cells by centrifugation at 105,000 *g* for 90 min [6]. To prevent inactivation of Rho proteins, $MgCl_2$ (3 mM final concentration) was added routinely to the β cell fractions [21] and stored at −70° until used.

Studies of Post-translational Modification of Low Molecular Mass GTPases

A summary of experimental approaches used for studying various post-translational modifications of β cell low molecular mass GTPases is provided in Table 1. Specific experimental procedures employed in these modification studies are described briefly below.

TABLE 1. Summary of various post-translational modifications of small molecular mass GTPases examined in the current studies

Modification type	Toxins used	Substrates used	GTPases studied	Purported functional consequence
Carboxyl methylation	None	SAM	Cdc42, Rap, or Rac	Activation
ADP-ribosylation	C3-exoenzyme	NAD	Rho	Inhibition
UDP-glucosylation	Toxins A, B, or LT	UDPG	Rho, Ras, Rac, Rap, Cdc42	Inhibition

ADP-RIBOSYLATION OF LOW MOLECULAR MASS GTPASES. ADP-ribosylation reaction was carried out using homogenates (or cytosolic fractions) of β cells as described in Refs. 20 and 21. The latter were incubated in buffer consisting of 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.3 mM PMSF, 50 mM Tris–HCl, pH 7.4, 0.3 μM [^{32}P]NAD in the presence of C3 exoenzyme (1 μg/mL) for 15 min at 30°. Reaction was stopped by the addition of Laemmli sample buffer, and labeled proteins were separated by SDS–PAGE (12%) and identified by autoradiography.

UDP-GLUCOSYLATION OF LOW MOLECULAR MASS GTPASES. UDP-glucosylation reactions were performed using normal rat islet or HIT cell homogenates (1.5 to 2 mg/mL) in a medium containing 30 μM [^{14}C]UDP-glucose (50 nCi), 3 mM $MgCl_2$, 0.3 mM GDP, 150 mM KCl, 50 mM Tris–HCl, pH 7.5, cell lysate, and 10 μg/mL toxin A or B or LT (5 μg/mL) or diluents (i.e. no added toxin), where indicated. Following incubation at 37° for 45 min, proteins were separated by SDS–PAGE (12.5%), and the amount of radioactivity in the 21–23 kDa range was quantitated by incubating the gel slices in H_2O_2 (30%) at 60° followed by scintillation spectrometry. Furthermore, islet or HIT cell GTPases were immunoprecipitated (following solubilization in the immunoprecipitation buffer consisting of 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, and 10 μg/mL each of aprotinin, pepstatin, leupeptin, and PMSF in phosphate-buffered saline, pH 7.4) using specific antisera directed against Cdc42, Rho, Rac, Rap, or Ras or preimmune serum as described in Ref. 6. In brief, cell lysates were incubated overnight at 4° with antisera directed against individual GTPases or preimmune serum (1:500 dilution), and immune complexes were captured using either protein A-agarose or pansorbin, and separated by centrifugation. Pellets were washed extensively with 20 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, and the radioactivity associated with immunoprecipitates (i.e. glucosylation) was quantitated by scintillation spectrometry [6]. All values were adjusted by subtracting non-specific incorporation of the label into the immunoprecipitates using the preimmune serum. Additional details of these methods, including efficiency of immunologic detection and immunoprecipitation methods used for various GTPases, have been described in our earlier publications (Ref. 6 and references therein).

PRENYL-CYSTEINE CARBOXYL METHYLATION OF LOW MOLECULAR MASS GTPASES. In cell-free preparations, protein carboxyl methylation assays were carried out at 37° for 45 min in a total volume of 100 μL consisting of β cell homogenates (20–50 μg protein), 50 mM sodium phosphate buffer, pH 6.8, 1 mM EGTA, and 100 μCi/mL (7 μM) [^{3}H]SAM as the methyl donor [6]. GTPγS and other reagents were present in their respective concentrations as described in the text. The reaction was started by the addition of SAM and was terminated by the addition of Laemmli sample buffer; labeled proteins were separated by SDS–PAGE (12% acrylamide). The degree of labeling was quantitated by a vapor-phase equilibration assay (see below). For the quantitation [6] of agonist-induced carboxyl methylation of low molecular mass GTPases in intact cells, intact HIT cells were prelabeled with [^{3}H]methionine (40–100 μCi/mL; 2 mL total volume) at 37° for 60 min in the presence of 0.1 mM glucose. Following this, cells were washed (two times) with an isotonic medium, and agonist-induced stimulation of the carboxyl methylation of individual GTPases was determined using immunoprecipitation methods as described in Ref. 6. Under these conditions, >75% of authentic Cdc42 and Rac, which had been carboxyl methylated by enzymes provided by addition of HIT cell homogenates, was recovered in the immunoprecipitates [Ref. 6 and additional data not shown].

Base-labile [^{3}H]Methanol Release Assay

The α carboxyl methyl groups on prenyl-cysteine residues of modified GTPases are base-labile [6, 11]. To demonstrate that such sites are specifically modified in insulin-secreting cells, methyl esters were quantitated by a vapor-phase equilibration assay (i.e. quantitation of base-labile [^{3}H]methanol release] as described previously [6, 11]. Briefly, individual lanes of dried gels were cut into 3- or 5-mm slices and were placed in 1.5-mL Eppendorf centrifuge tubes (without caps) containing 300–500 μL of 1 N NaOH. Tubes were placed in 20-mL scintillation vials containing 5 mL of scintillation fluid (Ultima Gold; Packard Instrument Co., Meriden, CT). The vials were capped and left at 37° overnight to maximize the base-catalyzed release of [^{3}H]methanol due to hydrolysis of methyl esters. Following this, the radioactivity was determined by scintillation spectrometry.

Metabolic Viability of Control and Toxin-Treated β Cells

Metabolic viability of (control or toxin-treated) intact β cells was monitored [22] using a [3-(4,5)-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium (MTS) assay kit (Celltiter 96TM) developed by Promega (Madison, WI). This assay measures colored formazan production in a reduction reaction driven by nutrient metabolism. This reduction reaction is felt to represent largely the increase in reduced pyridine nucleotides generated by cytosolic and mitochondrial dehydrogenases. Control or toxin-treated cells were preincubated at 37° for 30 min and then incubated for an additional 30 min in the MTS-containing Krebs–Ringer's medium (KRB) with or without glucose as indicated in the text. At the end of the incubation period, following centrifugation at 2900 *g* for 6 min, the supernatant was removed and used for measuring optical density and insulin secretion. Optical density was recorded at 490 nm using a microplate reader (EL 312, Biotek Instruments, Winooski, VT). Readings were corrected for background optical density by subtracting out readings from MTS-containing buffer incubated (without cells) under identical conditions [22].

Insulin Release Studies

Insulin release from isolated islets was quantitated by batch-type static incubations as described in Refs. 3, 4, and 6. Incubations in the presence of test compounds or appropriate diluents were carried out as described in the text. To assess insulin content, islets were extracted overnight in 1 mL of acid alcohol (77% ethanol, 22% water, and 1% 12 N HCl; by vol.) at 4°; then the supernatant was removed, diluted (1:10) in phosphate-buffered saline to a final dilution of 1:20, and assayed by radioimmunoassay [3, 4, 6]. For insulin release studies involving HIT cells, the latter were seeded in 24-well culture plates and cultured for 3–4 days in RPMI 1640 medium [6]. Test agents were included in the culture medium for various times as specified in the text. On the day of the experiment, the cells were washed with fresh KRB. After a 30-min preincubation period (in 0.5 mL KRB), the medium was replaced by one containing secretagogues (0.5 mL) and the cells were incubated for 30 min. The supernatant was used for measurement of insulin secretion, and attached cells were extracted for the determination of insulin content as described above.

Other Methods

Low molecular mass GTPases were identified by western blotting [6], and their relative mobilities were compared to purified Cdc42, Rac, and Rho (purified from bacterial cultures following expression of the respective plasmids, which were provided by Dr. Gary Bokoch, Scripps Institute, La Jolla, CA). $[Ca^{2+}]_i$ was measured using fura-2 (Molecular Probes Inc., Eugene, OR) as described in Ref. 23. Protein concentration was determined according to Bradford [24] using bovine serum albumin as the standard.

RESULTS

Characterization of C3 Toxin-Mediated ADP-Ribosylation of Rho in Broken Insulin-Secreting Cell Preparations

Initial experiments were directed to optimize conditions for ADP-ribosylation of Rho in four classes of insulin-secreting cells (e.g. rat and human islets, HIT cells, and INS cells). The degree of C3 exoenzyme-mediated ADP-ribosylation of Rho in HIT cell cytosol was time- and protein concentration-dependent, being saturable by 15 min of incubation at 37°, and linear up to 15 μg of protein/assay (additional data not shown). Under optimal conditions of assay, the magnitude of ADP-ribosylation of Rho was comparable in the homogenates of rat islets, human islets, and in HIT cells (4–5 densitometric units/μg protein). A similar but lower degree of ADP-ribosylation of Rho was also detected in INS cells (2 U/μg protein). We also observed that ADP-ribosylation of Rho was inhibited markedly by 0.03% SDS (−84%) and by 3 mM deoxycholate (−78%) compatible with the known inhibitory effects of these agents on C3 exoenzyme-mediated ADP-ribosylation of recombinant Rho [21].

Effects of Clostridial Toxin A or B on C3 Toxin-Mediated Ribosylation of Rho

It has been reported that prior glucosylation of Rho (at Thr-37) results in significant reduction in the subsequent ADP-ribosylation of Rho (at Asn-41) catalyzed by C3 exoenzyme [21]. Therefore, we examined C3 toxin-mediated ADP-ribosylation of Rho in intact HIT cells and intact rat islets pretreated with *Clostridium difficile* toxin A to determine the efficacy of toxin A effects. Using intact HIT cells, we observed that within a preincubation period of 3 hr with toxin A, the subsequent ADP-ribosylation of Rho mediated by C3 toxin was inhibited by >80% (data not shown). Similar inhibitory effects were also demonstrable in intact normal rat islets preincubated with toxin A (Fig. 1); however, in this case, prolonged exposure periods (up to 20–22 hr) and/or higher concentrations were required for optimal inhibitory effects of the toxin on the subsequent ADP-ribosylation of Rho, or on glucose- or potassium- (i.e. calcium-mediated) induced insulin secretion (see below). These data may suggest relatively slower rates of uptake of these toxins into intact rat islets (where β cells are sequestered largely in a central core) compared with dispersed and monolayer-cultured HIT cells. Microscopic examination of toxin A- or B-treated HIT cells indicated that virtually all cells rounded up, suggesting a substantial disturbance of β cell cytoskeletal architecture (additional data not shown).

FIG. 1. Inhibition of C3 toxin-mediated ADP-ribosylation of Rho in intact rat islets following preincubation with toxin A. Intact rat islets (300 islets/group) were incubated for 21 hr at 37° with either diluent (−) or 500 ng/mL toxin A (+). Following this, cells were washed (two times) with an isotonic medium, and C3 exotoxin-mediated ribosylation of Rho was carried out in lysates of control and toxin-treated islets using [^{32}P]NAD (see Materials and Methods). Labeled proteins were identified by autoradiography at −70°. Data are representative of two experiments with similar results.

Effects of Toxin A (or B) or LT on Glucosylation of G-Proteins in Intact or Broken β Cell Preparations

We next examined whether toxin A (or B) or LT-mediated inhibitory effects on ribosylation of Rho are indeed mediated via the prior monoglucosylation of the latter. These studies indicated that the remaining glucosylation (assessed by incorporation of [^{14}C]glucose from [^{14}C]UDPG) catalyzed by toxin A was reduced markedly in intact rat islets (−80%) or HIT cells (−50%) that had been pre-exposed to toxin A (additional data not shown). Additional immunoprecipitation studies (Fig. 2, A and B) indicated that toxin A specifically glucosylated Rho, Rac, and Cdc42 (but not Ras) in HIT cell homogenates incubated with [^{14}C]UDPG (Fig. 2A). Under these conditions, LT specifically glucosylated Ras, Rap, and Rac, whereas Rho and Cdc42 were not detectably glucosylated (Fig. 2B). Likewise, in isolated rat islet homogenates, toxin A catalyzed the glucosylation of Rac, Rho, and Cdc42 (Fig. 3A). Under identical conditions, in rat islet homogenates, LT glucosylated Rac and Rap (Fig. 3B). These glucosylation data using rat islet homogenates were similar to those obtained using HIT cell homogenates. However, we failed to detect any glucosylated Ras in normal rat islet homogenates, unlike HIT cell homogenates. Further studies by western blotting revealed no detectable Ras in normal rat islets, compatible with recent data indicating the lack of Ras presence in mouse islets (see below). Nonetheless, these data are compatible with reports of the specificity of toxin A (or B) and LT on glucosylation of these three small GTPases in other cell types [13–16].

Effects of Clostridial Toxins on Glucose- and Potassium-Induced Insulin Secretion

The effects of a preincubation period of 21 hr in different concentrations (0–20 ng/mL) of toxin B on subsequent glucose (16.7 mM)-stimulated insulin secretion from iso lated rat islets were examined. Data in Fig. 4A indicate that >70% inhibition of glucose-induced insulin secretion was demonstrable at 20 ng/mL. Furthermore, as shown in Fig. 4B, LT also inhibited glucose-induced insulin secretion from normal rat islets in a concentration-dependent manner. No significant effects of either toxin A, toxin B, or LT pretreatment were demonstrable on either basal insulin secretion or total insulin content (additional data not shown). Under identical conditions, preexposure of isolated islets (21–24 hr; 37°) to toxin A (500 ng/mL) also resulted in a marked reduction (−79%) in glucose-stimulated insulin secretion from isolated rat islets. Likewise, potassium (40 mM)-induced insulin secretion was inhibited −66% by toxin A, −46% by toxin B, or −58% by LT (Fig. 5). However, it may be noted that a higher concentration (100 ng/mL) of toxin B was required for inhibition of potassium-mediated secretion (Fig. 5) in contrast to 20 ng/mL of toxin B required for its inhibition of glucose-stimulated secretion (Fig. 4A). Reasons for this difference are not clear at the present time.

We then examined the effects of these toxins on glucose- or potassium-induced secretion from pure β (HIT) cells. Inhibitory effects of these toxins in HIT cells were demonstrable within 3 hr of exposure. Both glucose- and potassium-induced secretions were attenuated by toxin B (Fig. 6A). Interestingly, >50% inhibition by toxin B of glucose-stimulated insulin secretion was demonstrable after 60 min of exposure, at which point only 10% reduction in potassium-induced secretion was observed (Fig. 6A). After a 3-hr exposure, potassium-induced secretion was attenuated by >40%, under which conditions glucose-induced insulin secretion was reduced by >60% (Fig. 6A). Additional exposure times (up to 6 hr) did not inhibit either glucose- or potassium-induced secretion any further than what was observed at 3 hr (data not shown). Additionally, these toxins exerted similar inhibitory effects on glucose- (Fig. 6B) or KCl-induced (Fig. 6C) insulin secretion from HIT cells. Together, based upon glucosylation (Fig. 2) and insulin release data (Figs. 4–6), there are five identifiable candidate GTPases (i.e. Cdc42, Rap, Rac, Ras, and Rho) that might be involved in glucose- and potassium-induced insulin secretion from β cells.

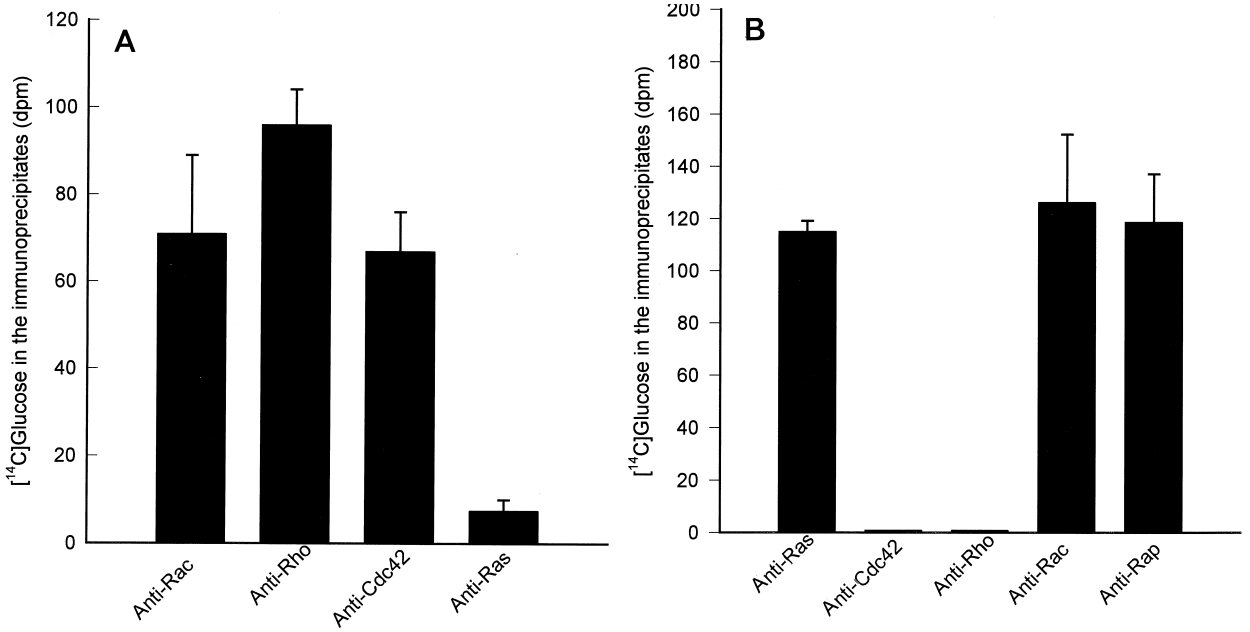

FIG. 2. Toxin A (panel A)- or LT (panel B)-mediated glucosylation of low molecular mass GTPases in HIT cell homogenates. HIT cell homogenates (2 mg/mL) were incubated with either toxin A (10 μg/mL) or LT (5 μg/mL) and [^{14}C]UDPG (30 μM) as described under Materials and Methods. Following this, proteins were solubilized in immunoprecipitation buffer, individual GTPases were immunoprecipitated using specific antisera, and the radioactivity in the immunoprecipitates was quantitated by scintillation spectrometry. Values were adjusted by subtracting non-specific incorporation of the label (representing 40–55 dpm) into immunoprecipitates, as assessed using preimmune serum. Data are means ± SEM of three determinations in each case. These data indicate that Cdc42, Rac, and Rho are substrates for toxin A, whereas Rac, Rap, and Ras are substrates for LT. In the absence of either toxin, no detectable incorporation of [^{14}C]glucose into small molecular mass GTPases was demonstrable.

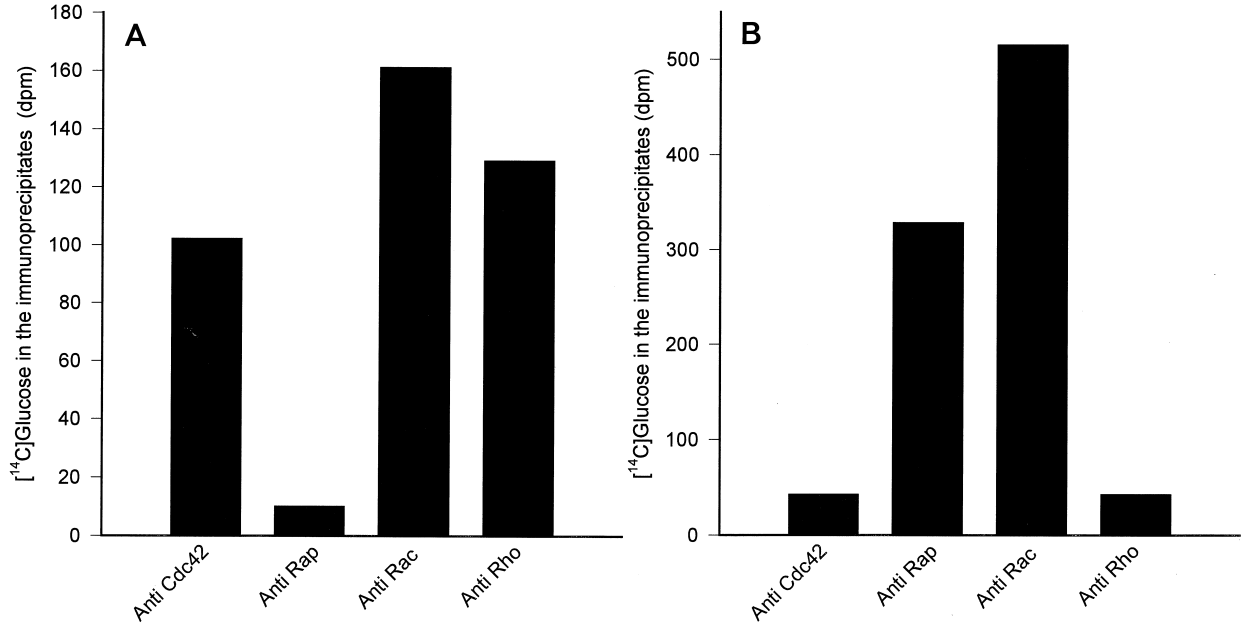

FIG. 3. Toxin A (panel A)- or LT (panel B)-mediated glucosylation of low molecular mass GTPases in isolated rat islet homogenates. Islet homogenate protein (1.4 mg, corresponding to 2700 islets) was incubated with either toxin A (10 μg/mL) or LT (5 μg/mL) and [^{14}C]UDPG (30 μM) as described above. Following this, proteins were solubilized in immunoprecipitation buffer, individual GTPases were immunoprecipitated using specific antisera, and the radioactivity in the immunoprecipitates was quantitated by scintillation spectrometry. Values were adjusted by subtracting non-specific incorporation of the label (representing 30–45 dpm for toxin A and 60–80 dpm for LT) into immunoprecipitates, as assessed using preimmune serum. Data are means of two individual immunoprecipitations in each case. In the absence of either toxin A or LT, no detectable glucosylation of low molecular mass GTPases was demonstrable.

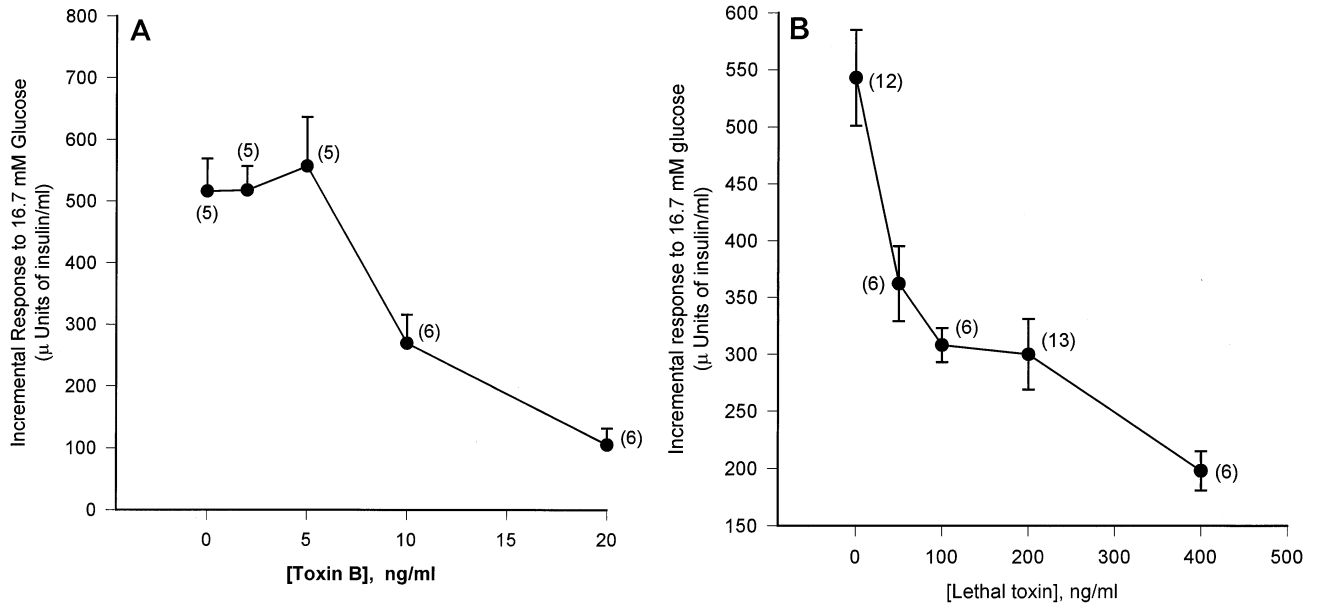

FIG. 4. (A) Concentration-dependent inhibition by toxin B of subsequent glucose-induced insulin secretion from normal rat islets. Isolated islets were cultured for 21 hr in the presence of various concentrations (0–20 ng/mL) of toxin B as indicated in the figure. Following a 45-min preincubation, insulin release was measured under static incubation conditions for 45 min in the presence of either 3 or 16.7 mM glucose. Data are expressed as the incremental response (i.e. stimulated release minus basal release) to 16.7 mM glucose and are means ± SEM from the number of determinations at each concentration of toxin B indicated in the figure ($P < 0.01$ vs control at 10 and 20 ng/mL). (B) Concentration-dependent inhibition by LT of subsequent glucose-induced insulin secretion from normal rat islets. Isolated islets were cultured for 21 hr in the presence of various concentrations (0–400 ng/mL) of LT as indicated in the figure. Following a 45-min preincubation, insulin release was measured under static incubation conditions for 45 min in the presence of either 3 or 16.7 mM glucose. Data are expressed as the incremental response to 16.7 mM glucose (see above) and are means ± SEM from the number of determinations at each concentration of LT as shown in the figure. As stated in the text, no significant effects of this toxin (up to 200 ng/mL) on basal secretion were demonstrable. However, at higher concentrations (i.e. 400 ng/mL), a modest increase in the basal secretion was observed [18 ± 3 μU of insulin/mL in the absence of toxin, N = 13, vs 79 ± 28 μU of insulin/mL in the presence of toxin B, N = 3).

Effects of Toxins on Metabolic Viability and Agonist-Induced Rises in $[Ca^{2+}]_i$

To exclude the possibility that these toxins are exerting non-specific toxic effects on fuel metabolism, we quantitated the latter by a formazan reduction method [22] in control and toxin A and/or LT-treated cells (100 ng/mL; 6 hr). The two toxins (singly or in combination) failed to affect significantly nutrient metabolism in HIT cells (Fig. 7A). Additionally, we examined whether toxin treatment affects either glucose- or potassium-induced rises in $[Ca^{2+}]_i$. Under the conditions used (6-hr exposure to concentrations as high as 100 ng/mL toxin), there were no detectable alterations in $[Ca^{2+}]_i$ induced by either toxin A or LT treatment (Fig. 7B). These data enable us to conclude that the inhibitory effects of these toxins on glucose- or potassium-induced insulin secretion are exerted distal to the elevation of $[Ca^{2+}]_i$ induced by the two agonists.

Effects of C3 Exoenzyme on Glucose- or Potassium-Induced Insulin Secretion

C3 toxin selectively modifies (and inhibits) Rho with much less, or no, modification of other Rho family GTPases such as Cdc42 or Rac. After exposure of intact HIT cells to (12.5 to 25 μg/mL) C3 exoenzyme for 48 hr at 37°, no subsequent ribosylation of Rho was demonstrable in cells during an acute exposure to C3 exoenzyme, indicating that most (if not all) Rho endogenous to HIT cells had been modified. Furthermore, C3 exoenzyme-treated cells also rounded up similarly to toxin A- or B- or LT-treated cells, indicating disturbances in cytoskeletal organization (not shown). To explore which of these GTPases is critical for glucose- or potassium-induced insulin secretion, we measured glucose (10 mM)- and potassium (40 mM)-induced insulin secretion from HIT cells previously exposed to C3 exoenzyme. Exposure of HIT cells to C3 exoenzyme did not affect significantly either (10 mM) glucose- or (40 mM) potassium-induced insulin secretion from these cells (Fig. 8). These data suggest that Rho is not critical for glucose- or potassium-stimulated secretion from β cells, even under conditions in which endogenous Rho is apparently fully ribosylated (and inhibited), and despite morphologic indications of alteration in cytoskeletal function. Furthermore, we observed no significant differences in insulin content in control and C3 toxin-treated cells (additional data not shown).

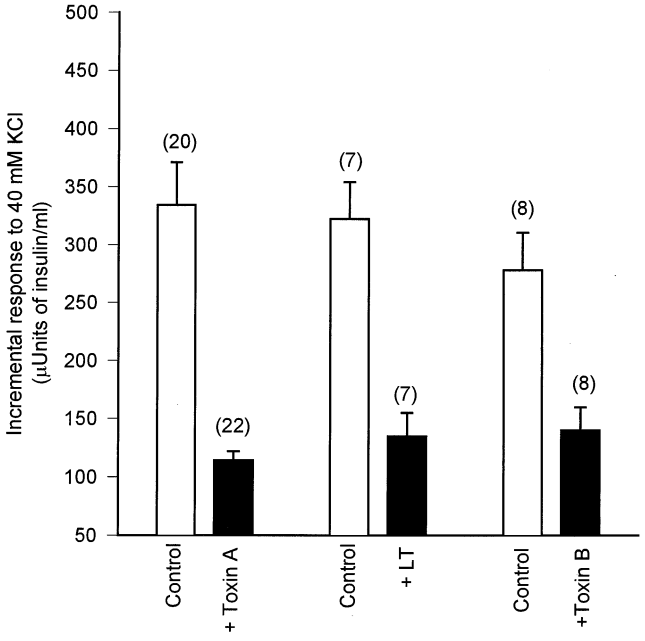

FIG. 5. Inhibition by toxin A, toxin B, or LT of potassium-induced secretion from normal rat islets. Isolated islets were cultured in the presence of diluent alone, toxin A (500 ng/mL), toxin B (100 ng/mL), or LT (200 ng/mL) for 22 hr as described under Materials and Methods. Insulin release was quantitated under static incubation conditions in the presence of 3 mM glucose alone or 3 mM glucose plus 40 mM KCl. Data (incremental response to 40 mM KCl) are means ± SEM from the number of determinations indicated in the parentheses. $P < 0.01$ for control vs toxin-treated cells.

Effects of Glucose or Depolarizing Concentrations of Potassium on the Carboxyl Methylation of Low Molecular Mass GTPases

In previous studies, GTPγS stimulated the carboxyl methylation of Cdc42 [6] and Rap [6, 25] in broken β cell preparations. In the present study, using similar immunoprecipitation protocols [6], we observed that the carboxyl methylation of Rac also was stimulated markedly (9.3 ± 1.5-fold; N = 4 determinations) by GTPγS (100 μM) in HIT cell homogenates. We have shown previously that in intact β cells, the carboxyl methylation of Cdc42 was stimulated only by glucose [6], whereas the carboxyl methylation of Rap was stimulated by either glucose or potassium [6, 25]. Based upon these data, we proposed that Cdc42 may be required in the early steps of signalling by glucose, whereas Rap may play a role(s) in a more distal calcium-mediated event(s). Data shown in Table 2 indicate that glucose markedly stimulated the carboxyl methylation of Cdc42 and Rap (but not Rac). In contrast, 40 mM KCl stimulated the carboxyl methylation of Rap and Rac (but not Cdc42; Table 2). These data clearly indicate a differential regulation of these GTPases by glucose and potassium (Table 3 and see below).

DISCUSSION

Through the use of clostridial toxins that selectively modify (glucosylate or ribosylate) specific GTP-binding proteins, the current studies identify, for the first time, putative GTPases involved in glucose- or potassium-induced insulin secretion. Several recent studies have utilized such toxins to decipher the roles of specific GTPases in cellular function. For example, using toxin B, Prepens *et al.* [26] identified Cdc42 as one of the G-proteins involved in agonist-induced [^{3}H]serotonin release from rat basophilic leukemic (RBL-2H3hml) cells. Recently, Schmidt *et al.* [27] utilized toxin B to probe the role of Rho GTPases in receptor-mediated activation of phospholipase D in human embryonic kidney cells. To our knowledge, the current study is the first to assess the functional role(s) of low molecular weight GTPases in β cell stimulus–secretion coupling through the use of these specific toxins.

In the present study, we found that at least three β cell GTPases (namely Rho, Rac, and Cdc42) underwent glucosylation catalyzed by *Clostridium difficile* toxin A (or B). Moreover, LT specifically catalyzed the glucosylation of Rap, Rac, and Ras but not of Cdc42 or Rho in HIT cells. We also observed that either toxin A (or B) or LT markedly reduced both glucose- or potassium-induced insulin secretion from intact HIT cells and normal rat islets, suggesting that toxin-mediated glucosylation of any (or all) of the five GTPases (i.e. Cdc42, Rho, Rap, Rac, or Ras) interferes with glucose- or potassium-induced insulin secretion. However, prior glucosylation of Rho mediated by C3 exoenzyme in β cells did not alter glucose- or calcium-induced insulin secretion from HIT cells. This toxin selectively ribosylates Rho and (to a much lesser degree) Rac, but not Cdc42 [21]. Thus, it is unlikely that Rho is required for glucose- or Ca^{2+}-induced insulin secretion. Data from these studies also indicate that Ras is glucosylated by LT in HIT cells. Even though recent evidence indicates the presence of this GTPase in neonatal rat islets [28] and in HIT cells (current study), it was undetectable in normal rat islets (Kowluru A and Metz SA, unpublished observations) and mouse islets [29]. Therefore, the precise role of Ras, if any, in normal β cell function remains to be determined. It seems more likely to be involved in mitogenesis [28] than in insulin secretion.

Based upon the current studies and our other recent data [6], we propose that Cdc42 may be required for glucose-induced insulin secretion for the following reasons. First, glucose (but not 40 mM K^+) stimulates the carboxyl methylation of Cdc42; conversely, selective inhibition of the methyl transferase step markedly reduces glucose-induced carboxyl methylation of Cdc42, glucose-induced phosphoinositide hydrolysis, and glucose-induced insulin secretion from normal rat islets [6]. Second, pharmacologic depletion of endogenous GTP markedly attenuated the ability of glucose to stimulate the carboxyl methylation of Cdc42, phosphoinositide hydrolysis, and insulin secretion [6]. These data thus support our proposal that Cdc42 may play a key regulatory role in nutrient-induced insulin

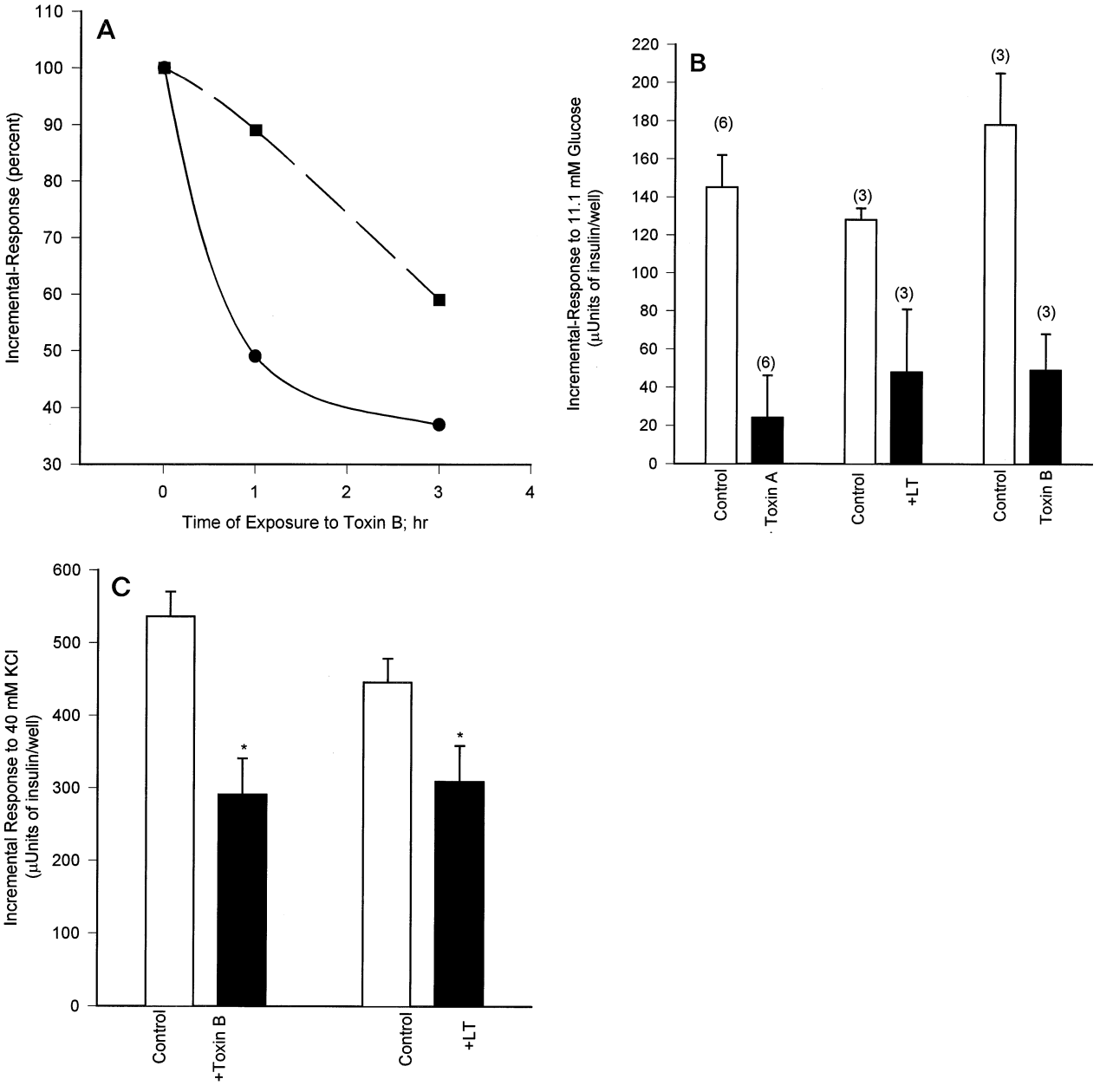

FIG. 6. (A) Time-dependent inhibition by toxin B of glucose- or potassium-induced insulin secretion from intact HIT cells. Intact HIT cells were cultured for different time intervals in the presence of diluent alone or toxin B (5 ng/mL) as indicated in the figure. Following a 30-min preincubation, insulin release was studied under static incubation conditions for an additional 30 min in the presence of zero glucose, 10 mM glucose, or 40 mM KCl. Data are expressed as percent of control (incremental) responses and are the means of at least three determinations at each point. Key: (●——●) glucose-induced, and (■----■) potassium-induced secretion. (B) Inhibition by toxin A, toxin B, or LT of subsequent glucose-induced insulin secretion from intact HIT cells. Intact HIT cells were cultured for 6 hr in the presence of diluent alone, toxin A (100 ng/mL), toxin B (5 ng/mL), or LT (100 ng/mL). Insulin release was quantitated for 30 min in the presence of zero glucose or 11.1 mM glucose. Data are expressed as the incremental response to 11.1 mM glucose and are means ± SEM from the number of determinations indicated in parentheses. $P < 0.01$ for control vs toxins A- or B-treated and <0.05 for control vs LT-treated cells. (C) Inhibition by toxin B and LT of subsequent potassium-induced insulin secretion from intact HIT cells. Intact HIT cells were cultured for 6 hr in the presence of diluent alone, toxin B (5 ng/mL), or LT (100 ng/mL). Insulin release was quantitated for 30 min in the presence of zero glucose or zero glucose plus 40 mM KCl. Data are means ± variance from two individual determinations. Key: (*) $P < 0.01$ for control vs toxin-treated cells.

secretion from the β cell, but not necessarily in calcium-induced exocytosis itself [6].

Interestingly, however, potassium-induced secretion from HIT cells and rat islets was also blunted by toxin A (and B) and LT treatment, indicating that calcium-induced exocytosis of insulin may require one or more GTPases. Based on the current studies showing a lack of effect of either toxin A or LT on glucose or potassium-induced rises

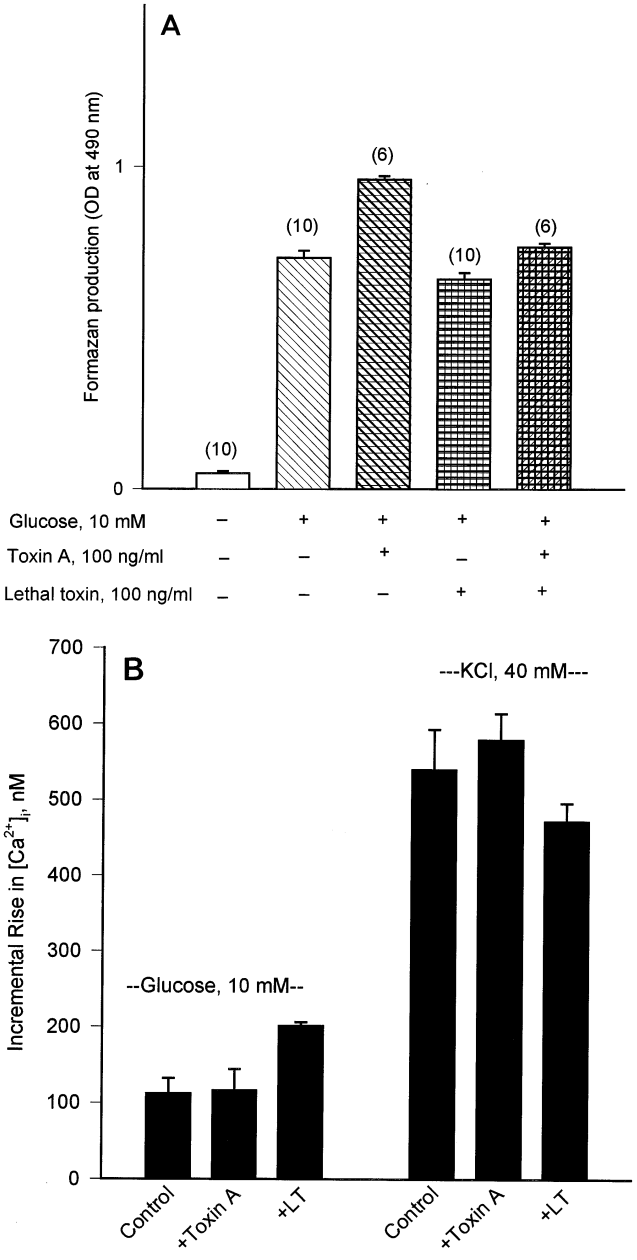

FIG. 7. (A) Effects of toxin A and LT pretreatment on metabolic viability of HIT cells. Intact HIT cells were cultured in the presence of diluent alone, toxin A (100 ng/mL), and/or LT (100 ng/mL) for 6 hr. Metabolic viability of control and toxin-treated cells was quantitated by the MTS assay (see Materials and Methods). Data are means ± SEM from the number of determinations indicated in parentheses. (B) Effects of toxin A or LT pretreatment on glucose- or potassium-induced rises in $[Ca^{2+}]_i$. Intact HIT cells were cultured in the presence of diluent alone, toxin A (100 ng/mL), or LT (100 ng/mL) for 6 hr. Glucose (10 mM)- or KCl (40 mM)-induced rises in $[Ca^{2+}]_i$ were quantitated using fura-2. Data are means ± SEM from three determinations in each case.

in $[Ca^{2+}]_i$, it seems likely that the effects of either toxin on potassium-induced secretion might be distal to increases in $[Ca^{2+}]_i$. Nonetheless, the relevant GTPase for potassium-mediated insulin release appears not to be Cdc42, since unlike glucose, the carboxyl methylation of Cdc42 is

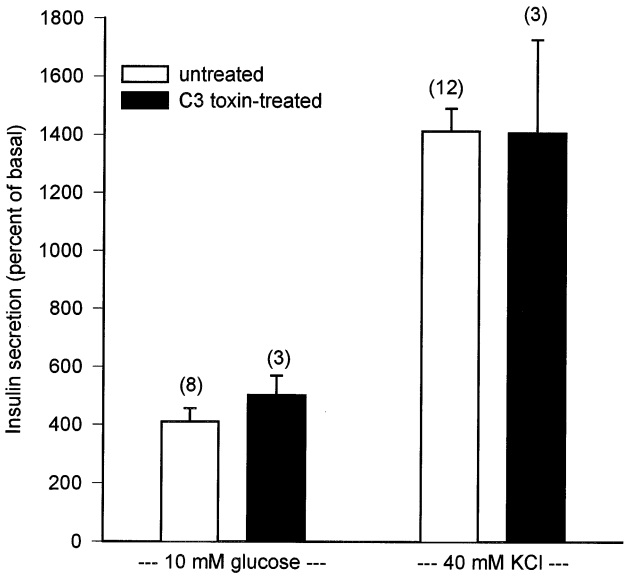

FIG. 8. Lack of effects of C3 toxin pretreatment on glucose- and potassium-induced insulin secretion from intact HIT cells. Intact HIT cells were cultured for 48 hr in the presence of diluent alone or C3-exoenzyme (12.5 to 25 μg/mL) as indicated in the figure. Following a 30-min preincubation, insulin release was studied under static conditions for an additional 30 min in the presence of zero glucose, 10 mM glucose, or 40 mM KCl. Data are expressed as percent of basal response (observed in the presence of zero glucose corresponding to 60 μU of insulin/well) and are means ± SEM from the number of determinations indicated in parentheses. There was no significant difference between the control and toxin-treated islets in their response to either glucose or potassium.

unaffected by high potassium [current study and Ref. 6]. In contrast, 40 mM K^+ preferentially stimulates the carboxyl methylation of Rap [current studies and Refs. 6 and 25] or Rac (current study). Although Rap is a substrate for LT [16], it is not a substrate for toxin A (or B) mediated

TABLE 2. Stimulation by glucose or potassium of the carboxyl methylation of low molecular weight GTPases in intact HIT-T15 cells

	Degree of carboxyl methylation of: (% of control)		
Agonist	Cdc42	Rap	Rac
Glucose, 10 mM	229 ± 21*	351 ± 45*	152 ± 22†
KCl, 40 mM	119 ± 14†	216 ± 33*	234 ± 46*

Intact HIT cells were prelabeled with [^{3}H]methionine for 1 hr at 37° as indicated in the text and then exposed to either glucose (0.1 or 10 mM) for 15 sec or KCl (40 mM) for 30 sec [6]. Following stimulation, proteins were solubilized using immunoprecipitation medium (see Materials and Methods). Cdc42, Rap, or Rac was immunoprecipitated using selective antisera directed against each of these GTPases. The degree of carboxyl methylation of these GTPases was quantitated in the immunoprecipitates by vapor-phase equilibration assay [6]. Non-specific precipitation of labeled proteins was determined using preimmune serum and was subtracted from experimental values, which are expressed as a percent of control (i.e. observed in the presence of 0.1 mM glucose. These values represented 5, 10, and 3 fmol of base-labile [^{3}H]methanol released for Cdc42, Rap, and Rac, respectively.) Data are means ± SEM from 5–8 individual immunoprecipitations.

* $P < 0.01$ vs control.

† Not significant vs control ($P < 0.1$).

TABLE 3. Regulation of Rho family GTPases in pancreatic β cells

Parameter(s) studied	Low molecular mass GTPases studied		
	Cdc42	Rap	Rac
Predominant localization (unstimulated state)	Cytosolic	Membranous*	Membranous*
Effect of GTP on translocation (broken cells)	Stimulatory	None	None
Effect of GTP on carboxyl methylation	Stimulatory	Stimulatory	Stimulatory
Effect of glucose on carboxyl methylation	Stimulatory	Stimulatory	None
Effect of 40 mM KCl on carboxyl methylation	None	Stimulatory	Stimulatory
Substrate for toxin A/B-mediated glucosylation	Yes	No	Yes
Substrate for LT-mediated glucosylation	No	Yes	Yes

* Data using β TC3 cell subcellular fractions indicated that both Rap and Rac are predominantly associated with a secretory granule-rich fraction [25]. Recent evidence indicates that Rap is predominantly localized in an insulinoma secretory granule fraction; however, Cdc42 was not detectable in this fraction (Kowluru A and Metz SA, unpublished results).

glucosylation. In contrast, Rac is a substrate for both toxin A and LT. By elimination, we conclude that Rac is likely to be involved in potassium-induced insulin secretion, although we cannot exclude an additional role for Rap.

Data from the current studies indicate that post-translational modifications differentially regulate the function of these GTPases. For example, the GTP-dependent carboxyl methylation of Cdc42 (which results in activation of this GTPase) seems to be required for insulin secretion, whereas glucosylation of Cdc42 (current study) seems to impede nutrient-induced insulin secretion. It is not likely that the glucosylation of Cdc42 resulted in a decrease in methylation at its C-terminus (and thereby inhibited insulin secretion), since we did not observe any significant difference in basal- or GTP-stimulated carboxyl methylation of Cdc42 between control and toxin A-treated HIT cells (1.53-fold stimulation by GTPγS in the control vs 1.62-fold stimulation in toxin-treated cells; mean of two determinations). Therefore, we believe that the observed inhibitory effects of toxin A or B on glucose-induced secretion may be due directly to glucosylation of islet G-proteins, specifically Cdc42 [6], in accord with the recent data suggesting that glucosylation inactivates the relevant GTPases [13–15]. Furthermore, the observed inhibitory effects of toxin A or B on insulin secretion are not likely due to non-specific or toxic effects since neither toxin altered glucose metabolism or the consequent rises in $[Ca^{2+}]_i$; conversely, C3 toxin altered cell morphology (i.e. cytoskeleton) similarly to LT or toxin A or B but did not reduce secretion, thus making the cytoskeleton an unlikely site of action of the latter.

These data, however, do not exclude the possibility that there may be an intermediary involvement of a heterotrimeric G-protein, which, in turn, regulates low molecular mass GTPases, or vice versa. Indeed, using human embryonic kidney cells, Schmidt *et al.* [27] recently demonstrated inhibition by *Clostridium difficile* toxin B of phospholipase D activity stimulated by aluminum fluoride. Since the latter stimulates heterotrimeric G-proteins [30], but only rarely stimulates low molecular mass GTPases, these data suggest that toxin B interferes with the signalling pathway to phospholipase D somewhere downstream of the receptor-mediated activation of heterotrimeric G-proteins. The current data also do not exclude the possibility that a cascade of several small GTPases (e.g. Cdc42 → Rac/Rap) is involved sequentially in physiologic insulin secretion. Indeed, a similar signal transduction mechanism(s), involving a cascade of GTPases (e.g. Cdc42 → Rho → Rac), has been proposed in other cell types for regulation of the actin cytoskeleton [31].

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