An Essential Role for Rac/Cdc42 GTPases in Cerebellar Granule Neuron Survival*

Rho family GTPases are critical molecular switches that regulate the actin cytoskeleton and cell function. In the current study, we investigated the involvement of Rho GTPases in regulating neuronal survival using primary cerebellar granule neurons. Clostridium difficile toxin B, a specific inhibitor of Rho, Rac, and Cdc42, induced apoptosis of granule neurons characterized by c-Jun phosphorylation, caspase-3 activation, and nuclear condensation. Serum and depolarization-dependent survival signals could not compensate for the loss of GTPase function. Unlike trophic factor withdrawal, toxin B did not affect the antiapoptotic protein kinase Akt or its target glycogen synthase kinase-3β. The proapoptotic effects of toxin B were mimicked by Clostridium sordellii lethal toxin, a selective inhibitor of Rac/Cdc42. Although Rac/Cdc42 GTPase inhibition led to F-actin disruption, direct cytoskeletal disassembly with Clostridium botulinum C2 toxin was insufficient to induce c-Jun phosphorylation or apoptosis. Granule neurons expressed high basal JNK and low p38 mitogen-activated protein kinase activities that were unaffected by toxin B. However, pyridyl imidazole inhibitors of JNK/p38 attenuated c-Jun phosphorylation. Moreover, both pyridyl imidazoles and adenoviral dominant-negative c-Jun attenuated apoptosis, suggesting that JNK/c-Jun signaling was required for cell death. The results indicate that Rac/Cdc42 GTPases, in addition to trophic factors, are critical for survival of cerebellar granule neurons.

Rho GTPases belong to the Ras superfamily of monomeric G proteins. The three most studied Rho GTPases, RhoA, Rac1, and Cdc42Hs, are best known as regulators of actin cytoskeletal dynamics (1). However, Rho GTPases also modulate other critical cell functions including cell cycle progression (2), gene transcription (3), and cell-cell or cell-matrix adhesion (4, 5). Recently, Rho GTPases have also been shown to influence cell survival. For example, Rho GTPases up-regulate expression of pro-survival Bel-2 family members in T cells and epithelial cells (6, 7). Constitutive activation of Rho GTPases with cytotoxic necrotizing factor protects epithelial cells from UV-induced apoptosis (8). Similarly, constitutively active Rac inhibits apoptosis induced by various stimuli in Rat1 fibroblasts, epithelial cells, and hematopoietic cells (9–11). However, the prosurvival effects of Rho GTPases are cell type- and paradigm-specific. For example, overexpression of Rac sensitizes NIH-3T3 fibroblasts to serum withdrawal-induced apoptosis (12). Furthermore, constitutively active Rho GTPase mutants enhance susceptibility of CHO cells expressing a chimeric CD4-Fas receptor to Fas-induced apoptosis (13). Thus, in non-neuronal cells Rho GTPases can display either prosurvival or proapoptotic functions. In contrast to non-neuronal cells, comparatively few studies have investigated a role for Rho GTPases in neuronal survival. Furthermore, the available data suggest that neuronal cell type-specific responses may exist. For example, dominant-negative mutants of Rac and Cdc42 protect sympathetic neurons from nerve growth factor withdrawal-induced apoptosis by suppressing activation of apoptosis signal-regulated kinase and c-Jun-NH₂-terminal kinase (JNK)³ (14, 15), suggesting that Rho GTPases can function as upstream activators of stress-activated protein kinase cascades during neuronal apoptosis. On the other hand, recent data obtained from primary cortical neurons suggest that Rho GTPases may also mediate pro-survival signaling in some neuronal cell types. Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (statins) decrease the plasma membrane localization of Rho GTPases and induce cortical neuron apoptosis (16). Similar results utilizing statins to induce apoptosis were also reported recently for rat brain neuroblasts (17). These latter findings provide indirect evidence that Rho GTPases may play a prosurvival function in some types of neurons.

In the current study, we utilized clostridial toxins, enzymes that covalently modify and inactivate Rho family GTPases in a highly specific manner (18), to investigate if Rho GTPases regulate the survival of primary rat cerebellar granule neurons (CGNs). Clostridial toxins provide a distinct advantage over statins in that they directly modify Rho GTPases rather than affecting mevalonate synthesis, a precursor required for the isoprenylation of many cellular proteins besides Rho GTPases. CGNs isolated from early postnatal rats provide a well-characterized in vitro model to study neuronal apoptosis in that their survival requires serum-derived and activity-dependent (membrane depolarization) signals (19, 20). The results show that inhibition of Rho GTPases, specifically Rac/Cdc42, promotes apoptosis of CGNs maintained in culture medium containing

* This work was supported by a Department of Veterans Affairs merit award (to K. A. H.) and by the Research Enhancement Award Program (to K. A. H. and D. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: JNK, c-Jun NH₂-terminal kinase; CGN(s), cerebellar granule neuron(s); DAPI, 4,6-diamidino-2-phenylindole; CMV, cytomegalovirus; DN, dominant-negative; GSK3β, glycogen synthase kinase-3β; MAP, mitogen-activated protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
serum and depolarizing potassium, indicating that serum-deprived and calcium-dependent survival signals cannot compensate for the loss of GTPase function. Moreover, apoptosis occurs independently of actin cytoskeletal disruption and requires phosphorylation of the transcription factor c-Jun. The data are the first to establish clearly a prosurvival function for Rho GTPases in a primary neuronal cell model.

**EXPERIMENTAL PROCEDURES**

**Materials**—Clostridium difficile toxin B (21), Clostridium sordellii lethal toxin (22), Clostridium botulinum C2 toxin (23), and C. botulinum C2 toxin (2C11)-C2 antibody (22) were isolated and characterized as described previously. SB203580 (4(4-fluorophenyl)-2-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole, cytochalasin D, ascinomyacin, dynactin A, and dyncholeximide were from Calbiochem. PDI69316 (4(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole) was provided by Dr. Alan Saltiel (Warner-Lambert/Parke-Davis, Ann Arbor, MI). RWJ67657 (4(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridyl)-1H-imidazol-2-yl)-3-buty1-1-ol) was obtained from Dr. Scott Wadsworth (R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ). Hoechst dye 33258 and DAPI (4,6-diamidino-2-phenylindole) were from Sigma. Latrunculin A and rhodamine-conjugated phalloidin were from Molecular Probes (Eugene, OR). Adenoviral-CMV was obtained from Dr. Jerry Schuman, University of Colorado Health Sciences Center (Denver, CO). CRM1 was obtained from the University of North Carolina Vector Core (Chapel Hill). Adenoviral dominant-negative (DN)-c-Jun (TAM67) was kindly provided by Dr. Jong Sung Park, Medical College of Virginia (Richmond). Polyclonal antibodies to c-Jun and caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Y27632 was from Upstate Biotechnology (Lake Placid, NY). Polyclonal, phospho-specific antibodies to c-Jun (Ser-63), Akt (Ser-473), glycogen synthase kinase-3β (GSKβ) (Ser-9), JNK (Thr-183/Tyr-185), and p38 MAP kinase (Thr-180/Tyr-182) were obtained from Cell Signaling Technology/New England Biolabs (Beverly, MA).

**Cell Culture**—Rat CGNs were isolated from 7–8-day-old SpragueDawley rat pups (15–19 g) as described previously (25). Briefly, neurons were plated at a density of 2.0 × 10⁶ cells/ml in basal modified Eagle’s medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, and penicillin (100 units/ml)-streptomycin (100 μg/ml) (Life Technologies, Inc.). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. Using this protocol, the cultures were 95–99% pure for granule neurons. In general, experiments were performed after 7 days in culture. Apoptosis was induced by either removing serum and decreasing the extracellular potassium concentration from 25 mM to 5 mM or by the direct addition of clastocidins to complete medium containing serum and 25 mM potassium.

**Quantitation of Apoptosis**—After induction of apoptosis as described above, CGNs were fixed with paraformaldehyde, and nuclei were stained with either Hoechst dye or DAPI. Cells were considered apoptotic if their nuclei were either condensed or fragmented. In general, experiments were performed after 7 days in culture. Using this protocol, the cultures were 95–99% pure for granule neurons. In general, experiments were performed after 7 days in culture. Apoptosis was induced by either removing serum and decreasing the extracellular potassium concentration from 25 mM to 5 mM or by the direct addition of clastocidins to complete medium containing serum and 25 mM potassium.

**Adenoviral Infection**—On day 5 in culture, CGNs were infected with either control adenovirus (adenoviral-CMV), adenoviral-CRM1, or adenoviral-DN-c-Jun each at a multiplicity of infection of 5–10. After infection, cells were returned to the incubator for 48 h at 37 °C and 10% CO₂. On day 7, the cells were then incubated for an additional 24 h with either vehicle (1 mM BSA in PBS) or 500 pg/ml C. difficile toxin B. Apoptosis was quantified as described above.

**Preparation of CGN Cell Extracts**—After incubation with clastocidins for the indicated times described in the text, the treatment medium was aspirated, CGNs were washed once with 2 ml of ice-cold PBS (pH 7.4), and cells were then placed on ice and scraped into lysis buffer (200 μl/35-mm well) containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM NaCl, 1 mM EGTA, 5 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell lysates were centrifuged at 10,000 × g for 15 min at 4 °C and the protein concentration of the supernatant was determined using a commercially available protein assay kit (Pierce Chemical Co.). Aliquots (~150 μg) of supernatant protein were diluted to a final concentration of 1 × SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 5 min, and electrophoresed through 10–15% polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride mem-
remarkably sensitive to Rho GTPase inhibition with \( \sim 80\% \) of the cells undergoing apoptosis after 48 h of incubation with 500 pg/ml toxin B. This latter observation is particularly striking given that cell death occurred in the presence of serum and a depolarizing concentration of potassium. Consistent with previous results that indicate that CGN apoptosis induced by withdrawal of serum and potassium is blocked by inhibitors of RNA or protein synthesis (19), toxin B-induced apoptosis was similarly prevented by coincubation with either cycloheximide or actinomycin D (Fig. 1C). Thus, apoptosis of CGNs after inhibition of Rho family GTPases apparently requires the synthesis of proapoptotic proteins.

Apoptotic cell death is orchestrated by the activation of a caspase cascade, proteases that cleave target proteins, including other caspases, at postaspartate residues (for review, see Ref. 27). Of the caspase family members, caspase-3 is commonly regarded as an "executioner" caspase because its cleavage from an inactive promolecule to an active protease by upstream caspases usually signifies commitment to apoptosis (28, 29). Caspase-3 activation is a key component of CGN apoptosis induced after the withdrawal of serum and potassium (30). In a like manner, prolonged inhibition of Rho GTPase function with toxin B led to a significant activation of caspase-3 (Fig. 2A). Moreover, adenoviral infection of CGNs with the known caspase inhibitor, CrmA (31), significantly attenuated toxin B-induced apoptosis (Fig. 2B). Collectively, these results indicate that inhibition of Rho family GTPases in CGNs evokes the activation of a caspase cascade that ultimately leads to apoptotic cell death.

Toxin B Elicits an Acute Increase in the Phosphorylation and Expression of c-Jun—Previous work has demonstrated that apoptosis of CGNs after withdrawal of serum and potassium requires the activity of the transcription factor c-Jun (32). Indeed, deprivation of serum and potassium for 4 h resulted in a substantial accumulation of phosphorylated c-Jun in the nu-
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RNA synthesis essentially abolished the increase in c-Jun expression elicited by either toxin B or deprivation of serum and potassium (Fig. 3C). Thus, apoptosis induced after either an inhibition of Rho family GTPase function or removal of trophic factors is associated with enhanced phosphorylation and expression of the proapoptotic transcription factor c-Jun.

Akt and GSK3β Are Regulated Differentially during Apoptosis Induced by Toxin B or Withdrawal of Serum and Potassium—Akt is a serine-threonine kinase that has been shown to be downstream of phosphatidylinositol 3-kinase in a prosurvival signaling pathway that is activated by both growth factors and integrins. The catalytic function of Akt is activated by phosphorylation on Ser-473 by upstream kinases (33, 34). Although withdrawal of serum and potassium from CGNs for 6 h promoted the dephosphorylation (inactivation) of Akt (Fig. 4A, last lane) and corresponding dephosphorylation (activation) of GSK3β (Fig. 4B, last lane), incubation with toxin B had no significant effect on the phosphorylation (i.e., activation) status of either kinase. These results indicate that Rho GTPases do not significantly regulate the kinase activity of GSK3β and thereby inhibit its proapoptotic function (25, 36). Phosphorylation of GSK3β on Ser-9 by Akt effectively inactivates the kinase activity of GSK3β and thereby inhibits its proapoptotic function (25, 36). Although withdrawal of serum and potassium from CGNs for 6 h promoted the dephosphorylation (inactivation) of Akt (Fig. 4A, last lane) and corresponding dephosphorylation (activation) of GSK3β (Fig. 4B, last lane), incubation with toxin B had no significant effect on the phosphorylation (i.e., activation) status of either kinase. These results indicate that Rho GTPases do not significantly regulate the phosphatidylinositol 3-kinase-Akt cell survival pathway in CGNs.

Selective Inhibition of Rac/Cdc42 Function with C. sordellii Lethal Toxin Mimics the Effects of Toxin B on c-Jun Phosphorylation and Apoptosis of CGNs: Dissociation of Actin Cytoskeletal Disruption and Cell Death—Lethal toxin from C. sordellii is similar to toxin B in that it also has monoglucosyltransferase activity; however, these two toxins differ in their substrate specificities. Whereas toxin B glucosylates all three Rho family members (Rho, Rac, and Cdc42), Rho is not a substrate for lethal toxin which selectively modifies Rac and to a lesser extent Cdc42, as well as some other Ras subfamily members (18, 22, 37). The glucosylation of Rac and Cdc42 catalyzed by lethal toxin occurs on the same threonine residue as that modified by toxin B.

An inevitable consequence of inhibiting Rho family GTPases is an eventual disruption of the actin cytoskeleton (26). Accordingly, phalloidin staining of CGNs after 24 h of incubation with either toxin B or lethal toxin revealed a significant disruption of the actin cytoskeleton.
of the cytoskeleton characterized by cell rounding and a perinuclear accumulation of F-actin aggregates (Fig. 5, A–C). Similarly, incubation with *C. botulinum* C2 toxin, an ADP-ribosyltransferase that directly modifies F-actin (23), resulted in a nearly complete loss of F-actin staining (Fig. 5D). Interestingly, although incubation of CGNs with each of the above three toxins led to disruption of the actin cytoskeleton, a significant number of condensed nuclei were only observed with toxin B or lethal toxin (Fig. 5).

In a manner analogous to toxin B, selective inhibition of Rac/Cdc42 with lethal toxin elicited acute increases in both nuclear staining for phospho-c-Jun (Fig. 6A) and total c-Jun detected in cell lysates (Fig. 6B). Lethal toxin also induced caspase-3 cleavage (data not shown), and prolonged incubation of CGNs with either toxin B or lethal toxin resulted in a similar degree of apoptosis (~45% apoptosis after 24 h) (Fig. 6C). In contrast, specific inhibition of either Rho (with *C. botulinum* C3 fusion toxin (24)) or Rho kinase (with Y27632 (38)) did not enhance c-Jun phosphorylation or induce CGN apoptosis (data not shown). Furthermore, direct disruption of F-actin with either C2 toxin, cytochalasin D, or latrunculin A was also insufficient to induce either c-Jun phosphorylation/expression (Fig. 6B and data not shown) or apoptosis (Fig. 6C and data not shown). These results indicate that a selective inhibition of Rac/Cdc42 function is entirely sufficient to evoke CGN apoptosis independently of Rho inactivation. Furthermore, the apoptosis induced after inhibition of Rac/Cdc42 also occurs independently of actin cytoskeletal disruption.

CGNs Possess High Intrinsic JNK Activity and Low Basal p38 MAP Kinase Activity That Are Unaffected by Toxin B—To determine if the increase in c-Jun phosphorylation observed after acute inhibition of Rho family GTPases was caused by an enhancement of stress-activated protein kinase activity, the degree of JNK and p38 MAP kinase activation was assessed in CGNs. CGNs exhibited high basal JNK activity indicated by the arrows in B and C. Note that although each toxin resulted in significant disruption of the actin cytoskeleton, a significant number of condensed nuclei were observed only in cells incubated with either toxin B or lethal toxin.

**Fig. 5.** Incubation with *C. difficile* toxin B, *C. sordellii* lethal toxin, or *C. botulinum* C2 toxin induces disruption of the actin cytoskeleton in rat CGNs: differential effects on nuclear morphology. Cells maintained in culture medium containing serum and a depolarizing concentration of potassium were exposed for 24 h to PBS/BSA vehicle (A), 500 pg/ml toxin B (B), 200 ng/ml lethal toxin (C), or 200 ng/ml C2 (C2IIa + C2I) toxin (D). After fixation, F-actin was visualized with rhodamine-conjugated phalloidin (shown in white), and nuclei were stained with DAPI (shown in blue). Scale bar = 20 μm. Cytoskeletal rounding and/or perinuclear accumulation of F-actin aggregates is indicated by the arrows in B and C. Note that although each toxin resulted in significant disruption of the actin cytoskeleton, a significant number of condensed nuclei were observed only in cells incubated with either toxin B or lethal toxin.

**Fig. 6.** Selective inhibition of Rac/Cdc42 function with *C. sordellii* lethal toxin mimics the effects of toxin B on c-Jun phosphorylation/expression and neuronal apoptosis: direct ADP-ribosylation of F-actin with C2 toxin is insufficient to induce cell death. A, granule neurons were incubated for 6 h with either vehicle (PBS containing 1 mg/ml BSA, left panel) or 200 ng/ml lethal toxin (right panel). After incubation, cells were fixed, and nuclear phospho-c-Jun was analyzed by staining with an antibody that specifically recognizes c-Jun phosphorylated on Ser-63, as described under “Experimental Procedures.” Scale bar = 20 μm. B, cells were incubated for 6 h with either vehicle (Veh), 500 pg/ml toxin B (ToxB), 200 ng/ml lethal toxin (LTox), 200 ng/ml activated binding component of C2 toxin (C2IIa), 200 ng/ml catalytic component of C2 toxin (C2I), or complete C2 toxin (C2IIa + C2I). After treatment, cell lysates were resolved by SDS-PAGE, transferred to polyvinylidene fluoride, and membranes were probed for total c-Jun. C, neurons were incubated as described in B for 24 h. Apoptotic cells were quantified by nuclear staining with Hoechst dye. Asterisks indicate a significant difference from the vehicle control (*p < 0.01, n = 4*).
Relative to JNK activity, the basal p38 MAP kinase activity of CGNs was low as demonstrated by a substantial increase after anisomycin exposure (Fig. 7B, first lane versus sixth lane). Yet, incubation of CGNs with toxin B did not result in a significant increase in the activation of p38 MAP kinase (Fig. 7B). Thus, inhibition of Rho family GTPases does not promote a detectable activation of stress-activated protein kinases in CGNs.

**Pyridyl Imidazole Inhibitors of p38/JNK Signaling and Adenoviral DN-c-Jun Attenuate CGN Apoptosis Induced after Inhibition of Rac/Cdc42 Function**—Recently, others have reported that CGNs exhibit high basal JNK activity that is largely unaltered by withdrawal of serum and potassium (32). However, Coffey et al. (39) have demonstrated that a small pool of active JNK may translocate from the cytoplasm to the nucleus in response to stress, where it can then phosphorylate c-Jun. To determine if the high basal JNK activity detected in CGNs was required for the enhanced phosphorylation of c-Jun after inhibition of Rac/Cdc42, we utilized several pyridyl imidazole compounds to inhibit JNK and p38 activities in CGNs. Although this class of compounds has previously been thought to inhibit p38 MAP kinase specifically, a recent report demonstrated that pyridyl imidazoles are also capable of blocking JNK activity in CGNs (40). Coincubation of CGNs with any one of three different pyridyl imidazole compounds (SB203580 (40), PD169316 (41), or RWJ67657 (42)) significantly attenuated the increase in total c-Jun induced by either toxin B (Fig. 8A) or lethal toxin (Fig. 8B). The two more potent compounds, PD169316 and RWJ67657, were also very effective at inhibiting the nuclear accumulation of phosphorylated c-Jun elicited by toxin B (Fig. 8C). In agreement with c-Jun playing a causative role in CGN apoptosis resulting from inhibition of Rac/Cdc42, the pyridyl imidazoles significantly blunted apoptosis in cells incubated with either toxin B (Fig. 9A) or lethal toxin (Fig. 9B). Moreover, adenoviral DN-c-Jun also significantly attenuated toxin B-induced apoptosis (Fig. 9C). Thus, an enhanced phosphorylation and expression of the transcription factor, c-Jun, are required for CGN apoptosis induced by inhibition of Rac/Cdc42 function.

**DISCUSSION**

Primary rat CGNs are a widely studied model of activity-dependent (depolarization-mediated) neuronal survival. Membrane depolarization elicits Ca$^{2+}$ influx through L-type voltage-gated channels, and inhibitors of Ca$^{2+}$ entry induce apoptosis of CGNs (20). Recently, one Ca$^{2+}$-dependent survival factor was identified as the transcription factor, myocyte-enhancer factor-2 (43). Transfection of CGNs with a constitutively active mutant of myocyte-enhancer factor-2 attenuates apoptosis induced by lowering extracellular potassium (43). In addition to Ca$^{2+}$-dependent survival signals, growth factors such as insulin-like growth factor-I are also known to protect CGNs from apoptosis induced by withdrawal of serum and potassium (19, 20). The principal sur-
Granule neurons were incubated for 24 h with 500 pg/ml toxin B (ToxB) (A) or 200 ng/ml lethal toxin (LTox) (B) alone or in combination with pyridyl imidazole inhibitors of p38/JNK as described in the legend to Fig. 8. Apoptosis was quantitated by nuclear staining with Hoechst dye. Asterisks indicate a significant difference from either toxin B (A) or lethal toxin (B) alone (p < 0.01, n = 3). C, cells were infected with either control adenovirus (Ad-CMV) or Ad-DN-c-Jun (each at a multiplicity of infection of 10) followed by incubation with toxin B; apoptosis was quantified by Hoechst staining. Asterisks indicate a significant difference from toxin B in the uninfected control (p < 0.01, n = 3).

Fig. 9. Pyridyl imidazoles and adenoviral DN-c-Jun attenuate apoptosis of CGNs induced by either toxin B or lethal toxin. Granule neurons were incubated for 24 h with 500 pg/ml toxin B (ToxB) (A) or 200 ng/ml lethal toxin (LTox) (B) alone or in combination with pyridyl imidazole inhibitors of p38/JNK as described in the legend to Fig. 8. Apoptosis was quantitated by nuclear staining with Hoechst dye. Asterisks indicate a significant difference from either toxin B (A) or lethal toxin (B) alone (p < 0.01, n = 3). C, cells were infected with either control adenovirus (Ad-CMV) or Ad-DN-c-Jun (each at a multiplicity of infection of 10) followed by incubation with toxin B; apoptosis was quantified by Hoechst staining. Asterisks indicate a significant difference from toxin B in the uninfected control (p < 0.01, n = 3).

Cortical neurons and rat brain neuroblasts the isoprenylation/plasma membrane localization of Rho GTPases correlates with neuronal survival (16, 17). Our data are the first to establish clearly a prosurvival function for Rho family GTPases in a neuronal cell model by utilizing highly selective inhibitors of Rho GTPase function (clostridial toxins). Collectively, these data indicate that as in non-neuronal cells, Rho GTPases can play either a prosurvival or proapoptotic role in neuronal cells depending on the type of neuron studied.

As mentioned above, growth factor-mediated survival of CGNs occurs primarily via a phosphatidylinositol 3-kinase-dependent activation of Akt (25, 34, 44). Akt, in turn, phosphorylates and inactivates several proapoptotic signaling molecules including GSK3β (36). Many studies have previously demonstrated a relationship between the Rac GTPase and phosphatidylinositol 3-kinase/Akt activity (45–47). Therefore, it was of interest to determine if inhibition of Rac function with C. difficile toxin B affected the activation status of either Akt or GSK3β in CGNs. In contrast to serum and potassium withdrawal, which led to an inactivation of Akt and a concurrent activation of GSK3β, toxin B had no effect on the activation status of either of these kinases. This result demonstrates that Rac does not influence phosphatidylinositol 3-kinase/Akt activity in CGNs significantly.

An inevitable downstream consequence of inhibiting Rho family GTPase function is the disruption of the actin cytoskeleton (26). In many non-neuronal cells, actin cytoskeletal disruption evokes apoptotic cell death (48–50). In the present study, apoptosis of CGNs induced by inhibition of Rho GTPases could be entirely dissociated from actin cytoskeletal disruption because agents that directly disassemble F-actin, C. botulinum C2 toxin, cytotoxicin D, and latrunculin A, did not induce cell death within 24 h. Similar results were observed in intestinal epithelial cells that also undergo apoptosis after incubation with toxin B but not cytotoxicin D (51). Our data indicate that the apoptosis observed in CGNs after blockade of Rac/Cdc42 function is likely the result of the inhibition of a specific downstream effector(s) of these GTPases rather than a global perturbation of CGN morphology. In this context, the serine/threonine kinase, p21-activated kinase, is a Rac/Cdc42 effector that has recently been shown to phosphorylate and inactivate proapoptotic signaling molecules such as the Bcl-2 family member Bad (52). Thus, future work will focus on the identification of Rac/Cdc42 effectors that promote survival of CGNs.
Inhibition of Rac/Cdc42 in CGNs induced a marked increase in the phosphorylation, expression, and nuclear accumulation of the transcription factor c-Jun. c-Jun is a required proapoptotic factor in both nerve growth factor withdrawal-induced death of sympathetic neurons (53) and in CGN death after withdrawal of serum and potassium (32). In primary sympathetic neurons, nerve growth factor withdrawal is associated with an increase in the activity of the stress-activated protein kinase JNK (53). In contrast, CGNs have been reported to contain high basal JNK activity and low p38 MAP kinase activity that are unaffected by withdrawal of serum and potassium (32). The observation that inhibition of Rac/Cdc42 function enhanced c-Jun phosphorylation is somewhat paradoxical in that Cdc42 and Rac have been implicated upstream of JNK activation by several groups (14, 54, 55). In the present study, we observed high basal JNK activity and low p38 activity in control cultures of CGNs, neither of which was significantly increased after incubation with toxin B. Thus, inhibition of Rac/Cdc42 in CGNs increases c-Jun phosphorylation independently of activating stress-activated protein kinase cascades. Moreover, these data suggest that JNK activity in CGNs is regulated by a mechanism that is distinct from Rac/Cdc42 signaling. Similar Rac/Cdc42-independent regulation of JNK has been observed in fibroblasts and endothelial cells in response to platelet-derived growth factor and interleukin-1 (56).

Finally, pyridyl imidazole compounds that inhibit both p38 and JNK activities in CGNs (40) significantly attenuated both the increase in c-Jun phosphorylation and apoptosis induced after inhibition of Rac/Cdc42 with either toxin B or lethal toxin. These results are in agreement with previous data that showed that pyridyl imidazoles also inhibit apoptosis of CGNs evoked by withdrawal of serum and potassium (40) and improve the survival of transplanted neurons in vivo (41). In addition, CGN apoptosis induced by toxin B was also attenuated significantly by adenosine DN-c-Jun. The above results suggest that the high basal JNK activity detected in CGNs is likely responsible for the enhanced phosphorylation of c-Jun after inhibition of Rac/Cdc42. Furthermore, c-Jun phosphorylation is required for apoptosis of CGNs in response to Rac/Cdc42 inhibition. The mechanism underlying the JNK-mediated phosphorylation of c-Jun in the absence of a detectable increase in JNK activity may involve translocation of a small pool of active JNK to the nucleus (39). Alternatively, modification of the association between JNK and the JNK-interacting protein JIP, which inhibits the JNK/c-Jun interaction, may also play a role (39). Interestingly, JIP has recently been shown to associate with a guanine nucleotide exchange factor for the Rho family GTPases (57). This latter observation suggests the possibility that active Rho GTPases may be required to maintain the JNK-JIP interaction, and therefore, inhibition of Rho GTPases may release active JNK from JIP, allowing for its interaction with and phosphorylation of c-Jun.

In summary, we have demonstrated that selective inhibition of Rac/Cdc42 with clostridial toxins induces apoptosis of CGNs. Cell death was observed in the presence of serum and depolarizing potassium, indicating that growth factor-mediated and Ca²⁺-dependent survival signals could not compensate for the loss of GTPase function. Apoptosis occurred independently of actin cytoskeletal disruption but required signaling via the transcription factor c-Jun. These data indicate that specific effectors of Rac/Cdc42 GTPases promote the survival of CGNs.

Acknowledgment—We thank Ron Bouchard for assistance with the preparation of the manuscript.
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