BOTULINUM TOXIN TYPE A TARGETS RHOB TO INHIBIT LYSOPHOSPHATIDIC ACID-STIMULATED ACTIN REORGANIZATION AND ACETYLCHOLINE RELEASE IN NGF-TREATED DIFFERENTIATED PC12 CELLS

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ABSTRACT

It is generally accepted that Botulinum toxin cleaves the 25-kDa synaptosomal-associated protein to inhibit acetylcholine release (neuroexocytosis). Since several reports suggest another mechanism, we investigated possibility that inhibition of neuroexocytosis by the toxin occurs through the RhoB signaling which controls actin cytoskeletal organization. We found that the G-protein activator lysophosphatidic acid (LPA) triggers actin reorganization followed by acetylcholine release in PC12 cells, and that botulinum toxin blocks both events through ubiquitin-dependent degradation of RhoB by the proteasome. Overexpression of wild-type RhoB caused actin reorganization and enhanced release of acetylcholine, and overcame the toxin's inhibitory effect on actin reorganization and exocytosis.

INTRODUCTION

The phospholipid lysophosphatidic acid (LPA) promotes actin cytoskeletal changes through a Rho-mediated signaling pathway in a variety of cells (4). This actin reorganization in neuronal cells is considered to be prerequisite to neurotransmitter release from presynaptic neurons (5). Botulinum toxin A (BoNT/A) inhibits ACh release at peripheral cholinergic synapses by the metalloprotease activity (Zn^{2+}-dependent) of its light-chain (L-chain, 50 kDa) on SNAP-25 (25 kDa synaptosomal-associated membrane protein. However, deletion of SNAP-25 does not result in a complete blockade of exocytosis (6), and toxin that has been stripped of Zn^{2+} still blocks exocytosis (7). These findings suggest that another pathway may exist through which BoNT/A inhibits exocytosis.

In this study, we focused on the role of Rho GTPases and the actin cytoskeleton on regulated ACh exocytosis in NGF-differentiated PC12 cells.

MATERIALS AND METHODS

Antibodies and materials. RhoA, RhoB, polyclonal antibodies, and protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SNAP-25 antibody was from Sternberger Monoclonals (Lutherville, MD). Botulinum toxin type A (BoNT/A) was procured from Wako (Richmond, VA).

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Cell Culture. PC12 cells were kindly provided by Gordon Guroff (NIH, Bethesda, MD

ACh release study ACh release stimulated by either KCl (80 mM) or LPA (0.2-100 μM) was measured according to the method of Ray et al. (8).

Actin localization and reorganization. Actin architecture was visualized by immunohistochemistry as described by Trifaro et al. (5). Immunocomplexes (actin filaments) were examined by Bio-Rad confocal and/or Nikon fluorescence microscopy imaging systems.

Immunoprecipitation and Western blotting. For immunoprecipitation followed by Western blotting to detect the ubiquitinated RhoB. Immunoreactive bands were visualized by ECL with use of appropriate antibodies.

Inhibition of RhoB degradation by proteasome inhibitors. Proteasome specific inhibitors (lactacystin, MG-132, and proteasome inhibitor I) were dissolved in 100% dimethylsulfoxide (DMSO). And used at 10 μM final concentration, with DMSO at 0.3% final concentration.

Expression constructs and overexpression of wRhoB. wRhoB was amplified from a rat brain cDNA by polymerase chain reaction (PCR). The PCR product was then cloned into pTracerCMV.

RESULTS

ACh release by LPA. At doses of 0.6 to 100 μM, LPA stimulated ACh release ~1.5 to 2.5-fold over the basal release rate (no LPA; Fig. 1). The maximum amount of ACh release by LPA was equivalent to levels (physiologically triggered by high K^+) known to trigger depolarization- and Ca^{2+}-induced exocytosis. Given that LPA interacts exclusively with G-protein-coupled receptors, these data suggest that exocytosis in NGF-treated differentiated PC12 cells may be regulated by a LPA-stimulated G-protein signaling pathway.

Fig. 1

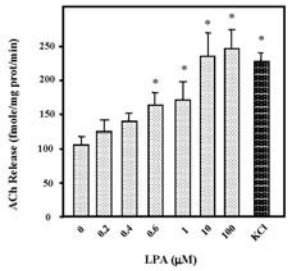
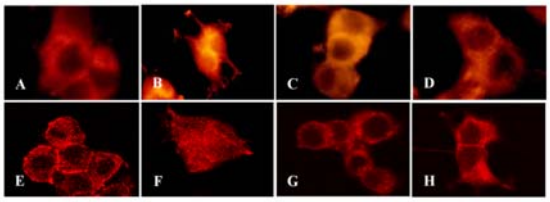


Fig. 2



Actin reorganization by LPA and the stabilization of actin architecture by BoNT/A. Actin reorganization is a prerequisite for exocytosis. Therefore, the effect of LPA on actin architecture in NGF-treated differentiated PC12 cells was investigated before and after treatment with LPA (10 μM) and/or BoNT/A (10 nM) using both fluorescence microscopy (FM) and confocal microscopy (CM). In untreated cells, strong actin rings were visualized along the outer limiting cell membranes (Fig. 2A, E) while very diffuse actin staining was apparent in the cytosol (Fig. 2E). In LPA-stimulated cells, FM revealed the absence of the ring and the distribution of actin structures throughout the cytosol (Fig. 2B). CM confirmed that actin reorganization resulted in the loss of the actin ring and the accumulation of filamentous actin structures throughout the cytosol within 10 min after the stimulation (Fig. 2F). When cells were pretreated with BoNT/A (10 nM) for 4 h followed by exposure to LPA, no such actin

reorganization was observed along the membrane (Fig. 2D) or in the cytosol (Fig. 2H) suggesting that BoNT/A stabilized the actin architecture. BoNT/A treatment alone did not promote actin organization (Fig. 2C, G).

Fig. 3A

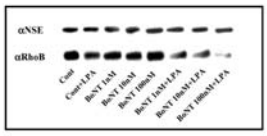
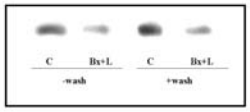


Fig. 3B



RhoB associated with LPA and/or BoNT/A treatment. RhoB levels were not altered when cells were treated with either LPA (10 μM) for 10 min or BoNT/A (1 to 100 nM) for 4 h (Fig. 3A). However, a marked dose-dependent decrease in RhoB immunoreactivity was evident in cells treated with

Fig. 4A

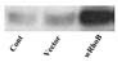
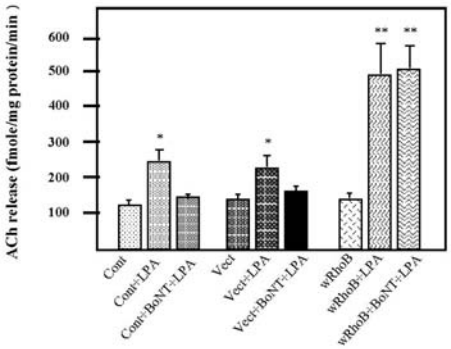


Fig.4B



BoNT/A followed by LPA stimulation for 10 min (Fig. 3A). No such difference in immunoreactivity was evident for neuron-specific enolase (NSE, internal control) suggesting that the decrease in RhoB was not due to a general decrease in protein levels (Fig. 3A). Since BoNT/A L-chain remains active (and therefore toxic) in the cytosol for several months (9), we examined whether the observed decrease

of RhoB was due to the activity of the L-chain. Cells were exposed to BoNT/A for 4 h followed by incubation for another 24 h in fresh medium lacking BoNT/A. The cells were then exposed to LPA (10 μM) for 10 min and RhoB levels were analyzed. Decreased RhoB immunoreactivity was still evident, suggesting that this decrease was due to the action of the BoNT/A L-chain (Fig. 3B). No changes were observed in the immunoreactivity of RhoA, an internal control Rho protein (Fig. 6B), or of RhoGDI (data not shown), the function of which is regulated by its protein level (10).

Fig.4 C

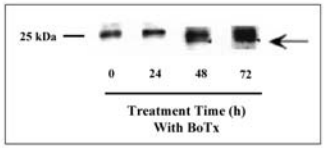
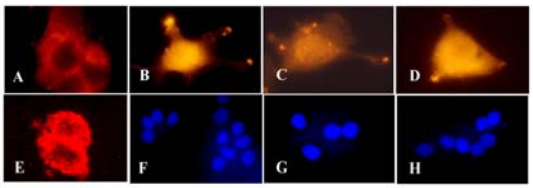
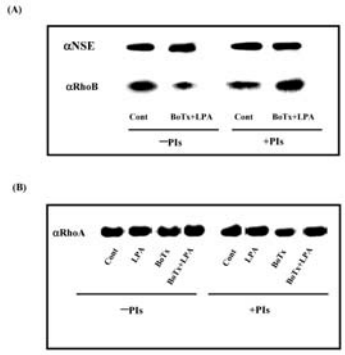


Fig. 5



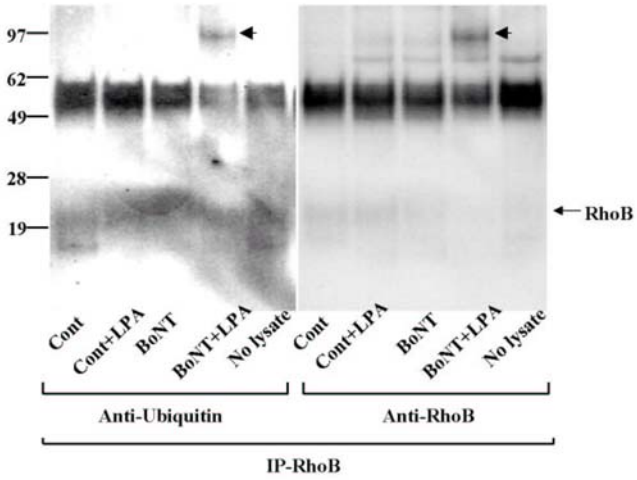
Effects of wRhoB overexpression on the inhibition of ACh release by BoNT/A. Western blotting confirmed that wRhoB was expressed at a high level 24 h after transfection (Fig. 4A). PC12 transfectants that overexpressed wRhoB released about 4-fold more ACh upon LPA stimulation compared to normal PC12 cells (Fig. 4B). There was no difference in the basal (no LPA) level of ACh release between the normal cells and wRhoB transfectants (Fig. 4A). PC12 cells transfected with vector alone showed the same amount of LPA-induced ACh release as untransfected cells (~2-fold over basal level) indicating that vector transfection had no effect on either basal or stimulated ACh release (Fig. 4B). Interestingly, when BoNT/A was tested, PC12 transfectants that overexpressed wRhoB did not show the expected decrease in ACh release after LPA treatment suggesting that wRhoB overexpression antagonized the toxin's inhibitory effect on exocytosis (Fig. 4B). Figure 4C shows a typical time course study of ACh release in the transfectants with or without BoNT/A treatment. It should be noted that SNAP-25 degradation in the presence of BoNT/A is appreciable only after the cells were exposed to the toxin for 24 h (Fig. 4D).

Fig. 6



Effects of wRhoB overexpression on actin reorganization by LPA and stabilization of actin architecture by BoNT/A. Figure 5 shows the effects of LPA and/or BoNT/A treatment on actin architecture in wRhoB overexpressing PC12 cells. Cells transfected with vector alone (Fig. 5E) showed no change in the basic actin architecture compared to control and vector-transfected cells (no treatment, Fig. 5A and E) in that strong actin staining was evident as a ring along the cell membrane. Interestingly, wRhoB overexpression alone resulted in the reorganization of actin without LPA stimulation, as the ring disappeared while the cytosol accumulated actin filaments (Fig. 5B). BoNT/A treatment did not interfere with this actin reorganization in the wRhoB-overexpressing transfectants regardless of LPA treatment (Fig. 5C, D). These data indicate that BoNT/A inhibits LPA-stimulated actin reorganization and that overexpression of wRhoB not only results in actin reorganization but also overcomes the toxin's inhibitory effect on actin reorganization by LPA. The data also strongly suggest that the RhoB signaling pathway plays an important role not only in actin reorganization but also in exocytosis stimulated by LPA (via regulating actin reorganization). Furthermore, BoNT/A may target RhoB thus interfering with ACh release by blocking LPA-stimulated actin rearrangement in NGF-treated differentiated PC12 cells. There was no significant decrease in cell number 24 h after transfection with either wRhoB or vector (data not shown), and DAPI staining showed that the nuclei of the wRhoB (5G) or vector (5H) transfectants were intact, as seen in control cells (Fig. 5F).

Fig. 7



RhoB degradation by proteasomes. Since many short-lived regulatory proteins involved in the cell cycle or apoptosis are degraded by proteasomes, we also investigated whether the decrease in RhoB levels was due to degradation by this pathway (1, 5). Therefore, we used combinations of the specific

PIs lactacystin, MG132, and proteasome inhibitor I to study whether the rapid decrease in RhoB associated with BoNT/A and LPA treatment was due to degradation by 26S proteasomes. When BoNT/A-treated cells were incubated for 2 h in medium A containing proteasome inhibitors and then stimulated with LPA, RhoB immunoreactivity returned to control levels (Fig. 6A) suggesting that the decrease in RhoB was due to proteasome-mediated degradation. In contrast, no change in RhoA immunoreactivity was evident in the control (no treatment) or in LPA- and/or BoNT/A-treated cells either in the presence or absence of PIs (Fig. 6B). These data suggest that RhoB but not RhoA is degraded by proteasomes.

Ubiquitination of RhoB. Since most proteins that undergo proteasome degradation are ubiquitinated (11), we tested whether treatment of cells with both BoNT/A and LPA induced ubiquitination of RhoB. We did not observe the typical ubiquitin-immunoreactive smear/ladder usually observed in Western blots of proteins undergoing ubiquitin-mediated degradation (12). However, both ubiquitin and RhoB antibodies detected an intense band (~95 kDa) in cells treated with both BoNT/A and LPA (Fig. 7). Another intense band (~50 kDa) corresponded to IgG heavy chain that was also detected in the negative control (no lysate).

DISCUSSION

Our data on LPA-stimulated ACh release from NGF-treated PC12 cells indicate that the release is controlled by the G-protein-related signal transductio. As shown in Fig.2, intense actin ring staining and diffuse distribution of actin in the cytosol was observed in untreated control cells under both FM and CM. Microscopy revealed the rapid disappearance of the ring and redistribution of actin throughout the cytoplasm following the addition of LPA to the culture medium, indicating that LPA induces the reorganization of the actin cytoskeleton.

Since only RhoB is both inducible and degradable, and this protein has a short half-life suggesting that its function in signaling processes, accordingly, we followed RhoB expression levels in cells following exposure to BoNT/A and/or LPA stimulation. As shown in Figure 3A, dose-dependent degradation of RhoB occurred in cells co-treated with BoNT/A and LPA. Further studies will address whether RhoB, like SNAP-25, is a substrate for L-chain-mediated degradation.

Surprisingly, overexpression of wRhoB caused actin reorganization in the absence of LPA stimulation, and enhanced ACh release upon LPA stimulation. These findings strongly suggest that actin reorganization is requisite for ACh release and that RhoB is involved in regulating this step, perhaps through changes in its expression level.

As Figure 6A demonstrates, proteasome inhibitors halted the decrease of RhoB in the presence of BoNT/A and LPA, strongly suggesting that RhoB is degraded by proteasomes. In contrast, under identical conditions RhoA levels were unchanged (Fig. 6B) suggesting that RhoB but not RhoA is responsible for actin rearrangement and ACh release in response to LPA. The ~95 kDa band is consistent with the expected size of RhoB conjugated to nine ubiquitin molecules, and was apparent only after treatment with BoNT/A and LPA suggesting that this treatment triggers RhoB ubiquitination.

Further study is necessary to clarify the ubiquitination mechanism for RhoB Degradation by BoNT/A, because our work does not address why BoNT/A+LPA treatment initiates ubiquitination.

CONCLUSIONS

Our results show that BoNT/A targets RhoB to promote accelerated ubiquitin-dependent RhoB degradation by proteasomes thereby blocking actin dynamics and ACh release. Our data also suggest that a RhoB-related signaling pathway is involved in neuroexocytosis in NGF-treated differentiated PC12 cells.

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