A Rho-related GTPase Is Involved in Ca\(^{2+}\)-dependent Neurotransmitter Exocytosis*

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Frédéric Doussau‡‡, Stéphane Gasman¶, Yann Humeau‡, Francesco Vitiello‡, Michel Popoff**, Patrice Boquet‡‡, Marie-France Bader‡§, Yann Humeau‡, Francesco Vitiello‡, Michel Popoff**, Patrice Boquet‡‡, Marie-France Bader‡§, and Bernard Poulain$$$§§

From the ‡‡Laboratoire de Neurobiologie Cellulaire, CNRS, UPR 9009 and $$INSERM, U-338 Biologie de la Communication Cellulaire, F-67084 Strasbourg Cédex, **Toxines Microbiennes, Institut Pasteur, F-75774 Paris Cédex 15, and $$$INSERM, U-452 Biologie Cellulaire et Moléculaire des Microorganismes et de leurs Toxines, F-06109 Nice Cédex 2, France

Rho, Rac, and Cdc42 monomeric GTPases are well known regulators of the actin cytoskeleton and phosphoinositide metabolism and have been implicated in hormone secretion in endocrine cells. Here, we examine their possible implication in Ca\(^{2+}\)-dependent exocytosis of neurotransmitters. Using subcellular fractionation procedures, we found that RhoA, RhoB, Rac1, and Cdc42 are present in rat brain synaptosomes; however, only Rac1 was associated with highly purified synaptic vesicles. To determine the synaptic function of these GTPases, toxins that impair Rho-related proteins were microinjected into Aplysia neurons. We used lethal toxin from Clostridium sordelli, which inactivates Rac; toxin B from Clostridium difficile, which inactivates Rho, Rac, and Cdc42; and C3 exoenzyme from Clostridium botulinum and cytotox necrotizing factor 1 from Escherichia coli, which mainly affect Rho. Analysis of the toxin effects on evoked acetylcholine release revealed that a member of the Rho family, most likely Rac1, was implicated in the control of neurotransmitter release. Strikingly, blockage of acetylcholine release by lethal toxin and toxin B could be completely removed in <1 s by high frequency stimulation of nerve terminals. Further characterization of the inhibitory action produced by lethal toxin suggests that Rac1 protein regulates a late step in Ca\(^{2+}\)-dependent neuroexocytosis.

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Rho proteins form a subfamily of highly conserved small GTPases belonging to the Ras superfamily. In mammals, Rho GTPases comprise Rho (A to H isoforms), Rac (Rac1 and Rac2 isoforms), Cdc42 (Cdc42Hs and G25K isoforms), and more distant members. Like other monomeric GTPases of the Ras family, Rho proteins act as molecular switches: upon receiving upstream signals, they are converted into an active GTP-bound form that is able to interact with downstream effectors. These comprise protein kinase N, Rho kinase, phosphatidylinositol 4-kinase, phosphatidylinositol 5-kinase, and the myosin-binding subunit of myosin phosphatase (for review, see Ref. 1). In fine, the activation of Rho-related GTPases leads mainly to a rearrangement of the actin-based cytoskeleton and/or to a regulation of phosphoinositide levels. Rho proteins have been implicated in a large variety of cellular functions, including chemotaxis, cell cycle progression, axonal guidance, and endocytosis (for review, see Refs. 1–3).

Ca\(^{2+}\)-triggered neurotransmitter release and hormone secretion are closely related mechanisms that involve proteins common to both neurons and secretory cells. For example, synaptobrevin, syntaxin, 25-kDa synaptosomal-associated protein, synaptotagmin, soluble N-ethylmaleimide-sensitive factor, and soluble N-ethylmaleimide-sensitive factor attached proteins act in concert to ensure docking and/or fusion in both dense-core granules and synaptic vesicle exocytosis. Consistent with the implication of Rab proteins in vesicle trafficking, the small GTPase Rab3 has been shown to regulate both neurotransmitter and hormone secretion (for review, see Refs. 4–10). On the other hand, despite the similarities between the release of the content of synaptic vesicles and large dense-core granules, several recent studies have revealed mechanistic differences between these two exocytotic processes (for review, see Refs. 7 and 11).

In endocrine cells, members of the Rho family have been proposed to regulate exocytosis. Indeed, Cdc42 and Rac control regulated secretion in pancreatic beta cells (12), basophilic leukemia cells (13), and mast cells (14). In chromaffin cells, RhoA localized on secretory granules controls subplasmalemmal actin and exocytosis by regulating a granule-associated phosphatidylinositol 4-kinase (15, 16). Rho-like GTPases have been implicated in actin filament dynamics and organelle movement in growth cones (for review, see Ref. 17). The aim of our study was to probe the presence of Rho proteins in nerve terminals and to determine their possible implication in neurotransmitter release.

We found that Rac1, RhoA, RhoB, and Cdc42 are present in nerve terminals, with Rac1 selectively associated with purified synaptic vesicles. The role played by these small GTPases in neurotransmission was addressed by monitoring acetylcholine (ACh)\(^3\) release from identified cholinergic neurons in the Aplysia buccal ganglion. The function of Rho-related GTPases was impaired by presynaptic microinjection of bacterial toxins known to selectively activate or inactivate subgroups of Rho proteins (for review, see Ref. 18). Our results suggest that a Rho-related protein, most likely Rac1, plays a major role in neurotransmission by controlling a yet undefined Ca\(^{2+}\)-dependent late step of synaptic vesicle exocytosis.

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† Present address: Dept. of Neurobiology, Duke University Medical Center, Durham, 27710 NC.
‡ Present address: Dipt. di Farmacologia e Fisiologia Umana, Facoltà di Medicina e Chirurgia, Università di Bari, I-70124 Bari, Italy.
§§ To whom correspondence should be addressed: Lab. de Neurobiologie Cellulaire, CNRS, 5 rue Blaise Pascal, F-67084 Strasbourg Cédex, France. Tel.: 33-3-88-45-66-77; Fax: 33-3-88-60-16-64; E-mail: oulaim@neurochem.u-strasbg.fr.
**EXPERIMENTAL PROCEDURES**

**Toxin Preparation**—Lethal toxin (LT) from *Clostridium sordellii* strain IP82 was purified as described previously (19). Recombinant C3 exoenzyme was expressed in *Escherichia coli* strain Sure (Strategene) from pMRP143, consisting of the DNA fragment coding for the mature C3 protein (20) under the control of the iotoxin gene promoter in vector pJIR750 (21). After sonication bacterial cells in 10 ml Tris-HCl (pH 8.5), the extract was clarified by centrifugation, treated with proteinase A (Sigma; Merck) for 30 min at 4 °C, and centrifuged again. The supernatant was loaded onto a QAE-Sepharose A50 column (Amersham Pharmacia Biotech) equilibrated in 10 ml Tris-HCl (pH 8.5). The flow-through containing the purified C3 enzyme showed a single band of 25 kDa on SDS-polyacrylamide gel electrophoresis. Cytoxic necrotizing factor 1 (CNF1), a 110-kDa protein toxin from pathogenic *E. coli* strains, was purified as described previously (22, 23). Stock solutions (20 μM) were prepared in 50 μM Tris-HCl (pH 7.4) containing 100 μM NaCl. A recombiant C-terminal 14.8–31.5 kDa peptide corresponding to the catalytic region of CNF1 was produced as a glutathione S-transferase fusion protein. It was purified according to a previously described procedure (24). A stock solution (20 μM) was prepared in sodium phosphate buffer (pH 7.4) containing reduced glutathione (20 mM). All toxins were stored at −80 °C in 3–5-μl aliquots. The various buffers used for storage of the toxins had no effect on evoked ACh release.

The biological activity of each batch of toxin was verified by the ability to induce morphological changes and cytoskeletal modifications (C3, ToxB, and LT: cell rounding and disruption of stress fibers; and CNF1: cell spreading and increase in stress fibers), visualized by staining of HeLa or Vero cells with fluorescein-conjugated phallidin (22, 23, 25). The activity of C3 was further determined by its ability to induce [3H]MDP-ribosylation of membrane-bound RhoB present in purified chromaffin granule preparations (15). The enzymatic activity of CNF1 on recombiant RhoA (22) or chromaffin granul-associated RhoA (data not shown) was demonstrated by an increase in the apparent molecular mass of the molecule on SDS gels.

**Isolation and Fractionation of Synaptic Vesicles from Rat Brain**—Synaptic vesicles were prepared from rat brains according to Huttner et al. (26). Cerebral cortices were dissected free of meninges, placed in an ice-cold homogenization buffer (320 mM sucrose and 4 mM Hepes-NaOH (pH 7.4)). From this point on, the material was kept at 4 °C. Cerebral corticals were dissected free of cerebellum, brainstem, and most of the midbrain and homogenized in the same buffer containing 1 mM phenethylmethylsulfonyl fluoride and 2 μM peptatin in a Teflon/glass homogenizer. The homogenate was then centrifuged for 10 min at 11,000 × g. The resulting supernatant was centrifuged for 15 min at 9200 × g, and the pellet was resuspended in 10 ml of buffered sucrose/brain and centrifuged for 15 min at 11,000 × g. The resulting pellet (synaptosomes) was resuspended in buffered sucrose, diluted with 9 volumes of ice-cold H2O (hypotonic lysis of synaptosomes), and immediately homogenized. Protease inhibitors (peptatin and phenethylmethylsulfonyl fluoride) and 1 mM Hepes (pH 7.4) at a final concentration of 7.5 mM were added, and the homogenate was incubated on ice for 30 min and then centrifuged for 20 min at 25,500 × g. The pellet containing synaptosomal membranes (i.e. plasma membrane, mitochondria, and granules) was saved. The supernatant was centrifuged for 2 h at 48,000 rpm in a Ti-60 rotor (Beckman Instruments). The resulting supernatant was cleared by centrifugation at 100,000 × g and saved (cytosol). The pellet (crude synaptic vesicles) was resuspended in 4 ml of 30 mM sucrose and 4 mM Hepes (pH 7.4), homogenized, passed five times back and forth through a 25-gauge needle, loaded on a continuous gradient of 50–800 mM sucrose in 4 mM Hepes-NaOH (pH 7.4), and centrifuged for 1 h at 25,000 rpm in a SW28 rotor (Beckman Instruments). After centrifugation, 2-ml fractions were collected. Fractions in the 200–400 mM sucrose region were pooled and chromatographed on a glycercyl-coated controlled-pore glass bead column (GLY 0300B, Electro-Nucleons Inc.) to obtain highly purified synaptic vesicles (26). Protein content of the various fractions was analyzed by the Bradford procedure (Bio-Rad).

**Gel Electrophoresis and Immunoblotting**—SDS-polyacrylamide gel electrophoresis was performed on 12% acrylamide gels in Tris/glycine buffer (27). Proteins were transferred to nitrocellulose sheets, and blots were developed with secondary antibodies coupled to alkaline phosphatase (Sigma); immunoreactive bands were detected with 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml) and nitro blue tetrazolium (0.3 mg/ml in 40 mM sodium carbonate and 5 mM MgCl2 (pH 9.8). In some experiments, blots were developed with secondary antibodies coupled to horseradish peroxidase (Amersham Pharmacia Biotech), and immuno-reactive bands were revealed with the ECL system (Amersham Pharmacia Biotech).

**Antibodies**—Mouse monoclonal antibodies against Rac1 (Transduction Laboratories) were used at 1:500 dilution. Mouse monoclonal antibodies against RhoA (Santa Cruz Biotechnology) and Cdc42 (Santa Cruz Biotechnology) were used at 1:50 and 1:100 dilutions, respectively. Mouse monoclonal anti-synaptogamin antibodies against the C2a domain (clone 1D12), a generous gift from Dr. Masami Takahashi (Mitsubishi Kasei Institute, Tokyo, Japan), were diluted 1:1000. Rabbit antibodies against the cytosolic epitope EKQGYPQNYGQ of synaptophysin (28) were kindly provided by Dr. Nicolas Morel (Laboratoire de Neurobiologie Cellulaire, CNRS, Gif-sur-Yvette, France) and utilized at 1:1000 and 1:2000 dilutions.

**ACh Release and Electrical Recordings at Aplysia Synapses**—Experiments were performed at identified cholinergic synapses (29) in buccal ganglia of *Aplysia californica* (70–120 g of body weight; Marinus Inc., CA) according to previously published procedures (30, 31). Briefly, two presynaptic cholinergic interneurons (100–150 μm in diameter) and one postsynaptic neuron (150–200 μm in diameter) in the buccal ganglion were impaled with two glass microelectrodes (3 M KCl and Ag/AgCl, 2–10 megaohms). ACh release from a presynaptic neuron was monitored by evoking an action potential at 40-ss intervals. In some experiments, 1- or 2-s trains of stimuli were generated by using supramaximal depolarizing pulses of 5 ms separated by a repolarizing phase of adequate duration (SMP-311 pulse generator, Bio-Legic S.A., Grenoble, France).

ACh release was estimated by measuring the amplitude of the evoked postsynaptic current (at these synapses, it is a Cl− current) using a conventional two-electrode voltage-clamp technique and subsequently converting it to an apparent membrane conductance by taking into account the null potential for Cl− (i.e. the reversal potential of the postsynaptic response). The holding potential of the postsynaptic neuron was maintained at 30 mV above ECl−.

Dissected buccal ganglia were maintained at 22 °C using a Peltier plate system and superfused continuously (50 ml/h) with a physiological control medium containing NaCl (460 mM), KCl (10 mM), CaCl2 (33 mM), MgCl2 (2 mM), and MgSO4 (28 mM) in 10 mM Tris-HCl (pH 7.5). This diffusion-rich medium has a high [Ca2+]i/[Mg2+]i ratio (0.42) to minimize fluctuations in evoked ACh release due to spontaneous neuron firing activity. To modify the extracellular CaCl2 concentration, the respective concentrations of CaCl2 and MgCl2 were calculated according to the following equations: [CaCl2] (mM) = [Ca2+]i/[Mg2+]i (Q + 1) and [MgCl2] (mM) = [Ca2+]i/([Ca2+]Q + 1)(W), where Q is the [Ca2+]i/[Mg2+]i ratio. When CaCl2 was added, it was added directly to the control medium. To reduce the intracellular concentration of Ca2+ ions, EGTA was applied intraneuronally by pressure injection (see below). Possible intracellular pH changes were avoided by preparing EGTA in Tris-HCl (pH 7.4) with a 2.2-fold excess of Tris base.

**Application of Toxins to Neurons**—To limit the toxin action to a given neuron without modifying ACh release by a change in the ACh receptor efficacy, neurons were microinjected into presynaptic neurons. The sample to be injected was mixed with a vital dye (10% (v/v) fast green; Sigma). The samples were air pressure-injected under visual and electrophysiological monitoring. The injected volume was in the range of 1% of the cell body volume. Following intracellular injection, only neurons with membrane potentials of −60 to −45 mV and with no alterations in the action potentials were utilized.

**Other Methods**—Unless indicated, data are presented as mean ± S.D. Statistical significance of the data was calculated by paired or unpaired t tests.

**RESULTS**

**Subcellular Distribution of Related Proteins in Synaptic Terminals**—The intracellular distribution of Rho proteins in presynaptic terminals was investigated in synaptosomes prepared from rat brains since the amount of neuronal tissue that can be collected from *Aplysia* does not allow subcellular fractionation. Fig. 1 shows a Western blot analysis of the soluble and membrane-bound fractions obtained from presynaptosomes by hypotonic lysis. Using specific antibodies raised against various members of the Rho family, we found that RhoA, RhoB, Rac1, and Cdc42 were present in the presynaptic terminals. In contrast to RhoA, which was largely cytosolic, and Cdc42, which was present in both cytosolic and membrane-bound fractions, RhoB and Rac1 were mostly detected in the
particulate fraction containing synaptosomal plasma membrane, mitochondria, large dense-core particles, and a huge amount of synaptic vesicles. To probe the direct association of Rho proteins with the membrane of synaptic vesicles, crude synaptic vesicles obtained by high speed centrifugation (see “Experimental Procedures”) were further fractionated on a 50–800 mM sucrose velocity gradient. Fig. 2 shows the distribution of two synaptic vesicle marker proteins estimated by immunoreplica analysis in the fractions collected from the sucrose gradient. Synaptic vesicles were distributed in fractions 7–18 as revealed by the immunosignal for synaptophysin and synaptotagmin. Fractions 7–18 were also labeled with anti-RhoB and anti-Rac1 antibodies (Fig. 2B), as revealed by the immunosignal for synaptophysin and synaptotagmin. Fractions 7–18 were also labeled with anti-RhoB and anti-Rac1 antibodies and polyconal anti-RhoB and anti-Cdc42 antibodies. B, C, and D, shown are the results from the quantitative analysis of the distribution of Rho proteins in synaptosomes. B, total protein content; C, Rho protein content estimated by immunodetection on nitrocellulose and scanning densitometry analysis. In B and C, the values correspond to the distribution of proteins and Rho immunoreactivity relative to the total amount detected in the synaptosomal lysates. Similar results were obtained in two separate experiments.

**FIG. 1.** Immunochemical detection of Rho-related proteins in subcellular fractions from brain synaptosomes. Brain synaptosomes were lysed by hypotonic shock and processed to separate the cytosol, the membrane-bound compartment, and the crude synaptic vesicle fraction. A, protein (10 μg) from total synaptosomes (Sy), synaptosomal membranes (Sy Mb), or the cytosol was subjected to gel electrophoresis and immunodetection on nitrocellulose sheets using monoclonal anti-RhoA and anti-Rac1 antibodies and polyclonal anti-RhoB and anti-Cdc42 antibodies. B, C, and D, shown are the results from the quantitative analysis of the distribution of Rho proteins in synaptosomes. B, total protein content; C, Rho protein content estimated by immunodetection on nitrocellulose and scanning densitometry analysis. In B and C, the values correspond to the distribution of proteins and Rho immunoreactivity relative to the total amount detected in the synaptosomal lysates. Similar results were obtained in two separate experiments.

**FIG. 2.** Rac1 is associated with purified synaptic vesicles. A, total protein profile of fractions collected from a sucrose velocity gradient layered with the crude synaptic vesicle fraction; B, immunoblot analysis of synaptic vesicle markers and Rho proteins in each fraction of the gradient (10 μg of protein/lane); C, immunoblot analysis of synaptic vesicles purified by permeation chromatography on a controlled-pore glass column (CPG).

specifically glucosylates Rac, Cdc42, and Rho (34); LT, which glucosylates Rac, but has no effect on Rho and Cdc42 (25); and C3, which ADP-ribosylates RhoA, RhoB, RhoC, and Aplysia Rho (35, 36) and, under certain circumstances, Rac (35, 37–39). Glucosylation or ADP-ribosylation in the effector domain of the various Rho, Rac, and Cdc42 isoforms disrupts their interaction with downstream effectors and thereby inactivates the intracellular pathways controlled by these GTPases. ToxB, LT, or C3 was pressure-injected into one presynaptic neuron, and the second presynaptic neuron was injected with the buffer used for toxin injection. In this way, we had an internal control for the stability of evoked neuroexocytosis for the duration of the experiments (up to 24 h). As shown in Fig. 3, all three toxins inhibited ACh release. More important, neither the action potential that triggers ACh release at nerve terminals nor the transmembrane resting potential and the membrane resistance of the injected neurons were significantly modified after injection of the toxins (data not shown). Therefore, the inhibition of ACh release induced by LT, ToxB, or C3 is not due to a modification of membrane excitability. The mean inhibition induced by the three toxins, calculated 3 and 20 h after injection, is shown in Fig. 3B. The three toxins induced an almost complete inhibition of neurotransmitter release 20 h after injection (Fig. 3B). Note, however, that high doses of C3 (2 μM final intraneuronal concentration) were required to abolish neurotransmission (Fig. 3B). Collectively, these results suggest that Rac is the most likely candidate to regulate a rate-limiting step of exocytosis in neurons since it is the sole GTPase inhibited by LT, ToxB, and high concentrations of C3.

**Is Rho Implicated in Neuronal Exocytosis?—**Rho isoforms can be constitutively activated by CNF1, which catalyzes the deamidation of glutamine 63. This residue is conserved in RhoA, RhoB, RhoC, and Aplysia Rho. Transformation of glutamine 63 to glutamic acid leads to inhibition of both intrinsic and Rho GTPase-activating protein-stimulated GTPase activity of Rho proteins (22, 40). Thus, the CNF1 action on Rho is equivalent to the amino acid substitution used to generate dominant-positive Rho mutants. Cdc42 can also be activated by a high concentration of this enzyme (22, 23, 40). To further evaluate the implication of Rho and Cdc42 in neuroexocytosis, dichainal CNF1 (200 nm final intraneuronal concentration) or a CNF1 recombinant catalytic moiety (data not shown) was mi-
injection or after 3 and 20 h); values for control neurons at 3 and 20 h were not significantly different from control neurons not injected with toxins are denoted (Cont.). The final concentration of LT in the cell body was ~50 nM. The second presynaptic neuron (C) was injected with control buffer and served as an internal control of release stability. B, shown is a comparison of the ACh release (mean ± S.D.) in gray bars) or 20 h (black bars) after injection of LT, ToxB, or C3. The final intrasynaptic concentrations of the toxins are indicated. Release values from control neurons not injected with toxins are denoted (Cont.). The decrease in ACh release that was observed after toxin injection is significant (p < 0.001) as compared with the various controls (before injection or after 3 and 20 h); values for control neurons at 3 and 20 h were not significantly different from values recorded before the time of injection.

crine injection into presynaptic neurons. As illustrated in Fig. 4A, no significant modification of ACh release was observed for at least 150 min. In longer experiments, CNF1 induced an alteration of membrane excitability. We also examined whether Rho activation by CNF1 would be able to restore ACh release previously inhibited by LT. LT (50 nM final concentration) was first injected into a presynaptic neuron, and after ACh release had stabilized, CNF1 (200 nM final concentration) was injected into the same neuron. As illustrated in Fig. 4B, CNF1 was unable to rescue the ACh release inhibited by LT treatment. These experiments suggest that neither Rho nor Cdc42 plays a crucial role in neurotransmitter release.

Effect of High Frequency Train of Stimuli on ACh Release Blocked by Clostridial Toxins—To further define the step in neurotransmitter release that is controlled by Rac, we examined the effect of high frequency stimulation on LT-induced blockage of ACh release. Trains of stimuli were elicited at 50 Hz for 1 or 2 s. Under control conditions, after a brief facilitation (31), ACh release declined slightly during a train stimulus (Fig. 5A, open circles), probably due to an imbalance between the replenishment of the readily releasable pool of vesicles and the number of vesicles that undergo exocytosis. In LT-injected neurons, the time course of ACh release evoked by a 50-Hz stimulation train was greatly modified (Fig. 5A, closed circles): ACh release stabilized for ~400 ms (404 ± 70 ms, n = 46) and then increased to reach within 1 s a level that was similar to that observed before LT injection (Fig. 5A, compare open and closed circles). This indicates that LT-induced inhibition of ACh release can be almost circumvented by high frequency stimulation. Note, however, that this recovery in response to 50-Hz trains declined after 10 h of LT-induced blockage (data not shown). The recovery of ACh release lasted 1.5–2 s after the end of the 50-Hz train; the synapse then returned to the blocked state with a time constant in the range of 15–60 s (see decay of ACh release in Fig. 5C). The short duration of the recovery phase indicates that this effect is distinct from the post-tetanic potentiation of ACh release that can be elicited by repetitive stimuli at Aplysia synapses and that lasts for >30 min (31). The restoration of neurotransmission depended largely on the stimulation frequency because 10-Hz trains after several seconds of stimulation only partially restored the activity of LT-inhibited synapses (data not shown). Interestingly, 50-Hz trains induced a similar recovery of ACh release in synapses inhibited by ToxB (data not shown) or high concentrations of C3 (Fig. 5B).

To determine if this release recovery was specifically related to the blockage induced by the inactivation of an intraneuronal GTPase, we examined the ACh liberation during 50-Hz trains in buccal ganglia in which ACh release had been previously depressed either by partial inhibition of Ca2+ channels (by adding CdCl2 to the superfusion medium) or by lowering extracellular Ca2+ concentration: superfusion of the preparation with a medium containing a Ca2+/Mg2+ ratio of 2.1 instead of 0.42 allowed influx of Ca2+ into presynaptic neurons in which ACh release was inhibited by preinjection of LT. We found that EGTA completely abolished the recovery of ACh release observed during 50-Hz stimulation in LT-injected neurons (Fig. 6A, compare open and closed circles). The calcium dependence of 50-Hz-induced recovery was further confirmed by increasing the extracellular Ca2+ concentration: superfusion of the preparation with a medium containing a Ca2+/Mg2+ ratio of 2.1 instead of 0.42 allowed a faster recovery of ACh release in response to 50-Hz trains (Fig. 6B, compare delays d1 and d2).

Effect of LT and C3 Injection on Paired-pulse Facilitation—Inhibition of ACh release by LT, ToxB, or high concentrations of C3 may result from a diminution of either the release probability (p) or the number of releasable vesicles (n) docked at the plasma membrane. An easy procedure to discriminate between p or n is to examine paired-pulse facilitation (PPF). PPF depends on the increase in amplitude of a second (test) response compared with a conditioning response following two successive stimulations. PPF amplitude depends mainly on p; a reduction of p is generally accompanied by an increase in PPF...
amplitude probably due to the fractional desaturation of the release machinery (41). For instance, lowering extracellular [Ca\(^{2+}\)] reduces \(p\). Accordingly, PPF was strongly increased when the \([Ca^{2+}]/[Mg^{2+}]\) ratio was reduced from 0.42 to 0.14 (Fig. 7A), an experimental condition that reduced ACh release to 28.5 ± 7%. To accurately determine PPF amplitude, ACh release levels must be stable. Hence, PPF protocols were elicited before and after LT or C3 injection, when ACh release was nearly stabilized. Fig. 7A shows that, despite the reduction of ACh release to 29.8 ± 2.5% by LT or to 33.5 ± 4.5% by 2 \(\mu\)M C3, PPF amplitude was not significantly modified, in contrast to the strong increase in PPF observed in low extracellular Ca\(^{2+}\) (Fig. 7A). This difference is further illustrated in an experiment performed in a synapse in which both experimental conditions were examined: PPF was first determined in low Ca\(^{2+}\) and then, after LT-induced inhibition, in physiological calcium (Fig. 7B). Whatever the time interval between the paired stimuli, we found that PPF was not significantly modified by the injection of LT, although it significantly increased when extracellular [Ca\(^{2+}\)] was reduced (see legend to Fig. 7B). Taken together, these results suggest that the inhibition produced by LT or C3 injection is likely to be linked to a decrease in the number of vesicles available for release rather than to a diminution of their release probability. Hence, a Rho-related GTPase appears to control the size of the pool of readily releasable synaptic vesicles in neurons.

**DISCUSSION**

**Rac1, RhoA, RhoB, and Cdc42 Are Present in Nerve Terminals**—The aim of this study was to determine the occurrence of Rho-related proteins in nerve terminals and their possible implication in neurotransmitter release. By subcellular fractionation of rat brain and immunoblot analysis, we found that RhoA, RhoB, Rac1, and Cdc42 are present in both cytosolic and membrane-bound compartments of purified synaptosomes. In addition, we demonstrated that only Rac1 is associated with synaptic vesicles. The absence of Rho on synaptic vesicles contrasts with the previously reported localization of RhoA on secretory granules in pituitary (42) and chromaffin (15) cells. This interesting difference might be related to mechanistic differences in the regulation of synaptic vesicle and secretory granule exocytotic processes.

**Are Rho GTPases Implicated in Neurotransmitter Exocytosis?**—To investigate the role of Rho-related proteins in neurotransmitter release, we injected into presynaptic *Aplysia* neurons various clostridial toxins (LT, C3, ToxB, and CNF1), which activate or inactivate specific members of the Rho family. We found that ToxB, which specifically ADP-ribosylates Rho isoforms at low concentrations (35, 36), but also affects Rac at high doses (35, 37–39). C3 inhibited neurotransmission only when injected at micromolar concentrations into the presynaptic neuron. Taken together, these observations suggest that Rac1 may participate in the molecular machinery underlying neurotransmitter release.

In addition to Rac, LT can also inactivate Ras (43) and, to a much lesser extent, Rap and Ral GTPases (25). Hence, the rapid inhibitory effect of LT compared with ToxB or C3 could indicate either that LT is much more efficient than ToxB or C3 in neutralizing intraneuronal Rac or that other small GTPases of the Ras superfamily participate in neurotransmission. Consistent with this latter possibility, Rap has been found on synaptic vesicles (33, 44), and translocation of Rap from secretory granules to plasma membrane has been reported (45, 46). Ras is present in neurons (33) and on synaptosomal membranes.
Rho-related GTPases Control Neurotransmitter Release

Fig. 6. Ca\(^{2+}\) dependence of ACh release recovery induced by high frequency stimulation in LT-injected neurons. A and B represent data from a typical experiment of a series of three to five performed as described in the legend to Fig. 5A. For comparison of the recovery effects under the various conditions, ACh release values were normalized against the amplitude determined at the end of the 1-s control trains recorded in each experiment (i.e. 100% recovery level). A, LT (50 nM in the cell body) was injected to block ACh release. Values (●) correspond to a 50-Hz response recorded 332 min after LT injection. Then, 1 mM EGTA was injected into the same neuron. Values (○) correspond to a typical train, recorded here 460 min after LT injection and 40 min after EGTA intraneuronal application. B, in this experiment, 50-Hz trains were recorded 242 min after LT injection in a physiological medium containing a [Ca\(^{2+}\)]/([Mg\(^{2+}\)]) ratio of 0.42 (●) or in the same experiment 340 min after LT injection in the presence of high Ca\(^{2+}\) (physiological medium containing a [Ca\(^{2+}\)]/([Mg\(^{2+}\)]) ratio of 2.1) (○). d1 and d2 represent the delays before the initiation of ACh recovery (47). However, the specific association of the Ras guanine nucleotide exchange factor CDC25M with postsynaptic densities (48) suggests a postsynaptic rather than a presynaptic role for Ras. Furthermore, it is noteworthy that LT, ToxB, and high doses of C3 produced similar responses in our experiments, i.e. a blockage of ACh release that could be completely suppressed in <1 s by 50-Hz trains. This suggests that the toxins interfere with neurotransmission most likely by inactivating the same target. The only common substrate of these three toxins is Rac. On the other hand, in view of the cross-talk between Rac- and Ras-dependent pathways (49, 50), we cannot completely exclude the possibility that inactivation of Ras participates to some extent in the LT-induced blockage of ACh release. For instance, the reduced efficiency of 50-Hz trains to restore ACh release 10 h after LT injection may well be due to the inactivation of both Ras and Rac.

Interestingly, functional Rac is needed for regulated exocytosis in rat basophilic leukemia cells (13), pancreatic beta cells (12), and mast cells (14). On the other hand, Rho is not considered to be an active regulator of secretion (12–15), despite the finding that activated RhoA stabilizes actin filaments around the secretory granules (15, 16). Thus, our hypothesis that Rac1 is involved in evoked neurotransmitter release correlates well with these data obtained in endocrine cells.

Clostridial Toxins Affect a Late Stage of Neurotransmitter Release—To characterize the origin of the ACh blockage induced by LT, ToxB, or C3, we examined ACh release during high frequency or paired stimulation. The main conclusions from these experiments can be summarized as follows. (i) Rho-related GTPases are well known organizers of the actin-based cytoskeleton (for review, see Ref. 2). However, the possibility that the inhibition of neurotransmission by the toxins is due to a remodeling of the synaptic connections is very unlikely because ACh release almost completely recovered in <1 s in response to high frequency stimulation. (ii) In view of its rapidity, it is rather unlikely that the recovery phase induced by repetitive stimulation results from a transient deglucosylation/de-ADP-ribosylation of the intraneuronal target(s) modified by the toxins. (iii) The inhibitory effect of LT on neurotransmitter release in response to a 50-Hz stimulation train was distinguishable from the effect induced by a blockage of Ca\(^{2+}\) channels. Thus, ACh blockage induced by LT, ToxB, or high concentrations of C3 is unlikely to result from an inactivation of Ca\(^{2+}\) influx. (iv) LT seems to block a Ca\(^{2+}\)-dependent step of neurotransmitter release because restoration of ACh release during 50-Hz trains is Ca\(^{2+}\)-dependent. (v) ACh release blocked by LT, ToxB, or high concentrations of C3 can completely recover within 1 s. This is clearly shorter than the time constant for vesicle recycling (6 s at hippocampal synapses) (51). Thus, the synaptic vesicles involved in the restoration of neurotransmission in response to high frequency stimulation must already be present in the toxin-blocked nerve terminals. (vi) Despite the fact that ACh release is strongly inhibited, paired-pulse facilitation is not significantly modified in LT- or C3-injected neurons. Hence, the blockade of ACh release induced by the toxins is likely to result from a decrease in the number of readily releasable vesicles.

Taken together, these conclusions are consistent with the proposal that the small G-protein modified by LT, ToxB, and high concentrations of C3 regulates a late Ca\(^{2+}\) dependent step of the neurotransmitter exocytotic process. Interestingly, high frequency firing has been demonstrated to allow a fast Ca\(^{2+}\)-dependent replenishment of the pool of readily releasable vesicles at the calyx of Held (52). In view of this observation, it is tempting to explain the recovery of neurotransmission induced by 50-Hz stimulation trains by the rapid replenishment of the

Fig. 7. Paired-pulse facilitation in LT-injected neurons. Paired stimuli were given at various interpulse time intervals in physiological medium and after stabilization of the inhibition of ACh release produced by lowering extracellular [Ca\(^{2+}\)]/([Mg\(^{2+}\)]) ratio (0.14) or by injection of LT (50 nM, 3–5 h, depending on the experiment). A, PPF induced by a 40-ms interpulse interval was determined from at least 25 recordings made for each condition in each experiment. For validity of comparison, PPF measurements were made at similar levels of ACh inhibition (ACh release reduced to 28.5 ± 7% in low Ca\(^{2+}\), to 29.8 ± 2.3% with LT, and to 33.5 ± 4.5% with 2 μM C3, n = 4 and n = 6, 3, and 4, respectively). The facilitation increment (mean ± S.D.) determined by subtracting control PPF from PPF calculated after treatment, is reported. A significant difference was observed when [Ca\(^{2+}\)] was lowered (p < 0.0001), but not after ACh blockage by LT (p = 0.5). B, in this representative experiment, the extent of PPF was determined in the same neuron in the presence of low Ca\(^{2+}\) (○), control physiological medium (□), and after LT (50 nM) had blocked ACh release (●). The time course of ACh release under these conditions is plotted in B2. In B1, the facilitation (mean ± S.E. from 12 recordings at each time interval) was significantly potentiated in low Ca\(^{2+}\) (** denotes p < 0.001; * denotes p < 0.01) except at an interval of 90 ms (p > 0.05). After blockage of ACh release by LT, PPF was not significantly increased for all intervals (p > 0.05; at 90 ms, p > 0.5).
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readily releasable vesicle population through a Ca\(^{2+}\)-dependent recruitment of synaptic vesicles that have been “frozen” close to the fusion sites, most likely by the inactivation of Rac1.

Control of Neurotransmitter Release by Rho-related G-proteins: Which Downstream Pathway?—A number of observations have implicated the Rho family in signal transduction pathways regulating the actin cytoskeletal network (for review, see Ref. 2). Rac1 and Rho can interact with Rho kinase (53, 54) and thereby control myosin phosphorylation (55). Since myosin II is localized within presynaptic terminals, where it controls neurotransmitter release (56), it is tempting to propose that Rac1 controls neurotransmitter exocytosis by regulating the actomyosin interactions involved in the movement of synaptic vesicles toward the docking/fusion sites. On the other hand, the effectors controlled by Rho-related proteins in neuroexocytosis might involve phosphoinositides. Indeed, Rac can stimulate directly several phosphatidylinositol-phosphate kinases (57, 58), and several steps of the exocytotic process are controlled by synaptic proteins that bind phosphoinositides. These include synaptotagmin (59), rabphilin (60), and FYVE finger proteins (61). Thus, it becomes quite interesting to determine whether synaptotagmin (59), rabphilin (60), and FYVE finger proteins II is localized within presynaptic terminals, where it controls pathways regulating the actin cytoskeletal network (for review, "References...

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