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# Botulinum toxin type A blocks the morphological changes induced by chemical stimulation on the presynaptic membrane of *Torpedo* synaptosomes

(acetylcholine release/potassium depolarization/synaptic transmission/ATP release/intramembrane particle)

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ABSTRACT **The action of botulinum neurotoxin on acetylcholine release, and on the structural changes at the presynaptic membrane associated with the transmitter release, was studied by using a subcellular fraction of cholinergic nerve terminals (synaptosomes) isolated from the *Torpedo* electric organ. Acetylcholine and ATP release were continuously monitored by chemiluminescent methods. To catch the membrane morphological changes, the quick-freezing method was applied. Our results show that botulinum neurotoxin inhibits the release of acetylcholine from these isolated nerve terminals in a dose-dependent manner, whereas ATP release is not affected. The maximal inhibition (70%) is achieved at neurotoxin concentrations as low as 125 pM with an incubation time of 6 min. This effect is not linked to an alteration of the integrity of the synaptosomes since, after poisoning by botulinum neurotoxin type A, they show a nonmodified occluded lactate dehydrogenase activity. Moreover, membrane potential is not altered by the toxin with respect to the control, either in resting condition or after potassium depolarization. In addition to acetylcholine release inhibition, botulinum neurotoxin blocks the rearrangement of the presynaptic intramembrane particles induced by potassium stimulation. The action of botulinum neurotoxin suggests that the intramembrane particle rearrangement is related to the acetylcholine secretion induced by potassium stimulation in synaptosomes isolated from the electric organ of *Torpedo marmorata*.**

Botulinum neurotoxin type A (BoNTx) is a clostridial protein that acts presynaptically impairing the release of acetylcholine (ACh) from cholinergic synapses (see ref. 1 as a review). The toxin inhibits quantal release (2) at the neuromuscular junction and at the electric organ of *Torpedo marmorata* (3), whereas some authors have reported that nonquantal release is also affected (4). These functional effects are not followed by morphological effects shortly after intoxication when electron microscope thin sections of poisoned neuromuscular junctions (2) or electroplaques (3) have been observed. However, Hirokawa and Heuser (5) reported that botulinum toxin prevents the increase of mitochondrial calcium spots in depolarized frog neuromuscular junctions. In freeze-fractured neuromuscular preparations, Pumplin and Reese (6) have studied the effect of BoNTx during electrical or brown widow spider venom stimulation. They observe that botulinum toxin decreases the number of vesicle fusion sites in the active zone induced by spider venom in the presence of external calcium.

*Torpedo* electric organ is homologous to the neuromuscular system, particularly enriched in cholinergic nerve terminals (7). It is a suitable model for correlation of structural and functional effects of the toxin, since it is a homogeneous and pure cholinergic innervated tissue. Furthermore, from this tissue, it is possible to isolate a subcellular fraction of cholinergic synaptosomes (8). We have previously shown that the electric organ, as well as the isolated synaptosomes, is sensitive to botulinum intoxication (3, 9).

On the other hand, several studies have analyzed the ultrastructural changes occurring at the presynaptic membrane of synaptosomes isolated from the electric organ of *Torpedo* during chemical stimulation. The main structural effect consists of a rearrangement of intramembrane particles (IMPs) on leaflets at either face—the protoplasmic freeze-fractured membrane face (PF) and the external freeze-fractured membrane face (EF)—of the synaptosomal plasma membrane (10–12). BoNTx might be a tool for correlation of these structural changes observed at the presynaptic membrane to ACh release from cholinergic synaptosomes.

## METHODS

*T. marmorata* specimens were caught from the Catalan coast and maintained alive in seawater in the Barcelona Marine Biological Station. Pure cholinergic synaptosomes were prepared from the electric organ of *T. marmorata* as described (8). Synaptosomes were recovered in a synaptosomal fraction that contained (in mM) NaCl, 280; KCl, 3; $MgCl_2$, 1.8; $CaCl_2$, 3.4; sucrose, 400; glucose, 5.5; Hepes/NaOH buffer (pH 6.8), 3.6 and buffered to pH 7 by $NaHCO_3$ (about 5 mM). All steps of the synaptosomal purification were performed at 4°C. The synaptosomal band represents 0.535 ± 0.018 g of initial tissue per ml and 0.194 ± 0.02 mg of protein per ml ($n$ = 17).

**Botulinum Neurotoxin Purification.** Botulinum complex was obtained from a culture of *Clostridium botulinum* type A (NCTC no. 2916). After acid precipitation of the culture, the toxin was purified by ion-exchange methods in DEAE-cellulose chromatography (13). Hemagglutinin was removed from the toxin complex by affinity chromatography and DEAE-cellulose chromatography as described (14, 15). Intraperitoneal mouse $LD_{50}$ of this purified neurotoxin was 0.66 ng/kg. Antiserum against BoNTx was made by injecting formalin-treated BoNTx emulsified with Freund adjuvant into a rabbit. The serum obtained from the animal contained 600 international units of type A antitoxin per ml of serum.

**Membrane Potential Measurement.** The membrane potential of the cholinergic synaptosomes was monitored by the fluorescent dye 3,3'-diethylthiadicarbocyanine iodide (4 μM) according to Meunier (16). The fluorescence was measured in

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Abbreviations: ACh, acetylcholine; BoNTx, botulinum neurotoxin type A; EF, external freeze-fractured membrane face; IMP, intramembrane particle; PF, protoplasmic freeze-fractured membrane face.

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a Kontron fluorimeter (excitation, 610 nm; emission, 668 nm) and displayed on a pen recorder. When the signal reached a constant level, valinomycin (Boehringer Mannheim; 0.25 μM, final concentration) was added to the synaptosomal membrane to increase the permeability to $K^+$ ions. The external $K^+$ ion concentration was then step increased and the changes in the membrane potential were recorded. Finally, gramicidin D (Boehringer Mannheim; 2.5 μM, final concentration) was added to obtain the reference value (0 mV). The Nernst equation allows us to calibrate in mV the fluorescent signals. The response to potassium depolarization was monitored by adding the dye to the synaptosomal suspension and increasing KCl concentration to 100 mM. The resting membrane potential and potassium depolarization were measured on poisoned (BoNTx, 125 pM, final concentration) and nonpoisoned synaptosomes.

**Lactate Dehydrogenase Activity.** To assess the synaptosomal membrane integrity after BoNTx poisoning, occluded lactate dehydrogenase (EC 1.1.1.27) activity was measured according to described procedures (17, 18).

**ACh and ATP Release.** ACh and ATP release were continuously monitored by chemiluminescent methods. ACh release was detected by a procedure described by Israel and Lesbats (19). This technique uses the specificity of two enzymes: acetylcholinesterase (EC 3.1.1.7; Sigma), which hydrolyzes the ACh liberated from the synaptosomes, and choline oxidase (EC 1.1.99.1; Sigma), which catalyzes in presence of $O_2$ the oxidation of previously formed choline producing betaine and $H_2O_2$. $H_2O_2$ formed is detected with a photomultiplier tube using luminol and peroxidase (EC 1.11.1.7; Sigma). As the luminol-peroxidase reaction needs an alkaline pH, synaptosomes were diluted by a factor of 2 in a modified physiological solution buffered to pH 8.6 with Tris·HCl (50 mM). ACh release was induced by addition of KCl (100 mM, final concentration). The synaptosomal fraction became intoxicated, during different periods and doses, in an incubation medium containing BoNTx prior to the addition of the isoosmotic alkaline luminescent medium. Controls were incubated under the same conditions, BoNTx being substituted by an equivalent volume of the physiological solution.

ATP was measured (20) by incubating the synaptosomal fraction in the presence of a luciferin/luciferase (EC 1.13.12.7) mixture (Sigma) in front of a photomultiplier tube. Samples of the synaptosomal suspension were preincubated for 10 min with BoNTx (125 pM). Then, luciferin (5 μg/ml, final concentration) and luciferase (40 μg/ml, final concentration) were added, and the light emission was monitored in the same dark chamber as for ACh detection. The ATP release was recorded as an increase of the light emission on the background light after addition of KCl (100 mM, final concentration). Both reactions were calibrated by adding known amounts of ACh or ATP to the same chemiluminescent mixture.

**Morphological Procedure.** The synaptosomal band became intoxicated with BoNTx (150 pM, final concentration) during 10 min at room temperature. After centrifugation, the pellet was resuspended in a small volume of physiological solution. Chemical stimulation of synaptosomes for freeze-fracture was performed as follows (10): an aliquot of concentrated synaptosomal fraction (intoxicated or nonintoxicated) was placed in a copper plate. Samples were incubated during 30 sec with modified physiological solutions containing high KCl concentration (100 mM after mixing and substituting NaCl) and frozen in liquid propane at −190°C cooled by liquid nitrogen. Membrane fracture was carried out in a Polaron E-7900 freeze-etching unit in a vacuum of $10^{-6}$ torr (1 torr = 133 Pa) at −110°C. The cleaved surfaces were shadowed with platinum and carbon at angles of 45° and 90°, respectively. The replica thickness was controlled by a quartz thin-film monitor. After thawing, the replicas were cleaned by sodium hypochlorite and rinsed in double-distilled water. A Philips EM 301 electron microscope was used to view the replicas. All electron micrographs were obtained at original magnification of ×45,000 with an accelerating voltage of 80 kV. Freeze-fracture faces from presynaptic membrane were identified as described (12). Membrane areas were enlarged photographically to a final magnification of ×150,000 to facilitate counting of IMPs and measuring of their diameter. Each experiment was performed in triplicate. Since the curvature of the fractured presynaptic membranes may introduce changes in the apparent size and density of IMPs, only flat regions of the membrane were selected for quantitative analysis.

**Statistics.** All results are given as means ± SEM and treated for statistical significance by the Student's unpaired *t* test.

## RESULTS

**Membrane Potential and Lactate Dehydrogenase Activity Measurements.** The hypothesis of a good preservation of the synaptosomal fraction during the poisoning is strengthened by the finding that BoNTx does not impair the synaptosomal occluded lactate dehydrogenase activity (76.7% ± 5.1% of the total activity in nonintoxicated synaptosomes, $n = 3$; 74.2% ± 5.3% in poisoned synaptosomes, $n = 3$). On the other hand, the measurements of synaptosomal membrane potential by the fluorescent method indicate that the toxin does not alter the resting potential (−52.0 ± 5.7 mV, control synaptosomes; −47.9 ± 7.6 mV, poisoned nerve endings). Moreover, the potassium-induced depolarization is not altered by botulinum intoxication [29.2 ± 5.7 mV ($n = 3$) nonintoxicated synaptosomes; 27.4 ± 4.8 mV ($n = 3$) poisoned synaptosomes] (Fig. 1).

**Effect of Botulinum Neurotoxin on the ACh and ATP Release.** BoNTx blocks the potassium-induced ACh release in a dose-dependent manner and saturates at a toxin concentration in the medium of 125 pM, reaching up to 70% of inhibition (Fig. 2*A*). The $IC_{50}$ is calculated at 25 pM. This effect can be prevented when synaptosomes are preincubated with the botulinum antiserum. The inhibition of ACh release from the isolated nerve endings can be detected after 2 min of BoNTx poisoning, reaching maximal inhibition after 6 min of incubation with the toxin (Fig. 2*B*). Since ATP is costored and coreleased with ACh (21), we have investigated the

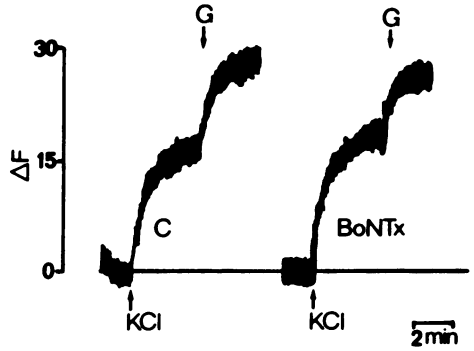


FIG. 1. Membrane potential was monitored by a fluorescent dye. Changes in the fluorescent signal (ΔF) in the course of depolarization were recorded. One milliliter of synaptosomal suspension was incubated with the dye until the fluorescent intensity reached a base line. KCl (100 mM, final concentration) was added to the cuvette and changes of fluorescence from this base line were recorded as a function of time. Finally, gramicidin D (G) (2.5 μM, final concentration) was added to completely abolish the membrane potential. "Intoxicated" synaptosomes (BoNTx) were pretreated with 125 pM BoNTx during 10 min at room temperature. C, control.

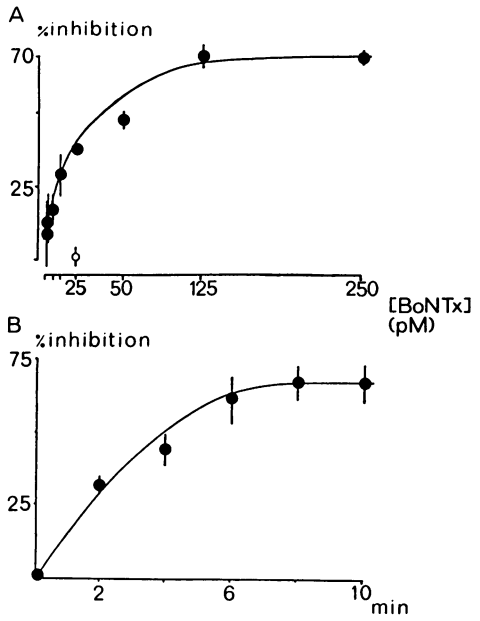


FIG. 2. Dose dependence (*A*) and time course (*B*) of botulinum neurotoxin action. ACh release from isolated nerve terminals was continuously measured by the chemiluminescent method. ACh release was triggered by adding KCl up to 100 mM (final concentration). Intoxicated synaptosomes were incubated with BoNTx at different concentrations during 10 min at room temperature. The results are expressed as percentage of ACh release inhibition. Prevention of the effect of the toxin by rabbit antiserum is also shown (open symbol in *A*). Results are expressed as means ± SEM ($n$ = four different experiments).

action of BoNTx on ATP release from cholinergic synaptosomes. Our results show that ATP release is not modified by the neurotoxin under the same experimental conditions (Fig. 3). BoNTx modifies neither the chemiluminescent reaction for ACh detection nor the luciferin-luciferase reaction and, thus, only ACh and ATP release is detected with these methods after poisoning.

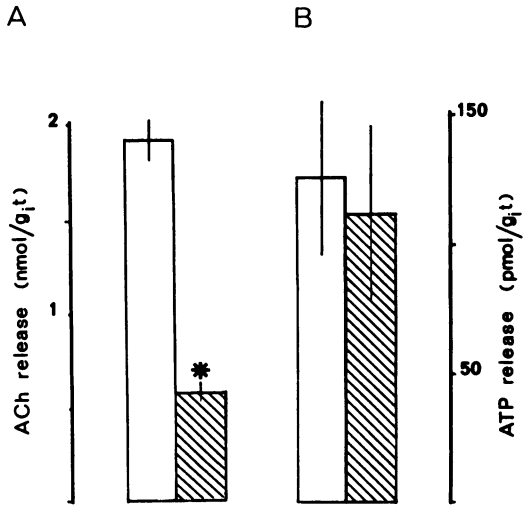


FIG. 3. Effect of BoNTx on ACh and ATP corelease. ACh and ATP release was triggered by KCl (100 mM, final concentration) and measured by either the choline oxidase-peroxidase (ACh release) or the luciferin-luciferase (ATP release) chemiluminescent method. Intoxicated synaptosomes were preincubated 10 min in 125 pM BoNTx prior to depolarization. (*A*) ACh release. (*B*) ATP release. Hatched bars, intoxicated synaptosomes; open bars, nonpoisoned nerve terminals. Results are presented as means ± SEM of released ACh and ATP per g of initial tissue ($g_i t$) ($n$ = six different experiments). Statistical significance is indicated by the asterisk: $P < 0.01$.

**Action of Botulinum Neurotoxin on the Rearrangement of IMPs.** Analysis of presynaptic membrane of stimulated synaptosomes shows a decrease in the number of IMPs per $\mu m^2$ at the PF (compare Fig. 4 *A–C*), whereas at the EF, the IMP density increases (Fig. 4 *B* and *D*). BoNTx treatment of unstimulated synaptosomes does not affect the density of IMPs either at the PF or the EF (Table 1). However, $K^+$ stimulation of intoxicated synaptosomes does not result in any modification in the IMP density at both faces (Fig. 4 *E* and *F*), resembling the particle distribution observed in resting conditions (Table 1).

Analysis of particle populations, with respect to their sizes, was performed on both presynaptic membrane faces, by plotting the number of small (≤9.5 nm) and large (>9.5 nm) IMPs. Table 2 illustrates that the density of small particles decreases at the PF of stimulated synaptosomes but that of large particles remains stable. Otherwise, at the EF, the large particle population increases. BoNTx does not modify the size of particles in resting conditions but inhibits the size distribution changes elicited by potassium stimulation. This inhibition of the rearrangement of IMPs at the presynaptic membrane produced by BoNTx is similar to that observed when synaptosomes are stimulated in a $Ca^2$-free medium (Tables 1 and 2).

## DISCUSSION

Isolated nerve terminals of the electric organ of *T. marmorata* are a pure cholinergic subcellular fraction that can be very appropriately used to correlate the effect of BoNTx on the release of ACh (9) and the structural changes elicited at the presynaptic membrane during synaptosome stimulation by chemical agents (10–12). We have previously shown (3, 9) that inhibition of ACh release from the electric organ of *T. marmorata* and from pure cholinergic synaptosomes by BoNTx is highly potent at very low concentrations, as occurs at the neuromuscular junction (2). Moreover, this inhibition by BoNTx is dose-dependent (maximal inhibition is achieved with a concentration of 125 pM), and its effect is prevented when the synaptosomes are previously incubated with botulinum antiserum. BoNTx acts very quickly on the synaptosomes (6 min), probably because in this preparation the delay due to the diffusion of the toxin within the extracellular space of the tissue is avoided, since >10 hr are required to intoxicate a single prism of *Torpedo* electric organ (3).

There are some effects of BoNTx on the cholinergic synaptosomes that are similar to those described in brain synaptosomal preparations (17). Even with a large dose of toxin, we only obtain a partial inhibition of ACh release up to 70%, but the toxin concentrations required to inhibit ACh release in brain synaptosomes are much larger (2.2 mM crude toxin) than those used in our experiments.

BoNTx blocks the release of ACh but not that of ATP from pure cholinergic synaptosomes stimulated by high potassium concentration (9), as occurs in brain synaptosomes (22). By using our preparation, it has been shown (16) that chemical stimulation with high external potassium concentration produces an increase in the synaptosomal membrane potential, which leads to an influx of $Ca^{2+}$ ions (23). Our results indicate that BoNTx does not inhibit either the membrane depolarization or the calcium uptake, and they agree with the results obtained previously at the neuromuscular junction (24), where the presynaptic ionic currents are not affected by the toxin. Occluded lactate dehydrogenase activity is not modified by the toxin. Taken together, these results indicate that after poisoning, the nerve terminals are still sealed and metabolically active.

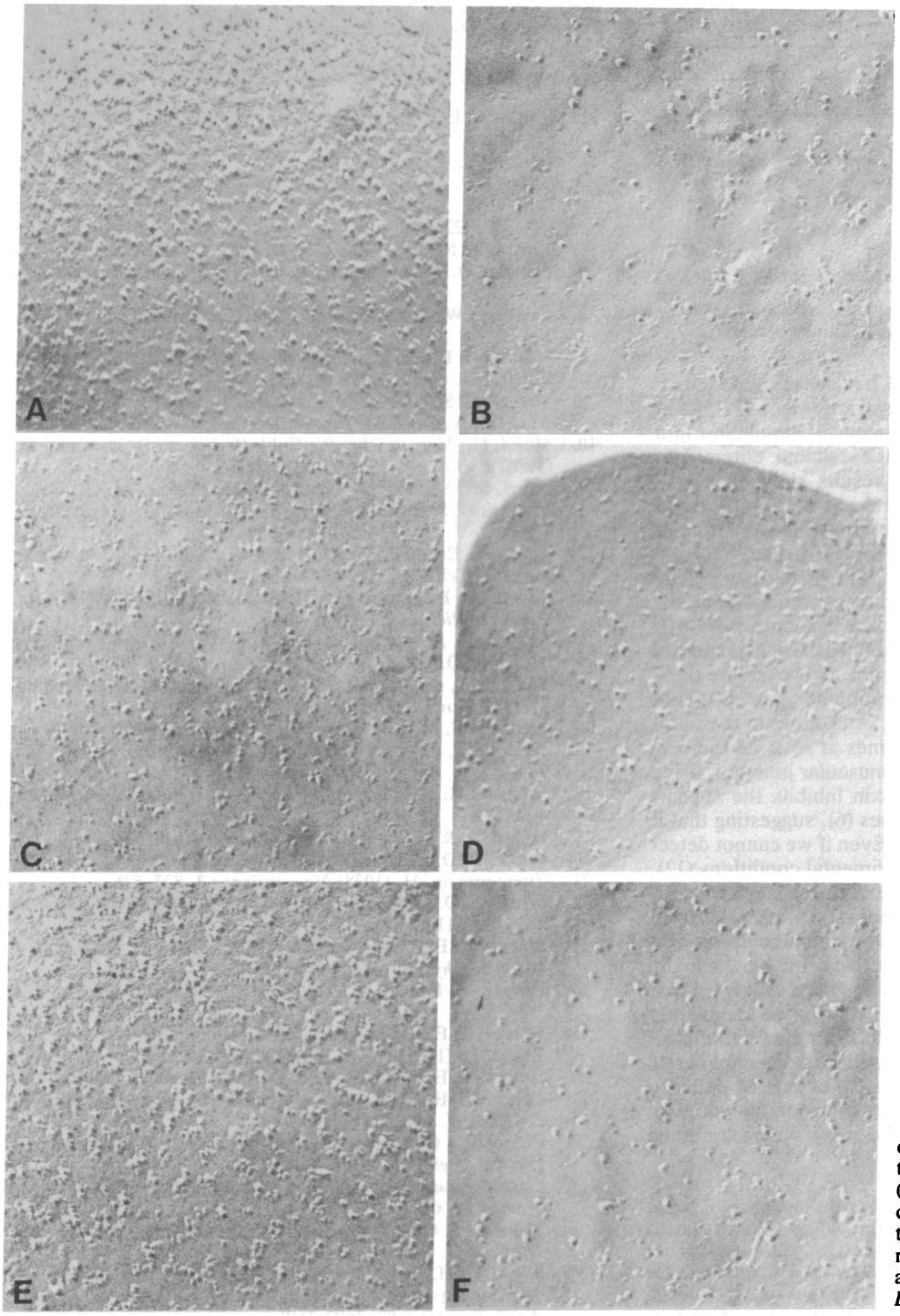


FIG. 4. Electron micrographs of quick-frozen synaptosomal presynaptic membrane in resting condition (PF, *A*; EF, *B*) and after potassium depolarization (100 mM, final concentration) (PF, *C*; EF, *D*). Botulinum neurotoxin-poisoned synaptosomes after potassium depolarization (PF, *E*; EF, *F*). (×77,400.)

In quick-freezing and freeze-fracture studies, during electrical stimulation of the frog neuromuscular junction in presence of 4-aminopyridine, the appearance of large IMPs at the PF during ACh release has been observed (25, 26). Other studies, using glutaraldehyde fixation (27, 28), reported that electrical stimulation induced IMP rearrangement at the PF according to their density and size distribution. Similar results have been described in *Torpedo* electric organ (29, 30)

Table 1. Density of IMPs before and after BoNTx intoxication

| | Nonintoxicated synaptosomes | | | Intoxicated synaptosomes | |
|---|---|---|---|---|---|
| | RC | $K^+$ | 0 $Ca^{2+}$, $K^+$ | RC | $K^+$ |
| PF | 1341 ± 50 (10.8) | 818 ± 41* (9.4) | 1270 ± 45 (11.2) | 1396 ± 62 (11.0) | 1246 ± 58 (15.3) |
| EF | 398 ± 24 (7.9) | 789 ± 40* (8.1) | 352 ± 35 (6.9) | 388 ± 76 (7.4) | 305 ± 38 (13.7) |

IMP density (number of particles per $\mu m^2$) is expressed as the mean ± SEM. The total area counted ($\mu m^2$) is indicated in brackets. RC, resting condition; $K^+$, potassium depolarization (KCl, 100 mM; $CaCl_2$, 3.4 mM); 0 $Ca^{2+}$, $K^+$, potassium depolarization in a calcium-free medium (KCl, 100 mM; $CaCl_2$, 0; 2 mM EGTA).
*Statistical significance: $P < 0.001$.

Table 2. Changes in the size distribution of IMPs before or after botulinum intoxication

| Synaptosomes | PF | | EF | |
|---|---|---|---|---|
| | ≤9.5 nm | >9.5 nm | ≤9.5 nm | >9.5 nm |
| Nonintoxicated | | | | |
| RC | 1002 | 345 | 185 | 230 |
| 100 mM $K^+$ | 509 | 302 | 293 | 442 |
| 0 $Ca^{2+}$ + 100 mM $K^+$ | 980 | 301 | 156 | 206 |
| Intoxicated | | | | |
| RC | 1176 | 304 | 192 | 247 |
| 100 mM $K^+$ | 927 | 390 | 186 | 243 |

The IMP density corresponds to a total area of 1 $\mu m^2$ of synaptosomal presynaptic membrane. RC, resting condition.

and synaptosomes (10–12). When ACh release is inhibited by BoNTx, there is a blockade of the rearrangement of IMPs at the PF and EF, as occurs during chemical stimulation in a calcium-free medium (12, 30). Since calcium enters into intoxicated synaptosomes (9), our results show that IMP rearrangement is related to the ACh release process triggered by chemical stimulation, and it is not linked to the influx of calcium into the nerve terminals or to depolarization.

It has been suggested (31) that BoNTx forms channels in planar lipid bilayers. Then, it might be possible that the formation of these channels could induce an increase of IMPs at the presynaptic membrane of the stimulated synaptosomes masking the normal IMP rearrangement. However, we think that this is not the case in our preparation because we do not observe an increase of IMP density, or changes in their size distribution, in poisoned synaptosomes at rest. At the presynaptic plasmalemma of the neuromuscular junction, using electrical stimulation, botulinum toxin inhibits the appearance of large IMPs at the active zones (6), suggesting that it blocks synaptic vesicle exocytosis. Even if we cannot detect synaptic vesicle fusions in our experimental conditions (12), it would be possible that the increase of large IMPs at the EF could be a consequence of the incorporation of particles from the synaptic vesicles during the vesicle membrane fusion, and then BoNTx would inhibit this increase by blocking the exocytotic process. Alternatively, it has been suggested that the increase of large particles at the EF during the stimulation is elicited by the translocation of IMPs from the PF to the EF at the moment in which ACh is released from the cholinergic synaptosomes (32). These changes could be related either to different states of membrane fluidity or to modifications in the patched-lipidic composition at the presynaptic membrane during the ACh release (33).

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