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EFFECTS AND MECHANISMS OF POLYPEPTIDE NEUROTOXINS THAT ACT PRESYNAPTICALLY

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This review discusses several polypeptide neurotoxins that act on nerve terminals to alter the storage and release of neurotransmitters. Table 1 lists some of these toxins and gives their potencies and molecular weights. The references given in Table 1 are other pertinent review articles, which should be consulted for descriptions of early studies of the toxins and for details of the clinical course of human patients affected by these toxins. In our discussion we exclude toxins that block propagation of nerve impulses, toxins that primarily act postsynaptically, and toxins whose pharmacology and biochemistry are insufficiently characterized to review.

BLACK WIDOW SPIDER VENOM

Characterization of Toxins

The black widow spider *Latrodectus mactans tredecimguttatus* and the brown widow spider *L. geometricus* produce toxins that alter the structure and function of cholinergic (10–13), noradrenergic (14, 15), and aminergic nerve terminals (16) in vertebrates and invertebrates. The toxins also depolarize crayfish stretch receptors, block the heartbeat of cockroaches, and kill houseflies (1). Almost all of the toxicity studies with black widow spider or brown widow spider have employed unfractioned venom or, more commonly, a supernatant fraction of a homogenate of venom glands from these organisms. It is now known that the pharmacological effects of a

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Table 1 Polypeptide neurotoxins that inhibit transmitter release

Source	Toxin	LD_{50}[a]	Toxin M_r	Subunit M_r	Reference
Black widow	*α-Latrotoxin*	0.01	130,000	130,000	1
Bacteria	Botulinum toxin	2×10^{-6}	150,000	100,000 + 50,000	2, 3
	Tetanus toxin	2×10^{-6}	150,000	100,000 + 50,000	4, 5
Snakes	β-Bungarotoxin	0.025	20,500	13,500 + 7,000	6–8
	Crotoxin	0.05	22,000	14,000 + 8,500	9
	Notexin	0.025	13,600	13,600	7, 8
	Taipoxin	0.002	46,000	18,000 + 14,000 +13,000	7, 8

[a] μg/g mouse.

venom gland extract are caused by several different toxins. One of these toxins has been named α-latrotoxin (17); it is a 130,000 dalton protein composed of a single polypeptide chain (18). This toxin acts on vertebrate cholinergic neuromuscular junctions and produces effects that are essentially the same as those produced on the neuromuscular junctions by the crude venom gland extract (18). Purified α-latrotoxin also induces an efflux of acetylcholine (ACh), norepinephrine, and γ-aminobutyric acid from mammalian brain slices (17, 19), but it is inactive on invertebrate nervous tissue (18). A second protein obtained from the venom gland appears to be responsible for the effects of the gland crude homogenate on lobster neuromuscular junctions (1) and the crayfish stretch receptor (18). Other toxic factors have also been described (1). It is not known which of these toxins are secreted in the venom.

For the purposes of this review BWSV (black widow spider venom) refers to crude venom or venom gland extract from black widow spiders; BrWSV refers to the same crude preparation from brown widow spiders. The following discussion is restricted to the effects of BWSV and BrWSV seemingly caused by α-latrotoxin, and we indicate below which studies employed the purified toxin.

Effects on Neuromuscular Transmission

A few minutes after application of BWSV to a vertebrate neuromuscular junction there occurs a sharp increase in the frequency of spontaneous miniature end-plate potentials (mepps) to a maximum of 500–1000 times the normal frequency (10). The BWSV-induced mepps, which are of normal amplitude, continue at peak frequency for an additional 5 min or so, and then gradually decline to zero frequency over the next 30–50 min. This decline is accompanied by a complete inhibition of evoked release of ACh. Electron microscopy of thin sections of neuromuscular junctions undergoing a burst of BWSV-induced mepps shows synaptic vesicles in various

stages of fusion with the presynaptic plasma membrane (20). Later the nerve terminals become swollen and almost completely depleted of synaptic vesicles (20–22). The vesicle depletion is generally believed to be due to an inhibition of the endocytosis process that is thought to function in the regeneration of synaptic vesicles after they have fused with the plasma membrane.

The freeze fracture technique has been used to identify the sites of the increased fusion of synaptic vesicles to presynaptic plasma membranes of frog neuromuscular junctions treated with BrWSV or BWSV (1, 23). When the incubation buffer contained Ca^{2+}, the sites of vesicle fusion were located mainly in the active zone, which is the specialized presynaptic region where vesicles fuse after electrical stimulation. In Ca^{2+}-free medium containing 23.5 mM Mg^{2+}, BrWSV-induced fusions occurred both at and outside the active zone with approximately equal frequency (23).

BWSV does not appear to inhibit ACh synthesis directly (24). BWSV has been reported to depress ACh synthesis in rat brain slices and mouse diaphragm, and it has been proposed that this effect is secondary to a decrease in synaptic vesicle sites (11, 24). Others have found little effect of BWSV on ACh synthesis in rat superior cervical ganglion (13) or diaphragm (C. Gundersen, unpublished observation).

Mechanism of Action

α-LATROTOXIN AS AN IONOPHORE The mechanism by which BWSV (α-latrotoxin) induces massive quantal release of ACh is not known with certainty. No proteolytic or lipolytic activity has been found to be associated with purified α-latrotoxin (18). The purified toxin increased the cation conductance of artificial lipid bilayer membranes (25) suggesting that α-latrotoxin can insert itself into the lipid bilayer. In this system the α-latrotoxin molecules appear to form ion channels rather than acting as ion carriers since the conductance increases occur in discrete steps of large and uniform size. The toxin-formed channels make the membrane permeable to Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}, and the organic ions methylamine and choline, but not glucosamine (1, 25).

The neurotoxicity of α-latrotoxin has been ascribed to an ability to act as an ionophore for Na^+ and Ca^{2+} in nerve terminal membranes (23, 25). It is known that electrically evoked release of neurotransmitter is triggered by an entry of Ca^{2+} into nerve terminals; the Ca^{2+} enters through voltage-sensitive channels that open as the terminals are depolarized by an influx of Na^+ (26). Apart from depolarizing the terminals, an influx of Na^+ (or an efflux of K^+) may also increase intraterminal level of free Ca^{2+} by displacing internal Ca^{2+} from bound stores (27–29). Nevertheless, while BWSV can depolarize nerve endings (30), it is questionable whether the

toxin exerts its effects at neuromuscular junctions only by depolarizing the nerve terminals or, for that matter, by directly promoting the entry of Ca^{2+} into the terminals. One argument against such a mechanism is that BWSV causes mepp discharge and vesicle depletion in Na^+-free, Ca^{2+}-free medium (31, 32). BrWSV differs from BWSV in this regard because BrWSV does not deplete synaptic vesicles in Na^+-free medium (23). It is not known whether this result reflects a subtle difference in experimental protocol (31) or a basic difference in the mechanisms of BWSV and BrWSV.

There is other evidence against BWSV's inducing release only by acting as an ionophore for Na^+ or Ca^{2+}. Hypertonic solutions are known to increase mepp frequency at frog neuromuscular junctions, perhaps by increasing the concentration of free Ca^{2+} within the nerve terminals. If BWSV were acting to increase plasma membrane permeability to Ca^{2+}, it should reduce the mepp frequency of neuromuscular junctions bathed in hypertonic buffers lacking divalent cations. However, under these conditions BWSV increases mepp frequency over that observed in the hypertonic medium alone (31, 32). It should be noted that, in contrast to the case with BWSV, K^+-induced depolarization of nerve terminals does decrease mepp frequency under similar hypertonic, Ca^{2+}-free conditions (33).

It is also unlikely that BWSV induces transmitter release by increasing K^+ permeability and thereby causing an efflux of K^+ from the nerve terminals; BWSV depletes nerve terminals of synaptic vesicles under conditions in which an efflux of K^+ should not occur (31).

Depolarization of nerve terminals can account for some of the effects of BWSV. Swelling of nerve terminals does not occur when frog neuromuscular junctions are treated with BWSV in medium in which Na^+ is replaced by glucosamine (31). More important, removal of BWSV from a neuromuscular junction after treatment in regular Na^+-containing medium rarely results in recovery of synaptic transmission, but recovery does occur if the initial treatment with BWSV is in a Na^+-free medium (31). These findings suggest that a BWSV-induced increase in permeability to Na^+ causes the swelling of nerve terminals and the irreversibility of the blockade.

INFLUENCE OF DIVALENT CATIONS The matter of whether BWSV induces transmitter release by acting as an ionophore is somewhat complicated by the influence divalent cations have on BWSV-induced release. High concentrations of Ca^{2+} increase the peak mepp frequency and decrease the duration of the mepp burst in frog neuromuscular junctions treated with BWSV (34). Mepp discharge does not occur in frog neuromuscular junctions treated with BWSV in isotonic media lacking divalent cations (31, 32, 35). This requirement for divalent cations is partially met by as little as 0.1 mM Mg^{2+} according to one report (32) and by even less

Mg^{2+} according to another study (31). With 4 mM Mg^{2+} there is the usual generation of mepps by BWSV; with lower concentration of Mg^{2+} the increase in mepp frequency occurs more slowly but lasts longer (31, 32). The requirement for divalent cations can also be met by Ca^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+} (32). Recent studies of Misler & Hurlbut (32) indicate that the divalent cations are not required for toxin binding or as cofactors for some enzyme activity of the toxin. These authors suggested that BWSV makes the plasma membranes permeable to all of the divalent cations so that they enter the terminal and directly trigger the release apparatus or, in the case of the ions other than Ca^{2+}, displace Ca^{2+} from intracellular binding sites. This mechanism implies that BWSV can induce release in at least some cases by acting as an ionophore.

However, the requirement for divalent cations is not an absolute one. Divalent cations also influence BWSV-induced release of ACh from mammalian diaphragm but in a temperature-dependent fashion (31). When the treatment with BWSV is at a temperature above 31°C, BWSV stimulates ACh release and depletes vesicles in mouse diaphragms in the absence of added divalent cations; however, below 30°C addition of a divalent cation is required for BWSV activity. It should be recalled that, as mentioned above, divalent cations are also not required for BWSV to induce release under hypertonic conditions (31, 32). Perhaps more consistent with these findings is the suggestion that the dependence of BWSV on divalent cations reflects a requirement of divalent cations for some normal function of a membrane involved in BWSV-induced release (36).

SPECIFICITY OF α-LATROTOXIN BINDING It has been argued that although α-latrotoxin forms ion channels in simple artificial lipid bilayers, the toxin cannot exert its effects on the nervous system solely by forming ion channels in cell membranes nonselectively; otherwise it should affect all nervous tissue, but invertebrate nervous tissue is unaffected (18). Thus, regardless of its mechanism of action, α-latrotoxin could be expected to exhibit some specificity in binding to nerve terminal membranes. Tzeng & Siekevitz (36) have recently obtained evidence for such binding specificity. They studied the binding of ^{125}I-labeled α-latrotoxin to synaptosomal membranes prepared from dog brain. The iodinated protein was able to cause neuromuscular blockade but the loss (if any) of potency due to iodination was not precisely determined. Nonspecific binding, which was estimated by co-incubation with an excess ($>$ 500-fold) of unlabeled toxin, accounted for less than 10% of the total binding of the labeled α-latrotoxin. The specific binding was (*a*) saturable; (*b*) noncooperative; (*c*) high affinity with an apparent K_D of 1 nM; (*d*) completely inhibited by prior treatment of the membranes with trypsin, suggesting that the receptor is a protein; (*e*)

unaffected by 2 mM Ca^{2+}; (*f*) increased (50%) by 5 mM Ca^{2+}; and (*g*) partially inhibited (40%) by 2 mM EDTA and (70%) by 10 mM EDTA or 10 mM EGTA. Thus, there is not a clear requirement of divalent cations for toxin binding. The labeled toxin did not exhibit specific binding to liver membranes.

OTHER MECHANISMS Since an increase in cation permeability does not easily account in all cases for the ability of BWSV to stimulate quantal release of transmitter, several workers have speculated that this stimulation of release occurs by some other mechanism that operates in addition to or independent of its ionophore activity. However, there is little evidence in support of any of the other mechanisms proposed for BWSV. Rubin et al (37) found that BWSV did not induce transmitter release from either tissue-cultured or adult neuromuscular junctions that had been preincubated with 50–300 μg/ml of the lectin concanavalin A (Con A) before exposure to the BWSV. Prior treatment of these preparations with colchicine, which is known to interfere with microtubule function, prevented the Con A inhibition of BWSV activity (37). Con A also blocks BWSV-induced transmitter release from rat brain synaptosomes (30, 38). While an effect of Con A on BWSV binding to nerve terminals was not excluded, Rubin et al (37) favored the notion that Con A blocks BWSV activity by a mechanism analogous to that by which Con A inhibits lymphocyte capping, an effect that is also prevented by colchicine (39). Accordingly, they proposed that BWSV induced a redistribution of plasma membrane components (37). This redistribution is purported to affect the relationship between the plasma membrane and cytoplasmic elements (e.g. microfilaments, microtubules), resulting in an increase in vesicle fusion and an inhibition of the normal vesicle recycling process. In this view Con A is thought to block the BWSV activity by also affecting the interaction between the plasma membrane and cytoskeletal elements, thereby altering the mobility of membrane components; colchicine would prevent this effect by directly acting on the cytoskeletal system.

The mechanism of BWSV action proposed by Rubin et al (37) is based on the inhibitory effects of Con A. Tzeng & Siekevitz (36) have recently reported that Con A, at the doses used by Rubin et al, inhibits the specific binding of α-latrotoxin to brain synaptosomal membranes. The membrane receptors for Con A are mannosides, e.g. mannose-containing proteins or lipids. It is unlikely that Con A and α-latrotoxin compete for the same receptor since α-methyl-D-mannoside does not interfere with BWSV activity at frog neuromuscular junctions (37) or rat brain synaptosomes (30, 38). Tzeng & Siekevitz (36) favored a nonspecific effect of Con A, e.g. blocking the availability of the toxin receptor by binding to nearby glycoproteins or

glycolipids. It will be interesting to learn whether colchicine prevents the Con A inhibition of α-latrotoxin binding. If it does, the postulated analogy between BWSV activity and lymphocyte capping (37) becomes less attractive.

It has also been proposed that BWSV activates a contractile network thereby stimulating exocytosis (36). Ceccarelli et al (40) suggested that BWSV might interact with the presynaptic plasma membrane at sites near the large intramembranous particles that line the active zone and then induce release by circumventing the Ca^{2+}-dependent step of normal secretion.

In summary, the ability of α-latrotoxin to act as an ionophore cannot at present account for all of its effects on neuromuscular transmission. However, the alternative mechanisms of toxin action proposed to date are not compelling. While a dual mode of action (31) cannot be excluded, it seems more likely that a toxin with the apparent specificity α-latrotoxin exhibits would have only one mechanism of action.

BOTULINUM TOXIN

Production and Structure

Botulinum toxin (BoTx) is produced by the anaerobic bacterium, *Clostridium botulinum.* At least eight immunologically distinct types (A, B, C_α, C_β, D, E, F, G) of BoTx have been recognized; most strains of *C. botulinum* produce only one type (2, 3). The various types of BoTx appear to be similar in structure ($M_r \simeq 135{,}000$–$170{,}000$) and in pharmacological properties (2, 3, 41–44). The most potent and thoroughly studied BoTx is type A, which has a molecular weight of 150,000. In many purified preparations it is found aggregated with a larger protein that has hemagglutinin activity (45). The hemagglutinin does not contribute to the neurotoxicity of BoTx and can be separated from the BoTx protein. No enzyme activity has been reported to be associated with BoTx.

BoTx appears to be synthesized as a relatively inactive protoxin, which is converted to an active form by proteolytic cleavage. The two cleavage products, which have molecular weights of approximately 50,000 and 100,000, respectively are covalently linked by at least one disulfide bond (3, 41–44). Neurotoxicity is lost upon reduction of the disulfide bond(s); there is one report that reconstitution results in partial recovery of activity (46). Some strains of *C. botulinum* use endogenous proteases to activate the toxin and in such cases, e.g. type A BoTx, the toxin is fully active as isolated (47). However, BoTx obtained from nonproteolytic strains is only weakly active until treated with some exogenous protease.

Pharmacological Effects

BoTx inhibits the depolarization-induced release of transmitter from cholinergic nerve terminals. Peripheral administration of BoTx to whole animals produces a flaccid paralysis and other symptoms consistent with an interference with cholinergic transmission (2, 3). While most of the symptoms of botulinum intoxication can be attributed to an inhibition of evoked release of ACh from nerve terminals in the peripheral nervous system, some effects have been ascribed to an action on the central nervous system (48). However, it has not been rigorously demonstrated that the toxin can cross the blood-brain barrier. In vitro, the toxin has been found to inhibit evoked ACh release from nerve terminals at neuromuscular junctions (49, 50), in autonomic ganglia (51), and in the central nervous system (52–54). There are reports that BoTx can depress the evoked release of transmitter from noradrenergic terminals (55, 56). However, conflicting results have been obtained (57, 58) and it is possible that the reported effects of BoTx on adrenergic secretion are only secondary to an inhibition of cholinergic transmission.

Very little BoTx is required to produce neuromuscular blockade; it has been estimated that no more than 10 molecules of toxin are required to block a single cholinergic synapse (59). The time required for complete blockade depends on the type of neuromuscular junction and the treatment conditions. However, even when conditions are optimized, 30–40 min are required for BoTx to fully inhibit transmission in a mammalian nerve-muscle preparation (60). Since BoTx binds rapidly to the neuromuscular junction (49, 60), the long delay to blockade appears to be due to events that occur after toxin binding. The characteristics of BoTx binding to nerve terminals have been reviewed (3). It is not known whether BoTx must enter nerve terminals to exert its effects. Results of kinetic studies of BoTx activity have been interpreted to indicate that BoTx is internalized (61). Numerous agents or treatments have been found to promote or delay the blockade of transmission caused by BoTx. The latent period can be shortened by repetitive nerve stimulation or by the addition of eserine to the bathing medium (60). Treatments that delay an in vitro blockade include reduced temperature (49, 60), media with low Ca^{2+} or high Mg^{2+} concentration (62), β-bungarotoxin (63), theophylline (64), and atropine (60).

Neuromuscular preparations that have been treated with BoTx exhibit a decrease in the frequency and amplitude of spontaneously generated mepps (59, 65, 66), while the postsynaptic response to ACh is not altered (49, 67). Eventually, BoTx causes a complete inhibition of ACh release evoked by single nerve impulses (49, 50).

ACh Synthesis in BoTx-Treated Terminals

The effects of BoTx on neuromuscular transmission cannot be attributed to a BoTx-induced depletion of ACh stores. Contrary to an early report (68), direct chemical measurements have shown that BoTx does not inhibit ACh synthesis in mammalian diaphragm (69), brain slices (54), or synaptosomes (53); nor does it depress the total levels of ACh in these preparations. Furthermore, BoTx has no direct effect on choline acetyltransferase activity in muscle (70) or on choline uptake by synaptosomes from mammalian brain (53, 54). Finally, BoTx-poisoned nerve terminals retain considerable stores of ACh that can be released by treatment with BWSV and other agents as described subsequently.

While the studies cited above clearly indicate that BoTx has no direct effect on ACh synthesis, other findings suggest that ACh synthesis can be indirectly influenced by BoTx. BoTx is able to inhibit ACh release from primary cultures of cortical neurons without inhibiting ACh synthesis in these cells; however, prolonged treatment of the cultures with BoTx does result in a depression of [^{3}H]ACh formation (71). Similarly, there is a decline in choline uptake by synaptosomes prepared from BoTx-treated brain slices (54). Both of these findings have been interpreted to be a consequence of a primary effect of BoTx on ACh release (54, 71), although an effect on ACh compartmentation could not be excluded (54).

BoTx-treated synaptosomes from mammalian brain have been reported to contain a substantially increased amount of ACh after exposure to media containing a high concentration of K^+ (53). It was argued that ACh synthesis is normally controlled by depolarization (e.g. by high K^+) rather than by release; thus, high K^+ would increase ACh levels in BoTx-treated synaptosomes by stimulating ACh synthesis without inducing release. This effect has not been observed in rat brain slices (54), cultured cortical neurons (71), or neuromuscular junctions (69).

Low Amplitude Mepps in BoTx-Treated Neuromuscular Junctions

Several alternative explanations have been offered (65) for the abnormally low amplitude mepps observed in BoTx-poisoned neuromuscular junctions. Harris & Miledi (65) considered the possibility that normal mepps are due to the synchronous release of multiple quanta of ACh while the mepps of decreased amplitude found in BoTx-treated preparations are due to the release of fewer quanta at a time. Subsequent studies of this possibility have yielded conflicting conclusions (72–76). Among other possibilities suggested is that the small mepps arise from a partial discharge of vesicle contents or a change in some property of an ACh gate (if release were

assumed not to be from vesicles). These mechanisms imply that BoTx affects the release apparatus, per se.

Additional attempts to explain the low amplitude mepps generated in BoTx-poisoned neuromuscular junctions rely on the finding that, using amplitude as a criterion, two classes of mepps can be demonstrated in BoTx-treated neuromuscular junctions. Addition of BWSV or BrWSV to neuromuscular junctions that had previously been paralyzed with BoTx elicits a burst of mepps, which have the amplitude of mepps recorded in normal neuromuscular preparations (77–79). Before the spider venom treatment the BoTx-poisoned preparations produce mepps of very low frequency and amplitude as mentioned above. Frequent mepps of nearly normal amplitude can also be detected in BoTx-poisoned preparations that are subjected to hyperpolarizing currents (59), tetanic stimulation (65), or a Ca^{2+} ionophore in the presence of a high concentration of Ca^{2+} (> 4 mM) in the bathing medium (79). The existence of a class of mepps that are apparently resistant to BoTx suggested that BoTx-treated terminals contain two different populations of synaptic vesicles and that BoTx affects only the population of vesicles that participate in the normal release-refill cycle (59). In this regard it is noteworthy that the number of mepps spontaneously generated during BoTx treatment of mouse diaphragm is reported to be of the same order of magnitude as the readily releasable store, i.e. 300–1000 quanta by one estimate (72). Moreover, this readily releasable store may contain a disproportionate share of newly synthesized ACh (80) and in rat brain slices BoTx preferentially inhibits the K^+-evoked release of newly synthesized ACh relative to the release of older stores of ACh (54).

Harris & Miledi (65) suggested the possibility that there are two classes of cholinergic synaptic vesicles, one class filled with ACh and the other only partially filled. The former would be predominantly involved in normal release while the latter would be involved in release from BoTx-poisoned terminals. Boroff et al (59) modified this suggestion by proposing that BoTx acts by inhibiting the loading of ACh into synaptic vesicles. Synaptic vesicles obtained from BoTx-treated synaptosomes from mammalian brain have been found to contain the same amount of total ACh and newly synthesized ACh as control vesicles (53). However, the techniques used in this study may not have distinguished any special BoTx-sensitive subpopulation of vesicles existing in the nerve terminals.

Electron microscopy using freeze-fracture techniques has shown that BoTx inhibits nerve impulse-induced fusion of synaptic vesicles with the plasma membrane (23). While this result could be interpreted as evidence against inhibition of vesicle loading by BoTx, it may be that vesicles in toxin-treated terminals cannot fuse because they have not been properly loaded with ACh. This argument can be countered with the finding that

BWSV or nerve stimulation induces the fusion of apparently empty vesicles in nerve terminals depleted of ACh by treatment with hemicholinium-3 (81). However, if it were necessary for synaptic vesicles to interact with some special component of the nerve terminal in order to be loaded and subsequently emptied during nerve impulse-induced release, BoTx may act by perturbing the interaction of vesicles with that component. Except for the failure of synaptic vesicle fusion mentioned above, the morphology of toxin-treated nerve terminals appears to be normal at the onset of blockade (82).

Evidence of heterogeneity of synaptic vesicles and/or release sites in BoTx-treated terminals has also been obtained from electron microscopic studies. Kao et al (77) found that the morphology of mouse nerve terminals that had been treated with BWSV alone differed from that of terminals that had been blocked first with BoTx and subsequently treated with BWSV. In both instances the terminals were swollen and almost depleted of synaptic vesicles as expected; however, in the latter case the terminals were also found to contain clumps of vesicles located at release sites opposite postjunctional folds. This observation is consistent with either of the two mechanisms of BoTx action discussed above, i.e. inhibition of the release apparatus or perturbation of vesicle interaction at an activation site so as to impair proper vesicle loading and subsequent emptying during exocytosis. The vesicle clustering observed by Kao et al (77) may be species-specific because it was not seen in frog terminals treated with BoTx and BrWSV. Intriguingly, a very similar morphology (vesicle clustering at release sites) was observed after incubation of frog neuromuscular junctions in Ca^{2+}-free buffers containing 116 mM K^+, 4 mM Mg^{2+}, and 1 mM EGTA (31). However, these terminals became totally depleted of vesicles after addition of BWSV to the preparation. Using the freeze-fracture technique, Pumplin & Reese (23) showed that, in normal Ca^{2+}-containing medium, BoTx depressed BrWSV-induced fusion of vesicles at active zones; however, when Ca^{2+}-free medium was used, BoTx did not affect BrWSV-induced fusion of vesicles either at the active zone or outside it.

The electron microscopic data (23, 77) just described could be interpreted in terms of a selective depression by BoTx of ACh release at normal sites. Release from regions of the terminal outside the active zone could produce low amplitude mepps as suggested by Harris & Miledi (65), but it should also result in potentials with longer rise times. Evidence on the latter point is conflicting, with some workers reporting normal rise times for the small mepps (72) and others a possible lengthening (66, 79).

In summary the small mepps observed in BoTx-treated neuromuscular junctions could be due to (*a*) asynchronous release, (*b*) partial discharge of vesicle contents, (*c*) abnormal gating of ACh, (*d*) inhibition of vesicle loading, (*e*) discharge from a subpopulation of partially filled vesicles, or

(*f*) release from sites suboptimally located with respect to postsynaptic ACh receptors. Clearly, knowledge of the actual mechanism by which BoTx causes the small mepps is the key to understanding how BoTx induces neuromuscular blockade.

Antagonism by Ca^{2+}

There is evidence that an increased concentration of Ca^{2+} can antagonize BoTx. Simpson (62) found that the time required for toxin-induced blockade of an isolated neuromuscular preparation increased when the concentration of Ca^{2+} in the bathing medium was raised. Thesleff (82) reported that a doubling of the normal concentration of Ca^{2+} in the bathing fluid restored nerve impulse-induced end-plate potentials (epps) in a neuromuscular preparation lightly intoxicated with BoTx. Transmitter release was also restored in a BoTx-paralyzed neuromuscular preparation by various agents (e.g. 4-aminopyridine) that are believed to increase the intracellular level of Ca^{2+} (79). The Ca^{2+} ionophore A23187 does not induce ACh release from BoTx-poisoned terminals bathed in a medium containing a normal concentration of Ca^{2+} (77, 79). Therefore, BoTx acts by inhibiting some process other than Ca^{2+} entry. Cull-Candy et al (79) proposed that BoTx decreases the sensitivity of the release apparatus to Ca^{2+}. This mechanism could explain the clustering (see above) of vesicles observed in thin sections of neuromuscular junctions that had been treated with BoTx and BWSV (77) or with a high K^+, Ca^{2+}-free buffer (31).

TETANUS TOXIN

Production and Pharmacological Effects

Tetanus toxin is produced by another anaerobic bacterium, *Clostridium tetani.* In mammals the toxin produces a spastic paralysis (4, 5), which has been attributed to a tetanus toxin-induced blockade of the release of transmitters that mediate inhibitory neuronal pathways in the central nervous system (83, 84). Support for this hypothesis comes from the ability of tetanus toxin to block the release of glycine, γ-aminobutyric acid, and other putative amino acid transmitters from nerve terminals of mammalian brain and spinal cord (85–89). Tetanus toxin acts peripherally as well, causing a flaccid paralysis by inhibiting the evoked release of ACh at neuromuscular junctions (5, 90, 91); in certain animals, e.g. goldfish, it produces only a flaccid paralysis (92). Tetanus toxin may also act on the autonomic nervous system since cardiovascular symptoms have been noted in tetanus patients whose spastic paralysis was controlled (93, 94). Finally, effects on muscle cells have been reported (4).

There has not been extensive study of the properties of nerve terminals after treatment with tetanus toxin. Mammalian nerve-skeletal muscle preparations that had been blocked with tetanus toxin generate mepps of reduced frequency and amplitude (5, 91). In these preparations mepps of normal amplitude can be elicited by tetanic nerve stimulation (5, 91) but not by raising the external K^+ concentration (91). The toxin-treated nerve terminals exhibit no striking morphological alterations (4, 5).

Structure

Tetanus toxin has a molecular weight of ~150,000 (95–98). The toxin from culture filtrates (extracellular toxin) is composed of two polypeptide chains, a light chain of 50,000 daltons and a heavy chain of ~100,000 daltons, covalently linked by at least one disulfide bond (96–98). Tetanus toxin obtained from broken *C. tetani* cells (intracellular toxin) consists of a single polypeptide chain, which can be cleaved by mild trypsin treatment yielding a product that is indistinguishable from the extracellular protein (97). The trypsin treatment increases the toxicity of the intracellular toxin threefold (97). It is assumed that the intracellular toxin is a protoxin that is normally converted to the extracellular form by cellular proteases. Toxicity of the extracellular toxin is lost upon reduction of the disulfide bonds by treatment with dithiothreitol but an active dimer can be reconstituted by removal of the dithiothreitol (99).

The Receptor for Tetanus Toxin

There is evidence that the receptor for tetanus toxin is selectively localized to neurons and has a ganglioside component. Tetanus toxin binds to neurons but not to non-neuronal cells in cultures of rodent and chicken brain, ganglia, and retina (100, 101). Plasma membrane–enriched subfractions of mammalian brain synaptosomes fix tetanus toxin to a much greater extent than do subfractions enriched in synaptic vesicles or mitochondria (102). Native tetanus toxin and isolated heavy chain bind to sialic acid–containing gangliosides (103–106) but isolated light chain does not (105, 106). It has been suggested that the heavy chain portion of native toxin contains the ganglioside binding site (105, 106).

Treatment of tetanus toxin with papain results in cleavage of the heavy chain and yields two peptide fragments (98). Fragment C (M_r ~45,000) consists of part of the heavy chain while fragment B (M_r ~97,000) contains the remaining portion of the heavy chain linked to the (apparently intact) light chain via a disulfide bond(s). Each fragment was found to be antigenic and elicited antibodies that neutralized the toxicity of native tetanus toxin. Interestingly, while neither fragment was able to induce a spastic paralysis

in mice, high doses of fragment B caused a slowly developing syndrome involving respiratory distress, weight loss, inability to take food, flaccid paralysis, and eventually death (107). This syndrome was not due to contamination by native toxin because antibodies to fragment C did not interfere with the ability of fragment B to induce the syndrome. It was suggested that fragment B has only limited toxicity (e.g. inability to cause spastic paralysis) because of an impaired capacity to bind to appropriate receptors. Implicitly, in native toxin the receptor binding site would be located in or near the portion of the heavy chain comprising the C fragment. Support for this hypothesis comes from the finding that fragment B was without ganglioside-binding activity while fragment C could bind gangliosides marginally (106). Furthermore, monovalent antibodies to fragment C interfered with the binding of native tetanus toxin to gangliosides but antibodies to the light chain did not (106).

The neuronal receptor for tetanus toxin intriguingly appears to be similar to the thyroid receptor for the peptide hormone thyrotropin. Under the binding conditions used, tetanus toxin reversibly binds to thyroid membranes, and this binding is blocked or reversed by thyrotropin but not by any of several other peptide hormones (108). Conversely, thyrotropin exhibits a reversible binding to rat brain membranes and this binding is blocked by tetanus toxin (109). However, the binding of tetanus toxin to the brain membranes is enhanced by thyrotropin (109). It has been suggested that some of the symptoms of tetanus toxicity may be caused by tetanus toxin–induced thyroid hyperfunction (108). Another similarity between tetanus toxin and thyrotropin is that each can affect the membrane potential of a target cell; tetanus toxin has recently been found to alter the ion permeability of synaptosomes from guinea pig brain (110).

Transport of Tetanus Toxin to the Central Nervous System

Tetanus toxin does not appear to reach the central nervous system through the circulatory system (111, 112). Rather the route is apparently via neurons that have axonal processes extending from the central nervous system. Tetanus toxin is taken up by adrenergic, sensory, and motor nerve terminals of the peripheral nervous system and then transported to the corresponding cell bodies by retrograde axonal transport (112–115). Using electron microscopic autoradiography it has been shown that 7–14 hr after intramuscular injection of rats with ^{125}I-labeled tetanus toxin, label appeared in the axons, perikaryon, and dendrites of motor neurons and also over neuronal terminals that were afferent to the motor neurons (116). This finding suggests that tetanus toxin can be transported transynaptically. Not all workers accept the view that axonal transport is the primary means by which tetanus toxin reaches the central nervous system (4).

Comparison of Tetanus Toxin and BoTx

Tetanus toxin and BoTx are similar with respect to bacterial genus of origin, structure, and effects on neuromuscular transmission. Tetanus toxin of course affects a broader range of nerve terminals. It is tempting to speculate that tetanus toxin and BoTx have a common molecular mechanism of action (e.g. enzymic activity) but with different target cells.

SNAKE VENOM TOXINS

Structure and Phospholipase A Activity

A variety of polypeptide neurotoxins are found in snake venoms. In addition to α type toxins, which act postsynaptically by binding to the ACh receptor (6), there are several different snake venom neurotoxins that act presynaptically to inhibit the evoked release of ACh. These presynaptically acting toxins exhibit phospholipase A_2 (PLA) activity.

Various studies indicate that neither is PLA activity simply a vestigial and irrelevant activity of the toxin molecule nor is it due to a contaminating enzyme. All known presynaptically acting neurotoxins from snake venoms have PLA activity (7–9) and, where examined, have been found to be homologous in amino acid sequence to well-established PLAs such as porcine pancreatic PLA (117–121). The neurotoxicity and PLA activity of these toxins have similar cation requirements (122, 123). All agents that inactivate the PLA activity of these toxins also inactivate their neurotoxicity (124–129). In some cases covalent modification of a single amino acid results in the loss of both activities (124, 127, 128, 130, 131). These studies all indicate that the PLA catalytic site of these toxins functions in their neurotoxicity. Paradoxically, most known PLAs are not neurotoxic in spite of having greater specific enzyme activity.

Table 1 gives the potencies and molecular weights of the four toxic PLAs that are best characterized. The comparative structure/function relationship of these four toxins is interesting. Notexin, from the Australian tiger snake *Notechis scutatus scutatus,* is a single polypeptide chain (118). Crotoxin, from *Crotalus durrissus terrificus,* is a complex of two noncovalently linked proteins: a basic PLA and a smaller acidic protein called crotapotin (9, 132–134). The acid protein by itself lacks neurotoxicity and PLA activity, but it potentiates the neurotoxicity of the PLA.

β-Bungarotoxin (β-BuTx) is from *Bungarus multicinctus,* the same snake that produces the postsynaptically acting α-bungarotoxin. β-BuTx is composed of two subunits that are linked by at least one disulfide bridge (135, 136). Both the PLA activity and neurotoxicity are lost upon reduction of the disulfide bonds (135). Attempts to reassociate an active protein have not been successful. The larger subunit of β-BuTx is homologous to other

PLAs (136). All of the β-BuTx studies discussed in this review have employed the toxin just described. However, it should be mentioned that *B. multicinctus* venom also contains other neurotoxic PLA isozymes that are less potent and have a somewhat different composition of subunits (137, 138). These isozymes, which are also called β-BuTx, have been assigned subscript designations (126) to avoid some of the confusion existing in the literature about which isozyme is being studied.

The fourth presynaptically acting neurotoxin from snakes is taipoxin, which comes from the taipan *Oxyuranus scutellatus scutellatus.* The lethality of taipoxin for mice is approximately 10 times greater than that of any of the other three presynaptically acting snake toxins. Taipoxin has three noncovalently linked subunits. The amino terminal portions of the three subunits have homologous amino acid sequences, which are in turn markedly similar to those of a basic PLA from *N. nigricollis* and the proenzyme of porcine pancreatic PLA (120). Only one of the subunits is toxic by itself, and it is 500-fold less potent on a molar basis than the complex. Interestingly, this subunit has greater PLA activity than the complex (128).

In summary it is noteworthy that comparable enzymatic and pharmacological activities are found in molecules with the following different structures: (*a*) a monomeric protein, (*b*) a complex of two noncovalently linked polypeptides, (*c*) a protein with two covalently linked subunits, and (*d*) a complex of three noncovalently linked polypeptides.

Effects on Neuromuscular Transmission

The PLA neurotoxins inhibit the evoked release of ACh from the terminals of motor neurons (6–9) and some but not all cholinergic neurons of the autonomic nervous system (139–143). These toxins are not known to affect noncholinergic terminals in the peripheral nervous system. Notexin blocks motor transmission in the guinea pig vas deferens and seminal vesicle but the mechanism of this effect is unclear (142).

The neurotoxic effects of β-BuTx have been studied more extensively than those of the other PLA toxins. It takes approximately 1.5 to 3 hr for β-BuTx to cause complete neuromuscular blockade when incubated with an isolated neuromuscular preparation (63, 144). The blockade occurs more quickly if the nerve is repetitively stimulated during the incubation (144). The time required for blockade of an isolated phrenic nerve-diaphragm preparation did not increase when the preparation was washed after a 15 min exposure to β-BuTx (139). After only a 5 min exposure to the toxin, a toxic activity equivalent to approximately one third of the dose of toxin initially added was still exhibited. Thus, β-BuTx either quickly binds to the preparation in an irreversible manner or quickly produces some irreversible

effect. Preincubation of an isolated neuromuscular preparation with β-BuTx that had been inactivated by chemical modification with bromophenacyl bromide caused an increase in the time required for blockade by subsequently applied native toxin (126, 129); thus, native toxin may bind to specific nerve terminal sites, which can be competitively blocked by the modified toxin. Similarly modified taipoxin does not confer protection against native taipoxin (128).

Electrophysiological analysis of the neuromuscular blockade caused by β-BuTx has revealed that the blockade occurs in three stages (126, 129, 144). Within 5 to 10 min after exposure of an isolated neuromuscular preparation to β-BuTx a slight reduction is observed in the amplitude of the evoked end-plate potential (epp). This first stage is followed by an increase over the subsequent 30 to 60 min in evoked epp amplitude. The third stage consists of a gradual decrease in evoked epp amplitude until epps are undetectable when the nerve is stimulated. During the third stage the frequency of mepps also gradually decreases until they are barely detectable at the onset of complete neuromuscular blockade. β-BuTx does not affect mepp amplitude.

Only the first stage of blockade is observed under conditions in which the phospholipase activity of β-BuTx is inactive, e.g. after modification with bromophenacyl bromide or when Ca^{2+} in the incubation medium is replaced by Sr^{2+} (126, 129). The PLA activity of the toxin is required for the second and third stages (126, 129). A conflicting result with Sr^{2+} has been reported (145).

The mechanism by which β-BuTx causes the first stage is unknown. The second stage has been attributed to an increase in the level of free Ca^{2+} in the cytosol of the nerve terminals (146, 147). The characteristics of this stage are consistent with that hypothesis. During the second stage there occurs a two to four fold increase in the frequency of mepps (144). In addition there is an increase in quantal content (144, 146) and in delayed release, i.e. an increase in the number of mepps observed just after an evoked response (146). Sometimes episodic bursts of mepps are observed (145, 147). β-BuTx and BoTx are mutually antagonistic; when a neuromuscular preparation is treated with both toxins simultaneously, the time required for blockade is greater than when the preparation is treated with either toxin alone (63). The basis for this mutual antagonism is unknown although β-BuTx could antagonize BoTx by increasing the internal concentration of free Ca^{2+} (79).

Under certain conditions β-BuTx can cause extensive destruction of the plasma membrane of frog nerve terminals as seen by electron microscopy (148). However, such damage is not required for toxin-induced neuromuscular blockade (149). Rat nerve terminals blocked by β-BuTx exhibit a

slight decrease in the number of synaptic vesicles and an increased number of invaginations of the plasma membrane (149). This morphology has been attributed to a toxin-induced inhibition of the endocytosis that functions to recycle vesicles after transmitter release (149–151). Swollen mitochondria are also sometimes seen (144). An early finding that β-BuTx–paralyzed nerve terminals contain a normal amount of ACh (144) is in conflict with a recent report that β-BuTx causes a two to three fold increase in the ACh level of isolated phrenic nerve-diaphragm preparations (152). The neuromuscular blockade induced by crotoxin, notexin, or taipoxin has characteristics similar to those described above for β-BuTx but in a species-dependent manner (123, 151, 153–157). For example, crotoxin increases mepp frequency in mouse diaphragm but not in rat diaphragm (154); the converse is true for taipoxin (151, 153, 155). Intriguingly, taipoxin is three to five times more potent than β-BuTx or crotoxin at causing neuromuscular blockade of the mouse diaphragm, but it is 30–100 times less potent than β-BuTx or crotoxin at blocking the chick biventer cervicis muscle preparation (155).

Two PLA neurotoxins, crotoxin and β-ceruleotoxin, from *B. ceruleus* venom cause desensitization of the ACh receptor when incubated in vitro with receptor-rich membranes from fish electric organ (158, 159). It is significant that the intact crotoxin complex of the basic PLA and crotapotin is more potent than the PLA component alone at desensitizing the ACh receptor; furthermore, the intact complex exhibits saturable binding to the receptor-rich membranes while the PLA component alone does not (159). These effects may be due to the toxins' binding to and hydrolysis of lipids of presynaptic membranes contaminating the ACh receptor-rich membranes used in these studies. It is known that the ACh receptor is desensitized by small amounts of fatty acids and lysophospholipids (160). Most electrophysiological studies of the neuromuscular blockade caused by these toxins have failed to show any postsynaptic effects of the toxins during the time required to produce blockade (154, 161, 162); however, there is one early report of a reduced response to ACh of a rat diaphragm preparation that had been treated with crotoxin (163).

Effects on the Central Nervous System

Animals do not exhibit symptoms of central nervous system involvement after intraperitoneal or intravenous injection with the PLA toxins, presumably because of the blood-brain barrier. However, at least one of these toxins, β-BuTx is lethal when injected intraventricularly into the brains of rats; indeed, the lethality of β-BuTx by this route of administration is greater than that for peripheral administration (164). Sublethal injections of β-BuTx into the brain cause widespread neuronal damage with no obvi-

ous selectivity toward cholinergic structures (164). For example, the time course of the decline in the activity of glutamate decarboxylase (a GABAergic marker) in β-BuTx–injected dentate gyrus parallels that of the decline in the same region of the activity of choline acetyltransferase (a cholinergic marker). However, it still remains to be determined whether the damage to noncholinergic structures results from an independent action of β-BuTx on these structures rather than being a secondary response to toxin action specifically on cholinergic nerve terminals. Studies of the effects of β-BuTx on synaptosomes to be discussed below suggest that the former is the case.

Effects on Muscle

Some neurotoxic PLAs are also myotoxic in that they induce a degenerative necrosis of skeletal muscle independently of their action on neurons. Among them are notexin (165–167), notechis II-5 from *N. s. scutatus* (167), and myotoxin VI from *Enhydrina schistosa* (168). The best characterized myotoxic effects are those produced by notexin. Application of notexin to a chronically denervated hemidiaphragm preparation from rat causes, with little latency, a progressive increase in muscle tension and decline in the amplitude of twitch evoked by direct muscle stimulation (166). Within 1 hr after injection of 2 μg of notexin into a rat hind limb, peripheral muscle fibers become edematous and by 12 hr fiber necrosis is well advanced (167). The fibers most affected are type I and that portion of type II fibers that are rich in mitochondria. Under these conditions the affected muscle regenerates. Covalent modification of a particular histidine of notexin results in the loss not only of its PLA activity but also its myotoxicity (167), suggesting that the PLA activity of notexin plays a role in its myotoxicity. The ability of some toxic PLAs to be both neurotoxic and myotoxic indicates that the neurotoxicity is not due to a block of some system that is specific for nerve terminals. We suggest below that the neurotoxicity and the myotoxicity of the PLA toxins are caused by a common molecular mechanism. The reason only some of the toxic PLAs are myotoxic is not known, but this difference in myotoxicity may have the same basis as the species-dependent differences in neurotoxicity described above.

Effects on Cell-Free Model Systems

MAMMALIAN BRAIN SYNAPTOSOMES Several cell-free model systems have been used to explore the mechanism of action of the toxic PLAs. β-BuTx produces a variety of effects on synaptosomes prepared from rat brain. After brief incubations with the toxin, the synaptosomes exhibit (*a*) a decreased ability to take up and retain several transmitter and non-transmitter compounds including γ-aminobutyrate, norepinephrine, serotonin, choline, and deoxyglucose (169–171); (*b*) a decreased level of

ATP (170); and (*c*) a decrease in plasma membrane potential as measured with fluorescent carbocyanine dyes (172, 173). The toxin produces these alterations without lysing the synaptosomes (169). The initial effect of β-BuTx is the decrease in synaptosome membrane potential; the decrease in synaptosomal ATP occurs only secondarily and is due at least in part to utilization of ATP by synaptosomal Na^+/K^+-ATPase in an attempt to reestablish the original membrane potential (173). The toxin-induced inhibition of various synaptosomal transport processes is also a secondary effect, which results from both the depolarization and the depletion of ATP stores. The ion fluxes involved in toxin-induced depolarization of synaptosomes have not been identified, although studies on mitochondria to be discussed below implicate Ca^{2+} transport. The toxin's activity on synaptosomes is independent of extracellular Na^+ concentration (173), indicating that the toxin does not depolarize synaptosomes by opening Na^+ channels of the plasma membrane or by inhibiting the Na^+/K^+-ATPase.

It has been proposed that β-BuTx causes neuromuscular blockade via the same mechanism by which it affects synaptosomal energy metabolism (173). A toxin-induced increase in intracellular free Ca^{2+} could account for the increased transmitter release occurring in the second stage of the blockade. The third stage of blockade could result from a subsequent depletion of energy stores in the terminals as occurs in toxin-treated synaptosomes.

A non-neurotoxic PLA, which is also present in ***B. multicinctus*** venom, produces effects on synaptosomes that are similar to those produced by β-BuTx (170, 173). Various comparative studies of β-BuTx and this non-neurotoxic PLA, called IVa PLA, indicate that the action of β-BuTx on synaptosomes is a mechanistically relevant correlate of its neurotoxicity at neuromuscular junctions while the action of the non-neurotoxic IVa PLA can simply be attributed to its nonspecific PLA activity (173). For example, even though the IVa enzyme has 20 times the PLA activity of β-BuTx, the IVa enzyme is less potent than β-BuTx in altering synaptosomal membrane potential and transport processes. Further evidence on this point comes from chemical modification of β-BuTx. The PLA activity and neurotoxicity of β-BuTx are both inactivated by treatment with ethoxyformic anhydride (EOFA), which acylates histidine residues and amino groups. The PLA activity of the toxin is resistant to EOFA treatment if the treatment is performed in the presence of dihexanoyllecithin (DiC_6), a substrate for the enzyme (125). However, even in the presence of DiC_6, EOFA destroys the lethality of β-BuTx and its ability to cause blockade of an isolated neuromuscular preparation. The net result of the treatment with EOFA and DiC_6 is to convert β-BuTx from a neurotoxic PLA to a PLA without neurotoxic activity. Howard & Truog (125) suggested that EOFA alters at least two sites on the toxin: the PLA active site and another site also required for neurotoxicity. Only the PLA active site can be protected by

DiC_6. The nontoxic β-BuTx created by treatment with EOFA and DiC_6 has apparently normal PLA activity even when the substrate in the enzyme assay is synaptosomes (173). However, the non-neurotoxic β-BuTx has lost the ability to alter synaptosomal membrane potential, ATP stores, and transport processes (173). Thus, the studies with EOFA and DiC_6 show that the activity of β-BuTx on synaptosomes correlates better with its in vivo neurotoxicity than with its PLA activity, indicating that its activity on synaptosomes is relevant to its neurotoxicity.

A different pattern is obtained when the non-neurotoxic IVa PLA is treated with EOFA and DiC_6. As is the case with β-BuTx, DiC_6 protects the PLA activity of the IVa enzyme. However, unlike the situation with β-BuTx, protecting the catalytic site of the IVa enzyme with DiC_6 was sufficient for retention of that enzyme's ability to reduce synaptosomal membrane potential and block transport processes (173). These results demonstrate that β-BuTx and the non-neurotoxic IVa enzyme alter synaptosomes by different mechanisms. A comparison made of the substrate specificities of β-BuTx and IVa PLA during their hydrolysis of synaptosomal phosphoglycerides has not revealed a molecular basis for this difference in mechanisms. β-BuTx and IVa PLA were indistinguishable with respect to the ratio of synaptosomal phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine hydrolyzed and the ratio of synaptosomal fatty acid types liberated (173).

MITOCHONDRIA AND SARCOPLASMIC RETICULUM β-BuTx and notexin inhibit Ca^{2+} uptake by isolated mitochondria from rat brain (174, 175) and by sarcoplasmic reticulum vesicles prepared from mammalian skeletal muscle (175, 176). These effects may also be mechanistically relevant to the toxicity of these enzymes. β-BuTx and notexin are more potent than the nontoxic IVa enzyme in inhibiting mitochondrial Ca^{2+} uptake even though they have less PLA activity than the IVa enzyme (175). Furthermore, the ability of β-BuTx to inhibit mitochondrial Ca^{2+} uptake is lost when the toxin is converted to a nontoxic PLA by treatment with EOFA and DiC_6 (175). The ability of various PLAs to inhibit Ca^{2+} uptake by sarcoplasmic reticulum vesicles from rat correlates better with their in vivo myotoxicity than with their PLA activity (175).

It has been proposed that the toxic PLAs act in vivo by entering the cytoplasm of the affected cell and exerting their effect on some internal structure (153). If this were indeed the case, the Ca^{2+} transport systems of nerve terminal mitochondria and muscle sarcoplasmic reticulum might be the actual targets in vivo of PLA neurotoxins and myotoxins, respectively. The pathological effects produced by these toxins in vivo are consistent with this mechanism. However, the toxins could also produce these pathological effects by acting at and altering Ca^{2+} extrusion across the plasma mem-

branes of nerve terminals and muscle. Until this alternative possibility has been excluded, it is perhaps better to consider mitochondria and sarcoplasmic reticulum only as model targets for the toxic PLAs. Nevertheless, whatever the actual target is for the toxin in vivo, it seems likely that any toxin-induced alteration of ion transport will occur via the same mechanism operating for the in vitro inhibition of Ca^{2+} uptake into brain mitochondria and sarcoplasmic reticulum.

TORPEDO SYNAPTOSOMES Several PLA toxins block choline transport into synaptosomes and nerve terminal sacs prepared from *Torpedo* electric organ (128, 177, 178) just as they do with synaptosomes from rat brain. Contrary to some suggestions (171, 177) it is unlikely that inhibition of choline transport into motor nerve terminals accounts for PLA toxin-induced neuromuscular blockade; the characteristics of the blockade (e.g. no change in mepp amplitude and a rise in ACh levels in β-BuTx-treated muscle) argue against this mechanism. The fact that some of these toxins are also active on noncholinergic structures such as GABAergic synaptosomes and muscle fibers also makes it unlikely that the choline carrier system serves as the specific binding target for the PLA neurotoxins in nerve terminals (128).

The effects of the PLA toxins on the model systems described above suggests that these toxins act by altering ion channels and/or ion pumps. Based on the well-established fact that degradation of membrane phosphoglycerides can inactivate membrane proteins (179), Ng & Howard (173) have proposed that the toxins hydrolyze some specific membrane phosphoglyceride(s) that functions in the activity of an ion channel or pump. This crucial phosphoglyceride could be expected to be somewhat resistant to nontoxic PLA by virtue of having a unique composition or location, e.g. an annular lipid for an ion channel. The toxic PLAs may be directed to lipids near the ion channel by specifically binding to the ion channel itself. Alternatively, the hydrolytic activity of these toxins may uniquely form some reaction product that is toxic to ion channels or pumps. Also, it is possible that the PLA catalytic sites of these toxins function only to accomplish specific binding of the toxins to a particular membrane lipid or to expose some molecular target whereas another toxin site actually acts to alter the affected ion channel or pump.

CONCLUSIONS

The polypeptide neurotoxins reviewed here produce a wide variety of effects on the storage and release of neurotransmitters. The morphological and electrophysiological aspects of these effects have been well characterized,

but too little is known about the actions of the toxins at the molecular level. There soon should be significant advances in this area, especially in characterizing the neuronal binding sites for the toxins. However, a word of caution seems appropriate in this regard. It is possible that these toxins do not inhibit transmitter release by a direct alteration of the release apparatus, itself. For example, changes in membrane permeability to ions have been implicated in the primary action of α-latrotoxin, tetanus toxin, and β-BuTx, suggesting that their effects on transmitter storage and release may be only secondary to the ion permeability changes. Thus, knowledge of the molecular mechanisms of action of these toxins will not necessarily lead to an understanding of the biochemistry of the release apparatus. Nevertheless, all of the toxins should be useful for labeling and characterizing presynaptic components that are important for some neuronal function.

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