Pflügers Arch (1987) 409:533–539

Pflügers Archiv
European Journal of Physiology

Distinct sites of action of clostridial neurotoxins revealed by double-poisoning of mouse motor nerve terminals *

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Abstract. (1) We investigated the effects of single- and double-poisoning with tetanus toxin (TeTx), botulinum neurotoxin type A (BoTx A) and botulinum neurotoxin type B (BoTx B) on spontaneous and nerve-evoked quantal transmitter release at motor endplates of the triangularis sterni preparation of the mouse. (2) Inhibitory effects of TeTx and BoTx B on spontaneous and nerve-evoked transmitter release were very similar, except that the action of BoTx B required 500-fold lower concentrations and was less dependent on temperature. BoTx A caused stronger inhibition of quantal release than TeTx or BoTx B, but was comparatively much easier counteracted by 4-aminopyridine (4-AP). (3) In contrast to BoTx A, with TeTx or BoTx B the increase of transmitter release following onset of 50 Hz nerve stimulation was delayed for a few seconds and synaptic latencies of quanta showed large variations. This release pattern was also evident in all double-poisoning experiments, regardless of intoxication sequence. (4) Inhibition of evoked release was found to be slightly stronger with TeTx than with BoTx B, so the amount of nerve-evoked quanta released after double-poisoning with any sequence of these toxins always approached that of TeTx. In no case supra-additive actions were observed. (5) A strong reduction of evoked quanta was observed when BoTx A was applied in addition to either of the two other toxins. With reversed poisoning sequences (BoTx A – TeTx or BoTx A – BoTx B) the resulting values remained at the extremely low level of BoTx A. (6) In the presence of 4-AP double-poisoning with any combination between BoTx A and TeTx or BoTx B (regardless of intoxication sequence) revealed supra-additive effects, since the number of quanta released was considerably lower than that obtained with any of the toxins alone (in the presence of 4-AP). (7) Our results indicate that tetanus toxin and botulinum toxin type B have a common site of action which is different and independent from that of botulinum toxin type A.

Key words: Neurotoxin – Transmitter release – Neuromuscular junction – Tetanus toxin – Botulinum toxin – 4-Aminopyridine

* This is part of the thesis of M. G. to be presented to the Fachbereich Humanmedizin, Justus-Liebig-Universität Gießen

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Introduction

The bacterial genus Clostridium produces the most potent polypeptide neurotoxins to inhibit transmitter release from peripheral as well as central synapses (for reviews see Mellanby 1984; Simpson 1986; Habermann and Dreyer 1986). In addition to the well characterized tetanus toxin, several immunologically distinguishable types of botulinal neurotoxins (A, B, C_1, D, E, F, G) have been described (Sugiyama 1980). Apparently, some of them influence acetylcholine release from neuromuscular junctions in a way that much resembles tetanus toxin, suggesting a common molecular mechanism at the same site in the chain of events leading to exocytosis.

However, while nerve-evoked quanta are asynchronously released with tetanus toxin (Dreyer and Schmitt 1981) and botulinum toxins type B (Sellin et al. 1983b), type D (Harris and Miledi 1971; own unpublished observations) and type F (Sellin et al. 1983a), this is not seen with botulinum toxin type A, where quanta show normal synaptic delays. Furthermore, botulinum toxin type A has a higher maximal efficacy of inhibition of spontaneous and evoked quantal release than tetanus toxin. But interestingly, botulinum toxin A treated endplates remain vulnerable to the releasing effects of subsequently applied black widow spider venom (Cull-Candy et al. 1976; Kao et al. 1976), whereas poisoning with tetanus toxin almost completely prevents the spider venom actions (Dreyer et al. 1984). Thus, it may be hypothesized that the action of botulinum toxin type A is not just a 'variation on a common molecular theme' but due to a rather different molecular mechanism.

To shed more light on this subject we have carried out experiments in which nerve-muscle preparations were sequentially double-poisoned with all possible combinations of tetanus toxin and botulinum toxins type A and type B. Our results indicate that tetanus toxin and botulinum toxin type B have a common site of action which is different from that of botulinum toxin type A.

Methods

Experiments were performed on the triangularis sterni nerve-muscle preparation of adult mice (McArdle et al. 1981). This preparation was found to respond to the clostridial neurotoxins in much the same way as the mouse hemidiaphragm preparation, but offered an easier and faster localization of endplates. Bath solution comprised (mM): NaCl 115, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, $NaHCO_3$ 25, Na_2HPO_4 1, glucose 11, gassed with 95% O_2/5% CO_2

Table 1. Effects of clostridial neurotoxins on spontaneous and nerve-evoked transmitter release during 50 Hz nerve stimulation

	spontaneous release (m.e.p.ps/min)	Nerve-evoked release (Quanta/100 pulses) control	4-AP (100 μM)
Control	473 ±24 (30)	112 ±15/pulse[a]	728±167/pulse[b]
TeTx	25.7± 1.9 (25)	8.9± 0.9 (24)	256± 21 (26)
BoTx B	19.0± 1.4 (25)	13.9± 0.7 (22)	319± 29 (23)
BoTx A	1.4± 0.1 (22)	0.9± 0.1 (24)	805± 63 (29)

Values reflect mean ± SE (number of endplates investigated is indicated in parentheses, number of preparations ranged between 2 and 5). Data were acquired at 37° C

[a] Value corresponds to the ratio of mean endplate current (e.p.c.) to the mean miniature endplate current (m.e.p.c.) from 17 muscle fibres voltage clamped at −70 mV. Currents were measured using conventional two-electrode voltage clamp techniques

[b] Value was calculated by scaling the mean number of quanta constituting a normal e.p.p. by a factor derived from the ratio of e.p.c. amplitude in test solution (100 μM 4-AP) to that in control solution ($n = 10$). In these experiments d-tubocurarine (1.5 μM) was present to avoid muscle twitching and to allow the measurement of the relative amplitude of endplate currents in the same muscle fibre under both conditions

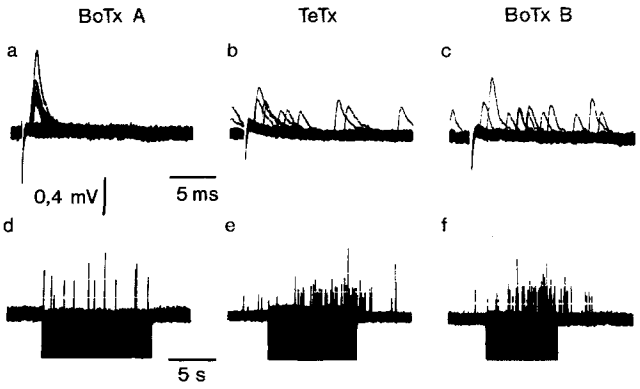

Fig. 1a–f. Effects of clostridial neurotoxins on quantal release evoked by 50 Hz nerve stimulation. **a–c**, superimposed traces in fast time sweep, revealing either synchronous or asynchronous release of quanta. 1000 traces (20 s) were sampled in **a**, while **b** and **c** reflect 100 traces (2 s). **d–f**, typical pattern of evoked release before, during and after brief periods of 50 Hz stimulation, recorded at slow time sweep. Downward deflection from the baseline corresponds to cutoff stimulation artifacts

(pH 7.3). Conventional microelectrode techniques were used to monitor spontaneous miniature endplate potentials (m.e.p.ps) and nerve-evoked endplate potentials (e.p.ps).

Botulinum toxin type A was a gift from Dr. Schantz (Food Research Institute, Madison, WI, USA). The neurotoxic component which was used in this study was separated from the hemagglutinin as described by Moberg and Sugiyama (1978). Tetanus toxin was provided by Dr. Bizzini (Institut Pasteur, Paris, France) and Botulinum neurotoxin type B was from Battelle Institut (Frankfurt, FRG). The LD_{50} values for all three toxins were in the range of 3–6 ng/kg (mouse, s.c. in the neck). Black widow spider venom was a crude extract from the venom glands of *Latrodectus mactans*. It was used at a final concentration of 0.04 glands/ml.

For in vitro intoxication nerve-muscle preparations were exposed to toxin for 1 h at room temperature and subsequently superfused with Ringer solution kept at 37° C. When employing appropriate toxin concentrations (TeTx: 5 μg/ml, BoTx A: 10 ng/ml, BoTx B: 10 ng/ml) this procedure ensured for any of the three toxins to produce maximal inhibitory effects on spontaneous and nerve-evoked transmitter release within 60–90 min. For double-poisoning experiments the same procedure was subsequently repeated using a second toxin.

Results

Effects of single clostridial neurotoxins

For correct evaluation of double-poisoning experiments, the maximal inhibitory effects of tetanus toxin (TeTx), botulinum neurotoxin type A (BoTx A) and botulinum neurotoxin type B (BoTx B) alone were characterized. The concentrations of toxins employed produced a degree of poisoning that may be regarded as supramaximal, since both tenfold lower or higher concentrations were equally effective at inhibiting transmitter release, eventually. They only affected the time course of paralysis. Comparable to the data obtained in other nerve-muscle preparations (Spitzer 1972; Duchen and Tonge 1973; Kryzhanowsky 1973; Cull-Candy et al. 1976; Dreyer and Schmitt 1981; Sellin et al. 1983b), all three toxins potently reduced spontaneous and nerve-evoked quantal release. TeTx and BoTx B reduced the average frequency of miniature endplate potentials (m.e.p.ps) of control cells (about 480/min) to values of 26/min and 19/min, respectively. An even stronger inhibition to 1.4 quanta/min was obtained with BoTx A (Table 1).

Nerve stimulation at frequencies lower than 10 Hz was ineffective in eliciting quantal release. When transmitter release was evoked by nerve stimulation at 50 Hz, release probability was increased, but values were still very low when compared with the number of about 100 quanta constituting a normal endplate potential (see Table 1). Yet, the above mentioned relative differences in efficacy between the three toxins were also evident here, i.e. BoTx A showed stronger inhibitory effects than TeTx or BoTx B.

Additional characteristic differences between BoTx A on one hand and TeTx and BoTx B on the other hand were revealed by the distribution of synaptic latencies of quanta released at high frequency nerve stimulation (Dreyer and Schmitt 1981; Sellin et al. 1983b). With BoTx A a constant and regular delay between stimulus and the rather few postsynaptic responses was consistently exhibited (Fig. 1a). This is in contrast to the effects of TeTx or BoTx B which both caused a characteristic desynchronization of nerve-evoked quanta (Fig. 1b and c). Further typical differences concern the release pattern following onset and offset of stimulation. With TeTx and BoTx B responses to trains of stimuli appeared after a considerable delay of 2–3 s. Furthermore, a period of a few seconds immediately following times of stimulation, was characterized by an elevated level of quantal release (Fig. 1e and f). This was not the case with BoTx A, where transmitter release after onset of stimulation was facilitated without any delay and spontaneous release following times of stimulation was found to remain unchanged at extremely low levels (Fig. 1d).

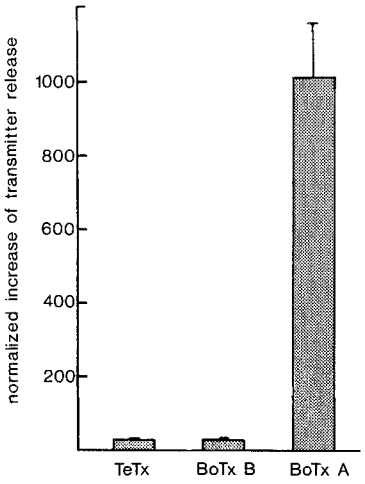

Fig. 2. Relative increase of nerve-evoked transmitter release due to 4-AP at single-poisoned endplates. The values correspond to the ratio of the number of quanta released by 50 Hz nerve stimulation in the presence of 100 μM 4-AP to that in control solution (Table 1)

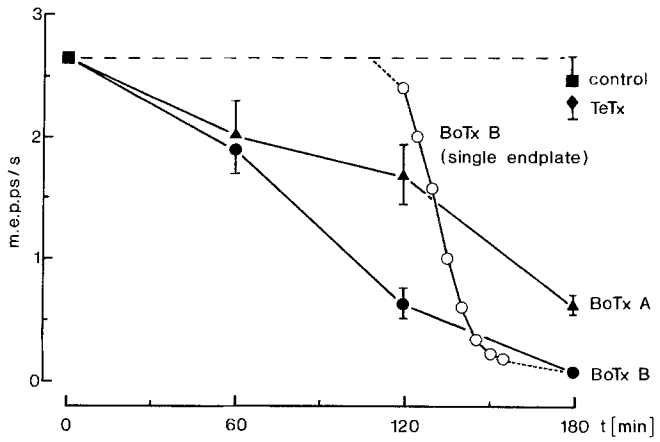

Fig. 3. Effects of clostridial neurotoxins on spontaneous transmitter release at 20° C. Nerve-muscle preparations received the regular doses of neurotoxins (see Methods) at time 0. *Points* correspond to the m.e.p.p. frequency (mean ± SEM) of 15–20 endplates out of three preparations. In addition, a typical time course of m.e.p.p. frequency in a single endplate treated with BoTx B is illustrated. In this case recording started 120 min after toxin application, but it should be noted that similar inhibitory effects of BoTx B started as early as 50 min and as late as 150 min after toxin administration

Potassium channel blockade by aminopyridines is one of several procedures which are believed to increase intraterminal levels of free Ca^{2+} and which are known to at least partially overcome the inhibitory effects of clostridial neurotoxins (Lundh et al. 1977; Dreyer and Schmitt 1981; Sellin et al. 1983b). Interestingly, inhibition by BoTx A, which was the strongest found among the toxins, was comparatively much easier counteracted by the addition of 4-AP than inhibition caused by TeTx or BoTx B. While normally BoTx A effected 100% failure of response to nerve stimuli applied at frequencies lower than 10 Hz, addition of 4-AP (100 μM) consistently elicited an average of 8 quanta per pulse regardless of the stimulation frequency. With TeTx or BoTx B the failure rate of 100% when applying pulses at a frequency lower than 10 Hz could not be reduced by 4-AP. The comparatively strong facilitatory effect of 4-AP on BoTx A treated preparations was also evident from the relative increase in the number of quanta released at 50 Hz stimulation (Fig. 2) and even the absolute number of nerve-evoked quanta was about three times higher than that of TeTx or BoTx B (Table 1). Thus, it appears that in the presence of TeTx or BoTx B even excessive Ca^{2+} entry only moderately increases transmitter release.

We have previously shown that black widow spider venom (BWSV) may be used as a tool to characterize clostridial neurotoxins, since preparations previously poisoned with TeTx or BoTx A respond considerably different to the spider venom (Dreyer et al. 1984). Addition of BWSV (0.04 glands/ml) to a preparation intoxicated with BoTx B produced very similar responses to those known from TeTx poisoned endplates (cf. Dreyer et al. 1986). Typically, the frequency of m.e.p.ps at BoTx B poisoned endplates (19/min) increased within 2–4 min to values of about 40–90/s for a period of about 10 min, after which it invariably declined to very low values (0.2–0.5/min). Within the subsequent observation period of 50 min release of 3–6 'anomalous m.e.p.ps' of variable amplitude (3–10 mV) and long duration (100–200 ms) occurred (not shown).

So far, the results presented suggest that TeTx and BoTx B have a similar, if not identical, mode of action. But we have also noticed one interesting difference between the two toxins. In agreement with previous studies (Habermann et al. 1980; Schmitt et al. 1981) the inhibitory action of TeTx was critically dependent on temperature. At 20° C no effect of TeTx on m.e.p.p. frequency was observed for 3 h after toxin application (Fig. 3). In contrast, BoTx B effectively reduced spontaneous release at this temperature. Similar effects were seen with BoTx A, although they developed considerably slower. The inhibitory effect of BoTx B on spontaneous release at 20° C in a single endplate could happen any time between 60 to 180 min, and its time course was rather fast, as illustrated in Fig. 3.

The development of inhibitory actions of BoTx B on spontaneous and evoked release appeared to proceed in parallel. In cells which had reached the low m.e.p.p. frequency of completely poisoned endplates, also no e.p.p. could be elicited. Likewise, endplates of neighbouring muscle fibres which still showed no reduction in spontaneous release, produced normal e.p.ps and muscle twitching.

Double-poisoning experiments

When TeTx intoxication was established first, subsequent poisoning with BoTx A or BoTx B showed no major alterations in spontaneous m.e.p.p. frequency (Fig. 4). Similarly, when BoTx B was the first toxin to act no significant modifications of spontaneous quantal release were observed with the other toxins (Fig. 4). Interestingly, the extremely low m.e.p.p. frequencies of endplates initially treated with BoTx A did not persist when TeTx or BoTx B were subsequently allowed to exert their actions. Instead, values rather tended to approach the ones normally found with TeTx or BoTx B alone.

Since release of quanta was either synchronized (BoTx A) or desynchronized (TeTx and BoTx B), it was of interest how double-poisoning would affect nerve-evoked release. In all experiments with preparations that were

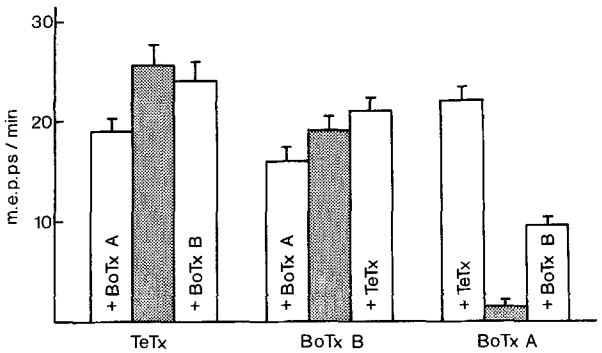

Fig. 4. Effects of double-poisoning on spontaneous transmitter release. Values correspond to the number of m.e.p.ps/min and are given as mean ± SEM (*n* ranged between 22 and 26 endplates out of three preparations). *Hatched middle bars* in each group correspond to the value of the initially applied toxin, while neighbouring *open bars* reflect the changes caused by subsequently applied toxins. *Bars* are labeled in the graph according to the treatment

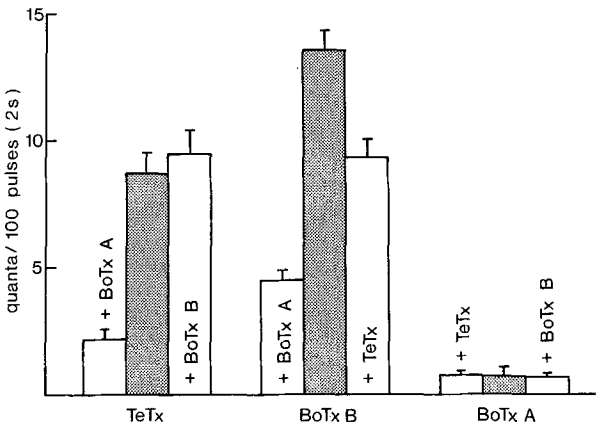

Fig. 5. Effects of double-poisoning on transmitter release evoked by 50 Hz nerve stimulation. Values correspond to the number of quanta/2 s (100 stimuli), measured 5 s after onset of stimulation. Data are given as mean ± SEM (*n* ranged between 19 and 26 determinations out of three preparations). *Hatched middle bars* in each group correspond to the value of the initially applied toxin, while neighbouring *open bars* reflect the changes caused by the subsequently applied toxins. *Bars* are labeled in the graph according to the treatment

sequentially exposed to two toxins (regardless of the intoxication sequence) TeTx or BoTx B determined the release pattern induced by nerve-stimulation at 50 Hz, i.e. increase of transmitter release following onset of stimulation was delayed for a few seconds and synaptic latencies of quanta showed large variations (cf. Fig. 1). It thus appears that this characteristic feature of TeTx and BoTx B, once established, cannot be reversed by BoTx A, nor does the latter prevent the actions of the former.

The lack of major alterations in the number of nerve-evoked quanta in double-poisoning experiments with the sequence TeTx – BoTx B indicate further analogies in the effects of the two toxins (Fig. 5). However, the reverse sequence (BoTx B – TeTx) showed a reduction of quanta to

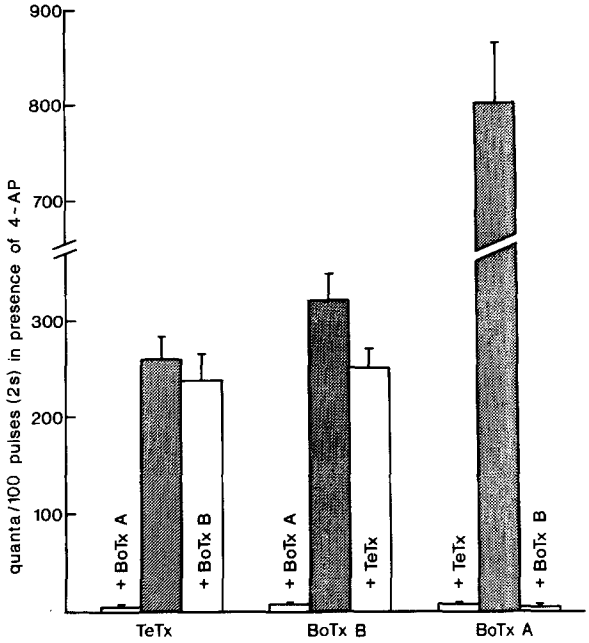

Fig. 6. Effects of double-poisoning on nerve-evoked transmitter release in the presence of 4-AP. Values correspond to the number of quanta elicited by 2 s of 50 Hz nerve stimulation in the presence of 4-AP (100 μM), measured 5 s after onset of stimulation. Values are the mean ± SEM (*n* ranged between 23 and 29 determinations out of three preparations). Values for initially applied toxins are represented by *hatched middle bars*, while neighbouring *open bars* reflect the changes caused by subsequently applied toxins. *Bars* are labeled in the graph according to the treatment

values normally found with TeTx alone. A strong reduction of released quanta was observed when BoTx A was applied in addition to either of the two other toxins (Fig. 5). When reversing poisoning sequences (BoTx A – TeTx or BoTx A – BoTx B), the resulting values remained at the extremely low level of BoTx A.

In the presence of 4-AP some remarkable effects on nerve-evoked quantal release were observed when preparations were sequentially double-poisoned. As has been mentioned above, evoked transmitter release was greatly enhanced when adding 4-AP (100 μM) to a BoTx A poisoned preparation. Subsequent application of TeTx or BoTx B resulted in a strong depression of transmitter release (Fig. 6). These actions were more than additive, since the number of quanta released was considerably lower than that obtained with any of the toxins alone (in the presence of 4-AP). Quite similar results were obtained when BoTx A was applied in addition to either TeTx or BoTx B (Fig. 6). In the presence of 4-AP double-poisoning with TeTx followed by BoTx B and vice versa showed quite similar results to those in the absence of the K channel blocker, i.e. BoTx B added to TeTx revealed no further inhibition of quantal release, whereas TeTx when added to BoTx B showed a reduction to values normally found with TeTx alone (in the presence of 4-AP).

Discussion

The results of the present study clearly suggest that the two neurotoxins tetanus toxin and botulinum toxin type B share

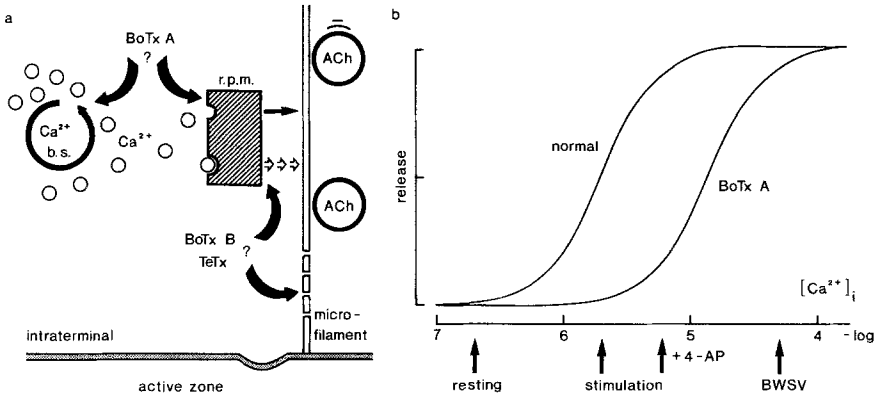

Fig. 7 a, b. Model of events involved in transmitter release process and possible sites of actions of clostridial neurotoxins. **a**, the model implies entry of Ca^{2+} into the cytosol, where it binds to a putative 'release promoting molecule' (r.p.m.). Subsequent activation of cytoskeletal microfilaments mediates transport of vesicles containing acetylcholine (ACh) towards the active zones, where vesicle fusion occurs. Intraterminal Ca^{2+}-level after stimulation is quickly brought to resting levels by Ca^{2+}-buffering systems (Ca^{2+} b.s.). **b** model suggests BoTx A to cause a shift of the intracellular Ca^{2+} concentration-response curve to right. *Arrows* on *abscissa* indicate resting, stimulation-induced (in the absence and presence of 4-AP) and BWSV-induced Ca^{2+} levels within the nerve terminal. Intracellular Ca^{2+} concentration, slopes of the curves and the actual location of *arrows* should be regarded as rough estimates, merely to provide and apprehensible notion of the hypothetic BoTx A action

a common mechanism of action at the same site in the chain of events leading to transmitter release. Both toxins have indistinguishable qualitative effects on transmitter release in as much as they produce a characteristic desynchronization of quanta evoked by 50 Hz nerve stimulation and they both protect against the releasing effects of BWSV. Also quantitative aspects of the inhibition of spontaneous and nerve-evoked release caused by TeTx and BoTx B are very similar. However, it appears that TeTx has a slightly higher maximal efficacy of inhibition of quantal release evoked by 50 Hz, both in the presence and absence of 4-AP. So not surprisingly, the values of the number of quanta released after double-poisoning with any sequence of BoTx B and TeTx always approached those of TeTx and in no case supra-additive actions were observed.

Two differences between TeTx and BoTx B are both worth to be mentioned. Compared with TeTx, the blocking effect of BoTx B to take place required about 500 times lower concentrations and was less dependent on temperature. While the former finding may reflect differences in acceptor density at the presynaptic membrane or different binding affinities of the two toxins, the latter may be mechanistically interesting. If one accepts that TeTx and BoTx B affect the same molecular process, differences in temperature dependence are likely to reflect toxin properties. From experiments in which TeTx was intracellularly injected into chromaffin cells (Penner et al. 1986), there is good evidence that the crucial temperature dependence in TeTx action is subsequent to binding and internalization. It is tempting to assume some enzymatic activity associated with the toxins, which may be modulated by temperature.

When compared with TeTx or BoTx B, three major characteristic differences of BoTx A are obvious. Nerve-evoked quanta are synchronously released, release probability can considerably be increased by 4-AP and the toxin does not protect against transmitter release induced by BWSV. These findings indicate that BoTx A on one hand and TeTx and BoTx B on the other hand may have different sites of action. Although at present, these sites cannot exactly be localized, we would like to discuss a hypothetic model which accounts reasonably well for the effects observed on nerve-evoked transmitter release (Fig. 7).

It is generally believed that transmitter release is initiated by Ca^{2+} entry into the nerve terminal. Let us assume that subsequent binding of Ca^{2+} ions results in activation of a 'release promoting molecule' which mediates transport of synaptic vesicles by contractile microfilaments (see Fig. 7). Since BoTx A does not inhibit Ca influx into nerve terminals (Gundersen et al. 1982; Dreyer et al. 1983) and the synchronized transport of vesicles still functions (although at a very reduced level), BoTx A presumably interrupts the exocytotic process at a step subsequent to Ca^{2+} entry and prior to the activation of contractile elements. It has been hypothesized that BoTx A reduces the sensitivity of a Ca^{2+}-binding protein towards Ca^{2+} (Mellanby 1984). Another hypothesis suggests that BoTx A increases the capacity of cytoplasmic Ca^{2+} buffering as a result of stimulating the efficacy of Ca^{2+} disposal mechanisms, thereby reducing the availability of intracellular Ca^{2+} (Molgo and Thesleff 1984).

These hypothetic mechanisms are both tantamount to a shift of the concentration-response curve of intracellular Ca^{2+} to the right (Fig. 7). This notion is particularly compelling in view of the ability to at least partially relieve inhibitory actions of BoTx A by 4-AP which is believed to functionally shift this curve to the left by increasing Ca^{2+} influx. Interestingly, the increase of the number of quanta released from BoTx A poisoned endplates by 50 Hz nerve stimulation was comparatively small. This may result from the inability of high frequency stimulation to sufficiently elevate intracellular Ca^{2+} level, whereas this is achieved by the action of 4-AP. It should be borne in mind, however, that regardless of the treatments, the release process is still strongly obstructed by the toxin (Table 1).

The effects of TeTx and BoTx B can be accounted for by assuming interaction with another, yet decisive step in exocytosis. Several steps of the secretory response are conceivable targets for the toxins' action. Interference with the coordinated transport of vesicles towards the membrane, appears as an attractive idea of how TeTx and BoTx B

might act. Disturbance of the transport mechanism (which is thought to be accomplished by cytoskeletal elements) may be achieved by damaging microfilaments directly, or by obstructing their activation through the putative 'release promoting molecule'. Both hypothetic mechanisms will strongly reduce transmitter release and conclusively explain desynchronization of nerve-evoked quanta. In any case the action of TeTx and BoTx B seems to happen at a stage that has comparatively low (if any) direct dependence on Ca^{2+}. This may be concluded from the effects of procedures which cause an increase of Ca^{2+} influx (frequent stimulation, 4-AP) but only moderately overcome inhibitory actions of the toxins. Likewise, if one assumes that the facilitatory action of BWSV at control endplates is caused by a massive Ca^{2+} influx into the nerve terminals (Grasso et al. 1980; Nicholls et al. 1982), this would also explain why BWSV is so little effective at TeTx or BoTx B poisoned preparations.

With the assumption of two independent sites of action one would expect, when two toxins are allowed to exert their maximal actions, that inhibition of transmitter release will be stronger than the inhibition caused by any of the toxins alone. However, this was only the case in experiments carried out in the presence of 4-AP, whereas in control solution the number of quanta released by 50 Hz nerve stimulation after BoTx A intoxication remained unaltered by TeTx or BoTx B. A possible reason for this finding may be that the output of quantal transmitter release after BoTx A treatment represents a basic minimal release which cannot be lowered by the other toxins. When raising nerve-evoked transmitter release above this minimal level by adding 4-AP, the inhibitory effects of TeTx or BoTx B are revealed and clearly become supra-additive, suggesting that TeTx and BoTx B had blocked those quanta which passed the block by BoTx A.

In experiments where BoTx A was subsequently applied to TeTx or BoTx B, a strong reduction of the number of quanta released by 50 Hz nerve stimulation was observed. While in the presence of 4-AP the inhibitory effect was again supra-additive, the degree of inhibition obtained in control solution did not quite reach the presumed minimal level usually achieved by BoTx A alone. This finding may possibly be due to pharmacokinetic implications of double-poisoning with the result that intoxication with the second toxin may be somewhat less effective. If this were a general phenomenon in our experiments, the data would slightly overestimate the minimal quantal release after double-poisoning.

The data acquired for spontaneous release cannot readily be interpreted in terms of the model proposed, since they do not reveal additive effects of the toxins. In fact, increases in m.e.p.p. frequency are registered with poisoning sequences BoTx A – TeTx or BoTx A – BoTx B. These phenomena may result from channel forming properties of the toxins (Borochov-Neori et al. 1984; Hoch et al. 1985).

In conclusion our data suggest that BoTx A has a site of action which is different and independent from that of BoTx B and TeTx. The results are consistent with the idea that BoTx A may influence the sensitivity of a Ca^{2+}-dependent 'release promoting molecule' or may affect processes that regulate the availability of free Ca^{2+}. In contrast, TeTx and BoTx B exert their actions at a site subsequent to Ca^{2+} binding, producing an essentially irreversible inhibition by directly interfering with the function of microfilaments or alternatively, by obstructing their activation by a putative 'release promoting molecule'. In view of the close relationship of clostridial neurotoxins this latter possibility appears particularly attractive as it infers the toxins to affect a common molecular target in different ways.

Acknowledgement. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 47).

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Received November 28, 1986/Accepted March 13, 1987