Model for the Interaction of Crotoxin, a Phospholipase A_2 Neurotoxin, with Presynaptic Membranes†

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ABSTRACT: Crotoxin is a phospholipase A_2 neurotoxin that impairs the release of acetylcholine at neuromuscular junctions, primarily at the presynaptic level. It associates a phospholipase A_2 subunit, CB, with a chaperon subunit, CA. We have shown elsewhere that the purely cholinergic synaptosomes from the *Torpedo* electric organ provided a convenient model to study the pharmacology of crotoxin and other related neurotoxins [Délot, E., & Bon, C. (1992) *J. Neurochem. 58*, 311–319]. In the present experiments, we labeled crotoxin with ^{125}I and demonstrated saturable binding to *Torpedo* presynaptic membranes. In the range of concentrations that was effective on synaptosomes, crotoxin bound to a single class of sites without cooperativity. The binding was affected by divalent cations, and its kinetics was rather complex. We observed a competition between crotoxin and related neurotoxins, but not CB. Although CA could not bind, it could compete efficiently with crotoxin. We compare our results with those previously obtained by others on guinea pig brain membranes. On *Torpedo* membranes, as on all models tested, CB was the major species bound to the membrane, while CA remained in solution. However, the mechanism underlying this phenomenon had never been clarified. The major question is the time scale of the events, *i.e.*, does CB bind before or after dissociating from CA? Our results indicate that the predominant pathway involves the formation of a ternary complex between crotoxin's subunits and the acceptor site preceding the release of CA.

Crotoxin, the major protein from the venom of the South American rattlesnake (*Crotalus durissus terrificus*), belongs to the class of β-neurotoxins. These neurotoxins from snake venoms block neurotransmission at neuromuscular junctions, primarily at the presynaptic level, by impairing acetylcholine release and cause death by respiratory failure. All β-neurotoxins are phospholipase A_2's. Their structures are, however, quite different. Some associate the enzymatic activity and the neurotoxicity in a single chain, such as ammodytoxin A, from the European *Vipera ammodytes* (Ritonja & Gubensek, 1985), or agkistrodotoxin, from the Asian viper *Agkistrodon blomhoffii brevicaudus* (Xu, 1990). In β-bungarotoxins, from *Bungarus multicinctus*, a phospholipase A_2 is linked covalently to a subunit analogous to pancreatic trypsin inhibitors (Kondo et al., 1982a,b). All other β-neurotoxins, including crotoxin, are noncovalent oligomers. Crotoxin associates a weakly toxic phospholipase A_2 subunit, CB, with a nontoxic, nonenzymatic subunit, CA (Hendon & Fraenkel-Conrat, 1971).

The structures of β-neurotoxins and their effects on nerve–muscle preparations have been studied extensively [for a review, see Strong (1987) and Hawgood and Bon (1991)]. However, the mechanism of recognition of their acceptor on neuronal membranes is not understood. Only for β-bungarotoxin has an acceptor been clearly identified. It is a multimeric protein with K^+-channel properties (Schmidt & Betz, 1989). Electrophysiological experiments show that crotoxin could also interact with a K^+-channel (Rowan & Harvey, 1988). However, much experimental evidence indicates that β-bungarotoxin and crotoxin interact with different acceptors: crotoxin and β-bungarotoxin are synergic at neuromuscular junctions (Chang & Su, 1980); no binding competition was ever observed on any membrane preparation (Rehm & Betz, 1982; Degn et al., 1991; Tzeng et al., 1986); these toxins also have different behavior on *Torpedo* synaptosomes (Délot & Bon, 1992).

Up to now, saturable binding of crotoxin has only been demonstrated on mammalian brain synaptosomal membranes (Degn et al., 1991; Tzeng et al., 1986) and *Torpedo* electric organ postsynaptic membranes (Bon et al., 1979). On these models, as well as on red blood cells (Jeng et al., 1978) and phospholipid vesicles (Radvanyi et al., 1989c), CA behaves as a chaperone toward CB, preventing its nonspecific binding. However, the brain membranes are not purely cholinergic, and the mechanism of toxicity may be different in the brain and at the neuromuscular junction. In fact, crotoxin is much more toxic by the intra-cerebroventricular route than by the systemic one (C. Bon and B. Saliou, unpublished observation). *Torpedo* postsynaptic membranes did not give indications of the effect of crotoxin on nerve terminals, where β-neurotoxins exert their primary effect. We have shown elsewhere that isolated nerve endings (synaptosomes) from *Torpedo* electric organ provide a convenient, purely cholinergic and presynaptic model to study the pharmacology of crotoxin and other β-neurotoxins (Délot & Bon, 1992). In the present experiments, we obtained fully functional radiolabeled crotoxin and studied its binding on this presynaptic cholinergic preparation.

On all models tested before, CB was the major species bound to the membrane, while CA remained in solution. However the mechanism underlying this phenomenon had never been clarified. Indirect evidence, obtained from binding experiments on phospholipid vesicles, indicated that, under some experimental conditions, the binding of crotoxin could occur before its dissociation (F. Radvanyi, personal communication). In the present study, we analyzed the kinetics of binding and of heterologous and homologous competitions in order to clarify

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whether CA dissociates from CB before or after CB has bound to the membrane.

MATERIALS AND METHODS

Toxins. Crotoxin was purified from *Crotalus durissus terrificus* venom, and its subunits were separated as described (Hendon & Fraenkel-Conrat, 1971). Isoforms of each subunit were further purified (Faure et al., 1991). Isoforms CA_2 and CB_d were used to reconstitute a crotoxin complex for labeling. The reconstitution was performed with a molar excess of CA over CB (1.2/1) in order to ensure total association of the phospholipase subunit (Hendon & Fraenkel-Conrat, 1971). Separation of CA, CB, and CACB was performed on a Mono-Q (Pharmacia, Uppsala, Sweden) anion-exchanging column as described (Faure & Bon, 1988). Electrophoresis on 15% polyacrylamide gels under denaturating conditions (SDS–PAGE) was performed according to Laemmli (1970). Ammodytoxin A was from Latoxan (Rosans, France), β-bungarotoxin was from Sigma Chemical Co. (St. Louis, MO) and agkistrodotoxin was a gift from Prof. Y.-C. Chen (Shanghai Institute of Biochemistry, China). All other reagents were of the best grade available.

Toxin Labeling. Iodination of crotoxin was performed with Iodo-gen (Pierce, The Netherlands) according to the manufacturer's instructions. Typically, 125 μg of Iodo-gen in chloroform was dried on the walls of a polypropylene microtube under a nitrogen stream. Crotoxin (50 nmol in sodium phosphate buffer (pH 7.0), 50 mM) was added, and the reaction was triggered by the addition of NaI. The reaction was allowed to proceed for 5–7 min at room temperature and stopped when the mixture was removed from the tube. Excess free iodine was retained by gel filtration on a 5-mL G25 fine Sephadex (Pharmacia) handmade column.

Different protocols were tried successively to separate labeled from unlabeled toxin, as this is a critical step to ascertain the homogeneous binding of the preparation. We first tried to label both subunits separately. CB, even with a moderate incorporation of iodine (0.75 mol per mole of CB), lost 50% toxicity and 40% enzymatic activity. When labeled under the same conditions, the whole toxin incorporated only 0.25 iodine per toxin and retained most of its enzymatic activity and full toxicity. Consequently, the whole toxin was labeled and its subunits were separated in 6 M urea (Hendon & Fraenkel-Conrat, 1971), which was subsequently removed by elution through a PD10 (Pharmacia) column. Unlike native subunits, labeled subunits were not able to renature properly or reconstitute a fully functional complex. This technique was, however, useful to assess the specific radioactivity of each subunit.

Because we could not separate functional labeled from unlabeled toxin, iodination was performed with a mixture of (cold) $Na^{127}I$ (Merck) and (radioactive) $Na^{125}I$ (Amersham). Reaching a 2/1 iodine to toxin ratio under these conditions ensured that, statistically, all subunits were iodinated, be it radioactive or not. Provided a treated toxin retains full activity and toxicity, one can reasonably assume all of the molecules will have the same binding behavior.

Presynaptic Plasma Membranes from Torpedo Electric Organ. The purification of presynaptic plasma membranes from *Torpedo* electric organ was performed according to Morel et al. (1985), with a few modifications for convenience. The early step of filtration of the freshly ground material through a nylon gauze was replaced by a low-speed centrifugation step. The tissue homogenate was centrifuged for 15 min in a Sorvall GSA rotor at 5000*g*. The supernatant was kept at 4 °C until further processing. To increase the yield, the soft pellet was homogenized once more (1 min with a Virtis homogenizer) and submitted to the same centrifugation. The second pellet was discarded, and the supernatant was added to the first one for immediate processing. Starting with 250 g of frozen tissue, we typically obtained 40 mg of presynaptic membrane proteins. The efficiency of purification was assessed at each step by measuring the acetylcholinesterase activity (Ellman, 1961) as a marker of presynaptic membranes. We obtained a specific activity of ≈ 10 μmol min^{-1} mg^{-1} protein as described (Morel et al., 1985). Proteins were assayed by the method of Bradford (1976) with the Bio-Rad reagent.

Binding Protocol. All binding experiments were performed at room temperature in Ultraclear polycarbonate microtubes (Beckman) in a final volume of 130 μL. The rapidity of the binding led us to mix buffer and labeled and unlabeled toxins before addition of the presynaptic membranes (80 μg of protein per assay). The binding buffer was 0.15 M NaCl, 3 mM KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.4). Except in the kinetics experiments, binding time was 20 min. The pellet and supernatant were separated with an Airfuge (Beckman) ultracentrifuge (28 psi, 4 min, room temperature) and counted separately for bound and free radioactivity, respectively, with a γ counter (LKB-Wallac, Uppsala, Sweden). Hill's coefficient of cooperativity was calculated as the slope of the log $(B/B_{max} - B)$ versus log F plot, where B and F are specifically bound and free toxin, respectively (Hill, 1910).

Pharmacological and Enzyme Assays. Intravenous toxicity was assessed by measuring the lethality of radiolabeled or native CB or crotoxin, with four doses differing by a factor of 2. The protein to be tested was injected in 200 μL of 0.15 M NaCl in the vein of the tails of three male Swiss mice for each dose (Charles River, Saint-Aubin-lès-Elbeuf, France).

Acetylcholine release by *Torpedo* electric organ synaptosomes was measured as decribed elsewhere (Délot & Bon, 1992).

Phospholipase A_2 activity was determined by the fluorometric technique described by Radvanyi et al. (1989a).

RESULTS

Crotoxin Labeling. A major problem that has always hampered binding studies of β-neurotoxins is the difficulty in obtaining labeled toxins that retain their enzymatic activity and toxicity. Analyzing our various attempts, we found a correlation between the ratio of incorporated iodine per toxin and the remaining activity (Figure 1). Below 2 mol of iodine incorporated per mole of toxin, crotoxin retained full intravenous toxicity and over 95% enzymatic activity and neurotoxicity toward *Torpedo* synaptosomes (not shown). These conditions were subsequently retained to radiolabel the crotoxin used throughout the following experiments. A specific radioactivity of 10 Ci $mmol^{-1}$ was used in most experiments. The radioactivity was incorporated in both subunits: 35–40% in CA and 60–65% in CB.

Scatchard Analysis of the Binding. An incubation time of 20 min was used for the binding studies, as preliminary experiments had shown that specific binding had stabilized by then. Between 1 nM and 4 μM, nonspecific binding (measured in the presence of an excess of unlabeled toxin) was proportional to the concentration of toxin. The difference between total and nonspecific binding revealed a saturable binding of crotoxin (Figure 2). Scatchard analysis of saturable binding data revealed a single class of sites with an apparent constant of dissociation, K_d, of 0.7 ± 0.07 μM. The total number of binding sites, B_{max}, was 0.24 ± 0.01 μmol per gram

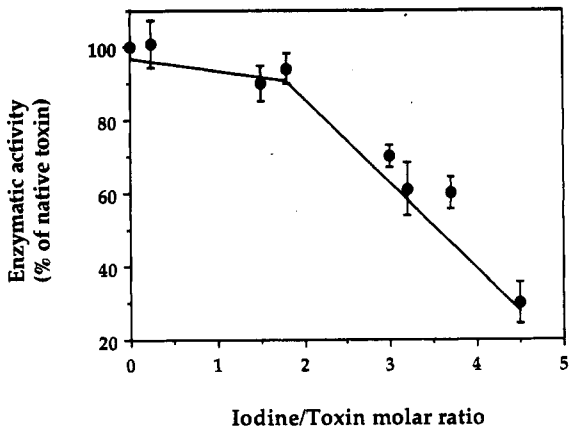

FIGURE 1: Correlation between the ratio of incorporated iodine per toxin and the remaining enzymatic activity.Crotoxin was iodinated with a mixture of (cold) $Na^{127}I$ and (radioactive) $Na^{125}I$ as described in Materials and Methods. The enzymatic activity of labeled crotoxin was measured with a fluorometric method (Radvanyi et al., 1989a) and is expressed as the percent activity of the native toxin. The data points represent the mean (± SE) of three determinations of the enzymatic activity.

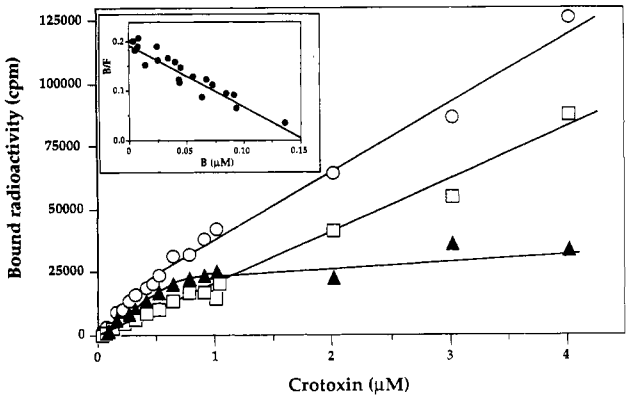

FIGURE 2: Binding of crotoxin to presynaptic membranes. Crotoxin was allowed to interact with presynaptic membranes from *Torpedo* electric organ for 20 min, at room temperature, in the presence of 5 mM $CaCl_2$. Bound radioactivity (*B*) was separated from free radioactivity (*F*) by centrifugation. Nonspecific binding (□) was determined in the presence of a molar excess of unlabeled crotoxin. Specific binding (▲) was obtained by subtracting nonspecific from total binding (○). Inset: A Scatchard graph was obtained by plotting *B/F* versus free (*F*) specific radioactivity. The figure illustrates the data of a typical experiment.

of presynaptic membrane proteins. The binding was not cooperative ($n_{Hill} = 1.03 \pm 0.03$). These values are the means of five independent determinations performed with two batches of labeled toxin on three preparations of presynaptic membranes.

These results are to be compared with those performed on guinea pig brain synaptosomal membranes (Degn et al., 1991; Tzeng et al., 1986). These authors also described a single class of sites without cooperativity. The values of both K_d and B_{max} were, however, higher on *Torpedo* membranes. The different B_{max} value indicates that *Torpedo* membranes are enriched in acceptors for crotoxin, as is expected for a presynaptic cholinergic toxin and a presynaptic cholinergic membrane preparation. The higher K_d value matches the range of doses shown to be effective on *Torpedo* synaptosomes (Délot & Bon, 1992). Nonspecific binding was higher in our experiments, but the percentage of saturable binding was comparable (15–20%) (Tzeng et al., 1986).

Two kinds of experiments were performed to assess which molecular species (CA, CB, or CACB) was actually bound

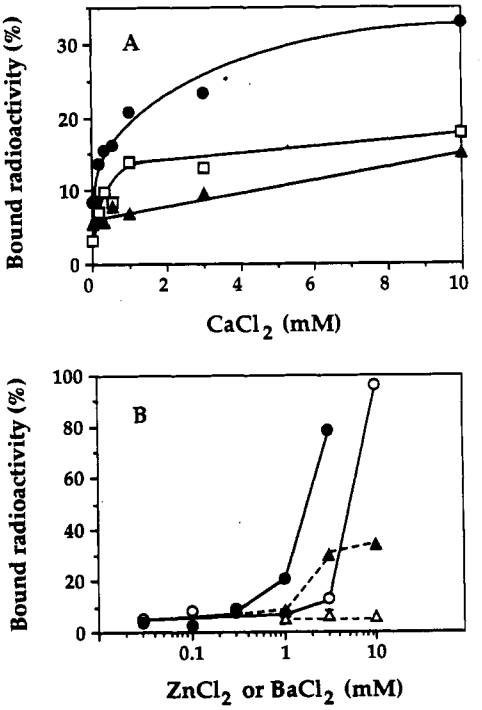

FIGURE 3: Effect of divalent cations on the binding of crotoxin. Labeled crotoxin (0.5 μM) was incubated with presynaptic membranes for 20 min at room temperature. Nonspecific binding was determined in the presence of 50 μM unlabeled crotoxin. Bound radioactivity is expressed as percent of total (bound plus free) radioactivity. The data points represent the mean ± SE of 3–4 independent determinations. (A) Effect of $CaCl_2$ on total (●), nonspecific (□) and specific (▲) binding of crotoxin. (B) Effect of $ZnCl_2$ (circles, solid lines) and $BaCl_2$ (triangles, dotted lines) on specific (closed symbols) and nonspecific (open symbols) binding of crotoxin, in the absence of $CaCl_2$.

to the membranes. SDS–PAGE of the pellet showed that no CA appeared to be bound. Chromatography of the supernatant before and after binding was performed on an anion-exchanging column, in conditions where all three species could be eluted separately (Faure & Bon, 1988). It indicated that, before binding, only CACB and the small excess of CA were observed. After binding, an decrease of radioactivity in the peak of CACB matched the increase in the peak of CA, indicating that CB was the only significantly bound species (data not shown). When labeled CA alone was allowed to interact with the membranes under the same conditions, no significant binding was observed (not shown).

Effect of Divalent Cations. The effect of $CaCl_2$, $BaCl_2$, and $ZnCl_2$ on the binding of crotoxin was observed. Calcium ions are compulsory cofactors of the phospholipase activity of crotoxin and CB. Barium ions cannot replace them and are even potent competitive inhibitors (Radvanyi et al., 1989b). In the absence of calcium, both specific and nonspecific binding were very low (Figure 3A). An increase in calcium to the millimolar range resulted in an increase in nonspecific binding without a significant effect on specific binding. Therefore, the concentration of calcium ions necessary to obtain optimal specific binding (*i.e.*, 1–10 mM) was the same as that necessary for optimal effects of crotoxin on *Torpedo* synaptosomes (Délot & Bon, 1992). $BaCl_2$ had no effect on nonspecific binding, but it increased specific binding up to 200% of the maximum binding obtained in the presence of $CaCl_2$ (Figure 3B).

Zinc ions have been shown to strongly promote the specific binding of OS_2, a single-chain neurotoxic phospholipase A_2 from snake venom, on brain membranes (Lambeau et al., 1989). Since crotoxin was the best competitor of OS_2 binding,

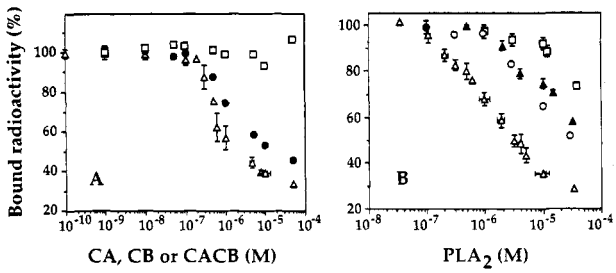

FIGURE 4: Homologous and heterologous competitions. (A) Competition with unlabeled crotoxin or its subunits. Labeled crotoxin (0.5 μM) was incubated in the presence of the indicated amount of unlabeled crotoxin (△), CA (●), or CB (□). (B) Competition with other β-neurotoxins. Labeled crotoxin (0.1 μM) was incubated in the presence of unlabeled crotoxin (△), ammodytoxin A (○), agkistrodotoxin (▲), or β-bungarotoxin (□). All incubations were performed at room temperature for 20 min in the presence of 5 mM $CaCl_2$. Bound radioactivity is expressed as percent of total radioactivity. The data points are the mean (± SE) of 4–5 (CA, crotoxin), 3–4 (ammodytoxin, agkistrodotoxin, CB), or 2–3 (β-bungarotoxin) determinations.

we tested the effect of zinc ions on the binding of crotoxin. In the presence of 1–3 mM $ZnCl_2$, specific binding increased dramatically (Figure 3B). At higher concentrations, the binding was no longer prevented by an excess of unlabeled crotoxin. In fact, the amount of bound radioactivity observed at 3 mM exceeded 80%, indicating that both CA and CB were bound under these conditions. We suggest that crotoxin can be bound to the phospholipids of the membrane via its divalent cation sites, as shown in the case of blood coagulation factor XII (Schousboe & Halkier, 1991).

Competition Experiments. Competition with unlabeled crotoxin performed at a concentration of labeled crotoxin close to its K_d (0.5 μM) showed an IC_{50} of 0.7 μM = K_d, indicating that crotoxin labeling did not modify its binding properties (Figure 4A). CB, the phospholipase A_2 subunit of crotoxin, was not able compete at all with crotoxin (Figure 4A). Although CA did not bind to the membranes at equilibrium, it was a very potent competitor of [^{125}I]crotoxin, as efficient as native toxin (Figure 4A). The inhibition by CA was of a mixed type, neither purely competitive nor purely noncompetitive, as demonstrated by the double-reciprocal plot of the competition data at two different concentrations of labeled crotoxin (not shown).

Agkistrodotoxin and ammodytoxin are two single-chain β-neurotoxins from *Viperinae* snakes which show structural, immunological, and pharmacological similarities with CB (Choumet et al., 1993; Délot & Bon, 1992). They were able to compete, at least partially, with crotoxin for its saturable binding on *Torpedo* presynaptic membranes (Figure 4B). β-Bungarotoxin was a weak competitor.

Kinetics. The association of crotoxin with its membrane acceptor showed complex kinetics (Figure 5). At 15 s, which was the resolution limit of our method, both nonspecific and specific binding had partly occurred. Specific binding then developed to reach a maximum at 20 min and showed little increase over the next 3 h (Figure 5B). This confirms that the previous experiments were carried out at equilibrium. Unexpectedly, nonspecific binding was stable for only a few minutes, after which time it started increasing linearly. This delayed increase was not observed when Ca^{2+} was omitted (Figure 5A) or when CA was used as a competitor instead of CACB (Figure 5C). Thus, this increase is most probably due to the addition of a large amount of phospholipase A_2 activity (unlabeled CACB) which hydrolyzes the membrane, creating new binding sites of low specificity.

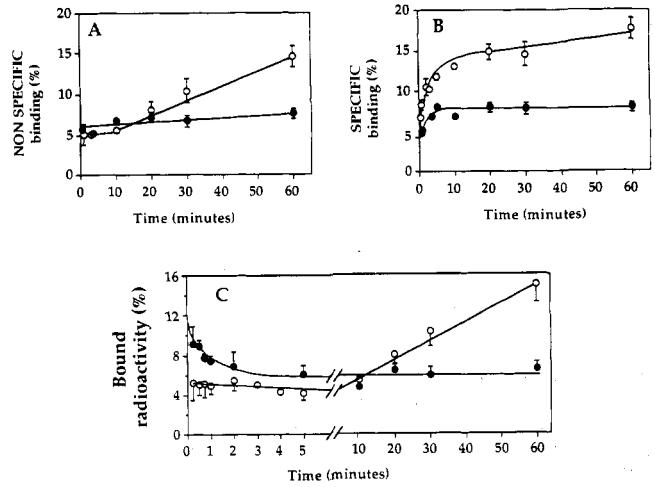

FIGURE 5: Kinetics of the binding of crotoxin. (A and B) Labeled crotoxin (0.1 μM) was allowed to interact with presynaptic membranes, with (○) or without (●) 5 mM $CaCl_2$, for the indicated time before centrifugation. Nonspecific binding was measured in the presence of 10 μM unlabeled crotoxin. Bound radioactivity is expressed as percent of total radioactivity. (C) Labeled crotoxin (0.1 μM) was allowed to interact with presynaptic membranes, with 5 mM $CaCl_2$, in the presence of 10 μM unlabeled crotoxin (○) or CA (●). The data points are the mean (± SE) of 3–4 independent determinations.

The early kinetics of the competition with CA and with crotoxin in the presence of calcium ions was also different (Figure 5C), confirming a different mechanism of inhibition. In the case of CA, the maximum inhibiting effect was reached after only 2–3 min. This indicates that some radioactivity had bound before CA could remove it from the membrane. In the presence of CACB, nonspecific binding is immediately basal, as expected in the case of pure competition.

When CACB had been allowed to bind for 20 min, addition of an excess of CA or CACB was not able to dissociate bound radioactivity within another 20 min (result not shown). The kinetics of the dissociation was not studied further.

DISCUSSION

The recognition by the dimeric β-neurotoxin, crotoxin, of its acceptor on neuronal membranes has long been a challenging biochemical problem. We obtained functional radiolabeled crotoxin and tested its binding to plasma membranes deriving from *Torpedo* electric organ, a model in which its pharmacology had been studied in detail (Délot & Bon, 1992). Crotoxin bound to a single class of sites without cooperativity, as observed on guinea pig brain synaptosomal membranes (Degn et al., 1991). The values of K_d and B_{max} were different however, which was expected due to the difference of preparations. B_{max} was higher on our purely cholinergic preparation (0.24 compared to 0.01 μmol g^{-1}). K_d also was higher, matching the range of pharmacologically effective concentrations on *Torpedo* synaptosomes (Délot & Bon, 1992). Nonspecific binding on *Torpedo* membranes increased with time in the presence of calcium ions. This increase was probably due to the creation of new low-affinity sites when the membrane is digested by the phospholipase. It could not be observed on brain synaptosomes (Degn et al., 1991), probably because the concentrations of toxin used were much lower. Ammodytoxin A and agkistrodotoxin, two single-chain β-neurotoxins, could partially prevent the specific binding of crotoxin while β-bungarotoxin was a poor competitor, as observed before (Rehm & Betz, 1982; Degn et al., 1991; Tzeng et al., 1986).

On *Torpedo* membranes, as on all models tested before, CB was the major species bound to the membrane while CA

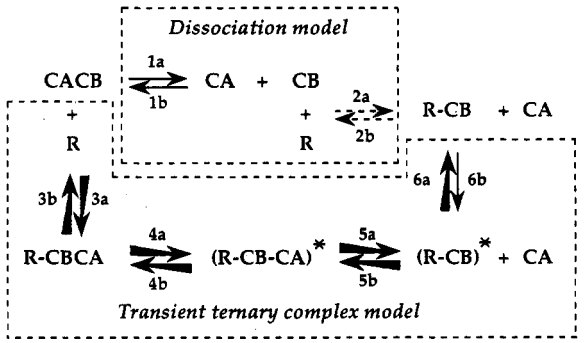

FIGURE 6: Dissociation and ternary complex models. The two possible pathways between crotoxin in solution (CACB) and its bound form (R–CB) are illustrated. Dissociation model: CA and CB dissociate before CB binds. Transient ternary complex model: Crotoxin binds before CA dissociates. R stands for the membrane acceptor of crotoxin. Dotted arrows indicate routes which were not observed under our experimental conditions. Thin solid arrows indicate slow events. Broad arrows indicate rapid reactions. The intermediate structural forms (*) of the ternary complex are referred to in the text.

remained in solution, indicating a dissociation of the crotoxin complex upon interaction with the membrane. However, the mechanism underlying this phenomenon has never been clarified. The major question is the time scale of the events, *i.e.*, does CB bind before or after dissociating from CA? The model in Figure 6 illustrates both hypotheses. The hypothesis which proposes that the complex dissociates first, before CB binds, will be referred to as the dissociation model (pathway 1–2), as opposed to the transient ternary complex model (pathway 3–6). Recently, the toxicity of the various isoforms was shown to be related to the affinity between their subunits (Faure et al., 1993). Complexes that dissociate easily are less toxic, *in vivo* by intravenous injection and *in vitro* on nerve–muscle preparations, than those with a high affinity constant. This confirmed that CA exerts the role of a chaperone toward CB, preventing nonspecific adsorption of the phospholipase on membranes. By lengthening CB's lifetime in the blood flow, CA will enhance the ability of the toxin to reach its target membrane. Thus, the higher the affinity between the subunits, the more toxin that can reach neuronal structures. However, when the toxin has reached neuronal structures or is artificially taken there (such as in presynaptic membrane or synaptosomal preparations), the ultimate mechanism could be quite different.

The dissociation model makes it easy to understand how CA can be such a good competitor: an excess of CA would displace the equilibrium in the 1b direction, reducing the concentration of free CB. There are, however, major arguments against this model coming both from the pharmacology on *Torpedo* synaptosomes and from binding results. On *Torpedo* synaptosomes, CB and crotoxin have qualitatively different effects on acetylcholine release (Délot & Bon, 1992). If dissociation was the primary event, one would expect only a difference in the kinetics, with crotoxin behaving like CB after a delay for the dissociation. In addition, no difference in efficiency could ever be detected on *Torpedo* synaptosomes between the various isoforms of crotoxin, despite their different association constants (E. Délot and C. Bon, unpublished results). The rapid time course of the pharmacological action, as well as that of the binding, also argues against the dissociation hypothesis: all of the effects of crotoxin were immediately observed and reached a maximum within a few minutes (Délot & Bon, 1992), while the half-time of dissociation for the isoform we used was 20 min (Faure et al., 1993). The last, and maybe strongest, evidence is the fact that CB was not able to compete with crotoxin. This indicates that reaction 2a is a highly improbable event. CB alone most probably binds to a large number of sites of low specificity on the membrane, as already demonstrated on postsynaptic membranes (Bon et al., 1979) or on red blood cells (Jeng et al., 1978).

Taken together, previous studies and the present results thus favor the hypothesis of a transient ternary complex. As a consequence, not only does CA chaperone CB *en route* to neuronal structures but it is also temporarily involved in CB's binding to the acceptor (routes 3a–5a in Figure 6). Some results remain to be interpreted in the light of this model, especially the competition by CA. When crotoxin had been allowed to interact with the membranes for 20 min, CA was not able to displace CB from its binding site. This means that route 6b is a slow, unlikely event in the time scale of the experiment. At equilibrium, CB can thus be considered to be irreversibly bound. This shows that the mechanism by which CA "competes" is not removal of CB from the membrane. CA was not able to directly compete with CACB for its binding site either. This was indicated by the double-reciprocal plots of the competition data and by the observation that labeled CA was not able to bind.

Thus, CA must act at an intermediate step, after the ternary complex has formed and before CB is irreversibly bound. In Figure 6, an intermediate structural form of the complex (termed [R–CB–CA]*) has been introduced. It represents the state when the binding of crotoxin to its acceptor has triggered a transient conformational state, from which CA is able to dissociate (route 5a). At this step, an excess of CA would favor route 5b and consequently displace all of the equilibria toward the unbound form of crotoxin. This requires the existence of an [R–CB]* conformational state in equilibrium with both [R–CB–CA]* (route 5b) and the "irreversibly" bound form, R–CB (route 6a). This also means that routes 3b, 4b, and 5b are rapid. If this model is correct, the kinetics of the competitions with CA and CACB should be different. CACB should prevent the binding of labeled toxin (equilibrium 3), while CA should displace the radioactivity bound on an intermediate form. In effect, immediate action of CACB is observed (within the first 15 s) while the action of CA is delayed (maximum displacement is reached after 2 min) (Figure 5C). The kinetics of direct binding is also in good agreement with the transient ternary complex model. The binding of CACB (route 3a) is a rapid (less than 15 s) event, followed by a slower isomerization step and the dissociation of CA. These events occur even in the absence of calcium, in good agreement with electrophysiological experiments at neuromuscular junctions, which showed that the initial binding step of β-neurotoxins is quick and calcium-independent (Rowan & Harvey, 1988).

The fact that CB was not able to compete with crotoxin had also been observed on guinea pig brain membranes (Degn et al., 1991). This is quite expected in the ternary complex model. It was, however, rather surprising since the single-chain β-neurotoxins agkistrodotoxin and ammodytoxin A could partially compete with crotoxin. CB shows 80% and 60% amino acid sequence similarity with agkistrodotoxin and ammodytoxin A, respectively (Aird et al., 1986; Kondo et al., 1982a; Ritonja & Gubensek, 1985). They also exert similar pharmacological action on *Torpedo* synaptosomes (Délot & Bon, 1992; Choumet et al., 1993). The competition experiments suggest that, in weakly neurotoxic CB, the "toxic site" (*i.e.*, the amino acids important for the binding to the acceptor) is different from that of homologous single-chain toxins.

Actually, immunochemical analysis of ammodytoxin A and CB indicated a difference in the amino acids involved in their toxic sites (Curin-Serbec, E. Délot, G. Faure, B. Saliou, F. Gubenšek, C. Bon, and V. Choumet, unpublished results). In CB, the very C-terminal stretch of amino acids is important for its toxicity. In crotoxin, either these amino acids are hidden or their three-dimensional structure is modified. The association of CA with CB would thus turn CB from a phospholipase (with affinity for membrane phospholipids) into a neurotoxin (with affinity for an acceptor on neuronal membranes) either by masking the domain responsible for low-affinity binding or by modifying the conformation of the toxic site. Comparative analysis of the structures of CB alone and CACB has been undertaken by NMR techniques and may reveal such a conformational difference. Further indications could also be obtained by analyzing the inhibition of crotoxin binding to membranes by site-directed antibodies.

The nature of crotoxin's acceptor remains the next question to be answered. β-Bungarotoxin is the only β-neurotoxin for which an acceptor protein has clearly been identified (Schmidt & Betz, 1989). In the case of crotoxin, complex—if not contradictory—results have been obtained (Tzeng et al., 1986; Hseu et al., 1990). The most likely candidate is an 85-kDa protein of unknown nature (Hseu et al., 1990; Lambeau et al., 1989). However, it has been proposed that negatively charged membrane phospholipids could be part of the target recognized by crotoxin on neuronal structures (Radvanyi et al., 1989c). The low specific radioactivity of our toxin did not allow us to identify any specifically labeled proteins by chemical cross-linking with *Torpedo* presynaptic membranes. In preliminary experiments, treatment of *Torpedo* membranes with phospholipase C strongly reduced the binding of crotoxin. Other investigations are needed to conclude whether phospholipase C simply competes with crotoxin or removes a phospholipid-anchored protein (such as acetylcholinesterase) or the polar head of specific lipids involved in the acceptor of crotoxin.

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REFERENCES

Aird, S. D., Kaiser, I. I., Lewis, R. V., & Krugger, W. G. (1986) *Arch. Biochem. Biophys. 249*, 296–300.

Bon, C., Changeux, J.-P., Jeng, T. W., & Fraenkel-Conrat, H. (1979) *Eur. J. Biochem. 99*, 471–481.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Chang, C. C., & Su, M. J. (1980) *Toxicon 18*, 641–648.

Choumet, V., Saliou, B., Fideler, L., Chen, Y.-C., Gubenšek, F., Bon, C., & Délot, E. (1993) *Eur. J. Biochem. 211*, 57–62.

Degn, L. L., Seebart, C. S., & Kaiser, I. I. (1991) *Toxicon 29*, 973–988.

Délot, E., & Bon, C. (1992) *J. Neurochem. 58*, 311–319.

Ellman, G. L., Diane, K., Courtney, V., Andres, J. R., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88–95.

Faure, G., & Bon, C. (1988) *Biochemistry 27*, 730–738.

Faure, G., Guillaume, J.-L., Camoin, L., Saliou, B., & Bon, C. (1991) *Biochemistry 30*, 8074–8083.

Faure, G., Harvey, A. L., Thomson, E., Saliou, B., Radvanyi, F., & Bon, C. (1993) *Eur. J. Biochem. 214*, 491–496.

Hawgood, B., & Bon, C. (1991) in *Handbook of natural toxins: Reptile and amphibian venoms* (Tu, A. T., Ed.) pp 3–52, Marcel Dekker, New York.

Hendon, R. A., & Fraenkel-Conrat, H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1560–1563.

Hill, A. V. (1910) *J. Physiol.* 40, 190.

Hseu, M. J., Guillory, R. J., & Tzeng, M.-C. (1990) *J. Bioenerg. Biomembr. 22*, 39–50.

Jeng, T. W., Hendon, R. A., & Fraenkel-Conrat, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 600–604.

Kondo, K., Toda, H., Narita, K., & Lee, C.-Y. (1982a) *J. Biochem. 91*, 1531–1548.

Kondo, K., Toda, H., Narita, K., & Lee, C. Y. (1982b) *J. Biochem. 91*, 1519–1530.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lambeau, G., Barhanin, J., Schweitz, H., Qar, J., & Lazdunski, M. (1989) *J. Biol. Chem. 264*, 11503–11510.

Morel, N., Marsal, J., Manaranche, R., Lazereg, S., Mazié, J.-C., & Israël, M. (1985) *J. Cell Biol. 101*, 1757–1762.

Radvanyi, F., Jordan, L., Russo-Marie, F., & Bon, C. (1989a) *Anal. Biochem. 177*, 103–109.

Radvanyi, F., Keil, A., Saliou, B., Lembezat, M.-P., & Bon, C. (1989b) *Biochim. Biophys. Acta 1006*, 183–192.

Radvanyi, F., Saliou, B., Lembezat, M.-P., & Bon, C. (1989c) *J. Neurochem. 53*, 1252–1259.

Rehm, H., & Betz, H. (1982) *J. Biol. Chem. 257*, 10015–10022.

Ritonja, A., & Gubenšek, F. (1985) *Biochim. Biophys. Acta 828*, 306–312.

Rowan, E. G., & Harvey, A. L. (1988) *Br. J. Pharmacol. 94*, 839–847.

Schmidt, R. R., & Betz, H. (1989) *Biochemistry 28*, 8346–8350.

Schousboe, I., & Halkier, T. (1991) *Eur. J. Biochem. 197*, 309–314.

Strong, P. N. (1987) in *Cellular and molecular basis of cholinergic function* (Dowdall, M. J., & Hawthorne, J. N., Eds.) pp 534–549, Ellis Horwood, Chichester, UK.

Tzeng, M.-C., Hseu, M. J., Yang, J. H., & Guillory, R. J. (1986) *J. Protein Chem. 5*, 221–228.

Xu, K. (1990) *Chin. Sci. Bull. 35*, 1145–1155.