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Use of Antibodies Specific to Defined Regions of Scorpion α-Toxin To Study Its Interaction with Its Receptor Site on the Sodium Channel

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ABSTRACT: Five antibody populations selected by immunoaffinity chromatography for their specificity toward various regions of toxin II of the scorpion *Androctonus australis* Hector were used to probe the interaction of this protein with its receptor site on the sodium channel. These studies indicate that two antigenic sites, one located around the disulfide bridge 12–63 and one encompassing residues 50–59, are involved in the molecular mechanisms of toxicity neutralization. Fab fragments specific to the region around disulfide bridge 12–63 inhibit binding of the ^{125}I-labeled toxin to its receptor site. Also, these two antigenic regions are inaccessible to their antibodies when the toxin is bound to its receptor site. In contrast, the two other antigenic sites encompassing the only α-helix region (residues 23–32) and a β-turn structure (residues 32–35) are accessible to their respective antibodies when the toxin is bound to its receptor. Together, these data support the recent proposal that a region made of residues that are conserved in the scorpion toxin family is involved in the binding of the toxin to the receptor.

The early studies on scorpion venoms were motivated by the problem of medical care of envenomation in several regions of the world (Vachon, 1952). The neurotoxic activity of the venom is due to the presence of small amounts (El Ayeb & Rochat, 1985) of basic proteins made up of 60–65 amino acid residues (Miranda et al., 1970), cross-linked by four disulfide bridges (Kopeyan et al., 1974). Although the immunogenicity of these compounds was recognized about 40 years ago (Grasset et al., 1946), the efficiency of the antiserum in therapeutic trials proved to be low (Balozet, 1971) when the whole venom was used as the immunogen. The venom of a definite species contains several α-neurotoxins that all bind to the same site on the sodium channel (Rochat et al., 1979; Catterall, 1984), even if they exhibit structural (Rochat et al., 1970) and antigenic polymorphism (Tessier et al., 1978; Delori et al., 1981; El Ayeb et al., 1983a). Moreover, the amount of each type of neurotoxin differs among individuals within a species (El Ayeb & Rochat, 1985), and finally, despite some structural homologies, toxins differ from species to species. Thus, this structural variability strongly complicates a rational design of polyvalent and efficient serotherapy.

Our laboratory has developed over the last few years several approaches to get a better understanding of the molecular features that are responsible for toxicity and antigenicity in the toxin family. A number of chemical modifications of the toxins have been performed to localize residues involved in the receptor binding or pharmacological properties of toxins. Lysines-56 for toxin I (Sampieri & Habersetzer-Rochat, 1978) and -58 for toxin II of *Androctonus australis* Hector (AaH I and AaH II)[1] and for toxin V of *Leiurus quinquestriatus quinquestriatus* (Lqq V), lysines-2 and -60 of Lqq V (Darbon

[1] Abbreviations: AaH II, toxin II of *Androctonus australis* Hector; Lqq V, toxin V of *Leiurus quinquestriatus quinquestriatus*; Bot III, toxin III of *Buthus occitanus tunetanus*; FAaH II, anti-AaH II Fab fragments; NRF BIII, anti-AaH II Fab fragments not retained on Bot III–Sepharose; RF BIII, anti-AaH II Fab fragments retained on Bot III–Sepharose; IgG, immunoglobulin G; PS I IgGs, PS II IgGs, PS III IgGs, and PS IV IgGs, anti-AaH II IgGs retained on the respective synthetic peptides; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

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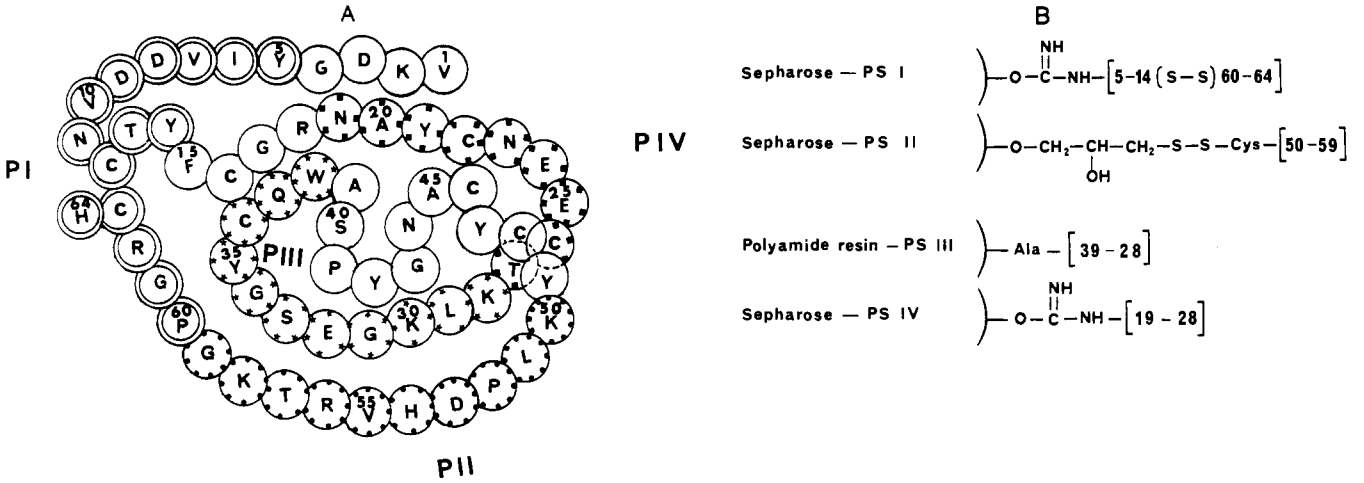

FIGURE 1: (A) Covalent structure of toxin II from *A. australis* Hector. (B) Immobilized peptides used in immunoaffinity chromatography.

et al., 1983), arginine-2 of toxin III of *A. australis* Hector (AaH III), and the N-terminal pentapeptide of toxin III of *Buthus occitanus mardochei* (Bom III) have been shown to be involved in the biological activity of these toxins (El Ayeb et al., 1986). All these residues are located in the conserved and relatively hydrophobic but nevertheless accessible region of these toxins proposed by Fontecilla-Camps (Fontecilla-Camps et al., 1981) to carry the "toxic site".

Detailed information related to the antigenicity of scorpion toxins has been accumulated only recently. When injected into rabbits, the scorpion α-toxins exhibit at least four major antigenic sites since they can bind simultaneously four antitoxin Fab fragments at their surface (El Ayeb et al., 1983b). The localization of these four major antigenic sites was attempted on AaH II [a representative model of scorpion α-toxins (Couraud et al., 1982)] by using an appropriate methodology based on (1) incomplete cross-reaction between AaH II and toxin III of *Buthus occitanus tunetanus* (Bot III) (El Ayeb et al., 1983b), (2) the effects of chemical modifications of AaH II on its antigenicity, and (3) the prediction of the location of antigenic sites by the method described by Hopp and Woods (1981). This has led to chemical synthesis of four model peptides that display significant antigenic activity (El Ayeb et al., 1984; Granier et al., 1984; Bahraoui et al., 1986a). Thus, we can now propose a model for scorpion toxins in which the antigenic sites are present in the variable exposed regions whereas the toxic site is in a conserved immunosilent area.

In this study, we have taken advantage of having five distinct antibody populations, each directed against a defined antigenic region of AaH II, to localize the neutralizing sites of the toxin and to examine its orientation when bound to the sodium channel.

Materials and Methods

Materials

$Na^{125}I$ was obtained from Amersham (13–17 mCi/μg). Lactoperoxidase (EC 1.11.1.7), papain (EC 3.4.22.2), and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) were purchased from Sigma. BrCN-activated Sepharose, protein A–Sepharose, Sephadex G-25, and protein A were purchased from Pharmacia Fine Chemicals. Tetrodotoxin was from Boehringer. All other products were reagent grade.

Methods

Preparation of Synaptosomes. Synaptosomes were prepared according to the method of Gray and Wittaker (1962). Whole brains were dissected from Wistar rats and homogenized with a Teflon pestle in a 0.32 M sucrose solution (0.1 g of tissue/mL). The homogenate was centrifuged for 10 min at 750*g*, and the resulting supernatant was centrifuged for 25 min at 10000*g*. Then, the pellet was suspended in Hepes–choline buffer (containing 140 mM choline chloride, 5 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM glucose, 25 mM Hepes, and Tris base to pH 7.2). Protein was measured by a modified Lowry method (Lowry et al., 1951).

Protein Iodination. (1) Iodination of Toxins. AaH II and Lqq V were iodinated by using the lactoperoxidase method of $Na^{125}I$ oxidation and purified by immunoprecipitation with AaH II antitoxin (neutralizing titer: 38 nmol/mL) and anti-Lqq G-50 fraction (neutralizing titer: 160 μg/mL) according to the procedure described by Rochat et al. (1977). The specific radioactivities for ^{125}I AaH II and ^{125}I Lqq V were 1100 and 700 Ci/mmol, respectively.

(2) Iodination of Protein A. Protein A was iodinated as previously described (Biberfeld et al., 1975). The iodinated protein was desalted from free $Na^{125}I$ by passage through Sephadex G-25 column (1 × 20 cm). The specific radioactivity was 157 Ci/mmol. Eighty to one hundred percent of the iodinated protein was still able to bind specifically with rabbit nonimmune IgG bound to Sepharose.

Toxins and Antitoxins. AaH I, AaH II, Bot III, and Lqq V were purified according to the general procedure established by Miranda et al. (1970). Carefully standardized conditions were used for the immunization of rabbits. Usually, each rabbit received a total of approximately 4 mg of pure antigen. The blood was removed by heart puncture as previously described (Delori et al., 1981).

Preparation of Antibodies and Fab Fragments. Antibodies specific to AaH II were purified in a single step by immunoaffinity chromatography on the immobilized toxin as previously described (El Ayeb et al., 1983b). Anti-AaH II Fab fragments were obtained by partial digestion with papain and purified from Fc fragments and undigested IgGs by filtration through a protein A–Sepharose CL-4B column as described by El Ayeb et al. (1983b). Anti-AaH II Fab fragment populations not retained on Bot III–Sepharose (NRF BIII) or retained on Bot III–Sepharose (RF BIII) have been purified by immunoaffinity chromatography (El Ayeb et al., 1983b). Also, NRF BIII has been shown to be specific to one antigenic site (El Ayeb et al., 1983b).

Preparation and Characterization of Antibodies Purified on Insolubilized Model Peptides. Anti-AaH II IgGs were fractionated on a series of four immunoaffinity chromatog-

raphy columns. Each column carried one of the four synthetic replicates of the antigenic sites of AaH II as shown in Figure 1. The specifically adsorbed IgG populations were purified and characterized as previously described (El Ayeb et al., 1984; Bahraoui et al., 1986a).

Inhibition Experiments with Fab Fragments. Fifty microliters of ^{125}I AaH II (10^{-9} M) and ^{125}I Lqq V (3×10^{-9} M), respectively, at 2×10^{-10} and 6×10^{-10} M final concentrations in the Hepes–choline buffer were incubated for 2 h at 37 °C in the presence of a variable concentration of Fab fragments. We used the following in separate experiments: anti-AaH II Fab fragments (from 10^{-13} to 10^{-6} M for AaH II and from 10^{-10} to 3.9×10^{-6} M for Lqq V), RF BIII fragments (from 10^{-13} to 10^{-6} M for AaH II and from 10^{-10} to 4.6×10^{-6} M for Lqq V), and NRF BIII fragments (from 10^{-13} to 10^{-6} M for AaH II and from 10^{-10} to 10^{-6} M for Lqq V). The final volume of incubation (225 μL) contained tetrodotoxin at 10^{-6} M, a selective blocker of the potential-dependent sodium channel (Narahashi et al., 1964). Then 25 μL of synaptosomes (0.6 mg of protein/mL) was added, and incubation was prolonged for 30 min. The reaction was stopped by centrifugation at 10000*g* for 1 min. The pellets were washed with 3 × 1 mL of Hepes–choline buffer, and radioactivity was counted. All assays were in duplicate. The 100% binding corresponds to the binding of ^{125}I AaH II and ^{125}I Lqq V in the absence of Fab fragments, and the 0% corresponds to the radioactivity bound in presence of an excess of the corresponding native toxins (2×10^{-7} M). Another control for nonspecific inhibition was carried out by using anti-AaH I Fab fragments, which do not cross-react either with AaH II or with Lqq V. The results are expressed as bound toxin (cpm) vs. log of Fab concentration.

Binding of Different Antibody Populations to Synaptosome-Bound AaH II. Two protocols have been followed: a variable concentration of specific IgG with a constant concentration of AaH II and vice versa.

First Protocol. Synaptosomes (225 μL) at 0.6 mg of protein/mL were incubated for 30 min at 37 °C with 25 μL of AaH II at 10^{-8} M (maximum binding) or with 25 μL of a mixture (v/v) of AaH II at 2×10^{-8} M and AaH I at 5×10^{-5} M (nonspecific binding). At the same time, 100 μL of a variable concentration of specific IgGs (from 5×10^{-10} to 10^{-7} M initial concentration) was incubated for 1 h at 37 °C with 100 μL of ^{125}I protein A at 6×10^{-8} M. Twenty-five microliters of IgG–protein A complex was added to the toxin–receptor complex, and the incubation was prolonged for 1 h at 37 °C. The reaction was stopped by centrifugation at 10000*g* for 1 min. The pellets were washed with 3 × 1 mL of Hepes–choline buffer, and the radioactivity was counted. Another negative control substituted rabbit nonimmune IgG for immune IgG. The results were expressed as bound ^{125}I protein A (cpm) vs. log of IgG concentration.

Second Protocol. The protocol is as above except that the initial concentration of specific IgG was constant (10^{-7} M initial concentration) whereas the concentration of AaH II was varied (from 2×10^{-9} to 10^{-8} M final concentration). The results were expressed as ^{125}I protein A (cpm) vs. concentration of AaH II (nM).

Results

(1) Characterization of IgG and Fab Fragment Subpopulations Specific to Defined Regions of AaH II. Three Fab fragments and four IgG fractions have been purified by immunoaffinity chromatography. FAaH II (anti-AaH II Fab fragments) were purified on AaH II–Sepharose (El Ayeb et al., 1983b). The fraction of FAaH II unretained on an immunoaffinity column of Bot III–Sepharose (NRF BIII) is believed to be specific to the single antigenic site of AaH II that is not shared with Bot III. This site is located near to the disulfide bridge 12–63. The antibody population NRF BIII was shown to bind to ^{125}I AaH II in a 1/1 molar ratio while FAaH II binds to the toxin in a 4/1 molar ratio (El Ayeb et al., 1983b). Conversely, the fraction of FAaH II that was bound by Bot III–Sepharose (RF BIII) is specific for all the antigenic sites shared by the two toxins. PS I IgGs are the fraction of the IgGs raised against AaH II that were retained by PS I, a synthetic peptide mimicking the region encompassing residues 5–14 and 60–64 (linked by a disulfide bridge between Cys-12 and -63) of AaH II (Figure 1). PS II IgGs were retained by the synthetic replicate of region 50–59 of AaH II (Figure 1) and therefore are thought to be specific for this area of the toxin (El Ayeb et al., 1984). PS III IgGs are antibodies from anti-AaH II IgGs that were retained by the synthetic replicate of the region 28–39 of AaH II, a region of the toxin that, according to the model of Fontecilla-Camps et al. (1981), must reside on "top" of the molecule between the face of the toxin that bears conserved residues and the face that bears the single α-helix strand. PS IV IgGs are those retained by a synthetic peptide mimicking the α-helix region (residues 19–29, Figure 1) of AaH II. All four affinity IgG populations were found to behave like single-site-directed antibodies (i.e., NR BIII IgGs) when compared to the multisite-directed anti-AaH II IgGs (El Ayeb et al., 1984; Bahraoui et al., 1986a).

Table I: Molar Fab Concentrations Giving 50% Inhibition[a]

	anti-AaH II Fab	RF BIII	NRF BIII
AaH II	2.5×10^{-9}	2.5×10^{-9}	1.3×10^{-8}
Lqq V	2.5×10^{-8}	1.4×10^{-8}	6.2×10^{-7}

[a] Inhibition of the binding of ^{125}I AaH II and ^{125}I Lqq V to rat brain synaptosomes with anti-AaH II Fab fragments and anti-AaH II Fab fragments retained (RF BIII) or not (NRF BIII) on a Bot III–Sepharose column.

(2) Inhibition of Binding of ^{125}I AaH II and ^{125}I Lqq V to Rat Brain Synaptosomes by Specific Fab Fragments. We tested the ability of three Fab fragment populations (FAaH II, RF BIII, and NRF BIII) derived from anti-AaH II IgGs to inhibit the binding of ^{125}I AaH II to its receptor site on the sodium channel. We similarly checked inhibition of the binding of ^{125}I Lqq V, a toxin closely related to AaH II (80% sequence homology). Fab fragments were used instead of the corresponding IgGs in order to minimize steric effects. The conditions for binding were those initially reported by Jover et al. (1980). Nonspecific binding of the iodinated toxins was determined by using an excess of the corresponding unlabeled toxins. The nonspecific inhibition by the different Fab fragments was assessed by using a high concentration (10^{-6} M) of anti-AaH I Fab fragments.

All three fragment populations assayed inhibited the binding of ^{125}I AaH II (10^{-9} M), but with different efficiencies (Figure 2 and Table I). The two polyspecific Fab populations (FAaH II and RF BIII) showed similar half-effects ($K_{0.5} = 2.5 \times 10^{-9}$ M), whereas the Fab population directed against a single antigenic site (NRF BIII) demonstrated lower inhibiting efficiency ($K_{0.5} = 1.3 \times 10^{-8}$ M). This could reflect the difference in the number of neutralizing antibody populations that are present either in FAaH II and RF BIII Fab fragments or in NRF BIII. Assuming that NRF BIII Fab fragments are specific for one single antigenic site, FAaH II as well as RF BIII may recognize more than one neutralizing site.

In a similar set of experiments, the three Fab fragment populations show their ability to inhibit the binding of ^{125}I Lqq

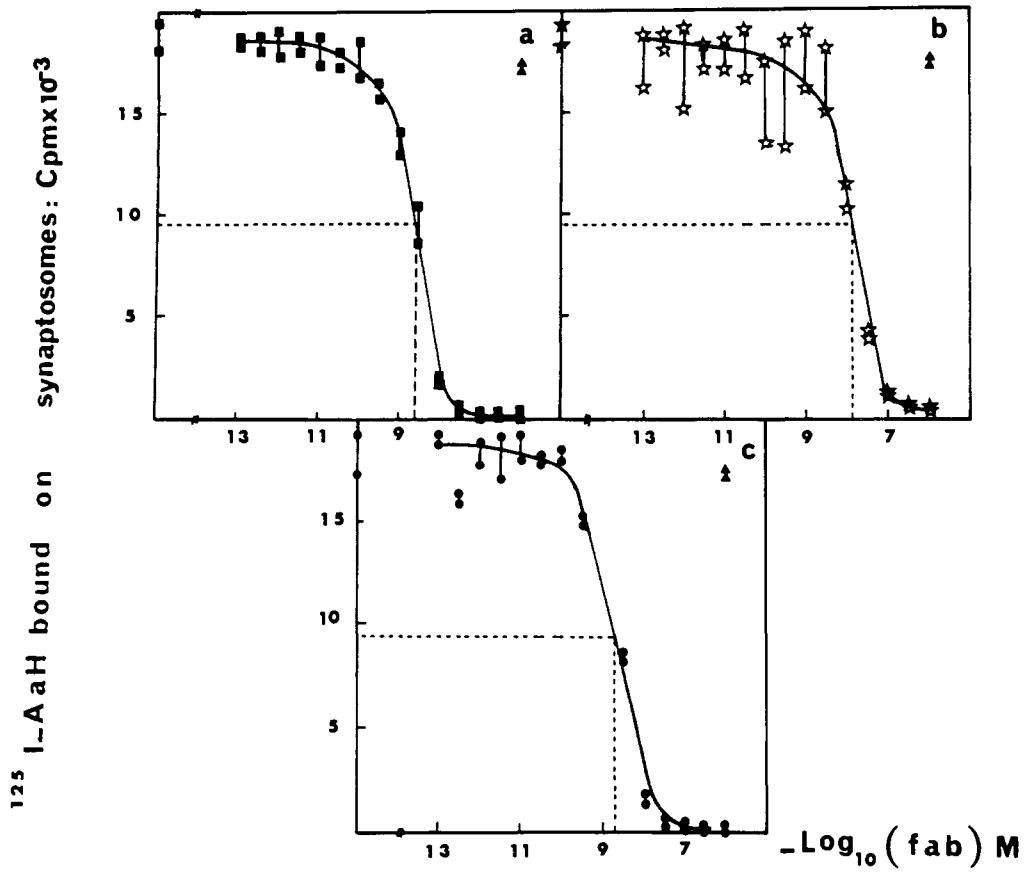

FIGURE 2: Inhibition of binding of ^{125}I AaH II to rat brain synaptosomes by Fab fragment populations. ^{125}I AaH II (50 μL) at 10^{-9} M was preincubated for 2 h at 37 °C with different Fab fragment populations: (a) anti-AaH II Fab fragments; (b) anti-AaH II Fab fragments not retained on Bot III–Sepharose (NRF BIII) at 10^{-13}–10^{-6} M; (c) anti-AaH II Fab fragments retained on Bot III–Sepharose (RF BIII). The final volume was 225 μL of 25 mM Hepes, 140 mM choline chloride, pH 7.2, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM glucose, 0.25% BSA, and 10^{-6} M tetrodotoxin. Rat brain synaptosomes (25 μL at 0.6 mg of protein/mL) were added, and the incubation was prolonged for 30 min. After centrifugation at 10000g for 1 min, the pellets were washed with 3 × 1 mL of the same buffer and then counted for radioactivity. (▲) The nonspecific inhibition control is obtained by using anti-AaH I Fab fragments at 10^{-6} M.

V to rat brain synaptosomes (Figure 3 and Table I). Here also, the monospecific Fab fragment populations (NRF BIII) inhibited less efficiently the binding than FAaH II or RF BIII populations.

It is worth mentioning that FAaH II Fab fragments were 10 times less potent in inhibiting the binding of ^{125}I Lqq V ($K_{0.5}$ = 2.5 × 10^{-8} M) than that of ^{125}I AaH II ($K_{0.5}$ = 2.5 × 10^{-9} M). This was expected since AaH II and Lqq V were previously found to exhibit different reactivities toward anti-AaH II IgGs in radioimmunoassays (El Ayeb et al., 1983a).

RF BIII were 6 times less potent in inhibiting the binding of ^{125}I Lqq V to synaptosomes than in inhibiting ^{125}I AaH II binding, a figure comparable to the ratio of 10 between the corresponding $K_{0.5}$ of FAaH II. However, NRF BIII inhibit the binding of ^{125}I AaH II 48 times more efficiently than that of ^{125}I Lqq V. This may be attributed to amino acid substitutions or deletions in the antigenic surface of Lqq V that are recognized by NRF BIII.

(3) Binding of Specific Antibodies to AaH II–Receptor Complexes. To determine whether or not the specific antibodies would recognize AaH II bound to synaptosomes, we measured the ability of ^{125}I protein A–IgG complexes to bind to synaptosomes preincubated with AaH II (total binding) or a mixture of AaH II and AaH I [nonspecific binding; AaH I competes with AaH II for binding to rat brain synaptosomes (Jover et al., 1980) but is not recognized by anti-AaH II antibodies (El Ayeb et al., 1983a)]. Figure 4 shows that ^{125}I protein A–anti-AaH II IgGs do exhibit specific binding to AaH II bound to its receptor. The nonspecific binding of ^{125}I protein A–IgG to synaptosome membranes, which was estimated by incubation with ^{125}I protein A–rabbit nonimmune IgGs, is lower than that obtained for the binding of ^{125}I protein A–anti-AaH II IgGs to AaH II/AaH I preincubated synaptosomes (Figure 4A). This observation probably reflects the binding of ^{125}I protein A–anti-AaH II IgGs to AaH II nonspecifically adsorbed to synaptosome membranes and not removed by a large excess of AaH I (5 × 10^{-5} M initial concentration) which is not recognized by anti-AaH II IgGs. Figure 4B shows the binding of a fixed concentration of anti-AaH II IgGs (10^{-7} M initial concentration) when the initial AaH II concentration was increased from 2.2 × 10^{-8} to 1.1 × 10^{-7} M.

Altogether, these results suggest that one or more antigenic sites do remain accessible to their specific antibodies after binding of the toxin to its receptor. Figure 5 further shows specific binding of PS III IgGs and PS IV IgGs to AaH II bound to rat brain synaptosomes. This strongly suggests that when the toxin is receptor-bound, these two antigenic sites, and perhaps others, remain free to bind specific IgG populations. The difference between the specific binding obtained at the plateau for anti-AaH II IgGs (18000 cpm) and for PS IV IgGs (6000 cpm) may be due to either (i) the binding of other IgG populations simultaneously with PS III and PS IV IgGs or (ii) the enhancement of the binding of one IgG population (PS IV IgGs) by fixation of the other one (PS III IgGs) on AaH II–receptor complex, resulting from a conformational change as demonstrated recently by Smith-Gill et al. (1984) for lysozyme or by Parham (1984) and Diamond et al. (1984)

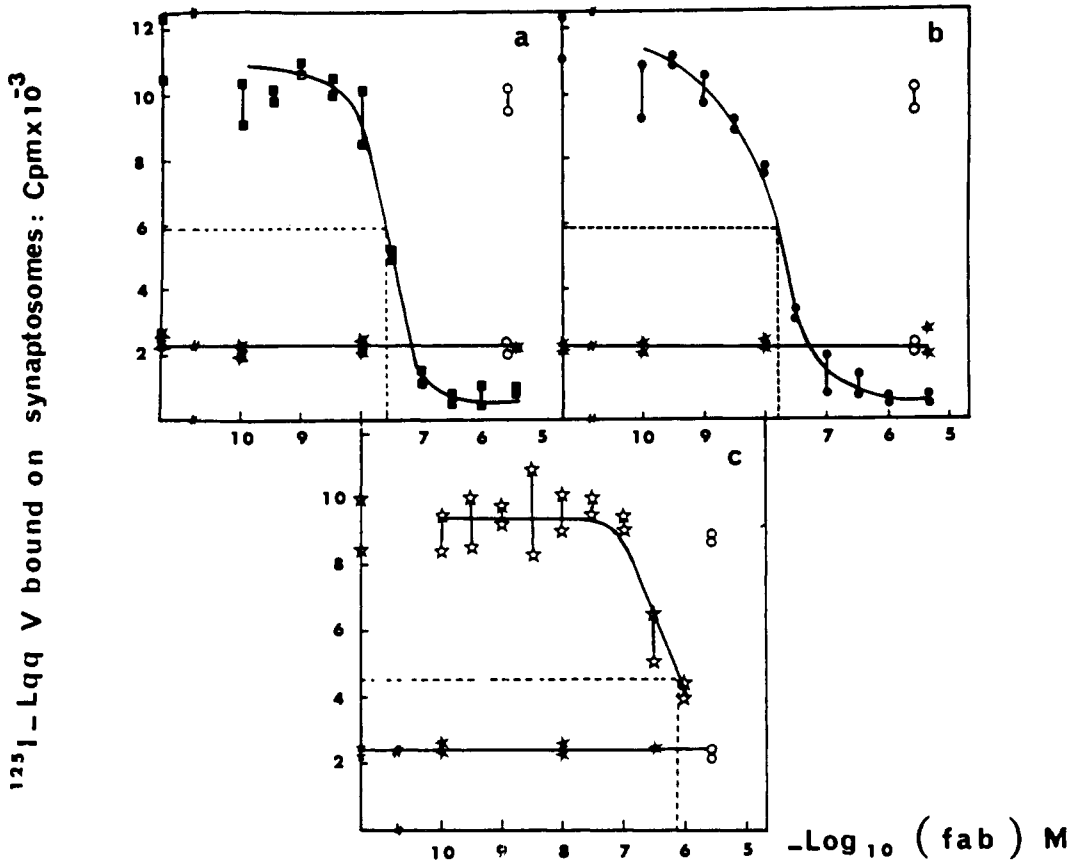

FIGURE 3: Inhibition of specific binding of ^{125}I Lqq V to rat brain synaptosomes by Fab fragment populations. ^{125}I Lqq V (50 μL) at 3 × 10^{-9} M was preincubated for 2 h at 37 °C with different Fab populations: (a) anti-AaH II Fab fragments from 10^{-10} to 3 × 9 10^{-6} M; (b) RF BIII from 10^{-10} to 4.6 × 10^{-6} M; (c) NRF BIII from 10^{-10} to 10^{-6} M. The experimental conditions used were the same as in Figure 2. (O) The nonspecific inhibition control was estimated by using anti-AaH I Fab fragments at 10^{-6} M. (★) The nonspecific binding of ^{125}I Lqq V on rat brain synaptosomes was measured by using Lqq V at 2 × 10^{-7} M.

for histocompatibility class I antigens. According to the specificity of the corresponding IgGs, antigenic amino acids in the sequences 19–29 (PS IV) and 28–39 (PS III) would be sufficiently far from the membrane receptor to remain accessible to an IgG molecule and, thus, may reasonably account for the binding of the corresponding IgG fractions to the toxin–receptor complex.

If the stoichiometry of protein A and IgG association in the experimental conditions used herein is assumed to be 1/1 (Langone, 1982) and if one also assumes a single toxin binding site per sodium channel, an approximate estimation of the amount of sodium channel per milligram of protein at the plateau (Figure 5B) can be calculated. Taking into account the specific radioactivity of protein A (157 Ci/mmol), the amount of protein in synaptosome membranes used per assay (135 μg), and the specific binding of PS IV IgGs at the plateau (Figure 5B), the amount of sodium channel is 75 fmol/mg of protein. This finding is in agreement with previous work of our laboratory concluding that there is a membrane capacity of 40–200 fmol/mg, estimated in the absence of batrachotoxin (Jover et al., 1980). However, in the presence of this alkaloid, Ray et al. (1978) found a membrane binding capacity 10 times higher on a highly purified synaptosome preparation.

As shown in Figure 6, no specific binding was found for PS I IgGs, PS II IgGs, and NR BIII IgGs, indicating the inaccessibility of their corresponding antigenic sites, which are presumably masked by the receptor. Thus, when the toxin is bound to its receptor, the region in the vicinity of the disulfide bridge 12–63 (which should have been recognized by PS I IgGs and by NR BIII IgGs) and that encompassing residues 50–59 (which should have been recognized by PS II IgGs) are located near to the receptor site.

Discussion

Antibodies are now widely accepted as powerful analytical tools. When their specificity is predetermined through affinity chromatography, they become determinant probes in structure–activity relationship studies (Lerner, 1982). In this study, antibodies were selected for their specificity toward various regions of the toxin II of *A. australis* Hector (Figure 1) by immunoaffinity chromatography either on an immobilized homologous toxin (toxin III of *B. occitanus tunetanus*) or on various immobilized synthetic peptides. The approach used to test the accessibility of toxin bound to its receptor on rat brain synaptosomes to antibodies specific to known regions is very similar to that used by Moyle et al. (1982) and Milius et al. (1983), using either monoclonal or polyclonal antibodies, respectively, and Arnheiter et al. (1983) to examine the orientation of either human chorionic gonadotropin or a human leukocyte interferon molecule on their respective receptors. While steric hindrance between antibody–toxin and toxin–receptor interactions may impair the use of selected antibodies to examine the precise location of the toxic site, they are particularly useful in determining antigenic sites that remain accessible to antibodies when the toxin is bound to its receptor. Accessibility of other toxins to their antibodies when bound to their receptors has also been examined for α-bungarotoxin (Merlie & Sebane, 1981) and toxin α of *Naja nigricollis* (Boulain & Menez, 1982), two snake toxins of molecular weight similar to that of scorpion toxins. In the case of α-bungarotoxin, antibodies are able to specifically immunoprecipitate the toxin–receptor complex whereas a monoclonal

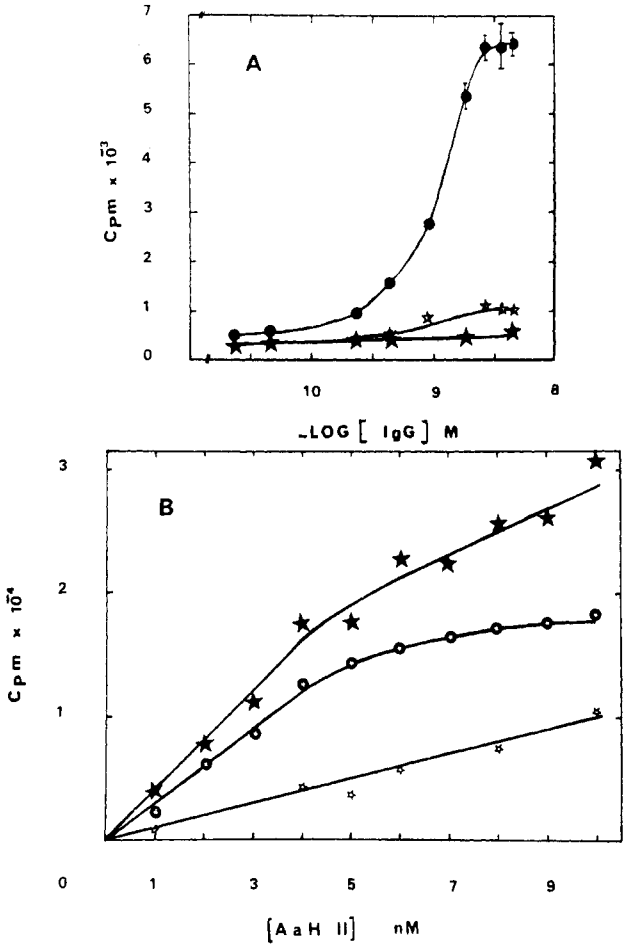

FIGURE 4: Binding of anti-AaH II IgGs to toxin-bound rat brain synaptosomes. (A) Synaptosomes (225 μL) at 0.6 mg/mL were incubated for 30 min at 37 °C with 25 μL of AaH II at 10^{-8} M (●) or with 25 μL of a mixture of AaH II at 2×10^{-8} M and AaH I at 5×10^{-5} M (☆). In a parallel experiment 100 μL of anti-AaH II IgGs (from 5×10^{-10} to 10^{-7} M) or rabbit nonimmune IgGs (★) was incubated for 2 h at 37 °C with 100 μL of ^{125}I protein A at 6×10^{-8} M. Twenty-five microliters of ^{125}I protein A–anti-AaH II IgG complex was added to the toxin–receptor complex, and the incubation was prolonged for 1 h at 37 °C. The reaction was then stopped by centrifugation at 10000*g* for 1 min, and the pellet was washed by 3 × 1 mL of Hepes–choline buffer and then counted for radioactivity. (B) The protocol was the same as for (A) except that the anti-AaH II IgG concentration was constant (10^{-7} M initial concentration) and the concentration of AaH II was varied (from 2.2×10^{-8} to 1.1×10^{-7} M initial concentration). (★) Total binding; (✪) specific binding; (☆) nonspecific binding measured with an excess of AaH I.

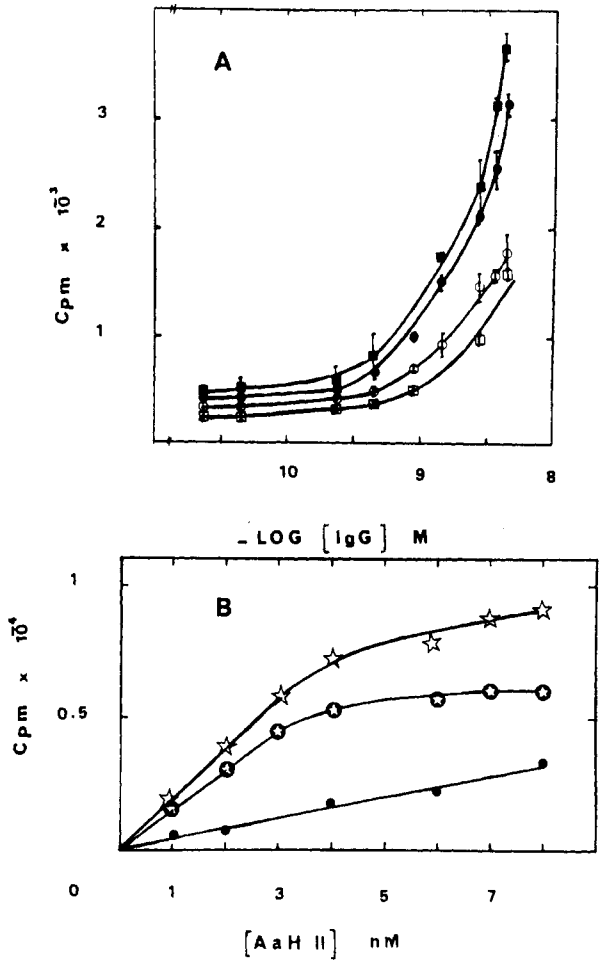

FIGURE 5: Accessibility to PS III IgGs and PS IV IgGs of the major antigenic sites of AaH II when bound to its receptor. (A) The experimental conditions were as in Figure 4. (■) Total binding of PS IV IgGs; (□) nonspecific binding of PS IV IgGs measured with a large excess of AaH I; (●) total binding of PS III IgGs; (○) nonspecific binding of PS III IgGs measured with a large excess of AaH I. (B) The experimental conditions were as in Figure 4. (☆) Total binding of PS IV IgGs; (✪) specific binding of PS IV IgGs; (●) nonspecific binding measured with an excess of AaH I.

antibody specific for toxin α of *N. nigricollis* is able to bind to the toxin–receptor complex and to promote an acceleration of the complex dissociation probably by changing the conformation of the toxin (Rousselet et al., 1984). The present immunochemical approach was expected to be successful since preliminary work from our laboratory had shown that sodium channels could be visualized by electron microscopy after binding AaH II followed by anti-AaH II IgGs (Cau et al., 1982). Moreover, as shown in the present study, scorpion toxin binding capacity on synaptosomes obtained by using antibodies was found to be of the same order of magnitude as that obtained by using direct titration of the receptor with labeled AaH II. PS III IgGs and PS IV IgGs probably recognize regions 29–39 and 19–29 of AaH II, corresponding respectively to the β-turn (residues 32–35) and α-helix (residues 23–32) regions as predicted by Fontecilla-Camps et al. (1982). Both IgG populations bind to their corresponding antigenic site when AaH II is associated with its receptor. However, anti-AaH II IgGs were more potent than PS III IgGs or PS IV IgGs in recognizing the remaining accessible antigenic sites. This could be explained either by a multideterminant binding effect or by the presence in anti-AaH II IgGs of additional subpopulations, other than PS III IgGs and PS IV IgGs, able to recognize the synaptosome-bound toxin.

A comparison of the inhibition of the binding of labeled AaH II and labeled Lqq V by either FAaH II, NRF BIII, or RF BIII suggests that (1) AaH II and Lqq V share at least two major neutralizing antigenic sites, (2) a greater neutralizing capacity is observed with FAaH II and RF BIII, polyspecific antibodies, compared to NRF BIII, which is specific to one antigenic site (probably around disulfide bridge 12–63) (El Ayeb et al., 1983b), and (3) the neutralizing site of AaH II and Lqq V recognized by NRF BIII is less structurally related in these two toxins than the other neutralizing sites. This is probably due to evolutionary substitutions where valine-10 and histidinamide-64 of AaH II are replaced by lysine and asparagine, respectively, in Lqq V. In addition, we have recently demonstrated (Bahraoui et al., 1986b) that polyclonal antibodies, generated by immunization with the synthetic peptide 50–59 of AaH II, are able to neutralize in vivo the activity of the toxin. Thus, at least two antigenic sites located around disulfide bridge 12–63 and in the region 50–59

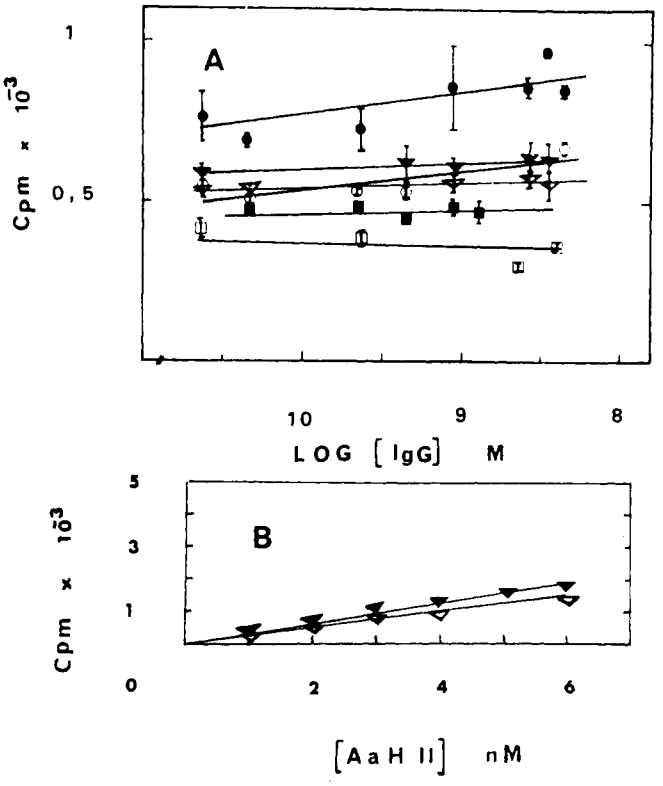

FIGURE 6: Inaccessibility to PS I IgGs, PS II IgGs, and NR BIII IgGs of the major antigenic sites of AaH II when bound to its receptor. (A) The experimental conditions shown in Figure 4A were used. (●) Total binding of NR BIII IgGs; (○) nonspecific binding of NR BIII IgGs measured in a large excess of AaH I; (▼) total binding of PS I IgGs; (▽) nonspecific binding of PSI IgGs measured in a large excess of AaH I; (■) total binding of PS II IgGs; (□) nonspecific binding of PSI IgGs measured in a large excess of AaH I. (B) The experimental conditions shown in Figure 4B were used. (▼) Total binding of PSI IgGs; (▽) nonspecific binding of PS I IgGs measured in a large excess of AaH I.

are involved in the molecular mechanism of neutralization of AaH II and Lqq V. However, the antigenic site encompassing sequence 50–59 seems to be more structurally homologous in AaH II and Lqq V than the site located around disulfide bridge 12–63. This finding is in agreement with our recent results (Bahraoui et al., 1986b) concerning the critical importance of lysine-58 in the antigenicity of AaH II. This residue is retained in Lqq V.

Fontecilla-Camps et al. (1981), starting from crystallographic data and a comparison of amino acid sequences, have proposed a structural model of scorpion toxins in which the unique conserved region is involved in the interaction with the sodium channel. The region of the α-helix (residues 23–32) and that of the β-turn (residues 32–35) are in the back view of the conserved region. Moreover, Darbon et al. (1983) and El Ayeb et al. (1986) have demonstrated that chemical modifications of selected amino acids abolish the binding capacity of these toxins only if these residues are enclosed in the conserved region. These findings further corroborate the previously proposed orientation of the toxin on its receptor site since antigenic sites recognized by PS III IgGs (β-turn region) and PS IV IgGs (α-helix region) were found to be accessible when AaH II is bound to its receptor. In contrast, antigenic sites encompassing residues around disulfide bridge 12–63 and residues 50–59, which are recognized respectively by PS I IgGs and PS II IgGs, are inaccessible in the AaH II–receptor complex and are thought to be involved in the molecular neutralization of toxicity, probably by a steric hindrance mechanism. For a more precise view of all the determinants that are involved in the neutralization mechanism or remain accessible when AaH II is bound to its receptor, the generation of monoclonal antibodies to the four synthetic peptides will be required. Some of these monoclonal antibodies as well as anti-AaH II IgGs could be used to purify, by the immunomatrix technique (Schneider et al., 1982), the toxin–sodium channel receptor covalent complex in a single step.

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Rate and Extent of Poly(ethylene glycol)-Induced Large Vesicle Fusion Monitored by Bilayer and Internal Contents Mixing†

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ABSTRACT: Poly(ethylene glycol) (PEG) of average molecular weight 8000 was used to mediate the fusion of large unilamellar vesicles composed of dipalmitoylphosphatidylcholine. Fusion was monitored by fluorescence assays of lipid mixing and aqueous contents mixing. The extent of lipid mixing, as monitored by DPHpPC fluorescence lifetime, indicated that large unilamellar vesicles underwent a single fusion cycle when incubated with PEG and subsequently diluted into buffer. The ANTS/DPX assays for contents mixing and leakage indicated that, while addition and dilution of PEG were accompanied by extensive contents leakage, this occurred on a much different time scale as compared to contents mixing. Both the lipid-mixing and contents-mixing assays gave comparable estimates for the number of rounds of fusion that occurred in a given time following PEG addition, although the contents-mixing assay always yielded an estimate 10–15% larger than the lipid-mixing assay. These assays were used to evaluate several factors purported to influence PEG-induced fusion. First, the initial rate of fusion was found to be dependent on PEG concentration in the range of 0–35 wt %, while the extent of fusion was not. In addition, a substantial rate enhancement occurred when vesicles were incubated with greater than 26% PEG. Second, the creation of an osmotic gradient upon dilution of vesicle–PEG mixtures was shown to have no effect on either the extent or the initial rate of fusion. Consistent with this observation, both contents and lipid mixing were found to occur prior to and independent of the dilution of the PEG–vesicle suspension. Third, impurities, either present in our commercially available PEG or added to vesicle–PEG mixtures, also had no effect on the rate or extent of fusion. Fourth, another dehydrating polymer, dextran (average mol wt 9000), was capable of promoting fusion, though at a much lower rate than PEG. These results suggest that even partial bilayer dehydration accompanied by vesicle collapse and close interbilayer contact may be sufficient to induce vesicle fusion.

The juxtaposition of cellular or vesicular membranes is widely accepted to be necessary for fusion. However, this close association may be insufficient to kindle the mixing of bilayer lipids and the interaction of trapped internal components, both of which occur during a fusion event. The ability of several agents to induce reversible vesicle or cell aggregation (i.e., not leading to fusion) supports this idea (Honda et al., 1981; Boni et al., 1981; Wilschut et al., 1981). These findings imply that the continuity of bilayer structure must be disrupted or altered by some agent or event before fusion can occur. Lateral-phase separations (Papahadjopoulos et al., 1976), fusogenic im-

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