Cloning and functional expression of B chains of β -bungarotoxins from *Bungarus multicinctus* (Taiwan banded krait)

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The cDNA species encoding the B chains (B₁ and B₂) of β -bungarotoxins (β -Bgt) were constructed from the cellular RNA isolated from the venom glands of *Bungarus multicinctus* (Taiwan banded krait). The deduced amino acid sequences of the B chains were different from those determined previously by a protein sequencing technique. One additional Arg residue is inserted between Val-19 and Arg-20 of the B₁ chain. Similarly the insertion of one additional Val residue between Val-19 and Arg-20 of the B₂ chain is noted. Thus the B chains should comprise 61 amino acid residues. Moreover, the residues at positions 44–46 are Gly-Asn-His, in contrast with a previous result showing the sequence His-Gly-Asn. Instead of Asp, the residues at positions 41 and 43 are Asn. The B chain was subcloned into the expression vector pET-32a(+) and transformed into *Escherichia coli* strain BL21(DE3). The recombinant B chain was expressed as a fusion

protein and purified on a His-Bind resin column. The yield of affinity-purified fusion protein was increased markedly by replacing Cys-55 of the B chain with Ser. However, the isolated B(C55S) chain became insoluble in aqueous solution after removal of the fused protein from the affinity-purified product, suggesting that protein–protein interactions might be crucial for stabilizing the structure of the B chain. The B(C55S) chain fusion protein showed activity in blocking the voltage-dependent K⁺ channel, but did not inhibit the binding of β -Bgt to synaptosomal membranes. These results, together with the finding that modification of His-48 of the A chain of β -Bgt caused a marked decrease in the ability to bind toxin to its acceptor proteins, suggest that the B chain is involved in the K⁺ channel blocking action observed with β -Bgt, and that the binding of β -Bgt to neuronal receptors is not heavily dependent on the B chain.

INTRODUCTION

β -Bungarotoxins (β -Bgt), the main presynaptic phospholipase A₂ (PLA₂) neurotoxin purified from the venom of *Bungarus multicinctus* (Taiwan banded krait), consist of two dissimilar polypeptide chains, the A chain and the B chain, cross-linked by an interchain disulphide bond [1–3]. Although several isotoxins have been isolated [2–5], their A chains are structurally homologous to PLA₂, B chains share sequence similarity with trypsin inhibitor, toxin I and dendrotoxins [6–9]. Several studies have suggested that the presynaptic neurotoxicity of β -Bgt is attributed to a selective inhibition of certain voltage-sensitive K⁺ channels that are concerned with regulating neuronal excitability and synaptic transmission [10–12]. Moreover, the β -Bgt-binding proteins, which were suggested to be a member of a family of voltage-gated K⁺ channels, have been isolated from chick and rat brains [13–19]. Nevertheless, the roles of the A and B chains in β -Bgt are still controversial. Although chemical modification studies revealed that the A chain is an active subunit responsible for the phospholipase activity and neurotoxic effect of β -Bgt [20–23], the co-existence of the A and B chains in the toxin molecule made these results ambiguous. Rugolo et al. [24] suggested that the toxicity of β -Bgt towards mammalian nerve terminals can be largely accounted for by specific site-directed PLA₂-induced permeabilization of the plasma membrane. An ESR study on the interaction of β -Bgt with liposomes suggested that β -Bgt-mediated liposome fusion was concerned with its PLA₂ activity, and as a consequence with the creation of point defects in the lipid structure and release from transmission [25].

Alternatively, Benishin [26] proposed that the B chain of β -Bgt might be responsible for the blockage of certain voltage-gated K⁺ channels in a manner that did not involve the A chain.

To characterize the roles of the A chain and B chain in the activities of β -Bgt, the effective and convenient way is to clone the genes encoding the A chain and the B chain, then subject them to expression. Although the cDNA species encoding the A chains have been constructed [27–30], the genes encoding the B chains have not yet been determined. In the present study the cDNA species encoding the B₁ and B₂ chains of β -Bgt were constructed from the cellular RNA of *B. multicinctus* venom glands by using reverse transcriptase-mediated PCR (RT–PCR) and rapid amplification of cDNA ends (RACE) PCR. The deduced amino acid sequences of the B chains indicate the need for revisions of amino acid sequences determined by conventional protein sequencing techniques [2,3,7,8]. The present paper also describes the expression of a recombinant B chain in *Escherichia coli* and an assessment of its activity in comparison with that of native β -Bgt isolated from the venom. These results give an insight into the roles of the B chains in β -Bgt.

MATERIALS AND METHODS

Preparation of mRNA from venom glands

Cellular RNA was isolated from *B. multicinctus* venom glands, which had been stored in liquid nitrogen immediately after killing. Two deep-frozen glands from one snake were homogenized to extract RNA with a guanidinium isothiocyanate/phenol/chloroform isolation kit (Stratagene).

Abbreviations used: β -Bgt, β -bungarotoxins; PLA₂, phospholipase A₂; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcriptase-mediated PCR.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers Y12100 (B₁ chain) and Y12101 (B₂ chain).

PCR amplification and cloning

Two degenerate oligonucleotide primers of sense and anti-sense orientations based on the N-terminal and C-terminal sequences of the B₁ chain of β -Bgt [2,3,7,8] with forward and reverse sequences 5'-MGNCARMGNCAYMGNGAYTG-3' and 5'-YYANGGRTANACNARRCAYTC-3' respectively were synthesized. A termination codon was added to the 5' region of the reverse primer.

RT-PCR was performed with 100 μ l of reaction buffer containing 100 mM Tris/HCl, pH 8.3, 1 mM dNTP, 25 μ M anti-sense primer and 200 ng of RNA template. In the reverse transcription, cDNA synthesis was started with rTth reverse transcriptase (5 units) and 2 μ l of 10 mM MnCl₂ at 70 °C for 15 min. Then 8 μ l of chelating buffer containing 50 % (v/v) glycerol, 100 mM Tris/HCl (pH 8.3), 1 M KCl, 7.5 mM EGTA and 0.5 % (v/v) Tween 20 was added to the reaction. After the addition of 8 μ l of 25 mM MgCl₂ containing 25 μ M sense primer, the amplification proceeded on a thermocycler for 35 cycles of 1 min at 94 °C, 1 min at 48 °C and 1 min at 72 °C.

The PCR products were cloned into pCRII vector by the TA-cloning procedure (Invitrogen, San Diego, CA, U.S.A.).

DNA sequencing

Sequence analysis was performed by the dideoxy method with a sequencing kit (Sequenase sequencing system; USB), labelling with [³⁵S]dATP (Amersham; more than 1000 Ci/mmol). The reaction products were sequenced in a 6 % (w/v) polyacrylamide gel, which was dried and exposed on a Kodak film for 1 day at room temperature.

RACE protocol for PCR amplification

A RACE protocol for PCR amplification was performed with a Marathon cDNA amplification kit from Clontech. cDNA mixtures were synthesized from poly(A)⁺ mRNA by Moloney murine leukaemia virus reverse transcriptase. The cDNA species were ligated overnight by T4 DNA ligase with the adaptor provided by the kit. After adaptor ligation, the ligated cDNA species were then used as a template for PCR amplification. The 3' RACE amplification was performed with the primers 5'-AGGCAACGTCACCGAGATTGT-3', designed from the N-terminal region of the B₁ chain (see Figure 1), and AP1 primer provided by the same kit. The 5' RACE amplification was performed with the primer 5'-TTAGGGATATAC TAAACACTCGCAGCG-3', designed from the C-terminal region of the B₁ chain (see Figure 1) and AP1 primer. The second 3' RACE was performed with the AP2 primer and the primer 5'-GCAAGCATTTTCAGTATCGCGGC-3'; the primers for the second 5' RACE were 5'-GCCGCGATACTGAAATGCTTTGC-3' and AP2. The conditions for the PCR reactions were essentially identical with those described in the manufacturer's protocol. The PCR products were cloned into pCRII vector by using the TA-cloning procedure (Invitrogen).

Cloning and expression of the B₁ chain of β -Bgt

Synthetic oligonucleotides were designed to produce a 195 bp amplified DNA fragment spanning the open reading frame of the B₁ chain of β -Bgt. Primer 1 introduced a 5' EcoRV site and an in-frame Met codon preceding Arg-1:

5'-GATATCATGAGGCAACGTCATCGGGATTGTG-3'

Met Arg Gln Arg His Arg Asp Cys

The sequence of primer 2 was 5'-TTAGGGATACACA-AGACACTCGCAGCG-3'. The PCR products were cloned into pCRII vector. The inserted DNA fragment was cut with EcoRV and EcoRI, then ligated into the large fragment of EcoRV/EcoRI-cut pET-32a(+). The entire sequence was confirmed by dideoxynucleotide sequencing.

The resulting plasmid pET-B₁ was transformed into *E. coli* strain BL21(DE3). Transformants were selected on Luria-Bertani agar plates supplemented with 50 μ g/ml ampicillin. For the induction of gene expression, *E. coli* BL21(DE3) cells harbouring pET-B₁ plasmid were grown at 37 °C in Luria-Bertani medium containing 50 μ g/ml ampicillin. After reaching a *D*₅₅₀ of 1.0, isopropyl β -D-thiogalactoside was added to a final concentration of 1 mM. The culture was induced for periods of up to 4 h. The cells were harvested and lysed by ultrasonication.

Preparation of the recombinant B₁ chain

The B₁ chain of β -Bgt was expressed as a fusion protein with thioredoxin. The His-tag in the fusion protein allowed us to purify the fusion protein on an affinity column (His-Bind resin; Novagen). After ultrasonication, the insoluble pellet was removed by centrifugation for 30 min at 27000 g. The supernatant was applied directly to the His-Bind resin column, and the B chain fusion protein was purified in accordance with the manufacturer's protocol. The affinity-purified fusion protein was hydrolysed with enterokinase at 21 °C for 20 h. The hydrolysates were further separated on a Cosmogel SP Glass column (8 mm $\times$ 75 mm; Nacalai Tesque), and eluted with a linear gradient of 0.05–1 M ammonium acetate, pH 6.8, for 60 min. The flow rate was 0.8 ml/min; the effluent was monitored at 280 nm.

Gel electrophoresis and immunoblotting of the B chain

SDS/PAGE was performed in a 15 % (w/v) polyacrylamide gel by the method of Laemmli [31]. Samples were denatured by boiling for 5 min in 0.125 M Tris/HCl/4 % (w/v) SDS in the presence of 4 % (v/v) 2-mercaptoethanol. After SDS/PAGE, the protein on the gel was transferred electrophoretically to Immobilon PVDF membrane by the method of Towbin et al. [32]. The immunoreactive protein was revealed by immunological analysis with anti-(β -Bgt) antibodies and stained with horseradish peroxidase-conjugated protein A.

Electrophysiological measurement

Electrophysiological measurement was performed essentially with the use of the procedure described by Lo et al. [33]. Rat thoracic aorta smooth-muscle cells (cloned cell line A7r5) were obtained from the American Type Culture Collection (CRL1446; Rockville, MD, U.S.A.). A7r5 vascular smooth-muscle cells were transferred to a recorder chamber mounted on the stage of an inverted microscope (TMS-F; Nikon, Tokyo, Japan) coupled to a video camera system with a magnification of up to $\times$ 1500. Cells were batched at room temperature (20–25 °C) and superfused continuously at a rate of approx. 2 ml/min with Tyrode's solution containing (in mM) NaCl 136.5, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.53, glucose 5.5 and Hepes 5, with the pH adjusted to 7.4 with NaOH. Ionic currents were recorded with patch pipettes in a whole-cell configuration. The patch pipette was connected to the input stage of a patch-clamp amplifier (RK-400; Biologic, Claix, France). The tip resistance varied between 3 and 5 M Ω when filled with the following internal solution (in mM): KCl 130, Na₂ATP 3, MgCl₂ 2, GTP 0.1, Hepes 5 and Na-EGTA 0.1 (pH 7.2). A three-dimensional oil-driven micromanipulator (MO-

103; Narishige Scientific Instruments, Tokyo, Japan) was used to position the pipette near the cell. The voltage-step command signals were digitally generated at a rate of 0.5 Hz by use of a programmable stimulator (SMP 310; Biologic).

In experiments designed to construct the current-voltage (I - V) relationships, voltage steps with a duration of 500 ms from the holding potential to various potentials were applied. The conditioning voltage pulses (500 ms in duration) to various membrane potentials between -80 and $+80$ mV were applied from a holding potential of -80 mV.

Synaptosome binding assay

Synaptic membranes were prepared from the whole brains of rats by the method described by Hulme and Buckley [34]. The Chloramine-T method of Greenwood et al. [35] was used for the radioiodination of β -Bgt. The standard incubation buffer used in binding experiments was 50 mM imidazole/HCl buffer, pH 7.4, containing 90 mM NaCl, 5 mM KCl and 1.5 mM CaCl_2 (or 1.5 mM SrCl_2). The concentration of synaptic membranes was 150 μg of membrane protein, and the ^{125}I - β -Bgt concentration was 3 nM. After incubation of the membranes with ligand for 2 h, bound and free ^{125}I - β -Bgt were separated by a centrifugation/washing assay, as described previously [13]. All binding data are corrected for non-specific binding determined in the presence of a 1000-fold molar excess of unlabelled β -Bgt over the labelled toxin and in the presence of 1.5 mM SrCl_2 , and represent the means of at least two experiments.

RESULTS AND DISCUSSION

Cloning and sequencing of cDNA of the B_1 chain

Although previous studies reported that the β -Bgt isotoxins consisted of B_1 and B_2 chains [2,3,7,8], the B-chain variants for which the N-terminal amino acid sequences were slightly different from those of the B_1 and B_2 chains were identified by Chu et al. [4]. A similar result was also observed by Kwong et al. [9]. These studies showed that the reported sequences of the B chains [2,3,7,8] needed to be revised; the best way of doing this is to deduce the amino acid sequence from the corresponding cDNA species encoding the B chains. Two degenerate primers for the RT-PCR reaction were designed from the N-terminal and C-terminal sequences of the B_1 chain. Because the nucleotide sequences of the primers were degenerate, high concentrations (25 μM) of primers were used in the PCR reaction. PCR amplification of the venom cDNA mixtures with the designed primers achieved the isolation of a PCR fragment estimated to be approx. 180 bp (results not shown). The DNA fragments were then subcloned by a TA-cloning kit. More than 10 positive clones were selected for nucleotide sequencing; the selected clones had a deduced amino acid sequence corresponding to that of the B_1 chain (Figure 1, upper panel). The deduced amino acid sequence of the B_1 chain shows that it comprises seven cysteine residues, as previously reported [2,3,7,8]. However, the deduced protein sequence is different from that determined by conventional protein sequencing techniques. One additional Arg residue is inserted between Val-19 and Arg-20: thus the B_1 chain is composed of 61, rather than 60, amino acid residues. Moreover, the residues at positions 44–46 are Gly-Asn-His, in contrast with previous results showing the sequence His-Gly-Asn [2,3,7,8]. Instead of Asp, the residues at positions 41 and 43 are Asn.

Sequence determination of cDNA by RACE PCR amplification

To validate the determined sequence by PCR on the basis of the N-terminal and C-terminal degenerate primers, we adopted 5'-

Figure 1 cDNA sequences of B_1 and B_2 chains

Upper panel: the nucleotide sequence of 425 bp is shown above the amino acid sequence of 85 residues, including a signal peptide of 24 residues. The mature B_1 chain of 61 residues starts at Arg. The underlines indicate the residues discrepant from the published sequence determined by protein sequencing [2,3,7,8]. The marked regions were used to design the primers for RACE PCR. Several bases at the N-terminus and C-terminus of the B_1 chain, which are different from the cDNA amplified by degenerate primers, are indicated by bold letters. Lower panel: the nucleotide sequence of 328 bp is shown above the amino acid sequence of 85 residues, including a signal peptide of 24 residues. The mature B_2 chain of 61 residues starts at Arg.

end RACE and 3'-end RACE protocols for the PCR amplification of B_1 chain cDNA. By the use of 5' and 3' RACE protocols, the ambiguities in the N-terminal and C-terminal segments are therefore resolved. No appreciably amplified products were observed in the first PCR amplification. However, the 5' and 3' RACE PCR products were notably amplified by the second PCR. The amplified PCR products were estimated to be 160 and 300 bp respectively (results not shown). More than 20

positive clones were selected for sequencing. As shown in Figure 1 (upper panel), the deduced amino sequence of the precursor of the B₁ chain shows that its signal-peptide region contains 24 residues. Several bases at the 5' end (N-terminus) and the 3' end (C-terminus) of the B₁ chain cDNA amplified by degenerate primers are revised (Figure 1, upper panel). Moreover, the polyadenylation signal (AATAAA) appears at positions 381–386 of the B₁ chain cDNA.

Cloning and sequencing of the B₂ chain

To clone the gene encoding the B₂ chain, several forward and reverse primers from the nucleotide sequence of the B₁ chain were designed for amplification of the cDNA encoding the B₂ chain. Among them, two primers were designed from the region at the beginning of the signal peptide (5'-ATGTCTTCTGAGGTCTTCTTCTCC-3') and the 3'-untranslated region (5'-TGGTCCAGGGCAAGAAGCAGGGTC-3') respectively. PCR amplification of the venom cDNA mixtures with the designed primers achieved the isolation of a PCR fragment estimated to be approx. 330 bp (results not shown). The cDNA fragments were then subcloned by a TA cloning kit and subjected to nucleotide sequencing. As shown in Figure 1 (lower panel), the precursor of the B₂ chain contains the signal peptide with 24 residues. The deduced protein sequence reveals that the B₂ chain also comprises 61 residues. There is one additional Val residue inserted between Val-19 and Arg-20. Moreover the residues at positions 44–46 are Gly-Asn-His, and the residues at positions 41 and 43 are Asn. These findings are similar to those for the B₁ chain. Because the A and B chains are separately encoded by different genes, it indicates that the combination of A chain and B chain to form β -Bgt should occur at a post-translational stage.

Expression of the recombinant B₁ chain

To subclone the B₁ chain into the expression vector pET32a(+), a new primer (5'-GATATCATGAGGCAACGTCATCGGGA-TTGTG-3', in which the underline indicates the *EcoRV* site and the ATG codon) was designed to create an *EcoRV* site and an in-frame Met codon preceding the beginning of the nucleotide sequence encoding the B₁ chain. Because the B chain itself is devoid of a Met residue, the Met preceding Arg-1 of the B chain serves as a CNBr-cleavage site. The anti-sense primer was designed in proximity to the stop codon. The amplified DNA was inserted into the pCRII vector and finally subcloned into the expression vector pET-32a(+) by digestion with *EcoRV*/*EcoRI*. Recombinant clones were initially grown as 10 ml cultures and induced with isopropyl β -D-thiogalactoside. Aliquots of cell extracts were analysed by SDS/PAGE. The size of the protein fragment observed with SDS/PAGE is in accordance with that expected from a fusion protein of thioredoxin and the B chain (results not shown). The B chain fusion protein was further purified by a His-Bind resin column. Although a large amount of fusion protein in the cell extracts was observed by SDS/PAGE analysis, a low yield was obtained after passage through the affinity column. Moreover, the results of the SDS/PAGE analysis showed that the B chain and thioredoxin were not separated by cleavage with CNBr (results not shown). In view of the fact that one of the Cys residues in the B chains (at position 55) forms an interchain disulphide linkage with the A chain [2,3,7,8], our results indicated that one of the Cys residues of the B chain probably formed an alternative disulphide linkage with the fused thioredoxin, thus impeding the interaction between the His-tag and the His-Bind resin. Alignments of the amino acid sequences of the B chains and dendrotoxins show that all of the Cys

Figure 2 SDS/PAGE and immunoblot analysis of the B₁ (C55S) chain fusion protein

SDS/PAGE (A) and immunoblot analysis (B) of the B chain fusion protein. Lane 1, molecular markers (prestained SDS/PAGE standards from Bio-Rad, Hercules, CA, U.S.A.: phosphorylase, 107 kDa; BSA, 76 kDa; ovalbumin, 52 kDa; carbonic anhydrase, 36.8 kDa; soybean trypsin inhibitor, 27.2 kDa; lysozyme, 19 kDa); lane 2, the purified fusion protein from a His-Bind resin column; lane 3, the hydrolysates of the fusion protein after digestion with enterokinase.

residues are located at equivalent positions with the exception of Cys-55. Thus another anti-sense primer (5'-TTAGGGATACA-CAAGACACTCGCTGCG-3') was designed, with the aim of replacing Cys-55 (TGC) with Ser-55 (AGC). The yield of the affinity-purified B(C55S) chain fusion protein markedly increased by at least 100-fold. However, minor contaminants were found to be co-eluted with the recombinant protein (Figure 2). The fusion protein was hydrolysed with enterokinase or was cleaved with CNBr. SDS/PAGE and immunoblot analysis showed that the B chain could be separated from the fused thioredoxin by both methods (Figure 2). The mutated B(C55S) chain was further purified by FPLC on a Cosmogel SP Glass column. Unfortunately, the B chain purified from the CNBr-cleavage products was not homogeneous, as revealed by N-terminal sequence determination (results not shown). It contained a species with a short N-terminal extension relative to the venom-derived B chain, because CNBr cleavage did not occur exclusively at the Met residue preceding Arg-1 of the B chain and also occurred at Met residues in the C-terminal region of the fused protein. Alternatively, the peptide bond between the fused protein and B chain was specifically hydrolysed with enterokinase. The purified B chain represented a homogeneous component as revealed by the results of SDS/PAGE, immunoblot analysis and chromatographic analysis (results not shown). Moreover, it contains an extra N-terminal segment, AMADIM, preceding Arg-1 of the B₁ chain. In contrast with the B chain fusion protein, the purified B chain became insoluble in aqueous solution after short-term storage or freeze-drying. Therefore the biological activities of the recombinant B(C55S) chain were measured without removal of the fused thioredoxin.

Effect on K⁺ current in A7r5 cells and binding ability for synaptosomal membranes

As shown in Figure 3, the B(C55S) chain fusion protein produced a notably downward shift when the potential was more positive than -30 mV. It revealed that the outward K⁺ current was effectively blocked by the fusion protein. In a control experiment, the same concentration of recombinant κ -bungarotoxin (thioredoxin fused with κ -bungarotoxin) prepared as described previously [36] did not exhibit any significant effect on the current of K⁺ ions. These results clearly indicate that the blockage of the

Figure 3 Current-voltage relationship of K^+ outward current in the absence or presence of the recombinant B chain or κ -bungarotoxin

The cell was held at -80 mV; command voltage pulses with a duration of 500 ms were applied at 0.5 Hz to various membrane potentials. The K^+ outward current was obtained at the end of each voltage step in the absence (○) or presence (●) of 200 nM recombinant B_1 (C55S) chain (A), or in the absence (○) or presence (●) of recombinant 200 nM κ -bungarotoxin (B).

Figure 4 Inhibition of ^{125}I - β -Bgt binding to synaptic membranes by increasing concentrations of native β -Bgt, His-modified β -Bgt or recombinant B chain

The procedure was as described in the Materials and methods section. Synaptosomes were incubated with native β -Bgt (■, □), His-modified β -Bgt (●, ○) or recombinant B_1 (C55S) chain (▲, △) respectively, in the presence of 1.5 mM Ca^{2+} (■, ○, △) or 1.5 mM Sr^{2+} (□, ●, ▲). The results (B) are shown relative to the specific binding observed in the absence of competing ligand (B_0).

outward K^+ current can be attributed to the action of the B chain.

As illustrated in Figure 4, in comparison with that in the presence of Ca^{2+} , the binding of ^{125}I - β -Bgt to synaptosomal membranes was more easily displaced by unlabelled β -Bgt in the presence of Sr^{2+} . However, it was found that, in the presence of Ca^{2+} , the binding of labelled β -Bgt to synaptic membranes was inhibited by unlabelled β -Bgt only in an intermediate range of concentrations, above which the unlabelled toxin induced an increase in ^{125}I - β -Bgt binding. Similarly, several PLA_2 neurotoxins (including crotoxin B, ammodytoxin c, agkistrodotoxin and ammodytin 12), which showed a slight inhibitory effect on the specific ^{125}I -crotoxin binding at low concentrations, apparently increased the toxin binding at high concentrations [37]. It was likely that non-specific binding sites for β -Bgt might be produced after extensive hydrolysis of phospholipid by β -Bgt. This proposal is supported by the finding (Figure 4) that the increment of ^{125}I - β -Bgt binding was no longer observed by distorting the PLA_2 activity of β -Bgt (i.e. by modification of His-48 of the A chain of β -Bgt with *p*-bromophenacyl bromide or replacing Ca^{2+} with Sr^{2+}). However, the recombinant B(C55S) chain did not show an appreciable inhibitory effect on the binding of β -Bgt to synaptic membranes. Previous studies showed that Sr^{2+} was able to substitute for Ca^{2+} in toxin binding [16]. In contrast with the finding that the ability of unlabelled β -Bgt to displace ^{125}I - β -Bgt from its binding sites in the presence of Ca^{2+} was different from that in the presence of Sr^{2+} , the capability of His-modified toxin to bind the acceptor proteins was similar regardless of the presence of Ca^{2+} or Sr^{2+} . Moreover, a His-modified derivative exhibited a decrease in the ability to bind with synaptic membranes. These results, together with the finding that the binding of Ca^{2+} and Sr^{2+} to PLA_2 caused differential conformational changes [38], suggested that a modification of the His residue of the A chain might alter the synaptosome-binding ability of β -Bgt as well as the fine structure of β -Bgt, which was susceptible to alteration by binding with Ca^{2+} or Sr^{2+} .

The functional role of the B chain in β -Bgt

Although previous studies suggested that neuronal K^+ channels were the target for β -Bgt binding [10–19], the involvement of the A chain, the B chain or both in binding with the acceptor proteins remains to be determined. Kini and Iwanaga [20] suggested that the B chain acted like a 'chaperone' to bring β -Bgt to interact with the presynaptic site of the neuromuscular junction on the basis of the presence of overlapping hydrophobic regions in the amino acid sequence of dendrotoxins and B chains. Although the structures of the B chains and dendrotoxins share a high degree of similarity [9], β -Bgt bound very weakly to the dendrotoxin-binding components [39]. In contrast, dendrotoxin inhibited the binding of ^{125}I - β -Bgt to synaptic membranes in a non-competitive fashion [40]. Heterogeneity of the acceptors for β -Bgt and dendrotoxin was observed in the rat brain region [40]. Moreover, synaptosome-binding studies on the isolated B chain suggested that the B chains were not exclusively related to the specific binding of β -Bgt to its acceptors [16]. These observations imply that the B chain is not the only essential subunit for the binding of β -Bgt to its target. In view of the findings that Ca^{2+} is required for the binding of β -Bgt with its receptors [16–18] and that the A chain of β -Bgt is the Ca^{2+} -binding subunit of β -Bgt [21–23], it is likely that the A chain is related to the binding of β -Bgt to its neuronal acceptors in addition to its functional role in PLA_2 activity. Meanwhile, the observations that modifying His-48 in the A chain affected the ability of β -Bgt to bind synaptosomal membranes (Figure 4) and that the removal of Ca^{2+} by

EDTA led to a marked decrease in toxin binding (results not shown) further support this suggestion. These currently available results raise the possibility that both chains of β -Bgt are involved in the toxin's binding to its target site, as proposed by Harris [41]. Lin et al. [42] and Chang et al. [43] suggested that interaction between the A and B chains was essential for maintaining the active conformation of β -Bgt. X-ray crystallographic analysis revealed that the interface between the A and B chains was stabilized by electrostatic and hydrophobic interactions of the two chains [9]. Possibly, without the A chain, the B chain by itself does not possess the native structure that it adopts in β -Bgt, and vice versa. As a consequence, an inability of the B chain fusion protein to inhibit the binding of β -Bgt to synaptosomal membranes was observed. Nevertheless the B chain exhibits an activity in blocking the outward K^+ current. Similar results reported by Benishin [26] showed that reduced and S-carboxymethylated B chain manifested this action. This permitted the inference that the blocking activity of B chain was independent of its intact tertiary structure. Alternatively, structure-function studies on dendrotoxin indicated that the integrity of disulphide bonds was critical for its activity [44]. The differences in structural features for exerting the K^+ channel blocking activity might be reflected by the observation that their binding sites were not the same [40]. On the basis of our results, it suggests strongly that the B chain is directly involved in the K^+ channel blocking action noted with β -Bgt, and it is likely that the binding of β -Bgt to its acceptors is not heavily dependent on the B chain. We propose that, if the B chain is involved in the binding of toxin to its acceptors, it should be regulated by the A chain through protein-protein interactions.

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