Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 183-188

POSSIBLE INTERRELATIONSHIP OF MULTIPLE α-BUNGAROTOXIN BINDING COMPONENTS IN THE BRAIN OF THE HORSESHOE CRAB, LIMULUS POLYPHEMUS

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Received March 28, 1979

SUMMARY: Three [^{125}I]α-bungarotoxin binding components have been detected in solubilized extracts of Limulus brain tissue. These components have sedimentation coefficients of 9.0S, 15.4S and 17.4S. All three components were degraded by α-chymotrypsin. The addition of butyl alcohol to brain extract suggests an interrelationship of the toxin binding proteins by promoting a simultaneous decrease in the 9S component and increase in the 15.4S and 17.4S components. This transition was also demonstrated by the readdition of isolated fractions of each component to brain extract.

Several of the biochemical correlates of cholinergic transmission have been identified in the central nervous system of the horseshoe crab, Limulus polyphemus (1-5). Recently, the binding of αBGT[1] to particulate preparations of Limulus brain tissue has been reported (6,7). The pharmacological properties of toxin binding in Limulus are consistent with specific binding to an acetylcholine receptor. However, the kinetic parameters indicate complex binding (i.e., binding to multiple sites). An analysis of αBGT binding in solubilized extracts of Limulus brain tissue suggests the presence of multiple binding components. The presence of multiple toxin binding components is not particularly supportive of

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1 αBGT, α-bungarotoxin; HEPES, n-2-hydroxyethyl-piperazine-N'-2-ethanesulphonate.

0006-291X/79/090183-06$01.00/0

specific binding. Moreover, several recent studies have challenged the specificity of αBGT binding in central nervous system preparations (8,9). Therefore, the present study was undertaken to investigate the nature and possible interrelationship of the toxin binding components in Limulus brain tissue.

METHODS AND MATERIALS

Preparation of Solubilized Brain Extract. Brain tissue (0.3-0.5 g per animal) was dissected fresh from horseshoe crabs prior to each experiment. The tissue was homogenized in 10mM HEPES[1] pH 7.4 and centrifuged 15 min. at 43,500 x g. The pellet was resuspended in the same buffer containing 1% Triton X-100 and stirred for 2 hr. at room temperature. Solubilization was terminated by centrifugation for 60 minutes at 100,000 x g. The supernatant was decanted and used directly to assay solubilized toxin binding activity.

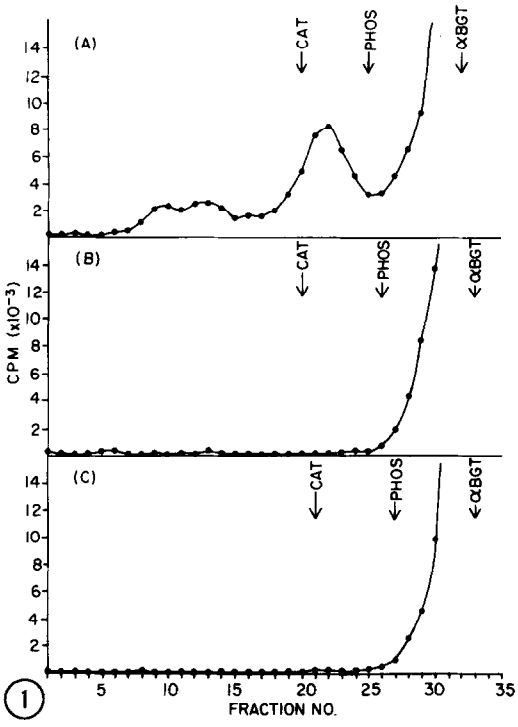
[^{125}I]αBGT Binding Reaction. αBGT was prepared and iodinated by methods previously described (6,10). One ml aliquots of solubilized extract, corresponding to 30-80 mg of whole brain tissue and containing 0.4 - 1.2 mg of protein, were incubated with [^{125}I]αBGT for 60 min. at room temperature. The concentration of [^{125}I]αBGT ranged from 23 to 31 nM.

Sucrose Gradient Sedimentation. Linear sucrose gradients (5-20%) containing 10mM HEPES pH 7.4 and 0.1% Triton were prepared with a total volume of 4.8 ml. Aliquots of 200 µl from toxin binding reaction mixtures were applied to the gradients along with two marker enzymes, alkaline phosphatase (EC 3.1.3.1) and catalase (EC 1.11.1.6). Following centrifugation for 10 hr. at 120,000 xg, gradients were fractionated and fractions counted and assayed for markers.

RESULTS AND DISCUSSION

The sedimentation profile of [^{125}I]αBGT binding activity in the solubilized extract of Limulus brain tissue is shown in Fig. 1A. Three peaks of toxin binding activity were detected with sedimentation coefficients of 9.0S, 15.4S and 17.4S. These three peaks of binding activity were absent in the sedimentation profiles of a control reaction mixture which was pretreated with excess unlabelled toxin (Fig. 1B) and [^{125}I]αBGT alone (Fig. 1C). The protein nature of the toxin binding activity was confirmed by exposure of the multiple binding components to the proteolytic enzyme α-chymotrypsin. The action of chymotrypsin was terminated with 1mM phenylmethylsulfonylfloride prior to the addition of labelled toxin. This treatment abolished all three peaks of toxin binding activity. A small amount (less than 10%) of binding activity was detectable at a position corresponding to a slightly lower molecular weight than the 9S peak.

The addition of a 10% concentration of butyl alcohol to the solubilized brain extract resulted in a large reduction in the 9S peak (Fig. 2B). This



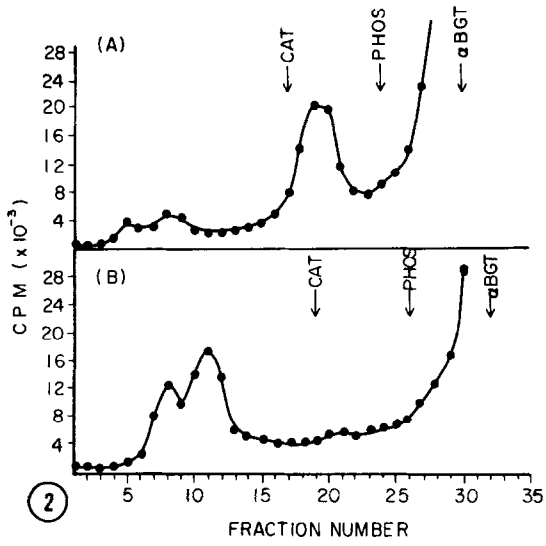


Fig. 1. Sedimentation profile of toxin binding activity in Limulus brain tissue. (A) Solubilized extract incubated with 26nM [^{125}I]αBGT for 60 min. (B) Solubilized extract was preincubated with 5.8 μM unlabeled toxin for 10 min. prior to incubation with 26nM labelled toxin. (C) A 26nM concentration of [^{125}I]αBGT incubated in buffer only. A sample of each reaction mixture was applied to a sucrose gradient and centrifuged as described in Methods. The position of marker enzymes is indicated by the arrows. αBGT denotes the position of unbound toxin.

Fig. 2. Effect of butanol on toxin binding components. Brain homogenate was solubilized in the presence of (A) no additives and (B) 10% butanol. A sample of each extract was incubated with 31nM [^{125}I]-αBGT and sedimented.

reduction in the 9S component was accompanied by a quantitatively equal increase in both the 15.4S and 17.4S components. This transposition of binding activity suggests that the formation of the 15.4S and 17.4S components is dependent upon the 9S component. This possibility was investigated further through the isolation of the 9S component, accomplished by pooling the gradient fractions containing the 9S peak. Resedimentation of aliquots of this component in fresh gradients showed it to be quite unstable resulting in both a large decrease in the amount of toxin

associated with this peak and the presence of a large peak of apparently unbound toxin. Although the isolated 9S peak was significantly diminished, it was still detectable. The treatment of aliquots of the isolated 9S component with 10% butanol prior to resedimentation produced results quite similar to the sedimentation profile of the untreated isolated 9S component (i.e., the alcohol did not produce a detectable shift of radioactivity from the 9S position to the 15,4S and 17.4S regions). This may have been attributable to the high degree of instability of the 9S protein making it difficult to detect this transition. However, the addition of the isolated 9S component back to fresh brain extract resulted in a slight increase in the stability of this component (Fig. 3B). Additionally, the inclusion of butanol in this mixture resulted in the formation of a 15.4S and 17.4S peak (Fig. 3C).

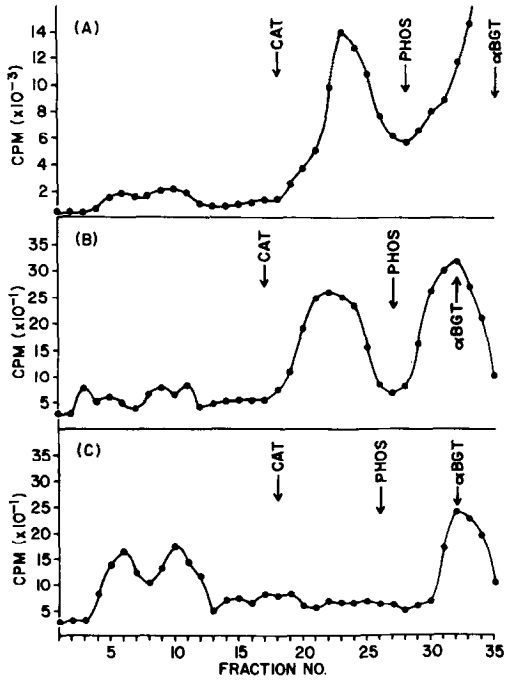


Fig. 3. The effect of butanol on the addition of the isolated 9S component to brain extract. (A) Sedimentation profile of brain extract and 23nM [^{125}I]αBGT. Fractions 23-25 were combined. (B) An aliquot of the combined fractions diluted fourfold with brain extract before resedimentation. (C) A 10% concentration of butanol was added to an aliquot of the combined fractions diluted fourfold with brain extract before resedimentation.

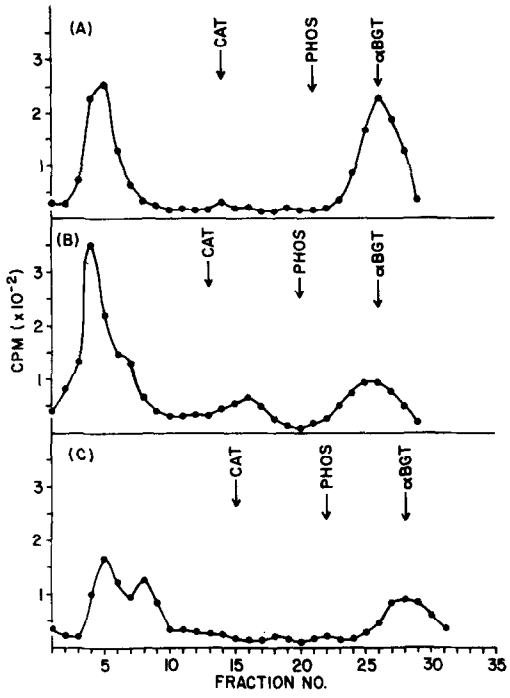


Fig. 4. The effect of butanol on the addition of the isolated 17.4S component to brain extract. (A) An aliquot of the 17.4S component diluted fourfold with buffer. (B) 17.4S component diluted fourfold with brain extract. (C) 10% butanol was added to an aliquot of the 17.4S component diluted fourfold with brain extract.

Isolation of the 17.4S component showed it to be more stable than the 9S component (Fig. 4A). The addition of this isolated component to fresh brain extract resulted in the production of a small peak in the 9S position (Fig. 4B). There was also a shoulder formed on the 17.4S peak which suggested the presence of a small amount of the 15.4S component. The addition of butanol to a mixture of the isolated 17.4S component and brain extract caused a reduction in the 17.4S peak (Fig. 4C). However, the small peak in the 9S region was absent. Instead, a prominent peak was present at the 15.4S position. Treatment of the isolated 15.4S component in a manner identical to the isolated 17.4S component produced very similar results.

The shift of radioactivity from the 9S peak to the 15.4S and 17.4S peaks, as well as the reverse, can be explained by at least three different models. (1) The 15.4S and 17.4S components may be the result of aggregation of the 9S

component with itself or other yet to be identified components. (2) All three peaks may represent a single protein with multiple conformational states. (3) A rapid exchange of bound toxin between the three proteins may occur, that is a dissociation of toxin from one component and reassociation with other components. In accordance with these three models, the action of butanol would be to promote aggregation or a conformational shift to the 15.4S and 17.4S entities or the exchange of bound toxin. It is not possible to convincingly confirm or eliminate either of these models based on the present data and further investigation is required. However, models (1) and (2) are supportive of an interrelationship of the three binding components and are therefore consistant with the specificity of toxin binding. This suggested interrelationship is supported by the observation that the exchange of bound toxin seems unlikely since the concentration of toxin that would be produced by dissociation is significantly less than the affinity range for binding to these proteins. It is therefore proposed that butanol may promote an aggregation or conformational change of the 9S component to form the 15.4S and 17.4S peaks, possibly by shifting the polarity of the medium.

The clear distinction of models (1) and (2) from (3) and the confirmation of an interrelationship between these proteins requires the demonstrative conversion of one isolated component to the other two components without the addition of brain extract. Attempts to achieve this are currently in progress.

ACKNOWLEDGEMENTS

We are grateful to Mrs. Rose-Marie Jean-Jacques for assistance in the preparation of the manuscript. This work was supported by NIH Grant No. 1 R01 HL24140 and MBS Grant No. RR08037.

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