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Insularity of cholinergic components in the central nervous system of *Limulus polyphemus*

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The major components of cholinergic transmission have been detected in the central nervous system (CNS) of the horseshoe crab, *Limulus polyphemus*. The synthetic enzyme for acetylcholine (ACh), acetyl CoA: choline-*O*-acetyltransferase (CAT: EC 2.3.1.6), has been characterized and purified from the subesophageal ganglia of *Limulus* [1]. Levels of CAT activity have also been determined in individual cells of the abdominal ganglion [2]. Acetylcholinesterase (AChE: EC 3.1.1.7) has been detected throughout the CNS of this animal [3]. The kinetic and pharmacological properties of AChE from the ventral nerve cord have been reported [4]. High levels of ACh have been reported in extracts of the subesophageal ganglia [5]. The uptake of choline (Ch) and subsequent conversion to ACh by *Limulus* nerve tissue were reported in 1976 by Townsel *et al.* [6]. More recently, a sodium-dependent high affinity uptake system for Ch has been characterized in the abdominal ganglia [7]. This uptake system exhibits those properties which have been hypothesized to subserve the selective uptake by cholinergic nerve terminals. Additionally, the Ch taken up by the abdominal ganglia has been shown to be rapidly converted to ACh. This ACh is released by the tissue in a depolarization-dependent manner requiring sodium and calcium ions [8]. Thus, *Limulus* nerve tissue has been shown to take up Ch via a high affinity uptake system suggestive of cholinergic terminals, to rapidly convert significant amounts of this Ch to ACh, and to release the ACh in a depolarization-dependent manner similar to *in vivo* neurotransmitter release.

The presence of the aforementioned components in the CNS of *Limulus* support the proposed role of ACh as a neurotransmitter in this system. Additional evidence to support this hypothesis has been provided by the characterization of α-bungarotoxin (αBGT) binding to *Limulus* nerve tissue [9, 10]. αBGT has been shown to bind to the acetylcholine receptor (AChR) in a specific manner in a variety of preparations (for a recent review, see Ref. 11). The pharmacological properties of αBGT binding in the subesophageal ganglia of *Limulus* are consonant with specific binding to an AChR. However, the kinetic parameters of toxin binding in particulate preparations are indicative of binding to multiple sites. This result appears to be explained by the identification of three separately sedimenting toxin binding proteins in solubilized extracts of the same tissue. The kinetics of toxin binding to the solubilized proteins appear to parallel those of binding in the particulate preparation.

The case for the specificity of αBGT binding in *Limulus* subesophageal ganglia is supported by recent findings which indicate that two of the three solubilized toxin binding proteins may be aggregates of the other one [12]. These findings, as well as the pharmacological properties, argue in favor of the specificity of αBGT binding in this preparation. However, in lieu of a physiological characterization of the specificity of a αBGT in this preparation, it appears useful to distinguish the toxin binding protein(s) from those components which can be viewed as most likely to serve as a source of non-specific binding, i.e. those components which are known to possess a recognition site for Ch or ACh. This would include CAT, AChE and the presumed Ch uptake protein. Therefore, the intent of this study was to show the separateness of the αBGT binding protein(s) from CAT, AChE and Ch uptake in the CNS of *Limulus*. Thus, the effect of αBGT on Ch uptake in abdominal ganglia was determined, and the relationship of solubilized toxin binding proteins from the subesophageal ganglia to AChE and CAT was investigated.

Choline uptake. Limulus abdominal ganglia were dissected out and split along the midline as described previously [7]. After blotting and weighing, each half ganglion was incubated in 210 μl of Chao's solution [13], buffered to pH 7.4 with 10 mM *n*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonate (HEPES), containing [^{3}H]Ch. The concentrations of [^{3}H]Ch used ranged from 0.2 to 2 μM. Half ganglia were incubated in the [^{3}H]Ch for 60 min. Following incubation, half ganglia were washed four times in 500 μl of Chao's solution. Each wash period lasted for 5 min. Ganglion halves were then solubilized and the radioactive content was determined as described previously [7, 8]. In all experiments, controls were performed by incubating one half of each ganglion at room temperature and the other half at 0–4°. Active uptake is expressed as uptake at room temperature minus the uptake at 0–4°. Uptake at 0–4° was always less than 10 per cent of that at room temperature.

[^{125}*I*]*αBGT binding*. The binding of iodinated toxin to half ganglia was determined by incubation in 210 μl of Chao's solution containing 55 nM [^{125}I]αBGT. Ganglia halves were incubated in this manner for 1 min to 24 hr. Following the incubation period, ganglia were washed four times in 500 μl of Chao's solution. Each ganglion was then transferred to a small test tube and counted in a Packard auto-gamma scintillation counter. The incubation of hemiganglia in Chao's solution containing 55 nM unlabeled αBGT was performed in the same manner for 16–24 hr. However, at the end of the incubation period, ganglia were transferred to [^{3}H]Ch uptake medium. The uptake of [^{3}H]Ch by these ganglia was determined as described above. The preparation of solubilized extracts of *Limulus* subesophageal ganglia tissue and the determination of [^{125}I]αBGT binding in these extracts were performed according to methods reported previously [9, 12].

Sucrose gradient sedimentation. Linear sucrose gradients (5–20%) were prepared according to Martin and Ames [14]. Sucrose solutions were prepared in 10 mM HEPES containing 0.1% Triton X–100 (v/v). Samples of 160 μl of the enzyme preparations and toxin binding reactions were applied to the top of the gradients along with 20 μl each of catalase and alkaline phosphatase. Gradients were centrifuged for 10 hr at 120,000 *g*. The gradients were then fractionated and the fractions were assayed for enzyme activity or counted in a gamma-counter. Marker enzymes, catalase and alkaline phosphatase, were assayed by the methods of Beers and Sizer [15] and Schilesinger and Barrett [16], respectively. CAT was assayed according to Fonnum [17], and AChE activity according to the method of Reed *et al.* [18].

In investigating the effect of αBGT on Ch uptake, abdominal ganglia were pre-equilibrated in 55 nM concentrations of toxin. This toxin concentration is well above

Table 1. Effect of αBGT on [^{3}H]Ch uptake*

[^{3}H]Ch concn (μM)	Ch uptake (pmoles Ch/mg tissue/60 min) No preincubation	16–24 hr Preincubation without αBGT	16–24 hr Preincubation with 55nM αBGT
2	5.12 ± 1.45	3.60 ± 0.47	4.24 ± 1.37†
1	2.79 ± 1.11	2.32 ± 0.81	1.91 ± 1.07†
0.5	1.26 ± 0.48	1.11 ± 0.62	1.02 ± 0.17†
0.2	0.38 ± 0.17	0.30 ± 0.08	0.18 ± 0.10†

* Values are the mean ± S.D. for three to six experiments. Ganglia halves were either used to determine uptake without preincubation or were preincubated for 16–24 hr in the presence or absence of 55 nM αBGT. [^{3}H]Ch uptake by all ganglia during a 60-min time period was determined as described. In all cases, the corresponding half of each ganglion was subjected to the same conditions and used as the 0–4° uptake control.

† Values were not significantly different from uptake after 16–24 hr of preincubation without αBGT at the corresponding Ch concentrations (*u*-test, 1 per cent significance level [19]).

that which has been shown to saturate proposed specific binding in *Limulus* subesophageal ganglia [9]. The kinetic and pharmacological properties of toxin binding in the abdominal ganglia of *Limulus* are similar to those reported for the subesophageal ganglia.* [^{125}I]αBGT binding to the abdominal ganglia halves in this study reached equilibrium after approximately 16 hr. This lengthy incubation period, required to achieve equilibrium, is the result of a significant decrease in the rate of toxin binding due to the high concentrations of salts in Chao's solution [10]. Additionally, the thorough penetration of toxin into the ganglia may also be a factor in the equilibration time.

The effect of αBGT on Ch uptake was determined by pre-equilibrating abdominal ganglia in unlabeled toxin for 16–24 hr prior to uptake analysis. However, it was determined that the level of Ch uptake in ganglia held at room temperature for periods up to 24 hr was slightly less than that observed in ganglia which were not preincubated (Table 1). The preincubation of ganglia for 16–24 hr appeared to have a deleterious effect on uptake whether αBGT was present or not. The uptake of choline by ganglia which were not preincubated was similar to that reported previously [8]. The level of Ch uptake by ganglia pre-equilibrated with αBGT was not significantly different from that of ganglia which were preincubated for the same time period without αBGT. Thus, although Ch uptake was always decreased after the long preincubation period, αBGT had no detectable effect on uptake at any of the Ch concentrations investigated. Therefore, on the basis of these results, αBGT does not appear to interact with the Ch recognition site involved in the uptake process.

The sedimentation profile of [^{125}I]αBGT binding activity in the solubilized extracts of *Limulus* subesophageal ganglia is shown in Fig. 1A. Three peaks of toxin binding activity are present with approximate sedimentation coefficients of 9.0S, 15.4S and 17.4S. The sedimentation profiles of AChE and CAT from the same tissue showed a single peak of activity for each enzyme (Figs. 1B and C). AChE activity sedimented with an approximate coefficient of 6.0S. The total of the enzyme activity in the 6.0S peak represented more than 80 per cent of the original activity applied to the gradient. The peak of CAT activity corresponded to an approximate sedimentation coefficient of 5.0S. In this case, more than 85 per cent of the original enzyme activity applied to the gradient was recovered in the peak fractions. The identity of the enzyme activity in the 6.0S peak was verified as AChE by the use of selective inhibitors. The fractions containing the 6.0S peak were pooled and assayed in the presence of these inhibitors. The cholinesterase inhibitor physostigmine inhibited the 6.0S activity by approximately 90 per cent at a concentration of 5 μM. The enzyme activity was inhibited greater than 95 per cent by a 10 μM concentration of the acetylcholinesterase inhibitor BW284c51. Concentrations as high as 5 mM of the pseudo-

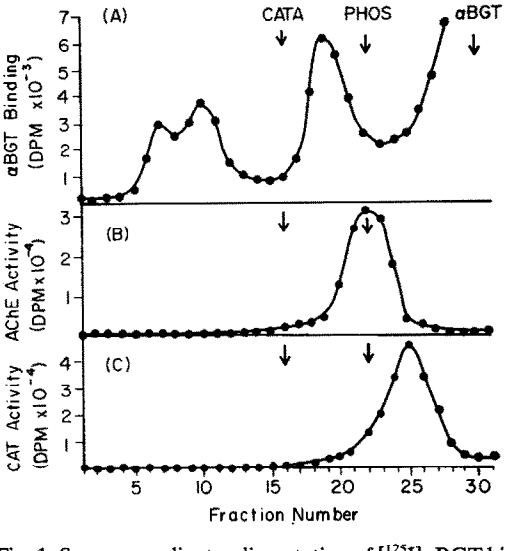

Fig. 1. Sucrose gradient sedimentation of [^{125}I]αBGT binding activity, AChE and CAT of *Limulus* subesophageal ganglia. (A) Solubilized extract incubated with 30 nM labelled toxin for 60 min prior to application to the gradient. (B) A 160-μl aliquot of a solubilized preparation of AChE applied to the gradient. (C) Sedimentation of a supernatant fraction of CAT. Gradients were centrifuged and fractionated as described in the text. The fractions were then counted to determine radioactive content or assayed for the respective enzymes. The positions of the marker enzymes catalase (CATA) and alkaline phosphatase (PHOS) are indicated by the arrows (αBGT in unbound toxin). Fraction number one corresponds to the bottom of the gradient.

* W. E. Thomas, unpublished observations.

cholinesterase inhibitor tetraisopropylpyrophosphoramide had no effect on the activity of the 6.0S enzyme. A similar confirmation of the 5.0S peak as CAT was not possible. The CAT of *Limulus* has been shown to be relatively insensitive to the only readily available specific inhibitors of CAT, the styrylpyridines [1, 20]. Even though a pharmacological confirmation of CAT was not possible, the 5.0S peak represents the majority of the ACh-synthesizing activity present in this tissue. Therefore, the CAT activity is essentially confined within the 5.0S peak, although its homogeneity is uncertain.

The sedimentation profiles of AChE and CAT clearly show the separation of these two enzymes from the solubilized toxin binding proteins. Additionally, αBGT exhibited no detectable effect on Ch uptake. Therefore, the results of this study suggest that αBGT does not interact with AChE, CAT or the site of Ch uptake in the CNS of *Limulus*. These findings are consonant with the specific binding of αBGT to an AChR in *Limulus*.

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Department of Biochemistry and Nutrition,
Meharry Medical College,
Nashville, TN 37208, *U.S.A.*
WILLIAM E. THOMAS

Department of Physiology and Biophysics,
University of Illinois Medical Center,
Chicago, IL 60680, *U.S.A.*
JAMES G. TOWNSEL*

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Microsomal peroxidase activities—effect of cumene hydroperoxide on the pyridine nucleotide reduced cytochrome b_5 steady state

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The oxidation of cytochrome b_5 during lipid peroxidation was noted by Bidlack *et al.* [1], who suggested the presence of a hydroperoxide peroxidase in hepatic microsomes. Hrycay and O'Brien [2, 3] and Bidlack and Hochstein [4] characterized the microsomal peroxidase activities, using either NADPH or NADH as reducing equivalents and cumene hydroperoxide as the lipophilic substrate. Most authors now agree that the hepatic mixed function oxygenase serves, at least in part, as the microsomal peroxidase, reducing the lipophilic hydroperoxides to alcohols over cytochrome P-450 [5–9]. Cytochrome b_5, on the other hand, has been implicated only in the transfer of electrons to the NADH mediated peroxidase activity [5–7]. However, immediate oxidation of reduced (NADPH or NADH) cytochrome b_5 occurs upon addition of cumene hydroperoxide [8, 9]. Similarly, using perfused livers and isolated hepatocytes, Sies and Grosskopf [10] reported that the cellular cytochrome b_5 was oxidized upon addition of cumene hydroperoxide.

The central role of cytochrome b_5 in NADH-dependent electron transfer has been examined in cytochrome *c* reductase [11], in stearyl CoA desaturase [12–14], in drug metabolism [15, 16], and in the peroxidase [5–7] activities. However, a more detailed knowledge of the redox state of cytochrome b_5 in the intact microsomal system may clarify the electron transfer through these various pathways. An initial examination of the effect of cumene hydroperoxide on the NADPH and NADH redox state of cytochrome b_5 in microsomes is presented.

Methods

Animal pretreatment. Male Sprague–Dawley rats (*ca.* 250 g) were maintained on Purina Rat Chow. Animals receiving phenobarbital pretreatment were given pheno-