ACETYLCHOLINESTERASE AND α-BUNGAROTOXIN: A STUDY OF THEIR POSSIBLE INTERACTION

B. ROBAIRE and G. KATO

Department of Pharmacology and Therapeutics, and Department of Research in Anaesthesia, McGill University, 3655 Drummond St., Montreal H3G 1Y6, Canada

Received 26 June 1974

1. Introduction

α-Bungarotoxin (α-BGT), a protein with a molecular weight of 8000, binds with high specificity to the acetylcholine nicotinic receptors of striated muscle [1–3], electroplax [4] and brain [5]. Several neurotoxins such as α-BGT have been used as tools to localize [6], purify [7] and assay [8] for these receptors. Changeux [4] stated that α-BGT has no effect on the kinetic parameters of acetylcholinesterase (Acetylcholine acetyl-hydrolase EC 3.1.1.7) obtained from the electric eel *Electrophorus electricus*, but no experimental results supporting this contention have thus far been presented.

Recently, Štalc and Župančič [9] have reported that α-BGT has two effects on membrane-bound acetylcholinesterase obtained from rat diaphragm: 1) at low substrate concentration (acetylcholine < 20 μM) the enzyme is inhibited by the toxin and the rate versus substrate concentration curve deviates from Michaelis–Menten kinetics, and 2) at higher substrate concentrations (acetylcholine > 20 μM). the enzyme activity in the presence of α-BGT is increased over control values without toxin. Furthermore, these investigators found that α-BGT did not alter the kinetics of commercial soluble acetylcholinesterase (electric eel; Worthington Biochem. Corp.).

Using endplate membrane acetylcholinesterase of rat diaphragm, Chang and Su [10] have recently shown that α-BGT (1 or 10 μg/ml) did not alter the rate of hydrolysis of 1 μM acetylcholine. These authors, however, did not report any of the kinetic parameters of acetylcholinesterase activity in the absence or presence of α-BGT.

As significant differences exist between the kinetic parameters of membrane-bound and solubilized acetylcholinesterase [11–13] we attempted to determine whether α-BGT had any effects on V_{max}, K_m, inhibition by excess substrate (%S I), or if it caused deviation from Michaelis–Menten kinetics. The possibility that α-BGT binds to a site distinct from the active center of the enzyme without affecting any of these kinetic parameters was also investigated.

We report in this letter that for both membrane-bound and solubilized acetylcholinesterase, none of the kinetic parameters studied are affected by α-BGT. Furthermore, the toxin does not bind to solubilized acetylcholinesterase while it does bind to electroplax membranes.

2. Methods

2.1. *Isolation of membrane-bound and solubilized acetylcholinesterase*

Fresh electric tissue from *Electrophorus electricus* was homogenized in a modified eel Ringer solution containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$, pH 7.2; the homogenate was filtered through a stainless steel sieve and centrifuged at 20 000 *g* for 30 min. The pellet was washed 3 times in the modified eel Ringer solution, resuspended in a volume of this solution equal to that of the original weight of the tissue and stored for 48 hr at 4°C. The suspension was then centrifuged at

North-Holland Publishing Company – Amsterdam

20 000 *g* for 30 min; the pellet was resuspended in modified eel Ringer and is referred to as membrane-bound acetylcholinesterase; the supernatant was centrifuged at 100 000 *g* for 2 hr and the resulting supernatant is referred to as solubilized acetylcholinesterase. All steps were done at 4°C.

2.2. *Assay of acetylcholinesterase activity by the pH-stat method*

Acetylcholinesterase activity was assayed with a pH-stat (Radiometer Corp.) using acetylcholine iodide (Sigma Chemicals) as substrate, under a nitrogen atmosphere at 30°C, pH 7.20, in 1.0 ml of a solution containing 2.0 mM phosphate in addition to the salts listed above in section 2.1.

2.3. *Purification and labeling of α-BGT*

α-BGT was isolated from the dried venom of *Bungarus multicinctus* and was purified by gel filtration on Sephadex G-50 followed by chromatography on carboxymethyl (CM) Sephadex (C-25). A solution containing 1 μg/ml of the toxin completely blocked the response of the frog's rectus abdominis to acetylcholine iodide (1 μg/ml) after 1 hr incubation with the toxin.

α-BGT was labelled with [^{125}I] according to the method of Greenwood et al. [14] as modified by Berg et al. [15]. The toxin had a specific activity of 12.8 × 10^6 CPM/μg protein; approximately 3% of all α-BGT molecules were labelled with [^{125}I]. Radioactivity was measured with a gamma counter (Model 1185, Nuclear Chicago).

2.4. *Sucrose sedimentation gradients*

The preparation of these gradients has been described previously [16]. Solubilized acetylcholinesterase was layered on linear sucrose gradients (8–17% sucrose, 5 mls) and then centrifuged at 290 000 *g* (49 000 rpm) for 7 hr. Membrane fragments were centrifuged for 30 min at 290 000 *g* (49 000 rpm) on linear gradients (5 ml) containing 12–42% sucrose. All centrifugations were done using an SW 50.1 Rotor in an L2-65B Beckman Ultracentrifuge.

Catalase and β-galactosidase were used as markers and were assayed as described previously [16]. Protein concentrations were determined by the method of Lowry et al. [17] and acetylcholinesterase activity was measured according to the method of Ellman et al [18].

3. Results

3.1. *Kinetics of acetylcholinesterase in the presence of α-BGT*

The kinetic data was fitted by a computer to a rectangular hyperbola, using Cleland's program [19] to obtain the K_m and V_{max} of acetylcholine as well as standard errors for these values; the standard errors are an indication of the goodness of fit of the data to a rectangular hyperbola.

The apparent lack of effect of α-BGT on the kinetic parameters of either membrane-bound or solubilized acetylcholinesterase is shown in fig. 1,A and B. At a concentration of the toxin 5 times that necessary to completely block the cholinergic nicotinic receptors, 5 μg/ml, there is neither an apparent increase in V_{max} nor is there sigmoidicity at low substrate concentrations.

The results obtained from the computer analysis of the data shown in fig. 1 are presented in table 1. Upon examination of these results, four observations are clear: 1) the projected V_{max} obtained by computer extrapolation to a rectangular hyperbola is not altered in the presence of α-BGT; 2) the K_m of acetylcholine for either membrane-bound or solubilized acetylcholinesterase is not changed with increasing amounts of the toxin; 3) all standard errors are smaller than 15% indicating a good fit of the data to a rectangular hyperbola, thus Michaelis–Menten kinetics and not sigmoidal kinetics are compatible with the hydrolysis of acetylcholine by membrane-bound or solubilized enzyme whether in the presence or absence of α-BGT, and 4) the degree of inhibition by excess substrate is also unchanged with increasing concentrations of α-BGT. A 1 hr preincubation of α-BGT with either membrane-bound or solubilized enzyme gave similar results to those described above.

3.2. *Binding study of [^{125}I] α-BGT to solubilized acetylcholinesterase and to membrane fragments*

Solubilized acetylcholinesterase was incubated with 1 μg/ml [^{125}I] α-BGT for 1 hr at 4°C and was then centrifuged on continuous sucrose gradients as

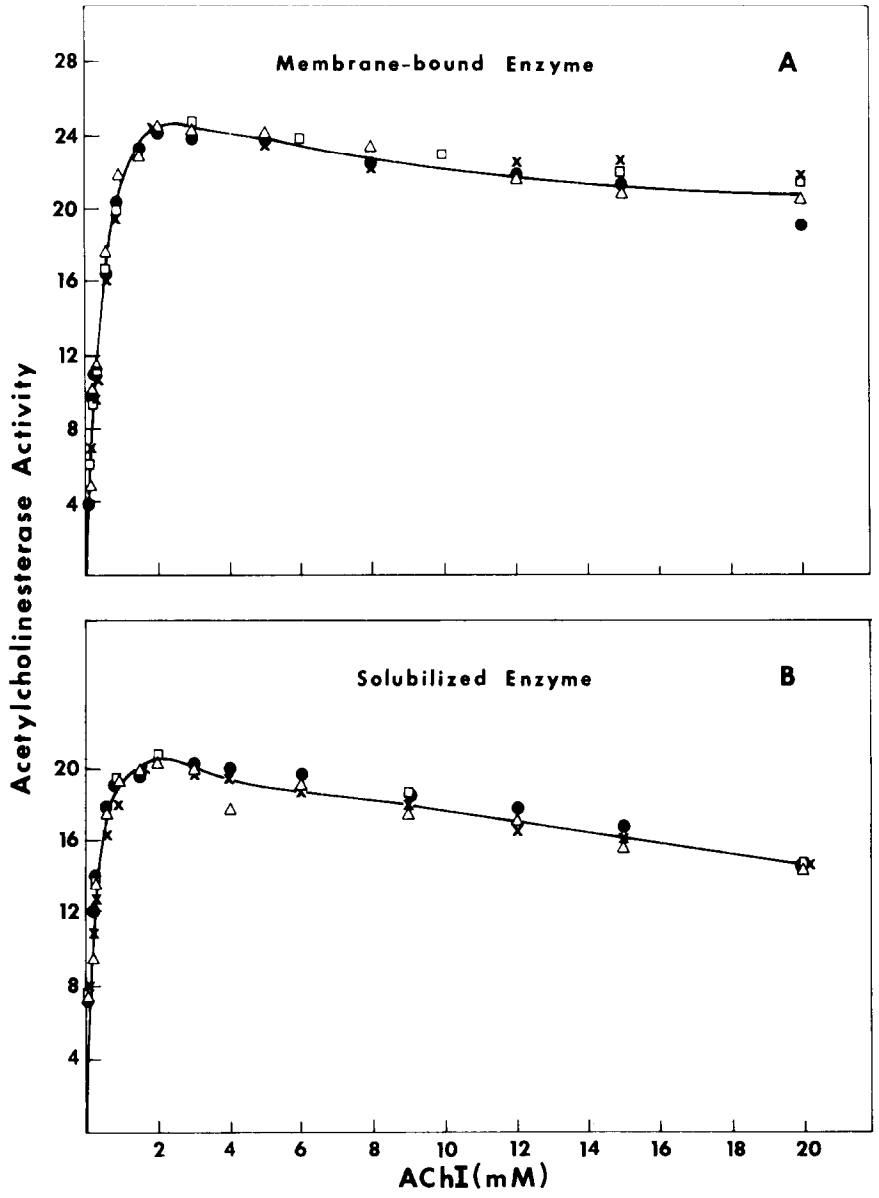

Fig. 1. Plot of initial reaction velocity of acetylcholine hydrolysis by acetylcholinesterase in the presence of α-BGT. Acetylcholinesterase activity was assayed by a pH state at 30°C, pH 7.20. The assay medium contained 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$, 2 mM phosphate. Rates of hydrolysis are expressed in terms of μmoles of acid formed/min/g wet weight tissue. The abscissa represents the concentration of substrate used in each assay. The rate of hydrolysis of acetylcholine is shown by A. membrane-bound acetylcholinesterase, and B. solubilized acetylcholinesterase. The concentration of α-BGT are: •——•, NO α-BGT; X——X, 0.2 μg/ml α-BGT; △—△, 1.0 μg/ml α-BGT; □—□, 5.0 μg/ml α-BGT.

Table 1
The effects of α-Bungarotoxin on V_{max}, K_m and %S1 of membrane-bound and soluble AChE from *Electrophorus electricus*

α-Bungarotoxin	V_{max}*		K_m (μM)		%S1**	
(μg/ml)	Membrane-bound	Solubilized	Membrane-bound	Solubilized	Membrane-bound	Solubilized
0	100.0±4.2	100.0±1.8	435±58	172±16	19	29
0.2	95.1±3.5	98.2±1.8	389±54	195±17	12	31
1.0	100.0±3.8	100.9±3.2	388±48	205±29	16	32
5.0	96.5±3.1	103.1±2.7	388±48	191±18	13	29

* The V_{max} is expressed as a percentage of the V_{max} in the absence of α-BGT and the standard errors (S.E.M.) as well as the K_m values and their S.E.M. are obtained from the computer analysis of Cleland (see text).

** % SI refers to the percent inhibition by excess substrate which is defined as $\frac{R_2 - R_{20}}{R_2}$ where R is the rate of hydrolysis of ACh and the subscripts 2 and 20 refer to the concentration of acetylcholine in mM.

described in section 2.4. The sedimentation profile is shown in fig. 2. It is clear from this figure that all the radioactivity remains near the top of the gradient while the acetylcholinesterase activity is distributed in two peaks with S values of 11.8 and 7.7. Some of the properties of these two forms of acetylcholinesterase have been described elsewhere [16,20].

When electroplax membrane fragments are incu-

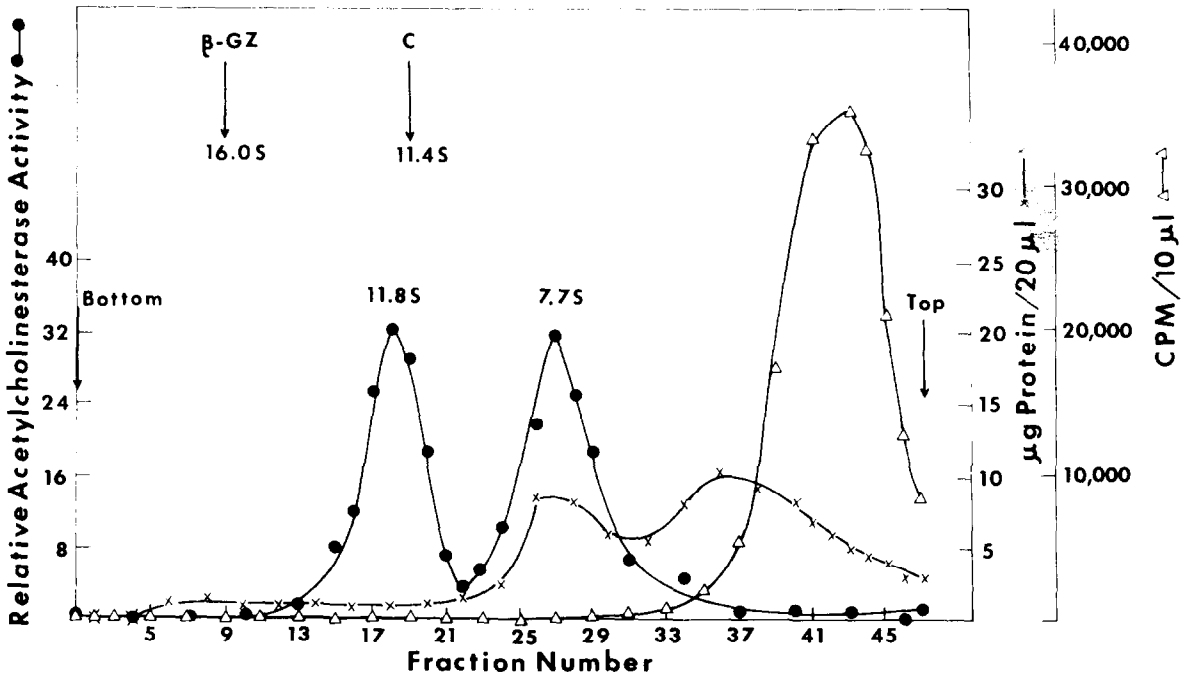

Fig. 2. Sucrose gradient centrifugation of acetylcholinesterase solubilized from electroplax membranes which has been incubated with [^{125}I]α-BGT (1 μg/ml) for 1 hr at 4°C. The gradient was prepared and centrifuged as described in the text. Acetylcholinesterase activity is represented by ●——●, protein concentration by ×——×, and CPM of [^{125}I]α-BGT by △——△. β-Galactosidase (β-GZ) and catalase (C) were used as markers.

Table 2
Binding of [^{125}I]α-BGT to electroplax membrane fragments*

	μg protein	CPM [^{125}I]/μg protein	AChE activity/μg protein
Top of gradient**	123***	1619	16.1
	176	1566	19.6
Pellet	690	3200	12.7
	450	1230	11.2

* Electroplax membrane fragments, free of soluble AChE, were incubated with 1 μg/ml [^{125}I]α-BGT for 1 hr at 4°C and centrifuged as described in section 2.3.
** The top of the gradient refers to the uppermost 15 per cent of the sucrose gradients
*** The two values shown represent results obtained from separate gradients.

bated with [^{125}I] α-BGT in a manner analogous to that described for solubilized acetylcholinesterase and these membranes are than centrifuged on a continuous sucrose gradient as described in section 2.4, all the acetylcholinesterase activity and the radioactivity was found either in the pellet or near the top of the gradient. These results are summarized in table 2. It is clear from the results that [^{125}I] α-BGT binds to membrane fragments since a large fraction of the total radioactivity was found in the pellet. Since the membrane preparation is essentially free of soluble enzyme [11], the radioactivity found near the top of the gradient is attributable to either one or both of the following: 1) free toxin; 2) toxin bound to membrane fragments containing sufficiently high lipid content to prevent their sedimentation in the sucrose gradients.

4. Discussion

The allosteric mechanism proposed by Štalc and Župančič [9] to explain the effects of α-BGT on membrane-bound acetylcholinesterase from rat diaphragm is thus not applicable to the electroplax enzyme since, in our experiments, α-BGT had no effect on the kinetic parameters of either membrane-bound or solubilized electroplax acetylcholinesterase. Furthermore, our experiments do not support the possibility that α-BGT binds to either the 11.8 S or 7.7 S forms of solubilized acetylcholinesterase without affecting the kinetic parameters of this enzyme. The binding of [^{125}I] α-BGT to membrane fragments is indicative of the presence of nicotinic cholinergic receptors as has been described by numerous other investigators [4–6].

Acknowledgements

We wish to thank P. Lavoie for a gift of [^{125}I]α-BGT. This work was supported by the Medical Research Council of Canada.

References

[1] Chang, C. C. and Lee, C. Y. (1963) Arch. Int. Pharmacodyn. Thér. 144, 241–257.
[2] Hartzell, H. C. and Fambrough, D. M. (1973) Develop. Biol. 30, 153–165.
[3] Miledi, R., Molinoff, P. and Potter, L. T. (1971) Nature, 229, 554–557.
[4] Changeux, J. P., Kasai, M. and Lee, C. Y. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1241–1247.
[5] Bosmann, H. B. (1972) J. Biol. Chem., 247, 130–145.
[6] Bourgeois, J. P., Ryter, A., Menez, A., Fromageot, P. and Changeux, J. P. (1972) FEBS Letters, 25, 127–133.
[7] Meunier, J. C., Olsen, R. W., Menez, A., Fromageot, P., Boquet, P. and Changeux, J. P. (1972) Biochemistry, 11, 1200–1210.
[8] Klett, R. P., Fulpius, B. W., Cooper, D., Smith, M., Reich, E. and Possani, L. D. (1973) J. Biol. Chem., 248, 6841–6853.
[9] Štalc, A., Župančič, A. O., (1973) Nature, 239, 91–92.
[10] Chang, C. C. and Su, M. J. (1974) Nature, 247, 480.
[11] Robaire, B., and Kato, G. (1973) FEBS Letters, 38, 83–86.

[12] Robaire, B. and Kato, B. (1974) Biochem. Pharmacol., in the press.
[13] Silman, H. I. and Karlin, A. (1967) Proc. Natl. Acad. Sci. U.S., 58, 1664–1668.
[14] Greenwood, F.C., Hunter, W. M. and Glover, J. S. (1963) Biochem. J. 89, 114–123.
[15] Berg, D. K., Kelly, R. B., Sargent, P. B., Williamson, P. and Hall, Z W. (1972) Proc. Natl. Acad. Sci. U.S. 69, 147–151.
[16] Robaire, B. and Kato, G. Submitted to FEBS Letters.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) Biochem. Pharmac. 7, 88–95.
[19] Cleland, W. W. (1967) Adv. Enzymol. 29, 1–32.
[20] Massoulié, J., Rieger, F., and Tsuji, S. (1970) Eur. J. Biochem. 14, 430–439.