DEMONSTRATION OF AN α-BUNGAROTOXIN-BINDING NICOTINIC RECEPTOR IN FLIES

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1. Introduction

Acetylcholine is believed to play a major role in nervous transmission in the central nervous system of insects [1]. It has been reported that flies do not contain a nicotinic receptor similar to that of vertebrates, but that their cholinergic receptor is of a mixed nicotinic and muscarinic nature [2–6] and does not bind the powerful nicotinic antagonist α-bungarotoxin [5,7]. I report here that flies do contain a nicotinic receptor that binds α-bungarotoxin specifically. The demonstration that the fruit fly, *Drosophila melanogaster*, contains such a receptor, could permit one to study the genetic control of the receptor and the effects of mutations on its expression and activity.

2. Materials and methods

Most of the experiments were performed on heads of *Drosophila melanogaster*, C-S strain. Flies were cultured under standard conditions [8]. Heads were separated from bodies by freezing and shaking, and homogenized (400 heads/ml) in 0.32 M sucrose, in a glass–glass dual homogenizer followed by a glass–Teflon homogenizer. Routinely the supernatant of 500 × *g* centrifugation for 10 min was used. House flies, *Musca domestica*, (Biological Institute, Ness-Ziona, Israel) were treated as above, except that 40 heads/ml were taken for homogenization.

α-Bungarotoxin (α-Btx) was purified from crude *Bungarus multicintus* venom (Miami Serpentarium Miami, USA) [9] and was iodinated according to Vogel et al. [10]. Binding of ^{125}I-labelled α-Btx was assayed by two methods:

(i) Aliquots of fly homogenate were incubated in 0.12 M NaCl, 2 mg/ml BSA, 0.05 M Tris–Cl, pH 7.4 (= Buffer 1), in total vol. 0.2 ml. Reaction was carried out at 25°C and terminated by diluting with 2 ml buffer 1 followed immediately by vacuum filtration through a Millipore EGWP filter as described by Vogel and Nirenberg [11]. The filter was then washed 3-times with 2 ml portions of Buffer 1 and counted. This method determines the amount of labelled toxin bound to membrane fragments.

(ii) Aliquots were incubated as above in Buffer 1 containing 0.1% Triton X-100, and reaction was terminated by precipitating the receptor–toxin complex in 35% saturated ammonium sulfate, as described by Aharonov et al. [12]. The precipitates were washed 3-times with 2 ml portions of 35% ammonium sulfate on a glass-fiber filter (GF/C, Tamar, Israel) and the filter counted. This method determines the amount of labelled toxin bound both to particulate and soluble receptor. The two assay methods were initially used to estimate soluble and particulate α-Btx-binding activity in fly homogenates, since the bulk of the cholinergic receptor in such preparations had previously been reported to be in the supernatant of 100 000 × *g* centrifugation [2]. However, since almost all α-Btx-binding activity was found to be particulate (see below), either method could be used, and both yielded similar results.

3. Results and discussion

Over the concentration-ranges used binding observed with given concentration of labelled toxin was linearly proportional to the amount of head homogenate present. Specific binding of toxin, defined as total binding minus the binding occurring in the

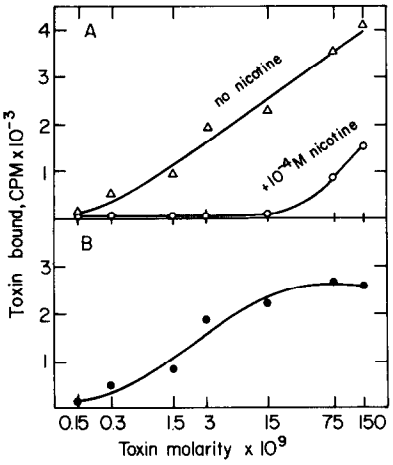

Fig.1. The level of ^{125}-labelled α-Btx-binding to *Drosophila* head homogenate after incubation for 1 h at various toxin concentrations. (A) Total binding in the presence and in the absence of 10^{-4} M nicotine. (B) Specific binding, calculated from A. Saturation corresponds to 0.9 pmol toxin-bound/1 mg protein.

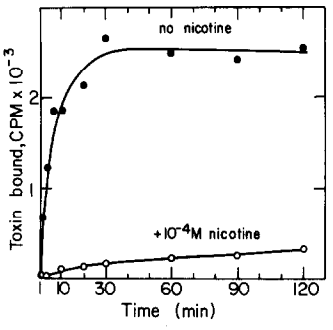

Fig.2. The time-dependence of ^{125}I-labelled α-Btx-binding to *Drosophila* head homogenate. Incubation medium contained 15 nM toxin and 120 μg head protein.

presence of 0.1 mM nicotine, was saturable (fig.1). After incubation for 1 h, maximum specific binding was obtained with toxin concentrations higher than 15 nM. Maximal specific-binding corresponds to 0.9 ± 0.4 pmol toxin bound/1 mg head protein. Concentration of toxin-binding sites in isolated bodies was found to be 0.1 pmol/mg.

Subcellular fractionation studies indicated that about 90% of the binding-sites sediment after centrifugation at 20 000 × *g* for 20 min, i.e., are located in a 'crude mitochondrial' fraction. Boiling at 100°C for 3 min abolished > 98% binding.

The time-dependence of binding is described in fig.2. Under the conditions employed, binding reached equilibrium in about 30 min. Assuming a simple bimolecular reaction between toxin and receptor, one can calculate the on-rate-constant for the formation of toxin–receptor complex to be $K_1 = 7 \times 10^{-5}$ M^{-1} s^{-1}.

Specificity of toxin binding and pharmacological properties were studied by testing the effect of various concentrations of cholinergic ligands on binding. Conditions were chosen so that binding in the absence of added ligand was within the initial linear-portion of the rate-curve [13]. Concentrations of various cholinergic agonists and antagonists that inhibited ^{125}I-labelled α-Btx-binding by 50% (I_{50}) are given in table 1. It can be seen that out of those tested nicotine is the most powerful ligand. DL-Muscarine had no effect at the concentrations tested. Dexetimide, a muscarinic antagonist reported to have a high affinity for fly cholinergic-receptor [6], was almost 3-orders of mag-

Table 1
Effect of various cholinergic ligands on ^{125}I-labelled α-Btx-binding to *Drosophila* head homogenate

Ligand	I_{50}
α-Btx	5×10^{-10} M
Nicotine	8×10^{-7} M
D-Tubocurarine	2×10^{-6} M
Acetylcholine[a]	2×10^{-5} M
Atropine	5×10^{-5} M
Carbamylcholine	9×10^{-5} M
Pilocarpine	8×10^{-5} M
Dexetimide	5×10^{-4} M
Decamethonium	9×10^{-4} M
DL-Muscarine[b]	$> 10^{-3}$ M

[a] Determined in the presence of 10^{-5} M diethylfluorophosphate, which completely inhibits acetylcholinesterase but has no effect on ^{125}I-labelled α-Btx-binding

[b] DL-Muscarine (Sigma) had no effect up to a concentration of 1 mM – Higher concentrations were not tested

Aliquots containing 100–150 μg head protein were preincubated for 25 min with the appropriate concentration of ligand. Reaction was started by addition of ^{125}I-labelled α-Btx (15 nM) and was carried out for 2 min at 25°C. I_{50} was determined from plots of relative reaction rate versus ligand concentration. Dexetimide was a generous gift of Janssen Pharmaceutica, Beerse, Belgium.

nitude less potent than nicotine in protecting against ^{125}I-labelled α-Btx-binding.

Similar experiments were carried out on heads of the house fly, *Musca domestica,* previously employed by various authors to study insect cholinergic-receptor. Again specific-binding of α-Btx was found to be linearly proportional to the amount of tissue present. I_{50}-Values were found to be: nicotine 3×10^{-6} M, D-tubocurarine 4×10^{-6} M, atropine 4×10^{-5} M, Dexetimide 4×10^{-4} M. Binding-site concentration for heads was found to be 0.8 pmol/mg protein.

The above data clearly indicate that flies do contain a nicotinic receptor that specifically binds α-Btx. The reaction kinetics and main pharmacological characteristics are essentially similar to those obtained with rat-brain [15], chick-embryo sympathetic neurons [16] and *Aplysia* ganglia [17]. Decamethonium, which is a powerful inhibitor of α-Btx-binding in vertebrate muscle, is much less effective in all the above systems, as well as in flies.

The concentration of α-Btx-binding sites reported above is one to two orders of magnitude below that reported for nicotinic–muscarinic binding sites in *Musca* head [2,6], but is in the range reported for α-Btx-binding sites in electric organ of the electric eel [18] and an order of magnitude higher than in mammalian brain [15]. The fly cholinergic-receptor ligand-binding measured in previous studies may be due to several classes of receptors [3,4], where by its specificity α-Btx-binding presumably reveals only the nicotinic-receptor. It is therefore plausible to assume that flies contain more than one type of cholinergic-receptor and that the nicotinic-receptor comprises only a small fraction of the total. Such situation, as well as differences in methods employed in preparation of homogenates, may account for the fact that α-Btx-binding sites in insects were not previously detected [5,7].

The demonstration of α-Btx-binding sites in *Drosophila* is of interest not only because of its phylogenetic significance but also because the system is readily amenable to genetic analysis. Thus, the effect of genetic lesion, receptor- and other cholinergic macromolecule, -coding genes on the development and behavior of the organism, and on the structural properties of the molecules themselves, could be studied. In this respect it is of interest to note that the structural gene for acetylcholinesterase has already been identified [19] and that effect of the mutated-gene on fly acetylcholinesterase molecules and behavior are under investigation [14].

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References

[1] Pitman, R. M. (1971) Comp. Gen. Pharmacol. 2, 347–371.
[2] Eldefrawi, A. T. and O'Brien, R. D. (1970) J. Neurochem. 17, 1287–1293.
[3] Eldefrawi, M. E., Eldefrawi, A. T. and O'Brien, R. D. (1971) Mol. Pharmacol. 7, 104–110.
[4] Aziz, S. A. and Eldefrawi, M. E. (1973) Pest. Biochem. Physiol. 3, 168–174.
[5] Donnellan, J. F., Jewess, P. J. and Cattell, K. J. (1975) J. Neurochem. 25, 623–629.
[6] Jewess, P. J., Clarke, B. S. and Donnellan, J. F. (1975) Croat. Chem. Acta 47, 459–464.
[7] Clarke, B. S. and Donnellan, J. F. (1974) Biochem. Soc. Trans. 2, 1373–1375.
[8] Lewis, E. B. (1960) Dros. Inf. Ser. 34, 117–118.
[9] Changeux, J. P., Kasai, M. and Lee, C.-Y. (1970) Proc. Natl. Acad. Sci. USA 67, 1241–1247.
[10] Vogel, Z., Sytkowski, A. J. and Nirenberg, M. W. (1972) Proc. Natl. Acad. Sci. USA 69, 3180–3184.
[11] Vogel, Z. and Nirenberg, M. (1976) Proc. Natl. Acad. Sci. USA 73, 1806–1810.
[12] Aharonov, A., Kalderon, N., Silman, I. and Fuchs, S. (1975) Immunochem. 12, 765–771.
[13] Schmidt, J. and Raftery, M. A. (1974) J. Neurochem. 23, 617–623.
[14] Dudai, Y. (1977) Dros. Inf. Ser. 52, in press.
[15] Eterović, V. A. and Bennett, E. L. (1974) Biochim. Biophys. Acta 362, 346–355.
[16] Greene, L. A., Sytkowski, A. J., Vogel, Z. and Nirenberg, M. W. (1973) Nature 243, 163–166.
[17] Shain, W., Greene, L. A., Carpenter, D. O., Sytkowski, A. J. and Vogel, Z. (1974) Brain Res. 72, 225–240.
[18] Weber, M. and Changeux, J.-P. (1974) Mol. Pharmacol. 10, 1–14.
[19] Hall, J. C. and Kankel, D. R. (1976) Genetics 83, 517–535.