GENETIC VARIANTS IN AN ACETYLCHOLINE RECEPTOR FROM *DROSOPHILA MELANOGASTER*

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1. Introduction

The central nervous system of the fruit fly *Drosophila melanogaster* has been shown [1–6] to be a rich source of an α-bungarotoxin-binding component with the properties expected of a nicotinic acetylcholine receptor. In view of the present confusion over the relation of α-bungarotoxin-binding activity to the nicotinic acetylcholine receptor in the vertebrate central nervous system [7,8], it is important to note that α-bungarotoxin blocks synaptic transmission in the insect central nervous system and thus appears to be binding to a functional acetylcholine receptor. This blockade has been demonstrated in the cercal nerve, giant fiber synapses in the terminal abdominal ganglion of the cockroach *Periplaneta americana* when the desheathed ganglion is bath-perfused with 10^{-6} M toxin. Resting potentials and action potentials recorded within the ganglion are unchanged after complete blockade of synaptic transmission (D. B. Sattelle, B. Hue, I. D. Harrow, J. I. Gepner and L. M. H., unpublished observations). The primary motivation for extending α-bungarotoxin binding studies to *Drosophila* is to open the possibility of isolating mutants with altered receptors that can be used in the analysis of receptor structure, function and role in behavior and development.

We describe here an experimental strategy for the detection of genetic variants that affect acetylcholine receptor structure. As a first step we have screened for nicotine-resistant flies in order to enrich for genetic variants affecting receptor structure. Nicotine, one of the oldest insecticides [9], is very effective for killing *Drosophila*, presumably because of its interaction with the acetylcholine receptor. Binding studies [2,3,5,6] have shown that of all the cholinergic ligands tested, nicotine is the best inhibitor of ^{125}I-labeled α-bungarotoxin binding to *Drosophila* extracts indicating that nicotine itself has a high affinity for the acetylcholine receptor. Once nicotine-resistant strains have been identified, isoelectric focusing of the acetylcholine receptor–^{125}I-labeled α-bungarotoxin complex was used to distinguish mutants that affect receptor structure from those which are resistant due to other mechanisms such as alterations in nicotine metabolism, changes in gut or neural sheath permeability, or even alterations in the membrane environment surrounding the receptor.

2. Materials and methods

2.1. *Nicotine-sensitivity testing*

We have found that the most reproducible way to administer nicotine to *Drosophila* is to add it as nicotine hydrogen tartrate directly to the culture medium. Drug-containing medium was prepared by mixing equal volumes of Instant *Drosophila* Medium (Carolina Biological Supply Company Formula 4-24) and a solution at twice the final concentration of nicotine hydrogen tartrate (Gallard-Schlesinger catalogue no. 29331). The nicotine-containing solutions were all adjusted to pH 7.0, with NaOH before adding the Instant Medium. Eggs were collected from overnight egg lays, counted, and transferred to nicotine-containing medium. Cultures were grown at 25°C and the number of hatching adults were counted to determine % survival. Nicotine hydrogen tartrate in

the medium at 3 mM was used for routine screening for nicotine-resistant strains.

2.2. *Isoelectric focusing studies*

Extracts were prepared by homogenizing heads (40 mg heads in 4 ml) in homogenization medium (5 mM sodium phosphate buffer (pH 7.3), 1% (w/v) sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml pepstatin, and 20 KIU aprotinin). The homogenate was centrifuged at 2000 × *g* for 10 min and the supernatant was collected. The pellet was washed twice by resuspending it in homogenization medium and centrifuging. The combined supernatants were filtered through nylon mesh and centrifuged for 30 min at 20 000 × *g*. The pellet was resuspended in 1 ml extraction medium (5 mM sodium phosphate buffer (pH 7.3), 500 mM sodium chloride, 0.1 mM phenylmethylsulfonylfluoride, 0.1 μg/ml pepstatin, and 20 KIU aprotinin). Triton X-100 was added to final conc. 1% and the mixture was homogenized and incubated in the dark for 1 h at 0°C. Insoluble debris was removed by centrifuging at 30 000 × *g* for 2 h. The supernatant was desalted through a Sephadex G-50 column (15 × 1 cm) eluted with extraction medium containing 1% Triton X-100 and lacking NaCl. This solubilized extract was incubated with mono-^{125}I-labeled α-bungarotoxin for 30 min at 22°C and then 2 vol. extract were mixed with 1 vol. sample buffer (4% LKB ampholytes, pH 5–8, 10% sucrose, 2.5% Triton X-100, 0.005% Evan's blue dye). Samples of 50 μl were applied to gels and run for 2.5 h at 4°C. The gels (8 × 0.5 cm) were prepared in glass tubes and consisted of 2.91% acrylamide, 0.09% *N,N'*-methylene-bis-acrylamide, 1.5% Triton X-100, 2% LKB ampholytes pH 5–8 and 7% glycerol and were polymerized with 0.06% ammonium persulfate. The upper (cathode) reservoir buffer was 0.4% ethylenediamine and 1.5% Triton X-100; the anode buffer was 0.2% sulfuric acid and 1.5% Triton X-100. Gels were prerun for 30 min after overlaying them with 50 μl solution containing 2.5% sucrose, 4% ampholytes pH 5–8, and 1.5% Triton X-100, and then the samples were applied. At the end of the sample run, gels were frozen at −75°C, removed from the tubes, and sliced into 1 mm slices using equally-spaced razor blades. Slices were placed into tubes containing 1 ml 10 mM NaCl and were counted in a gamma counter. This isoelectric focusing procedure was adapted from [10]. The monoiodo-α-bungarotoxin was prepared as in [2,11].

3. Results and discussion

The sensitivity of the Canton-S wild-type strain to nicotine hydrogen tartrate in the medium is illustrated by the open circles in fig.1. The drug at 3 mM is sufficient to kill 96% of the Canton-S strain. We have used this concentration to screen for nicotine-resistant strains in wild-type populations and to date have identified 8 strains that are resistant to nicotine. The dose response curve for one of our confirmed nicotine resistant strains is shown in fig.1. This strain has been designated *HR* and was isolated from the Hikone-R wild-type stock (obtained from the Bowling Green Stock Center). Half of the resistant *HR* strain survives at 3 mM nicotine hydrogen tartrate which kills 96% of the Canton-S strain.

Selection for nicotine-resistant strains provides a way to enrich for mutations which affect the nicotinic acetylcholine receptor structure, but not all nicotine-resistant mutants are expected to have altered receptors. In order to identify those resistant strains with altered receptors, we developed the procedure described in section 2 for solubilizing the receptor and then subjecting the receptor–^{125}I-labeled α-bungarotoxin complex to isoelectric focusing.

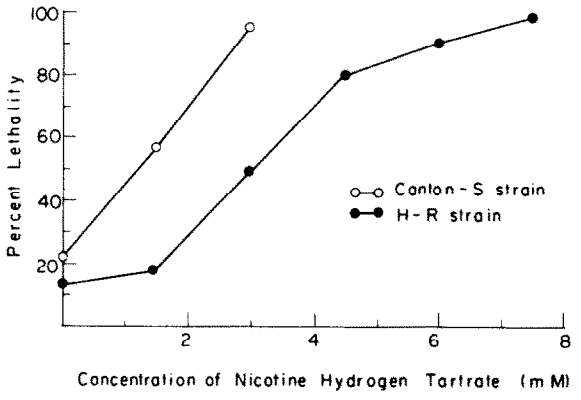

Fig.1. Comparison of sensitivity to nicotine hydrogen tartrate of the Canton-S (○—○) and the nicotine-resistant *HR* (●—●) strains of *Drosophila melanogaster*. Medium containing nicotine hydrogen tartrate was prepared as described in section 2. 200 individuals were tested for each point shown.

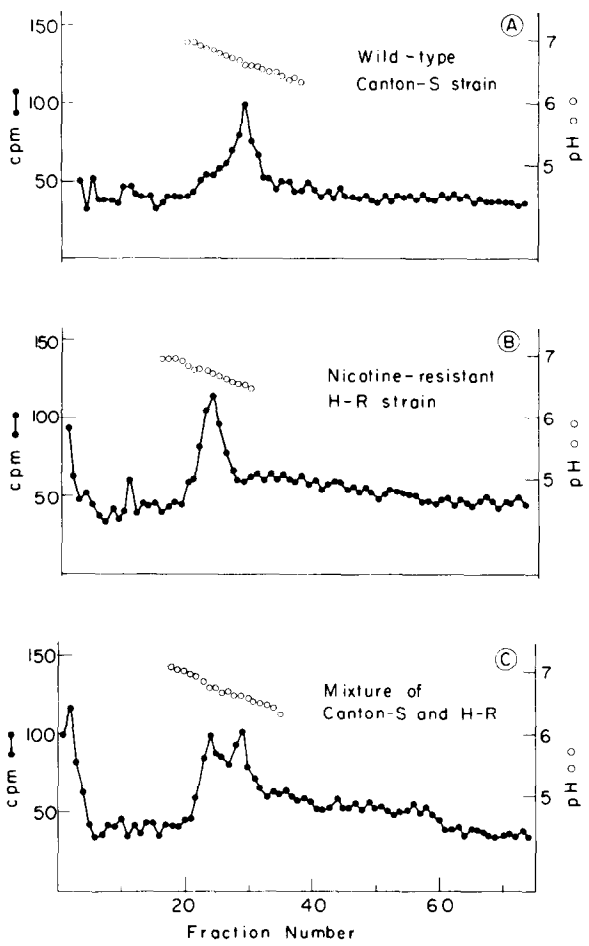

Fig.2. Isoelectric focusing of the acetylcholine receptor–α-bungarotoxin complex from wild-type Canton-S and nicotine resistant *HR* strains of *Drosophila*. Extracts were prepared and run on isoelectric focusing gels as described in section 2. (A) Canton-S strain alone; (B) nicotine-resistant *HR* strain alone; (C) mixture of extracts of Canton-S and *HR* strains.

As shown in fig.2A, the receptor–toxin complex in extracts from the Canton-S strain reproducibly focused as a single peak with an isoelectric point of 6.60. The receptor–toxin complex from the nicotine-resistant *HR* strain also focused as a single peak but, as shown in fig.2B, its isoelectric point was shifted to pH 6.69. In order to demonstrate that this difference in isoelectric point was not due to variation between gels, we prepared a mixture of extracts from the two strains and ran them together on the same gel after pre-incubating them with ^{125}I-labeled α-bungarotoxin. As shown in fig.2C, there were two easily distinguishable peaks: one with pI 6.60 corresponding to the Canton-S receptor–toxin complex and one with pI 6.72 corresponding to the *HR* receptor–toxin complex. Thus, we have used the isoelectric focusing procedure to identify a hereditary alteration in acetylcholine receptor structure.

We have used this isoelectric focusing procedure to analyze the other 7 nicotine-resistant strains. Of these 7, 5 show profiles indistinguishable from Canton-S, either as an extract run alone or as a mixture with Canton-S. The remaining two stocks show shifts in isoelectric point relative to Canton-S. The isoelectric focusing point variants may represent mutations in the structural genes which code for the polypeptide subunits of the receptor. Alternatively, they may be mutations in genes coding for enzymes involved in modification of the receptor complex. In any event, these mutations which we have identified to date cause changes in the receptor structure which do not drastically affect its function since all of these strains have apparently normal locomotor behavior.

Both the nicotine-resistance phenotype and the alteration in isoelectric point of the receptor–toxin complex that we have observed in the *HR* and other nicotine-resistant strains could be due to alterations in the structure of the acetylcholine receptor. Genetic mapping experiments are in progress that will allow us to determine whether the two phenotypes are caused by a change in the same gene. It should be emphasized, however, that irrespective of whether the same gene is responsible for both the nicotine-resistance phenotype and the shift in isoelectric point of the receptor, the isoelectric focusing procedure presented here provides us with a direct way to identify mutants with altered acetylcholine receptor structure. The isoelectric focusing variants will be used to map genes affecting receptor structure. Knowledge of gene location will make it possible to design efficient genetic schemes for the isolation of lethal and temperature-sensitive lethal mutants which alter receptor structure in such a way as to render it inactive. Temperature-sensitive mutants in which the receptor is active at a permissive temperature and inactive at a nonpermissive temperature will be especially useful for behavioral studies and for defining the role of acetylcholine receptors in normal neural development.

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