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AN ELECTROPHYSIOLOGICAL INVESTIGATION OF TARGET SITE INSENSITIVITY IN PERMETHRIN-RESISTANT AND SUSCEPTIBLE STRAIN OF *ANOPHELES STEPHENSI*

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Abstract

It was shown that permethrin resistance in adult *Anopheles stephensi* is due to at least three separate mechanisms. Cytochrome P-450s and esterases are responsible for two-thirds of the 8-fold enhanced tolerance of the DUB-APR resistant strain as compared with a sensitive strain, IND-S. It was postulated that target site insensitivity is also involved. To test this hypothesis, we conducted a neurophysiological study with the thoracic nerves of adult female *An.stephensi* under perfusion with saline. Spontaneous neural activity was recorded with saline alone and in the presence of permethrin. There was no change in the frequency of spontaneous firing during a 1h application of saline in either strain. Application of a single dose of 10^{-13} Molar permethrin rapidly produced a 3-fold increase in firing rate in the susceptible strain. In contrast, the resistant strain did not respond to this concentration. In a cumulative dose assay, the susceptible strain responded to the lowest concentration of permethrin (10^{-13} Molar), but the resistant strain required a much higher dose (10^{-8} - 10^{-7} Molar) to produce the same rise in frequency of action potentials. This strongly suggests that nerve insensitivity contributes to permethrin resistance in this mosquito.

Introduction

An.stephensi is an important malaria vector in the Persian Gulf, the

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Middle-East and Indian subcontinent. As a result of the intensive application of insecticides in these regions, some populations of this species have become resistant to several classes of insecticides including DDT (6), dieldrin (26) and malathion (10). In recent years the synthetic pyrethroids , with their high insecticidal activity, low mammalian toxicity, and degradability in the environment have been used increasingly in vector control programmes, especially for impregnation of bed nets. There have been several reports of pyrethroid resistance in *An.stephensi* based on laboratory selections (5,11,12,13,18,22) but only one report of pyrethroid resistance in a field population (12), from Dubai, United Arab Emirate.

We have studied the basis of resistance to permethrin in *An.stephensi* derived from this partially resistant field population. Following laboratory selection of adult female mosquitoes through 8 generations, the LT50 of the resistant strain was 300 min following exposure to papers impregnated with 10μg/cm^2 permethrin (11). Since the original selection this strain has been periodically reselected with permethrin but, at the time of the experiments reported here, the LT50 stood at 82 min (95% C.I.= 77.4-87.0 min, slope $\pm$ SE[1]=(standard Error) 5.57 $\pm$ 0.41) compared with an LT50 of 10.6 min (95% C.I.= 9.4-12.1 min, slope $\pm$ SE= 3.74 $\pm$ 0.42) in the susceptible IND-S strain.

To monitor the involvement of enzyme activity, synergist studies were conducted using inhibitors of esterases and mixed function oxidases; TPP and PB. Results indicated that cytochrome P-450s and esterases are responsible for two-third of the enhanced tolerance of the DUB-APR resistant strain as compared with a sensitive strains, IND-S. It has also been shown that ^{14}C-permethrin penetrated equally well in resistant and susceptible strains and there were no significance differences between susceptible and refractory strain with respect to the dynamic of permethrin penetration (unpublished data). The fact that some resistance remains following synergism by inhibitors of these enzymes suggests that another component of resistance may be a target site insensitivity mechanism.

It has been known for many years that the nervous system of insects is highly susceptible to poisoning by pyrethroids (15). Although the precise sites of lethal action of these compounds are poorly defined , effects upon both central and peripheral nervous activity are well documented. Pyrethroids show

1- Standard Error

neurotoxicological activity on sensory neurones, motor neurones, and interneurons. At the molecular level, they have been found to affect several types of ion channel including GABA-gated chloride channels, voltage-sensitive Na^+ and K^+ channels, Ca^{2+} channels, and membrane pumps such as the Ca^{2+} and Ca^{2+}-Mg^{2+}-ATPase (26,1), although voltage sensitive Na^+ channels are considered to be the major target site for pyrethroids.

Several forms of resistance to pyrethroids have been found in insects. One such, knock-down resistance (*kdr*), is associated with nerve insensitivity and was first recognised by Busvine in 1951 (4). Various kinds of indirect evidence have been taken to indicate the involvement of nerve insensitivity in pyrethroid resistance, including the existence of cross-resistance between DDT and pyrethroids (20), the ineffectiveness of synergists (8), lack of evidence of delayed penetration (21) or metabolic factors (7,3), the presence of high levels of unmetabolized insecticides at the target site and the absence of behavioral symptoms of toxicology in the initial post-treatment period (2). However, a more informative approach is to study the effect of insecticide directly on nerve preparations, thereby bypassing behavioral, penetration and metabolic resistance mechanisms.

To investigate the possibility of target site insensitivity, a neurophysiological study was undertaken to examine the response of the nervous system to the synthetic pyrethroid permethrin in resistant and susceptible mosquitoes.

Materials and methods

Technical grade permethrin, [3-phenoxybenzyl (1RS)-*cis-trans*-3-(2,2,dichlorvinyl)-2,2,-dimethylcyclopropanecarboxylat], of 97% purity, and with a *cis/trans* ratio; 60/40 obtained from Riedel-de Haën company.

A stock solution of 10^{-5} Molar permethrin was prepared from technical grade permethrin dissolved in acetone (Analar grade) and diluted directly in saline to give a final logarithmic range of concentrations of 10^{-13} to 10^{-7} Molar. The concentration of acetone present in each solution never exceeded 1%.

The newly emerged adults of permethrin resistant (DUB-APR) originated from Dubai and susceptible (IND-S) originally from India strains of *An.stephensi* were supplied with 10% sucrose on a cotton pad. Unfed, 2-6 day old females (30 resistant and 33 susceptible) were used for the neurophysiology assays.

Fresh insect saline (19) was prepared each day at the start of the neurophysiological experiments.

Sylgard dishes provide an inert resin for neurophysiology assays. Sylgard resin is prepared by mixing Sylgard 184 silicon elastomer (Dow corning) and curing agent in a ratio of 10:1. After mixing, the resin was poured into 50-mm diameter plastic petri dishes to a depth of 3-5 mm. Preparations were left to air dry for 48 h. Once dried, the dishes were placed into a bath of distilled water for a further 48 h then washed once more to remove any remaining surface oil. The dried Sylgard dishes were then used for neurophysiology assays.

For the neurophysiology assay, 2-6 day old females of *An.stephensi* were chilled for 30 seconds and pinned out on a layer of saline-washed Sylgard resin in a 50mm plastic petri dish. Legs, wings and head were removed. The thorax was dissected and the ventral nerve cord and was picked up with a 27 gauge stainless steel, hypodermic needle, electrically insulated on the external surface. This needle serve as a recording electrode. A stainless steel entomological pin (0.1 mm diameter X 15 mm length) grounded the preparation and served as a reference electrode. The preparation was placed on a low-vibration table at a constant temperature (25 ± 1°C). The recording electrode was connected to the remote head of a Neurolog high-gain, amplifier and conditioning system (Neurolog; Digitimer, UK). Spontaneous nerve activity was monitored on an oscilloscope and activity recorded on Maclab/2e data recorder and analyzed on a Macintosh LCII computer (14).

In a single dose assay, the preparations of susceptible and resistant strains were bathed in control saline for 1 hour followed by saline solutions containing a supra-threshold concentration of permethrin (10^{-13} Molar). In a cumulative dose assay, the number of action potentials in a 5 minute control period was first recorded and then saline was removed with a disposable glass pipette. Preparations whose firing rate remained relatively stable over the control period were then treated with permethrin. An identical volume of fresh saline containing the test compound was applied, first the lowest concentration (10^{-13} Molar) and subsequently, at further five minutes intervals, successively higher concentrations up to 10^{-7} Molar. The mean number of action potentials for each concentration was recorded and analyzed. For each strain, the frequency and SE of response at each concentration in the assay was calculated.

Results and discussion

The mosquito nerve preparations used here remained viable for at least 1 h. A single dose application of 10^{-13} Molar permethrin rapidly produced a 3-fold increase in action potentials rate in the susceptible strain. In contrast, the nervous system of the resistant strain did not respond to this concentration (Fig. 1). The results of a single dose application of permethrin indicated that the nervous system of the susceptible strain is highly sensitive to permethrin. In a cumulative dose assay, the susceptible strain responded to the lowest concentration of permethrin (10^{-13} Molar), whereas the resistant strain required a much higher dose (10^{-8} - 10^{-7} Molar) to produce the same rise in frequency of action potentials (Fig. 2).

It was found that insensitive or tolerant nerves of *Heliothis virescens* either required longer periods of exposure or higher concentration of insecticide to induce the same poisoning symptoms as susceptible preparations (17).

Our neurophysiology results are in agreement with studies upon susceptible and *kdr* strains of house fly, *Musca domestica* (16,17,19). These authors found that the lowest concentration of permethrin required to elicit a response in susceptible strain was below 10^{-10} Molar, but as high as 10^{-7} Molar for *kdr* resistant larvae. Thus the resistance to permethrin conferred on the *kdr* strain was over 1000-fold. It was found that larvae of a permethrin-resistant strain of *An.stephensi* required a 20-fold higher concentration of insecticide to bring about an increase in the spontaneous action potential rate in comparison with a susceptible strain (23). For larvae of resistant and susceptible strains of *Culex quinquefasciatus* the equivalent figure was 2000-fold . Neurophysiological studies on the *kdr*-type resistant larvae of *An.stephensi* (18) revealed that the resistant strain needed 20 times more permethrin than the susceptible strain to induce an increase in the frequency of miniature end-plate potentials. In a neurophysiology assay with permethrin the EC_{50} value for adults of a strain of house fly and 10^{-12} Molar, for *kdr* strain (9).

In our study we found a very wide difference in nerve sensitivity between the resistant and susceptible strains. This difference in sensitivity is much greater than the 8-fold resistance shown by *in vivo* bioassays and is comparable to data obtained with *Musca domestica* (25). This difference could reside in factors present in the intact insect and enhanced in the *in vitro* direct application.

It is not known where in the nervous system permethrin acts to cause the neurophysiological effects observed. The neurophysiology extracellular recording technique developed here is not specific for a single nerve cell. Action potentials of several nerves could be recorded and nerve insensitivity detected by the assay may be a function of ion channels or synaptic neurotransmitter release.

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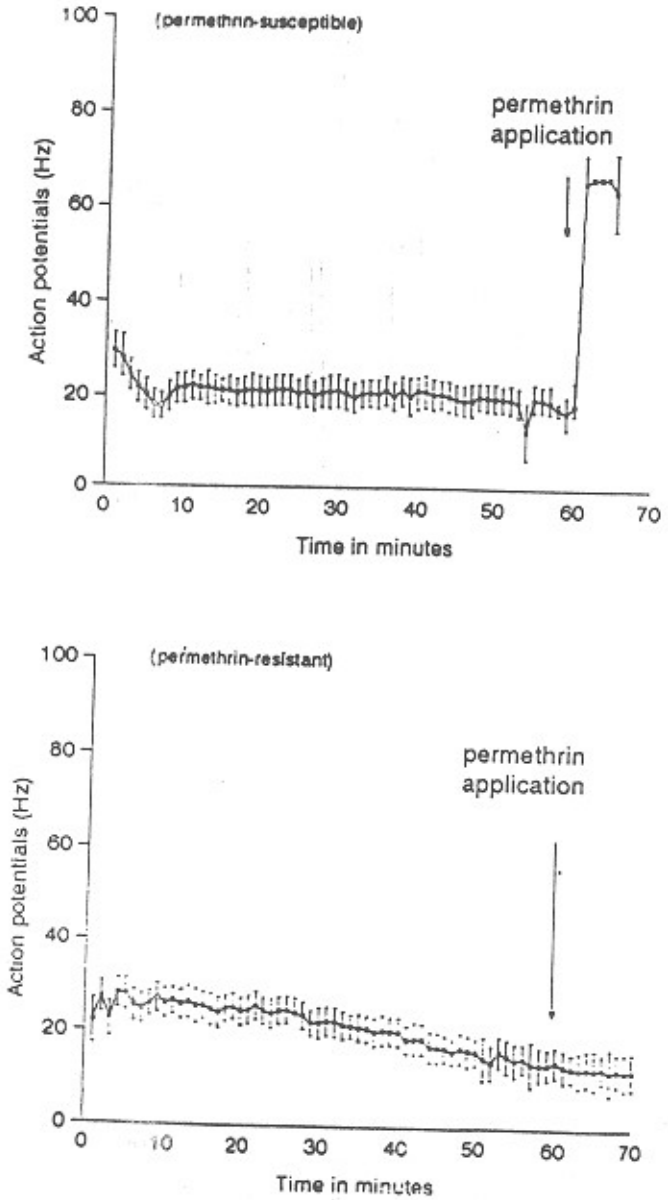

Fig. 1- Neural response of permethrin-susceptible and resistant strains of *An.stephensi* to a single dose (10^{-13} Molar) of permethrin. vertical bars= SE

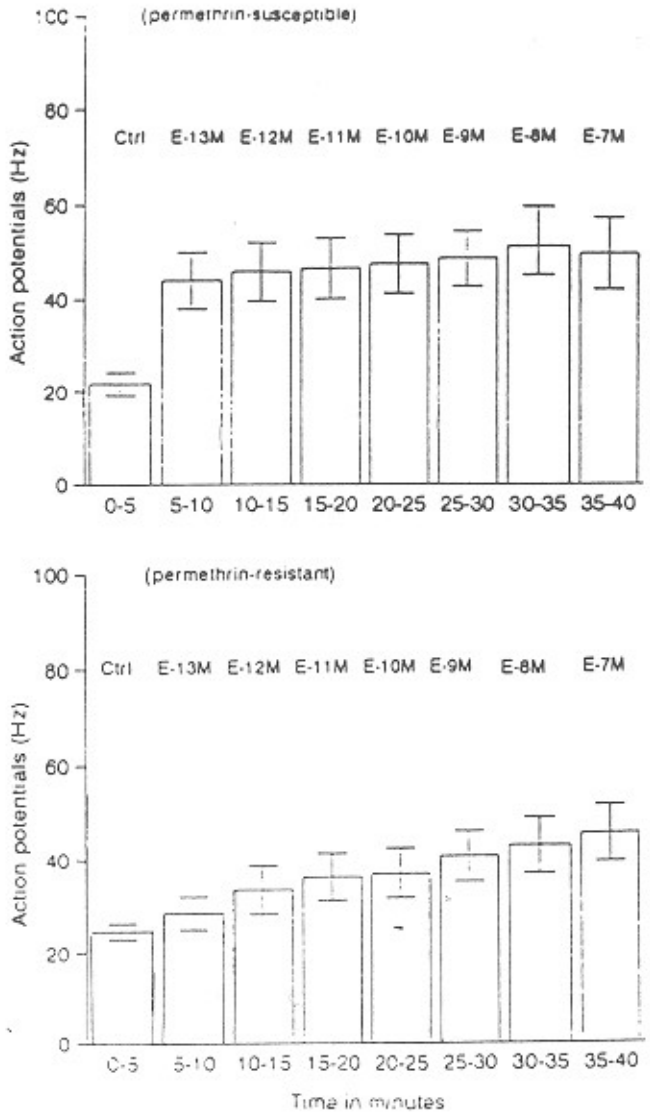

Fig. 2- Neural response of permethrin-susceptible and resistant strains of *An.stephensi* to permethrin in a cumulative dose assay (concentrations shown above bars range from 10^{-13}-10^{-7} Molar). vertical bars= SE

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