Innovations

Using the Techniques of Molecular Biology to Search for Novel Insecticides

Searching for safe, new methods to control insects is a difficult and challenging task. There is increasing public pressure to reduce the quantity of chemicals applied to farmland and to replace several effective but somewhat toxic pesticides with less harmful materials. Much research has been carried out recently on use of predaceous insects and parasitic organisms to control pest insects. Genetic engineering of insect-specific bacteria and viruses to increase the speed at which they kill pests is also feasible and may eventually lead to the development of novel organic insecticides. At present, however, even the most ardent supporters of biological control agree that there is a continuing need for insect-specific, environmentally friendly chemical pesticides. New strategies are therefore necessary to speed up the process of finding chemicals that are lethal to pest insects, but not to other animals, and that do not persist in the environment. Rapid advances in molecular biology are now providing exciting new tools to study insects and identify potential insecticides.

Conventional methods of insecticide discovery involve testing large numbers of chemicals in several different species of insects, then carrying out detailed studies on the mode of action and mammalian toxicity of the chemicals. Scientists at Rhône-Poulenc and other agricultural companies are attempting to improve this process by identifying important target sites within the insect and using this information to develop *in vitro* assays that can be carried out at the same time as traditional screening methods. A suitable target site might be any enzyme or receptor that is vital to the normal functioning of the insect, and it should be a site that is found only in insects or that differs in insects and vertebrates. This type of targeted approach to drug discovery is frequently used in the pharmaceutical industry, but it has only recently been successfully applied to insecticide research.

Identifying Potential Target Sites

Many insecticides now in use act on the nervous system of the insect. Disruption of nervous activity usually results in rapid cessation of feeding, giving good protection of crops. The problem is that there are many similarities between the nervous systems of insects and vertebrates, so finding insect-specific chemicals can be difficult. One area of the nervous system that differs somewhat in vertebrates and invertebrates is the receptor for the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). GABA binds to the receptor protein, causing a channel to open which forms an integral part of the receptor. When the channel opens, negatively charged chloride ions flow into the nerve or muscle cell, and this influx lowers the electrical potential of the cell, making it less likely to fire in response to other stimuli. Several insecticides, including the cyclodienes, such as dieldrin and endrin, act by blocking the chloride channel and preventing nervous inhibition, thus causing prolonged, uncontrolled firing of nerve cells. Most of these insecticides are no longer registered for use in the United States because of their long persistence in soil, but it may be possible to find other chemicals that act at the same receptor yet have more favorable environmental profiles.

Some useful information on the insect nervous system has come from electrophysiological experiments in which recordings are made from insect nerve and muscle cells impaled with tiny electrodes. The effects of chemicals that act on the GABA receptor in nerve or muscle can be measured, but it is only possible to carry out this type of experiment on a few large model insects such as cockroaches or grasshoppers. Due to their small size experiments on commercially important pest insects are often difficult to perform; these experiments are time consuming and therefore cannot be carried out on large numbers of test chemicals.

Larger numbers of chemicals can be screened using radioligand binding studies. A number of radioactively labeled chemicals that bind specifically to certain receptor proteins are commercially available, and the ability of test chemicals to compete with these chemicals at the receptor can be measured. This method can be used for routine screening of chemicals, but tedious and time-consuming dissection of the insect nervous system is still necessary to provide sufficient tissue for testing. Despite these limitations, radioligand binding has been used to demonstrate some pharmacological differences between vertebrate and invertebrate GABA receptors.

Vertebrate GABA receptors have been studied extensively. There is more than one subclass of GABA receptor, and the responses of these receptors to certain drugs are different. This type of information is not yet available for insect receptors. Detailed study of receptor proteins has been hampered by the lack of an abundant source of insect nerve tissue. This problem has led many scientists to search for the insect GABA receptor gene. Once a gene is cloned, there are a number of techniques available for synthesizing large amounts of the protein encoded by the gene. Cloning of the insect GABA receptor gene would make it possible to produce sufficient protein for detailed pharmacological studies and would allow the development of large-scale *in vitro* assays to screen for chemicals acting at this site.

Cloning a Gene for a Target Site

One novel approach to the search for the insect gene was pioneered by Richard Roush of Cornell University and Richard ffrench-Constant of the University of Wisconsin, in collaboration with Rhône-Poulenc. The starting point for this work was the fact that many insects develop resistance to cyclodiene insecticides, and experiments suggested that the mechanism of resistance was an alteration of the target site (the GABA receptor). To take advantage of the vast amount of knowledge on the genetics of the fruit fly, *Drosophila.* Roush and ffrench-Constant collected large numbers of field strains of *Drosophila* and screened them for resistance to dieldrin. Although fruit flies are not a major pest, they are extremely widespread, and are therefore likely to come in contact with insecticides destined for other pests. Contact had occurred in several areas where cyclodienes had been heavily used, and large numbers of *Drosophila* with some resistance to dieldrin were discovered.

The next step was to find out which chromosome contained the genes conferring resistance. To do this, the researchers cross-bred the most resistant field-collected insects with laboratory-reared insects that had recognizable physical traits. The laboratory-reared insects have been extensively studied, and the approximate position of the gene conferring the physical characteristic is known. These "marker genes" have been used to construct a partial map of the *Drosophila* genome. By examining which traits were most often co-expressed with resistance, it was possible to demonstrate that the resistance gene was carried at map unit 25 on the left arm of chromosome 3.

Further localization of the resistance gene involved a more complex series of experiments. Large numbers of resistant insects were crossed with a variety of laboratory strains that had small regions of

DNA missing from the third chromosome (deletion mutants). The offspring of these crosses were tested for resistance. If the deletion occurred in the region of the gene of interest, it would prevent expression of the gene.

The offspring of the crosses would therefore have only one copy of the gene, which would have come from the resistant parent. These insects would thus be fully resistant to dieldrin. If the deletion was anywhere else on the chromosome, the susceptible form of the gene would be unaffected and would be passed on to the offspring. The insects resulting from these crosses would have one copy of the resistant and one copy of the susceptible gene and would not be fully resistant. This type of painstaking experiment allowed the researchers to assign the gene to a region of the chromosome spanning about 155 kilobases of DNA.

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Looking for insects' Achilles heels. Alison Chalmers searches for insect target sites for use in developing insect-specific pesticides.

Individual genes from this region were identified using previously constructed cosmid libraries. Cosmids are bacterial plasmids (circular pieces of extrachromosomal DNA that are capable of replicating) engineered to contain 35–40 kilobase segments of foreign DNA. Cosmids are constructed to contain overlapping regions of the genome, so it is possible to sequence a small region from one cosmid, look for that same sequence in a different cosmid, and use this technique to "walk" down the chromosome from an area where gene sequences are known to a region that has not previously been sequenced. Sequences obtained in this way can be used to construct genetic probes (short lengths of single-stranded DNA). These probes can then be used to search libraries of *Drosophila* genetic material. These libraries are collections of plasmids or phages containing complementary DNA sequences constructed *in vitro* using individual strands of messenger RNA as a template. Each of these messenger RNA strands codes for a different *Drosophila* gene. Sequences that exactly match the genetic probes will bind tightly to the probe and can be isolated and sequenced.

Experiments on Target Sites

One of the *Drosophila* genes discovered showed some homology with a beta-subunit of a rat GABA receptor (45% of the bases sequenced were identical in both genes, and 64% of the encoded amino acids were similar). This homology suggested strongly that the gene (designated *Rdl*; *R*esistant to *d*ie*l*drin) did indeed contain the code for an insect GABA receptor subunit, but this had to be confirmed by making the protein encoded by the gene, then demonstrating that this protein responded to GABA. The method used first was expression of the insect gene in oocytes (unfertilized eggs) of the African clawed frog, *Xenopus laevis*. This technique, which has been used extensively in experiments on vertebrate receptors and ion channels, takes advantage of the capacity of these cells to translate foreign RNA and produce large amounts of protein. Small portions of the ovary are removed from a female frog, and individual oocytes are teased away from the surrounding tissue. Messenger RNA can be made *in vitro* from a cloned gene and injected into individual oocytes. In many cases, the oocyte will translate the message and produce proteins that are correctly folded and processed. The technique is particularly useful for membrane-bound receptors and ion channels because it is likely that these proteins will be correctly positioned in the membrane of the oocyte. These large cells can be easily impaled with recording electrodes, and electrical changes in the cell can be monitored.

Most studies on vertebrate receptors have demonstrated that at least two different subunit proteins are necessary before functional GABA receptors can be assembled in oocytes. Most vertebrate GABA receptors are composed of five individual subunits, of at least three distinct types. Surprisingly, injection of messenger RNA synthesized from the single cloned *Drosophila* subunit resulted in the expression of functional GABA receptors. Uninjected frog oocytes show no response to GABA, but oocytes injected with RNA from the *Drosophila Rdl* gene responded to the application of GABA with electrical currents of up to 5 microangstroms, caused by chloride ions flowing through GABA-mediated channels in the membrane. Pharmacological studies showed that these receptors were also sensitive to muscimol, an agonist of vertebrate and invertebrate GABA receptors. The response to GABA was not modified by bicuculline, which is an antagonist of the vertebrate receptor, but is inactive in several insect preparations. The response to GABA was, however, greatly reduced by the drug picrotoxinin, which blocks GABA-mediated chloride channels, and was reduced by dieldrin and other cyclodiene insecticides.

The fact that a single subunit is sufficient for functional expression of an insect GABA receptor does not necessarily imply that this is the form of the receptor present in insects. Recent studies by David Soderlund of Cornell University and David Sattelle of Cambridge University in England have revealed the existence of other subunits, and it is not yet known whether these will co-express with the *Rdl* gene or whether they form a separate class of insect GABA receptors. This single subunit expression does provide a useful model system for studying chemicals that bind to the insect GABA receptor. Recent experiments on vertebrate retinas have revealed the presence of a novel vertebrate GABA subunit, which also assembles in oocytes without additional subunits. The

pharmacology of these receptors appears to be similar to that of the insect receptor, but the DNA sequence of this retinal receptor is no more similar to the insect receptor than any of the other subunits.

Initial pharmacological experiments were carried out on the dieldrin-susceptible form of the gene, but at the same time, studies were continuing on the mutation in this gene that resulted in resistance. Roush and ffrench-Constant investigated resistant insects from several continents and demonstrated that the majority of these insects had only one mutation in the gene, and this resulted in the substitution of one amino acid for another (one serine was replaced by one alanine). The *Xenopus* oocyte expression system was used to directly compare the responses of the dieldrin-susceptible and dieldrin-resistant proteins. It appeared that, while the responses to GABA and muscimol were apparently unchanged by the mutation, the sensitivity to picrotoxin and dieldrin was greatly reduced. This implies that the altered amino acid is present at or near the binding site for these two compounds, and this information, combined with other studies, may help in constructing models of the receptor, which could eventually be used in insecticide and drug design.

Although *Xenopus* oocyte studies provide a useful way of studying receptor pharmacology, they are not ideal for screening large numbers of chemicals because oocytes must be injected individually. Other methods of expression involve incorporating the insect gene directly into the chromosomes of cells in a continuously growing cell line (permanent transformation), or incorporating it into a baculovirus (insect-specific virus) and using this to infect insect cells grown in culture. Both methods have been successfully used in the study of vertebrate GABA receptors, and both offer great promise as ways of producing the quantities of protein necessary to set up high-capacity, automated screens. This should greatly increase the efficiency of searching for chemicals acting on the insect GABA receptor.

This novel approach to the search for insect genes has several other important implications. Experiments are in progress to see if this same mutation has occurred in commercially important pest insects. If it has, it should be possible to use molecular biological techniques to develop assays to detect resistance in field populations of insect pests. Also, the resistant gene could be used alongside the susceptible gene in assays to allow selection of novel, active chemicals that do not show cross-resistance.

The techniques developed during this study can also be applied to the study of other insect receptors and enzymes. In the short term, these receptors can be used to develop rapid, automated screens for large numbers of chemicals, which should increase the efficiency of searching for novel insecticides. In the long term, it may be possible to use information on receptor structure to build molecular models of the binding sites and to use these models to design novel, insect-specific chemicals.

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Suggested Reading

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