PATCH-CLAMP ANALYSIS OF THE EFFECTS OF THE INSECTICIDE DELTAMETHRIN ON INSECT NEURONES

BY M. AMAR, Y. PICHON*
Département de Biophysique, Laboratoire de Neurobiologie Cellulaire et Moléculaire du CNRS, F-91198, Gif sur Yvette Cedex, France

AND I. INOUE
Institute for Enzyme Research, Tokushima University, Tokushima, 770, Japan

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Summary

1. The mode of action of the pyrethroid insecticide deltamethrin on inexcitable embryonic cultured cockroach neurones has been investigated using the patch-clamp technique.

2. Whole-cell recordings of the current induced by step depolarizations of the cell membrane showed that concentrations of deltamethrin ranging from $10^{-8}$ to $5 \times 10^{-6}$ mol l$^{-1}$ induced a small tetrodotoxin (TTX)-sensitive inward current that peaked at around $+10$ mV and reversed at around $+60$ mV. The activation and inactivation kinetics of this current were much slower than those of the axonal sodium current in this same species and were relatively insensitive to membrane potential. Steady-state inactivation was almost absent.

3. Single-channel activity associated with the action of the insecticide was analyzed using the cell-attached configuration. Three distinct patterns of activity were found: (1) discrete single-channel events of relatively short duration, (2) long events of comparatively small amplitude and (3) complex bursts made up of a succession of openings and closings to several levels. These three patterns were analyzed quantitatively using specially designed programs.

4. The first pattern of activity could be seen in most patches. It consisted of short (1–10 ms) rectangular events of comparatively small amplitude (1.5 pA at rest) and very low open time probability (around 0.001). The current–voltage relationship of these small events was linear over the voltage range studied and the (extrapolated) reversal potential approximated $E_{Na}$.

5. The second pattern of activity was observed less frequently. The channels could stay open for very long periods (up to several seconds) and occasionally flickered between two or more levels.

6. The third pattern of activity was observed in many patches. During the burst, which could last from a few milliseconds to a few hundred milliseconds, the single-channel current jumped almost continuously between several levels (up to 7 or 8).

* To whom reprint requests should be addressed

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7. The size of the different (single-channel) current levels was the same for the three patterns of activity. Furthermore, these levels corresponded to multiples of the same minimum level (around 1 pA at the resting potential).

8. These results suggest that deltamethrin activates several states of one or several populations of sodium channels.

Introduction

Agents that modify the properties of membrane ionic channels are useful tools for deciphering channel structure and function. Several molecules are known to activate the sodium conductance and are often referred to as agonists by analogy with receptor-gated channels; they include toxins such as grayanotoxin and batrachotoxin, alkaloids such as aconitine, veratridine and cevadine and insecticides such as pyrethroids and DDT. Veratridine, pyrethroid insecticides and DDT have very similar effects on the membrane: they induce repetitive firing, depolarizing afterpotentials and/or maintained depolarizations (see Ulbricht, 1969; reviewed by Hille et al. 1987). This resemblance is suggestive of a common mode of action. Thus, in his paper on the node of Ranvier, Hille (1968) suggested that DDT and veratridine act similarly in maintaining the sodium channels in the ‘open’ position. A few years later, Vijverberg et al. (1972) proposed that pyrethroids and DDT have a similar mode of action on sodium channel gating in myelinated nerves. The same conclusions were reached more recently by Leibowitz et al. (1987) for frog skeletal muscle treated with several insecticide and alkaloid agonists of the sodium channel. Investigations with single-channel and whole-cell voltage-clamp recordings of N18 neuroblastoma cells also support the hypothesis that veratridine modifies open channels (Barnes and Hille, 1988).

In contrast with the preceding conclusion, early voltage-clamp experiments on isolated cockroach axons revealed that micromolar concentrations of veratridine and cevadine (the two major components of veratrine sulphate) exerted their effects through a slow voltage-dependent sodium channel activation independent of the normal fast sodium activation and inactivation processes responsible for spike production (Pichon and Boistel, 1969; Pichon, 1974). Under similar experimental conditions, the main effect of DDT was to maintain in the open position a certain proportion of the sodium channels (Pichon and Boistel, 1969), as in the node of Ranvier (Hille, 1968) and lobster axons (Narahashi and Haas, 1968). More recent experiments on the same preparation confirmed the previous observations with DDT and showed that the mode of action of several pyrethroid molecules was different, resembling that of veratridine (Laufer et al. 1984; Pichon and Pelhate, 1985; Pichon et al. 1985, 1987). In a recent study of the kinetic properties of sodium channels of rat neuroblastoma cells modified by the pyrethroid fenvalerate, Holloway et al. (1989) reached a similar conclusion. Our recent observation that micromolar concentrations of veratridine induce a TTX-sensitive inward current underlain by a complex single-channel activity in inexcitable cultured embryonic cockroach neurones strengthens this hypothesis (Amar et al. 1991).
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Part of the observed discrepancy could be due to species differences and also, as discussed by Amar et al. (1991), to the use, in the former experiments, of high concentrations of agonists dissolved in most cases in strong solvents such as dimethylsulphoxide (DMSO). Since it is known that, in insects, as in most other preparations, the major effect of deltamethrin is to depolarize the nerve membrane slowly at submicromolar concentrations, even in the absence of nerve stimulation (Heilig, 1984; Laufer et al. 1984; Roche et al. 1985; Guillet et al. 1986; Pichon et al. 1986, 1987), it was important to study in detail the effects of such concentrations on an insect preparation. The present paper describes the effects of the pyrethroid molecule deltamethrin on embryonic cockroach neurones in culture. The goals of this study were (1) to determine whether deltamethrin could activate a sodium conductance in otherwise inexcitable neurones and to characterize this conductance and (2) to analyze the single-channel activity that underlies membrane depolarization in deltamethrin-treated neurones.

A preliminary report of some of these results has been published elsewhere (Amar and Pichon, 1989).

Materials and methods

Cultures

Neuronal cultures were prepared from the brains of 21-day-old embryos of Periplaneta americana (L.) as described by Dewhurst and Beadle (1985). Briefly, the egg cases (which contained up to 16 embryos) were sterilized with absolute ethanol for 10 min and opened and the heads of the embryos removed and stored in Schneider's revised Drosophila medium. The brains were then extracted from the head capsules and the cells dissociated by gentle trituration with a Pasteur pipette. The cultures were initiated in a second medium made up of five parts of Schneider’s medium and four parts of Eagle’s basal medium containing 100 i.u. ml<sup>-1</sup> penicillin and 100 μg ml<sup>-1</sup> streptomycin: three drops of the cell suspension were placed in the middle of a 50 mm Falcon light-seal plastic Petri dish and covered with a sterile glass coverslip. After 1 h, the culture dishes were turned upside down, to separate the live cells from the debris, and stored in an incubator at 29°C. After 7 days, the culture medium was replaced by a third medium made up of equal parts of Leibovitz’s L-15 and Yunker’s modified Grace medium containing penicillin and streptomycin supplemented with foetal calf serum (7%). In the second medium, the vast majority of the glial cells did not survive and little growth was seen. The third medium enabled the fast development of neuritic processes into a dense network. All culture media were obtained from Gibco Ltd (Life Technologies), Paisley, Scotland.

Experimental procedure

Immediately before an experiment, the culture medium was replaced by a standard saline containing 210 mmol l<sup>-1</sup> NaCl, 3.1 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> CaCl<sub>2</sub> and 10 mmol l<sup>-1</sup> Hepes buffer, adjusted to pH 7.2 using NaOH. The dish
was placed directly onto the stage of a Nikon TMS inverted microscope and the cells were observed under phase contrast. All experiments were performed at room temperature (24–26°C).

For the experiments using the whole-cell configuration of the patch-clamp technique (Hamill et al. 1981), the electrodes were filled with a solution containing 140 mmol l\(^{-1}\) potassium gluconate (normal-K\(^+\) internal solution) or caesium aspartate (K\(^+\)-free internal solution), 10 mmol l\(^{-1}\) EGTA, 1 mmol l\(^{-1}\) CaCl\(_2\), 10 mmol l\(^{-1}\) MgCl\(_2\) and 10 mmol l\(^{-1}\) Hepes buffer, adjusted to pH 7.2 using NaOH. Whenever needed, tetraethylammonium (TEA\(^+\)) chloride (100 mmol l\(^{-1}\)) and/or 4-aminopyridine (4-AP) (10 mmol l\(^{-1}\)) was added to the solution to block the potassium current and tetrodotoxin (TTX) (3 \(\mu\)mol l\(^{-1}\)) was used to block the sodium current. Deltamethrin was first dissolved in a small volume of ethanol. Appropriate volumes of this stock solution were added to the saline contained in the patch pipette (internal solution). Deltamethrin (1R,3R,6S) was a gift from Procida (Roussel UCLAF, Marseilles); the other compounds were purchased from Sigma.

**Electrophysiological techniques**

Electrodes were pulled from 1.5 mm borosilicate microhaematocrit tubing, using a Narishige micropipette puller, and fire polished. Their resistance ranged from 2 to 5 MΩ. The electrode was advanced towards the cell soma (10–15 μm in diameter) until gentle contact was established. A small negative pressure was then usually sufficient to create a seal of 1–10 GΩ. For whole-cell recordings, the membrane patch was subsequently destroyed by applying a short (0.1–0.5 ms) 1.5 V pulse across the membrane.

The patch electrode was connected to the input stage of an Axon Instruments (Axopatch 1C) patch amplifier. The outputs of the amplifier were connected to a Tektronix oscilloscope, to the input stages of a modified Sony digital audio processor (PCM 701) connected to a Sony Betamax video recorder (Lamb, 1985) and to the input amplifier of a laboratory-made data-acquisition and stimulation system (DAISY-12C) connected to a Hewlett Packard series 9000 model 310 microcomputer.

For the whole-cell clamp experiments, the command voltage steps were supplied by the Hewlett-Packard-based system and the resulting currents were digitized, after amplification and partial analogue compensation of the capacitive artefacts, and stored onto 3.5 inch floppy disks. For cell-attached experiments, the membrane potential was changed using the holding potential command of the patch amplifier.

**Data analysis**

Whole-cell currents were analyzed on the Hewlett Packard microcomputer: the leak current corresponding to a 20 mV hyperpolarizing voltage pulse was sub-
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tracted, after scaling, from the stored current traces and the resulting current displayed on the screen and plotted on a digital plotter. The corrected records were stored for further analysis.

Single-channel data were analyzed on an IBM-AT-compatible microcomputer. Twenty-second-long recordings were selected from the video tape, digitized using a data translation (DT 2801A) A/D converter and stored directly onto the hard disk. The resulting 12-bit binary files were then analyzed. In the early experiments, the program called IPROC-2, originally developed by Sachs et al. (1982), was used (see Beadle et al. 1989). Later analyses were performed using an original program called PAT2, a program derived from ANALYSIS (Pichon and Pichon, 1989) and designed specifically for fast analysis and display of long recordings with complex channel behaviour. The outputs of both programs consisted of a series of files that were subsequently used, in connection with a statistical library, to build histograms.

All results are given as mean±s.d. unless stated otherwise.

Results

Whole-cell clamp experiments

140 mmol l⁻¹ K⁺ internal solution

Under these experimental conditions, embryonic cockroach neurones in culture had a relatively low resting potential of −45±6.9 mV (N=25) and were electrically inexcitable. Under most conditions, however, strong voltage depolarizations in the current-clamp mode gave rise to small (less than 15 mV) biphasic spikes originating from the axonal processes, indicating that, as in most adult insect neurones, the cell bodies were inexcitable and lacked fast voltage-sensitive sodium channels although normal sodium spikes were present in the axons (see Pichon, 1974). Under voltage-clamp conditions, depolarizing voltage steps failed to elicit any significant inward current but gave rise to a large outward current. Varying the holding potential between −100 mV and −40 mV or using long-duration (200–400 ms) hyperpolarizing conditioning prepulses failed to de-inactivate a potentially inactivated sodium conductance.

The addition of low concentrations of deltamethrin (1−5 μmol l⁻¹) to the solution contained in the patch pipette had a slight depolarizing effect on the resting potential (−39±7.5 mV, N=9 for 5 μmol l⁻¹ deltamethrin) and induced a small voltage-dependent inward current. This current turned on soon after membrane depolarization, reached a peak value of 50−100 pA (mean −81±26 pA, N=7) during the first few milliseconds, then decreased and was replaced by a large outward potassium current (Fig. 1A). The maximum current was obtained for command steps to a membrane potential of about +10 mV and the apparent reversal potential (calculated by extrapolation from the linear portion of the I–V curve) was around +60 mV. This inward current was sensitive to the addition of tetrodotoxin to the bath.
Fig. 1. Ionic currents recorded from the soma of cultured cockroach neurones in the whole-cell clamp configuration in the presence of 10^{-6} mol l^{-1} deltamethrin. (A) Family of membrane currents corresponding to step membrane depolarizations from a holding potentials of -60 mV to membrane potentials between -50 and +60 mV in 10 mV increments (internal solution: potassium gluconate). (B) Family of membrane currents corresponding to step membrane depolarizations from a holding potential of -60 mV to membrane potentials between -50 and 0 mV in 10 mV increments (internal solution: caesium aspartate). (C) Current–voltage relationship for the peak sodium current recorded before (▲) and after a 3 min perfusion with 3 μmol{l}^{−1} TTX (▲); holding potential -60 mV. (D) Current–voltage relationship for the peak sodium current corresponding to step depolarizations to the indicated potentials from three different holding potentials: -50 mV (▲), -60 mV (▲) and -70 mV (▲). In B, C and D, the outward current was blocked with 10 mmol{l}^{-1} TEA^{+} and 0.1 mmol{l}^{-1} 4-AP.
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K⁺-free internal solution containing caesium

To analyze the time course of the deltamethrin-induced sodium current, most of the outward current was removed by replacing potassium gluconate in the internal solution with caesium aspartate. The mean resting potential under these conditions was $-47.7 \pm 2.6$ mV ($N=3$) in the absence and $-42 \pm 14.4$ mV ($N=6$) in the presence of potassium channel blockers in the bathing solution. The current–voltage relationship of the deltamethrin-induced peak inward current, measured under voltage-clamp conditions for neurones in which the turning on of the current was graded with potential, was basically the same as with potassium gluconate; the maximum current was larger, as was to be expected from the inhibition of the outward current (maximum inward current around $-120$ pA). Here again, the peak current was obtained for a membrane potential of about +10 mV and the mean (extrapolated) equilibrium potential was around +60 mV (Fig. 1C). This current was partly inhibited by bath application of TTX (3 μmol L⁻¹) (Fig. 1C).

Kinetics of the deltamethrin-induced current

In nearly 70% of the 20 neurones internally perfused with caesium aspartate, the turning on of the deltamethrin-induced inward current was not graded and some current traces exhibited notches, both features reflecting poor control of the membrane potential of the cell. These cells were discarded and the analysis was performed on the remaining 30%. In these remaining cells, the time course of the sodium current was characterized by a comparatively fast rising phase followed by a slow inactivation (Fig. 1B). For membrane potentials between $-40$ mV and $+20$ mV (values for which the inward current was large and the outward current was negligible), the current traces were fitted with a combination of an exponentially rising phase, an exponentially falling phase and a plateau. In most cases, as in the experiments illustrated in Fig. 1B, the time constants exhibited little, if any, voltage sensitivity between $-30$ and 0 mV (mean time constant of activation $3.6 \pm 0.64$ ms, mean time constant of inactivation $46 \pm 20$ ms for the cell illustrated in Fig. 1B).

Steady-state inactivation

Changing the holding potential of the cells from $-80$ mV to $-40$ mV was found to have little effect on the time course and the intensity of the deltamethrin-induced current. This feature is illustrated in Fig. 1D (for the intensity). Long-lasting changes in the holding potential (several minutes) were, however, found to modulate the size of the current in the direction expected for long-term inactivation; thus, the peak current was consistently smaller for a holding potential of $-60$ mV if the membrane had been held for some time at $-40$ mV. Conversely, the peak current was larger than the control if the membrane had previously been held at $-80$ mV.

Single-channel experiments

Single-channel activity induced by deltamethrin was studied using the cell-
Fig. 2. Various patterns of single-channel activity induced by applying 0.5\( \mu \text{mol}l^{-1} \) deltamethrin onto the membrane of a cockroach neurone. Cell-attached configuration of the patch-clamp technique. Patch held at H90 mV (i.e. hyperpolarized by 90 mV compared to rest). Outward current blocked with TEA\(^+\) (10 mmol\( l^{-1} \)) and 4-AP (1 mmol\( l^{-1} \)). Low-pass filter set at 3 kHz; sampling frequency 10 kHz. Note the presence in the same recording of rectangular events (\( \blacktriangledown \)), of long openings (■) and of complex bursts of activity (●).

attached configuration of the patch-clamp technique. Deltamethrin was added to the solution contained in the patch pipette at concentrations ranging from 0.01 to 10 \( \mu \text{mol}l^{-1} \).

Under normal conditions (i.e. in the absence of any agonist in the bathing medium or in the patch pipette and with 10 mmol\( l^{-1} \) Ca\(^{2+}\) in the external solution), the single-channel activity observed in these neurones was outwardly directed and corresponded to that of voltage-dependent and/or calcium-dependent potassium channels (Christensen \textit{et al.} 1988). It was blocked in the present experiments using high concentrations of TEA\(^+\) and 4-AP so that no detectable activity could be recorded.

Addition of deltamethrin to the pipette solution induced characteristic, but complex, single-channel activity (Fig. 2) that was totally abolished in the presence of TTX. Three distinct patterns of activity could be observed: relatively simple rectangular openings of variable duration, long openings of variable amplitude and bursts of complex events. The three patterns were often mixed in the same recording and were not characteristic of any given patch. The effects of membrane potential on the first pattern of activity (which is easier to analyze) are described in the following section. The properties of the other two patterns are briefly described in the last two sections.
Effects of membrane potential

The effects of deltamethrin on single-channel activity were investigated for a wide range of potentials ranging from 150 mV in the hyperpolarizing direction to 100 mV in the depolarizing direction (approximately −190 mV to +60 mV absolute membrane potential, based on an estimated resting potential of −40 mV). However, for depolarized membrane potentials, reliable estimates of the amplitude and kinetics of the single-channel events were seriously hampered by two factors: the comparatively large background noise (1–1.5 pA peak to peak) at 3 kHz and the presence of a small population of (outward) potassium channels that were not totally blocked by TEA⁺ and 4-AP. The quantitative analysis was, therefore, limited to potentials ranging from 150 to −20 mV (i.e. approximately −190 mV to −20 mV absolute membrane potential).

Effect of membrane potential on the mean single-channel current and on single-channel amplitude distribution. As in the experiment illustrated in Fig. 3, the

Fig. 3. Effect of membrane potential on single-channel activity induced by the addition of 0.1 μmol l⁻¹ deltamethrin to the solution contained in the patch pipette. Cell-attached configuration of the patch-clamp technique. Outward current blocked with TEA⁺ (10 mmol l⁻¹) and 4-AP (1 mmol l⁻¹). The activity was recorded at five different potential levels, as indicated; hyperpolarized by 20–60 mV compared with rest. Low-pass filter set at 3 kHz, sampling frequency 10 kHz. In this recording, only two categories of events could be distinguished: rectangular openings and short unresolved events. These two categories of event, which could be superimposed, as shown for H40 mV, increased in size but not in duration with membrane hyperpolarization.
The current amplitude of the induced single-channel activity increased following membrane hyperpolarization. The relationship between the mean single-channel amplitude and membrane potential was approximately linear, with a slope of around 20 pS and an extrapolated reversal potential between +20 and +90 mV (absolute potential) (Fig. 4).

Amplitude histograms were made for different potential values. As a rule, the amplitude histograms were not symmetrical (Fig. 5) and could therefore not be fitted with a single Gaussian curve, but only by multiple Gaussian curves corresponding to the various conductive states of the channels. Measurement of the amplitude of the first peak in an experiment in which this amplitude could be clearly defined for several potential values yielded the following values: 0.882, 1.372, 1.764 and 1.960 pA for pipette potentials, respectively, of 0, +40, +60 and +70 mV. These four values fell on a straight line ($r=0.992$) with a slope of 15.2 pS and a reversal potential of −56 mV (around +20 mV absolute membrane potential).

**Effect of membrane potential on the relative mean open time and the open time histogram.** The effect of membrane potential on the open time was studied systematically for five patches where the number of events at each potential level was sufficiently large. Under these conditions, the mean single-channel duration, which varied between 0.1 and 1 ms, was insensitive to changes in membrane potential (Fig. 6A). A possible change in the open time distribution was estimated...
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Fig. 5. Amplitude histogram of the 'single-channel' activity induced by 1 \( \mu \text{mol} \text{l}^{-1} \) deltamethrin. Holding potential \( +70 \text{mV} \) (70 mV hyperpolarized compared with rest). The histogram shows a main peak at approximately 1.3 pA but cannot be fitted with a single Gaussian curve, reflecting the existence of multiple conducting states.

Fig. 6. Effects of membrane potential on the relative mean open time (A) and the relative open time probability (B) of the deltamethrin-induced single-channel activity from different cell-attached patches. Despite a large scatter, the same general trends can be observed. (A) The mean open time was in the millisecond range and showed little voltage-dependency. (B) The relative open time probability was very low (0.001) at the resting potential level and increased significantly with membrane hyperpolarization. The interrupted line in B corresponds to an exponential fit of the pooled data \( (N=46) \) and had a correlation coefficient of 0.627.
by comparing the duration histograms of the same patch held at five different holding potentials. It was found that there was no significant change in the distribution between 0 and 80 mV (not shown).

Effects of membrane potential on the relative open time probability. The effect of membrane potential on the relative open time probability was studied for five membrane patches as above and the results are summarized in Fig. 6B. The relative open time probability, which was very low (around 0.001) at the resting level, increased significantly to approximately 0.01 when the membrane was hyperpolarized by 100 mV.

Long-duration channel openings

Long-duration channel openings were studied on selected records. The sampling frequency was reduced fivefold to 2 kHz and the data were filtered at 1 kHz. Selected records of some of these events are illustrated in Fig. 7A. Their open time and amplitude varied widely even in the absence of any change in the holding potential (here 60 mV). The amplitude histogram illustrated in Fig. 7B is typical: it is asymmetrical and exhibits several peaks. As illustrated in Fig. 7C for the same data, the open time histogram of these channels resembled that of normal events on a 50 times expanded time scale.

Bursting channels

The properties of the bursts were studied on selected records filtered at 1 kHz and acquired at 10 kHz. During a burst, two categories of events could be distinguished: very short triangular events (i.e. events that were not adequately resolved owing to the limited bandwidth of the recording system), during which the trace jumped directly from zero to one level or another and jumped back almost immediately to zero, and longer complex events during which the trace moved almost continuously from one level to another (Fig. 8A). Analysis of such bursts gave amplitude histogram distributions very similar to those observed normally but included many more events. The histogram is again asymmetrical, sometimes consisting of a succession of reasonably well-resolved peaks. In the analysis illustrated in Fig. 8B, the peaks were located at 0.976, 1.954, 2.93 and 4.1 pA. These values, which are multiples of 1 pA, corresponded almost exactly to the levels reached by the brief events. The duration histogram (Fig. 8C) could be fitted by two exponentials with time constants almost identical to those of normal events.

Discussion

The data presented here clearly indicate that low concentrations of deltamethrin activate a small TTX-sensitive sodium conductance in cultured embryonic neurones of the cockroach.

Whole-cell recordings

The kinetics of the current are much slower and less sensitive to changes in
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Fig. 7. Long-duration single-channel activity induced by 0.5 \( \mu \)mol l\(^{-1} \) deltamethrin in a cell-attached patch held hyperpolarized by 60 mV compared with rest. Outward current was blocked with TEA\(^+\) (10 mmol l\(^{-1} \)) and 4-AP (1 mmol l\(^{-1} \)). (A) Selected non-consecutive traces of the single-channel activity. Low-pass filter set at 1 kHz, sampling frequency 2 kHz. The interrupted line indicates zero current. Note that the size and the duration of the single-channel events were variable. (B, C) Amplitude (B) and duration (C) histograms of the events illustrated in A analyzed using the PAT2 program with a minimum open time of 2 ms and a minimum closed time of 25 ms. As expected from A, the amplitude of the single-channel activity could not be fitted by a simple Gaussian function. The duration histogram was best fitted by two exponential functions with a fast time constant of 4.3 ms and a slow time constant of 86 ms \((r=0.9979)\) (interrupted line).

membrane potential than the normal sodium currents. In some respects, the kinetics of the deltamethrin-induced current are reminiscent of those of the calcium current in adult cockroach neurones (M. Amar and Y. Pichon, in preparation). A further important difference between the deltamethrin-induced current and the fast axonal sodium current is the absence of steady-state inactivation.

As expected from our previous experiments with pyrethroid insecticides, the current–voltage relationship differs significantly from that of the normal axonal
Fig. 8. Bursting activity induced by 5 μmol l⁻¹ deltamethrin in a cell-attached patch held hyperpolarized by 60 mV compared with rest. Outward current was blocked with TEA⁺ (10 mmol l⁻¹) and 4-AP (1 mmol l⁻¹). Low-pass filter set at 1 kHz, sampling frequency 10 kHz. (A) Selected non-consecutive tracings showing the single-channel activity during a burst. For each recording, the upper interrupted line corresponds to zero current and the four others to the main peaks of the amplitude histogram illustrated in B. As in Fig. 2, the events that constituted the bursts could be divided into two categories: simple short unresolved events and complex events during which the current jumped between up to six or seven distinct levels, probably corresponding to substates of the sodium channel. (B,C) Amplitude (B) and duration (C) histograms of the events illustrated in A analyzed using the PAT2 program with both minimum open and minimum closed time set to 0. Here again, the amplitude histogram was asymmetrical with a main peak at 0.976 pA and a succession of smaller peaks at 1.954, 2.93 and 4.10 pA (▽). The duration histogram was fitted by a double exponential function with a fast time constant of 0.73 ms and a slow time constant of 4.4 ms (r=0.999) (interrupted line).

(Pichon and Boistel, 1967; Pichon, 1968, 1974) or neuronal (Lapied et al. 1990; M. Amar and Y. Pichon, in preparation) sodium currents. The peak current is displaced by 20–30 mV towards more depolarized potentials. As illustrated in Fig. 9, the voltage-dependency of the deltamethrin-induced sodium current is
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Fig. 9. Relationship between membrane potential and the pyrethroid-induced sodium conductance. The open triangles correspond to the tail current induced by S-bioallethrin in cockroach axons (modified from Pichon and Pelhate, 1985), the filled triangles correspond to the whole-cell current induced by 1 μmol l−1 deltamethrin in cultured cockroach neurones. The interrupted line corresponds to the best fit of the axonal data of equation 1 of Hodgkin and Huxley (1952) \((y=1/[1+e^{(V-V_m)/R}]\)), the dotted line represents the voltage-dependency of the fast sodium current of cockroach axons (Pichon, 1974).

almost identical to that of the tail current recorded after application of S-bioallethrin (Pichon and Pelhate, 1985) or methanotetramethrin (Laufer et al. 1984; Pichon et al. 1987) to the cockroach axonal preparation. The current density (around 100 μA cm−2 assuming a cell diameter of 10 μm) is very close to that reported for similar concentrations of insecticide molecules on the axon (Pichon et al. 1987). These results are in full agreement with our proposed hypothesis concerning the mode of action of pyrethroid insecticides.

In most, but not all, respects the effects of deltamethrin were reminiscent of those of similar concentrations of veratridine on the same preparation (Amar et al. 1991). The origin of the induced conductance remains speculative. The simplest interpretation of our data, which fits in well with experiments by other authors on non-excitable tissues or cultured cells (Romey et al. 1979), would be that the insecticide unveils ‘silent’ channels (i.e. channels that are present in the membrane but are not functional). The sensitivity to TTX supports this interpretation. Another interpretation would be that functional sodium channels are present in the membrane but their opening time is so short that they contribute very little to the whole-cell current and they are not detectable under patch-clamp conditions because of the limited bandwidth of the patch amplifiers. The effect of the insecticide molecule would be to lengthen the open time of these channels so that the net current increases and the single-channel activity becomes detectable.

The shift from the current–voltage relationship of the deltamethrin-induced sodium current to that of the fast axonal or neuronal sodium currents observed in
the same species is reminiscent, as for veratridine (Amar et al. 1991), of that observed by Goldin et al. (1990) for the sodium current induced by rat brain IIα subunit of the voltage-gated sodium channel injected into Xenopus oocytes. It is also suggestive of a modification of the (voltage-sensitive) S4 segment of domain II of the channel protein.

Single-channel recordings

Concentrations of deltamethrin ranging from $10^{-8}$ to $5 \times 10^{-6}$ mol$^{-1}$ induced complex but characteristic single-channel activity. Despite this complexity, the current–voltage relationship of the mean and peak currents could usually be fitted with a straight line with an extrapolated reversal potential between 30 and 90 mV (i.e. not very different from $E_{Na}$). The corresponding mean single-channel conductance (20 pS) is very similar, although slightly lower, than that observed with similar concentrations of veratridine on the same preparation (25.6 pS, Amar et al. 1991) and is of the same order of magnitude as that reported for normal sodium channels in neuroblastoma cells (Quandt and Narahashi, 1982; Nagy et al. 1983; Yamamoto et al. 1984; Chinn and Narahashi, 1986, 1990; Nagy, 1987; Barnes and Hille, 1988), for vertebrate muscle sodium channels (Sigel, 1987a), for neuronal sodium channels from wild-type Drosophila (Stühmer et al. 1987) and for mutant Drosophila neuronal sodium channels (Stühmer et al. 1989) expressed in Xenopus oocytes. It is slightly lower than the 21–25 pS reported for chick neuronal sodium channels expressed in Xenopus oocytes (Sigel, 1987b).

Another similarity between our single-channel data and those obtained on other preparations with or without treatment with sodium channel ‘agonists’, such as batrachotoxin, veratridine or pyrethroid insecticides, is the presence of channels with more than one conducting state. The conclusion that the recorded activity corresponds to multiple states of the same channel rather than to the superimposition of a number of independent channels is based on several considerations. The first is that, if one considers the extremely low open time probability of the single-channel events under our experimental conditions (around 1%, Fig. 6), the number of superimposed events greatly exceeds that predicted from the random superimposition of independent channels. The second argument is the observation that the number of transitions exceeding one or two unitary levels is several orders of magnitude larger than predicted on the basis of independent channels. The third argument is based on the statistical distribution within the bursts of the various time courses of ‘complex’ events (see Colquhoun and Hawkes, 1983). This was particularly obvious in the case of ‘bursting’ channels, where up to seven or eight ‘levels’ could be clearly separated, the lowest level having a calculated conductance of 6 pS, i.e. about one-third of the mean conductance value. Quite unexpectedly, and at variance with previous reports and our own observations on veratridine-treated neurones, the levels appeared to be exact multiples of the smallest unitary conductance. This observation is consistent with the hypothesis proposed by Sigel (1987b) that the small conductance could represent the real unitary conductance of the sodium channel and that the normally observed
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Fig. 10. Example of the distribution of the transitions and the conductance levels during a burst of activity induced by $10^{-6}$ mol l$^{-1}$ deltamethrin. (A) Number of occurrences of each transition category plotted against its size. (B) Number of occurrences of each conductance level plotted against its size. The interrupted lines correspond to fits of the experimental data with one exponential, the dotted line corresponds to a fit of levels 3–10 with one exponential. The transition size was taken as the size of an abrupt change in current from one level to another (size 2 would, for example, correspond to a direct transition from level 0 to level 2 or to a direct transition from level 3 to level 1). The level size was taken as the value of the current after each step. Both sizes were expressed as multiples of the unitary current, which was taken as one. For explanations, see text. Membrane potential hyperpolarized by 60 mV.

Conductances reflect the cooperative opening of several elementary channels (corresponding, for example, to three of the four $\alpha$-subunits of the sodium channel protein).

The large number of substates observed in our experiments could reflect the complexity of a primitive sodium channel and the fact that our experiments were performed at a comparatively high temperature (24–26°C). Thus, in recent experiments on deltamethrin-treated neuroblastoma cells, Chinn and Narahashi (1990) observed that the number of subconducting states was greater at room temperature than at 11°C. To test these hypotheses, it would be of interest to repeat our experiments on dissociated adult cockroach neurones that express functional sodium channels and to repeat the experiments at a lower temperature.

One interpretation of our data could be that the observed activity results from the transient aggregation of a variable number of (possibly identical) subunits (Pichon and Amar, 1991). The amount of cooperativity between the elementary channels can be estimated from the number of occurrences of each subconductance level and the distribution of sizes of the transitions. Thus, from the recording partly illustrated in Fig. 8, the distribution of the number of transitions of each category plotted against their size can be fitted with a single decaying exponential with a slope of 0.59±0.03 (Fig. 10A). The corresponding distribution of the number of occurrences of each conductance level against its size can be fitted by a plateau (between levels 1 and 3) followed by a decaying exponential (between levels 3 and 10) with a slope of 0.76±0.03 (Fig. 10B). These results suggest (1) that
the cooperativity between each individual opening is the same for all subunits, (2) that the probability of recording conductance levels 1, 2 and 3 is comparatively higher than that of recording higher conductance levels. It would be of interest to establish the correlation between these results and the structure of the putative sodium channel in embryonic cockroach neurones.

Mode of action of deltamethrin

Our experiments suggest that deltamethrin activates 'silent' sodium channels. The origin of these channels remains unknown. It is interesting to note, however, that, in the central nervous system of arthropods and particularly of insects, cell bodies are generally inexcitable (Hoyle, 1970; Pichon and Ashcroft, 1985), which agrees with our findings in cultured neurones, but may become excitable and give fairly normal overshooting sodium spikes following colchicine treatment (Pitman, 1975) or dissociation (Wu et al. 1983; O'Dowd and Aldrich, 1988; Gundel et al. 1989; M. Amar, unpublished observation). This indicates that, at least in these neurones, fast sodium channels may exist in the membrane in a non-functional configuration and may be transformed into functional channels under certain conditions. Insect neurones may prove to be very useful in the study of the expression of functional sodium channels during development and following various physical and/or chemical treatments.

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References

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