Biochimica et Biophysica Acta, 600 (1980) 882—897

BBA 78890

INTERACTION OF PYRETHROIDS WITH THE Na^+ CHANNEL IN MAMMALIAN NEURONAL CELLS IN CULTURE

Y. JACQUES, G. ROMEY, M.T. CAVEY, B. KARTALOVSKI and M. LAZDUNSKI

Centre de Biochimie du CNRS, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex (France)

(Received February 15th, 1980)

Key words: Pyrethroid; Na^+ channel; Neuroblastoma cell

Summary

The interaction of a series of pyrethroids with the Na^+ channel of mouse neuroblastoma cells has been followed using both an electrophysiological and a $^{22}Na^+$ influx approach. By themselves, pyrethroids do not stimulate $^{22}Na^+$ entry through the Na^+ channel (or the stimulation they give is too small to be analyzed). However, they stimulate $^{22}Na^+$ entry when used in conjunction with other toxins specific for the gating system of the channel. These include batrachotoxin, veratridine, dihydrograyanotoxin II or polypeptide toxins like sea anemone and scorpion toxins. This stimulatory effect is fully inhibited by tetrodotoxin with a dissociation constant of 1.6 nM for the tetrodotoxin-receptor complex. Half-maximum saturation of the pyrethroid receptor on the Na^+ channel is observed in the micromolar range for the most active pyrethroids, Decis and RU 15525. The synergism observed between the effect of pyrethroids on $^{22}Na^+$ influx on the one hand, and the effects of sea anemone toxin II, *Androctonus* scorpion toxin II, batrachotoxin, veratridine and dihydrograyanotoxin II on the other, indicates that the binding component for pyrethroids on the Na^+ channel is distinct from the other toxin receptors. It is also distinct from the tetrodotoxin receptor.

Some of the pyrethroids used in this study bind to the Na^+ channel but are unable to stimulate $^{22}Na^+$ entry. These inactive compounds behave as antagonists of the active pyrethroids.

An electrophysiological approach has shown that pyrethroids by themselves are active on the Na^+ channnel of mammalian neurones, and essentially confirm the conclusions made from $^{22}Na^+$ flux measurements.

Pyrethroids are also active on C9 cells in which Na^+ channels are 'silent', that is, not activatable by electrical stimulation. Pyrethroids chemically activate the silent Na^+ channel in a manner similar to that with veratridine, batracho-

toxin, or polypeptide toxins, which are known to slow down the inactivation process of a functional Na^+ channel.

Introduction

The natural insecticides, pyrethrins, and their synthetic pyrethroid derivatives, have been clearly demonstrated to act on the neuromuscular system of insects and crustaceans, inducing hyperexcitability, convulsions and eventual paralysis.

Direct application of pyrethroids to excised insect tissues causes an immediate hyperexcitation of the nerve activity and a block at higher concentrations [1—4].

Pyrethroids also interact with synaptic transmission [5—7]. It has even been suggested that synapses are the initial sites of action of these neurotoxins since they are affected at lower concentrations than nerve fibers [5,8].

Detailed electrophysiological analyses [9] have shown that these neurotoxins induce a marked prolongation of the sodium permeability during the action potential, leading to an increased negative after-potential which can be accompanied by repetitive after-discharges. At high concentrations, they depress both the sodium and potassium permeabilities, resulting in a complete blockade of nerve excitability. This mode of action has been demonstrated on cockroach giant axons [10], squid giant axons [11] and crayfish giant axons [12].

While numerous studies have been carried out on arthropod nerves, there is little information on the action of pyrethroids on vertebrate excitable membranes. Relatively large doses of pyrethroids are required to produce toxic symptons in mammals [13—15], which may be related to the high oxidative metabolism of pyrethrins in these animals [16]. Pyrethroids, which are very active on arthropod nerve fibers, have virtually no blocking action on peripheral nerves of the rat and frog [6,17]. However, allethrin induces a large negative after-potential and a marked repetitive activity in sensory fibers of the frog [17]. Sense organs are also sensitive to allethrin [18], and repetitive activity of presynaptic origin is produced at the motor end-plate by concentrations as low as 0.1 μM [19]. A similar action was reported for NRDC 119 (cismethrin), another synthetic pyrethroid [20].

Mammalian cells in culture have recently been shown to be very useful to study the mode of action of drugs which affect ionic channels [21,22]. The present work describes the properties of interaction of a series of pyrethroids with Na^+ channels of neuronal cell lines in culture.

Materials and Methods

Cell cultures. The adrenergic neuroblastoma NIE115 cells were grown essentially as described previously [21]. Fetal calf serum was reduced to 5% in stock cultures and the medium used to induce morphological differentiation of cells contained 1% fetal calf serum and 1.5% Me_2SO.

C9 cells were propagated as previously described [22] in a medium containing 5% fetal calf serum.

$^{22}Na^+$ uptake measurements. Na^+ uptake measurements with neuroblastoma cells were carried out at 37°C. After suction of the culture medium, cells were preincubated for 10 min in an Na^+-free medium buffered with 25 mM Hepes-Tris (pH 7.4) and containing 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 140 mM choline chloride, 5 mM glucose, 0.1 mg/ml bovine serum albumin and the neurotoxins under study. $^{22}Na^+$ uptake was initiated by replacement of the preincubation medium with a $^{22}Na^+$-labelled medium which was identical to the preincubation medium except that it contained 10 mM NaCl and 130 mM choline chloride, 0.5 mM ouabain and 0.6 μCi/ml of $^{22}NaCl$. Termination of uptake and determination of the initial rates of $^{22}Na^+$ influx were performed as described previously [21].

Na^+ uptake measurements with C9 cells were carried out at 37 and 5°C as described for neuroblastoma cells, except that the uptake medium contained 140 mM NaCl instead of 10 mM. Each experimental point in a series of experiments to obtain a given curve has been performed at least twice. Experimental errors were always within 5%.

Electrophysiological measurements. Culture dishes containing NIE115 and C9 cells were used directly for electrophysiological analysis after replacing the culture medium with an Earle medium (140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 25 mM Hepes-Tris, 5 mM glucose) buffered at pH 7.4. The culture dish was placed on the warm stage of an inverted microscope (Leitz-Diavert) and the temperature was maintained between 35 and 37°C. Glass capillary microelectrodes were used for intracellular recording and stimulation. They were filled with 3 M KCl and has resistance ranging between 10 and 30 MΩ. The reference electrode was an Ag/AgCl half-cell placed in the external medium. The microelectrode was connected to a negative capacitance electrometer amplifier (WPI, M707) with an active bridge network which enabled simultaneous injections of current and recording through the same microelectrode. Electrical activity was simultaneously digitized and stored by a digital computer (Intertechnique-Plurimat S) and displayed on storage oscilloscope (Tektronix 5103N).

Purification of the sea anemone toxin II. The purification of toxin II from the sea anemone, *Anemonia sulcata*, was carried out according to the method of Beress et al. [23,24] with minor modifications [25].

Chemicals and other toxins. Veratridine was purchased from Aldrich; ouabain, choline chloride and bovine serum albumin (fraction V) from Sigma; tetrodotoxin (citrate-free) from Sankyo; Dulbecco's modified Eagle's medium (catalogue No. H21) and fetal calf serum from Gibco; $^{22}NaCl$ from the Commissariat à l'Energie Atomique (Saclay).

Scorpion neurotoxin II from *Androctonus australis Hector* was given to us by Professor Lissitzky and his colleagues. Batrachotoxin was kindly provided by Dr. Daly (Bethesda, NIH) and Dr. Tokuyama (Osaka, Japan). Dihydrograyanotoxin II was a generous gift from Dr. Nakajima (Kyoto, Japan).

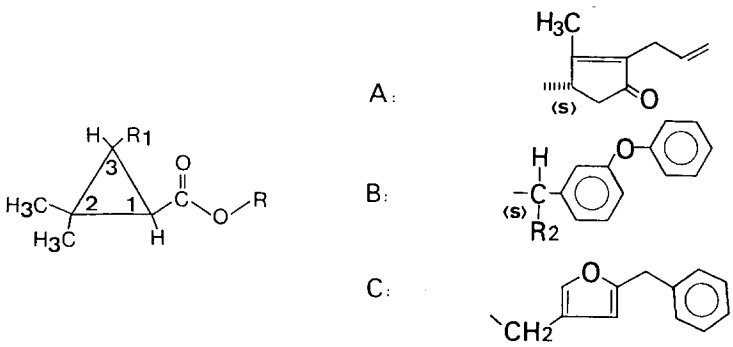
Pyrethroid compounds studied in this work were kindly donated by the Procida Company. They were dissolved in dimethyl sulfoxide (Me_2SO) or ethanol to make stock solutions of 10 mM. Concentrations of Me_2SO in experiments with neuroblastoma cells were kept constant at 1.5% Me_2SO, a concentration identical to that present in the culture medium used for differentiation.

There was no difference in flux measurements or electrophysiological results between 0 and 1.5% Me_2SO. In experiments with C9 cells, stock solutions of pyrethroids were made in ethanol; the final concentration of ethanol in $^{22}Na^+$ flux or electrophysiological measurements did not exceed 1%, a concentration which had no effect on the electrical or flux properties of these cells.

Results

The effect of pyrethroids on $^{22}Na^+$ influx into neuroblastoma cells

The structures of pyrethroids used in this work are presented in scheme I.



Name (Roussel-Uclaf) terminology	other names	R_1	R	R_2	Stereochemistry
	bioallethrin	$CH{=}C(CH_3)_2$	A	-	1R, 3R
Decis	NRDC161 : decamethrin	$CH{=}C(Br)_2$	B	CN	1R, 3S
RU 22090	cispermethrin	$CH{=}C(Cl)_2$	B	H	1R, 3S
RU 11484	NRDC107 : bioresmethrin	$CH{=}C(CH_3)_2$	C	-	1R, 3R
RU 12063	NRDC119 : cismethrin	$CH{=}C(CH_3)_2$	C	-	1R, 3S
RU 11679	ethanochrysanthemate	H C=	C	-	1R, 3R
RU 15525 RU 25475	'kadethrine'	H C= (E)	C	-	1R, 3R
		$CHBr{-}CRrCL_2$ (R+S)	B	CN	1R, 3S

SCHEME I. Nomenclature and structure of pyrethroids.

All have been shown to be active on crustacean axons (crab and crayfish), on which they produce (i) a decrease in amplitude of the action potential and a slowing down of the repolarisation phase, and (ii) the appearance of a negative after-potential after a repetitive stimulation of the excitable membrane. These toxic compounds are active at the following threshold concentrations: RU 15525 and RU 22090, 10 nM; RU 11484 and RU 12063, 0.1 μM; RU 11679 (and bioallethrin), 1 μM (Cavey, M.T., Romey, G., and Lazdunski, M., unpublished results).

In the presence of 10 mM external Na^+ and 0.5 mM ouabain (the latter to inhibit the Na^+ efflux catalyzed by ($Na^+ + K^+$)-ATPase), neuroblastoma cells accumulate $^{22}Na^+$ at an initial rate of 3 nmol $^{22}Na^+$/min per mg protein (Fig. 1A). The alkaloid, veratridine, which is well known to interact with the Na^+ channel of various excitable systems [26,27], increases the initial rate to 13 nmol $^{22}Na^+$/min per mg protein at a concentration of 100 μM. This stimu-

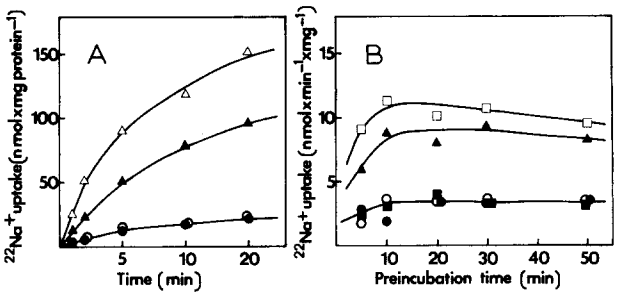


Fig. 1. Stimulatory effects of veratridine and Decis on $^{22}Na^+$ uptake by neuroblastoma cells: influence of preincubation. (A) Cells were preincubated for 10 min in Na^+-free medium, and $^{22}Na^+$ uptake was measured at the indicated times as described in Materials and Methods. Preincubation and uptake media were as follows: control (●———●); 10 μM Decis (○———○); 100 μM veratridine (▲———▲); 10 μM Decis + 100 μM veratridine (△———△). (B) Initial rates of $^{22}Na^+$ uptake by neuroblastoma cells were measured after different preincubation periods in Na^+-free medium. Media contained either no toxin (●———●), 10 μM Decis (○———○), 10 μM RU 15525 (■———■), 50 μM veratridine (▲———▲) or 50 μM veratridine + 2 μM RU 15525 (□———□).

latory effect, in contrast to the basal influx observed in the presence of ouabain only, is fully reversed upon addition of 100 nM tetrodotoxin (data not shown). Pyrethroid molecules alone have no significant effect on the rate of $^{22}Na^+$ uptake by neuroblastoma cells (Fig. 1). However, in the presence of veratridine, some of these compounds induce an increase in the initial rate of $^{22}Na^+$ influx. For example (Fig. 1), the addition of 10 μM Decis in the presence of 100 μM veratridine stimulates the initial rate from 13 to 26 nmol $^{22}Na^+$/min per mg protein. The most active compounds with this type of assay are Decis and RU 15525.

In order to ensure the association of the neurotoxins with their membrane receptors without any intracellular Na^+ accumulation, cells were preincubated in an Na^+-free medium before the $^{22}Na^+$ uptake measurements. Maximal activation by veratridine, and a maximal synergistic effect between 50 μM veratridine and 2 μM RU 15525 were observed after 10 min of preincubation (Fig. 1B). Except when indicated otherwise, these conditions of preincubation have been adopted throughout this work. Data in Fig. 1B also show that even after 50 min of association in an Na^+-free medium, Decis and RU 15525 hardly influence the rate of $^{22}Na^+$ influx in the absence of veratridine.

The dose-response curve depicting the effect of veratridine is shown in Fig. 2A. A maximum rate of 24 nmol $^{22}Na^+$/min per mg protein can be extrapolated at saturating concentrations of the toxin. A half-maximum effect ($K_{0.5}$) is observed at about 100 μM. In the presence of 10 μM Decis or 10 μM RU 15525, the maximum stimulatory effect of veratridine is increased without any significant alteration of the $K_{0.5}$ value. Conversely, Decis and RU 15525, which alone are not active, stimulate the initial rate of $^{22}Na^+$ influx in the presence of 100 μM veratridine (Fig. 2B). Their half-maximum effects are observed at 2 and 0.9 μM, respectively.

Alkaloid neurotoxins such as veratridine, batrachotoxin or aconitine, and dihydrograyanotoxin II, have been shown to compete for the same receptor site on the Na^+ channel in neuroblastoma cells [28]. Batrachotoxin is a better activator of the Na^+ channel than veratridine, inducing a rate of $^{22}Na^+$ influx

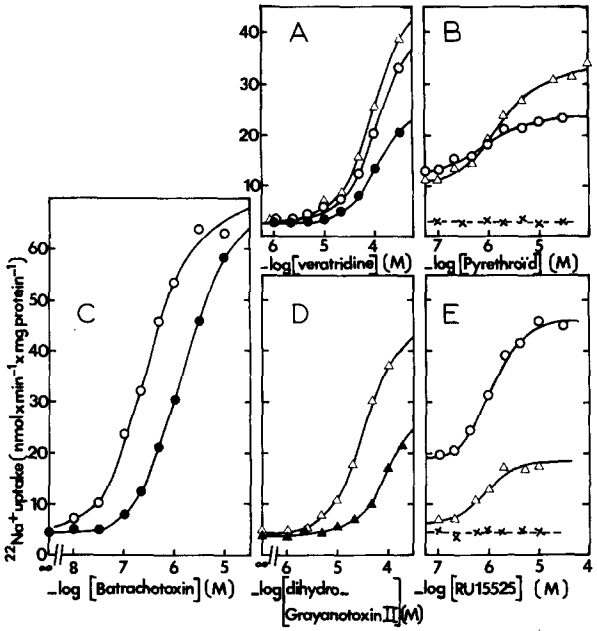


Fig. 2. Synergistic effects of pyrethroid and alkaloid neurotoxins in stimulating $^{22}Na^+$ uptake by neuroblastoma cells. (A) Dose-response curve for veratridine: control (●———●), in the presence of 10 μM RU 15525 (○———○), in the presence of 10 μM Decis (△———△). (B) Dose-response curves for the action of Decis in the absence (X———X) and presence (△———△) of 100 μM veratridine. The dose-response curve for RU 15525 was determined in the presence of 100 μM veratridine (○———○). (C) Dose-response curves for batrachotoxin in the absence (●———●) and presence (○———○) of 10 μM RU 15525. (D) Dose-response curves for dihydrograyanotoxin II in the absence (▲———▲) and presence (△———△) of 10 μM RU 15525. (E) Dose-response curves for RU 15525 alone (X———X) and in the presence of either 20 μM dihydrograyanotoxin II (△———△) or 0.5 μM batrachotoxin (○———○).

of 65 nmol $^{22}Na^+$/min per mg protein, with a $K_{0.5}$ value of 1.3 μM (Fig. 2C). Like veratridine, this alkaloid acts in synergy with pyrethroids. However, the synergy has different properties from those seen with veratridine. In the presence of 10 μM RU 15525, the dose-response curve relative to batrachotoxin is shifted towards lower concentrations ($K_{0.5}$ = 0.3 μM) but there is almost no effect on the maximum rate of $^{22}Na^+$ influx attained at saturating batrachotoxin concentrations. There also is a synergy of action between dihydrograyanotoxin II and RU 15525 (Fig. 2D). With these, the effects are intermediary between those observed with veratridine and batrachotoxin. The concentration required for half-maximum effect of dihydrograyanotoxin II ($K_{0.5}$ = 90 μM) is decreased upon addition of 10 μM RU 15525 ($K_{0.5}$ = 30 μM) and the maximum stimulatory effect at saturating concentrations of dihydrograyanotoxin II is enhanced from 30 to 48 nmol $^{22}Na^+$/min per mg protein in the presence of RU 15525. Like veratridine, batrachotoxin and dihydrograyanotoxin II stimulate the effect of pyrethroid on $^{22}Na^+$ influx (Fig. 2E). Half-maximum effects of RU 15525 are observed at 1.1 and 0.9 μM in the presence of 0.5 μM batrachotoxin and 20 μM dihydrograyanotoxin II, respectively.

The $^{22}Na^+$ influx activated by either alkaloid neurotoxins alone or by pyrethroid plus alkaloids are fully inhibitable by tetrodotoxin. Fig. 3A illustrates

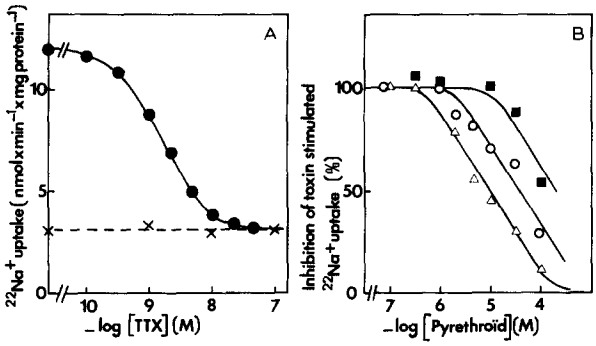


Fig. 3. (A) Inhibition by tetrodotoxin of the $^{22}Na^+$ uptake stimulated by the synergistic action of RU 15525 and veratridine on neuroblastoma cells. The initial rate of $^{22}Na^+$ influx was measured in the presence of 50 μM veratridine plus 10 μM RU 15525 after the addition of increasing concentrations of tetrodotoxin (●———●) (TTX). The basal $^{22}Na^+$ influx measured in the absence of neurotoxins was not affected by tetrodotoxin (X———X). (B) Inhibition of the $^{22}Na^+$ influx stimulated by the active compound RU 15525 by 'inactive' pyrethroids. Stimulation of the initial rate of $^{22}Na^+$ influx by RU 15525 is calculated from the difference between the rate obtained in the presence of 30 μM veratridine + 2 μM RU 15525 (100% stimulation) and that measured in the presence of 30 μM veratridine alone (0% stimulation). Influx was then measured in the presence of both toxins and increasing concentrations of RU 11484 (△———△), RU 22090 (○———○) or RU 12063 (■———■). The percent of the stimulatory effect induced by 2 μM RU 15525 remaining is plotted as a function of the concentration of the additional pyrethroid.

this property. Tetrodotoxin inhibits the $^{22}Na^+$ influx stimulated by 50 μM veratridine plus 10 μM RU 15525. The apparent dissociation constant for the tetrodotoxin-receptor complex estimated from Fig. 3A is 1.6 nM. This constant is nearly equal to the true constant for the tetrodotoxin-receptor complex in these cells [21], showing that pyrethroids do not alter the interaction of tetrodotoxin with its binding site.

Some pyrethroid molecules had no apparent effect on $^{22}Na^+$ uptake by neuroblastoma cells even in tne presence of 100 μM veratridine. The effect of increasing concentrations of these apparently inactive pyrethroids on the initial rate of $^{22}Na^+$ uptake stimulated by 2 μM RU 15525 in the presence of 30 μM veratridine is demonstrated in Fig. 3B. Most of these pyrethroid molecules are able to antagonize the stimulatory effect induced by RU 15525 in a dose-dependent manner. For example, RU 11484 gives a complete inhibition of the stimulatory effect with an apparent dissociation constant of 10 μM. RU 22090 and RU 12063 are less active, with half-maximum inhibition observed at 45 and 120 μM, respectively.

Interactions between pyrethroids and polypeptidic neurotoxins, sea anemone toxin II and scorpion toxin II

Sea anemone toxin II, one of the most abundant of the toxic polypeptides isolated from *A. sulcata* [23], has been demonstrated to interact with the Na^+ channel of neuroblastoma cells [21] and to interact in a synergistic manner with veratridine on this cellular system. As shown in Fig. 4, there is a similar type of synergy between the pyrethroid RU 15525 and sea anemone toxin II. Sea anemone toxin II alone stimulates the $^{22}Na^+$ uptake from 3 to 5 nmol/min per mg protein, with a half-maximum effect ($K_{0.5}$) of 0.3 μM. In the presence

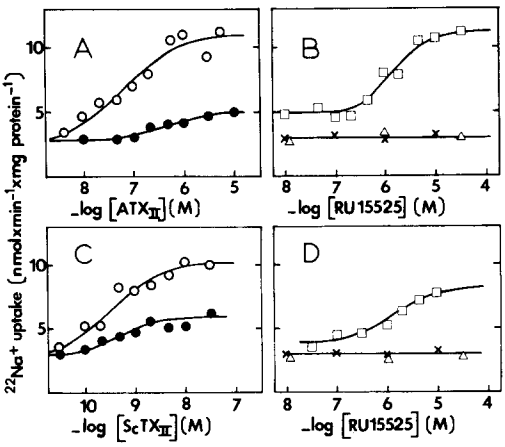


Fig. 4. Synergism between pyrethroids and polypeptide neurotoxins on $^{22}Na^+$ uptake by neuroblastoma cells. (A) Dose-response curves for sea anemone toxin II (ATX_{II}) in the absence (●———●) or in the presence (○———○) of 10 μM RU 15525. (B) Dose-response curve for RU 15525 in the absence (X———X) or in the presence (□———□) of 10 μM sea anemone toxin II, and in the presence of 10 μM sea anemone toxin II + 1 μM tetrodotoxin (△———△). (C) Dose-response curve for scorpion toxin II (S_cTX_{II}) in the absence (●———●) or in the presence (○———○) of 10 μM RU 15525. (D) Dose-response curve for RU 15525 in the absence (X———X) or in the presence (□———□) of 1 nM scorpion toxin II and with 1 nM scorpion toxin II plus 1 μM tetrodotoxin (△———△).

of 10 μM RU 15525, the rate of $^{22}Na^+$ uptake stimulated by sea anemone toxin II is raised to 11 nmol $^{22}Na^+$/min per mg protein. The $K_{0.5}$ value for sea anemone toxin II shifts from 0.3 to 0.1 μM in the presence of RU 15525.

In the presence of 10 μM sea anemone toxin II, increasing concentrations of RU 15525 stimulate $^{22}Na^+$ uptake from 5 to 11 nmol $^{22}Na^+$/min per mg protein (Fig. 4B). The concentration of RU 15525 giving a half-maximum stimulation of $^{22}Na^+$ influx is approx. 1 μM.

A similar type of interaction has been found between the receptors of pyrethroid molecules and the receptor of a polypeptide neurotoxin isolated from the venom of *A. australis Hector* (Fig. 4C and D). Scorpion toxin II stimulates the initial rate of $^{22}Na^+$ uptake from 3 to 6.5 nmol $^{22}Na^+$/min per mg protein in neuroblastoma cells. Its half-maximum effect is observed at 0.5 nM. In the presence of 10 μM RU 15525, the maximum rate of $^{22}Na^+$ influx is raised to 10 nmol $^{22}Na^+$/min per mg protein, whereas the $K_{0.5}$ value for scorpion toxin II is hardly affected (0.4 nM). In the presence of 1 nM scorpion toxin II, increasing concentrations of RU 15525 enhance the initial rate of $^{22}Na^+$ uptake from 3 to 8 nmol $^{22}Na^+$/min per mg protein with a half-maximal effect at 1 μM. As shown in Fig. 4 (B and D), tetrodotoxin blocks the stimulatory action of pyrethroids.

Interaction of pyrethroids with the Na^+ channel of C9 cells

C9 cells have Na^+ channels which remain silent upon electric stimulation but which can be chemically activated by sea anemone toxin II and also revealed by veratridine [22].

The action of pyrethroids on the initial rate of $^{22}Na^+$ uptake by C9 cells is illustrated in Fig. 5. None of the pyrethroids tested were able by them-

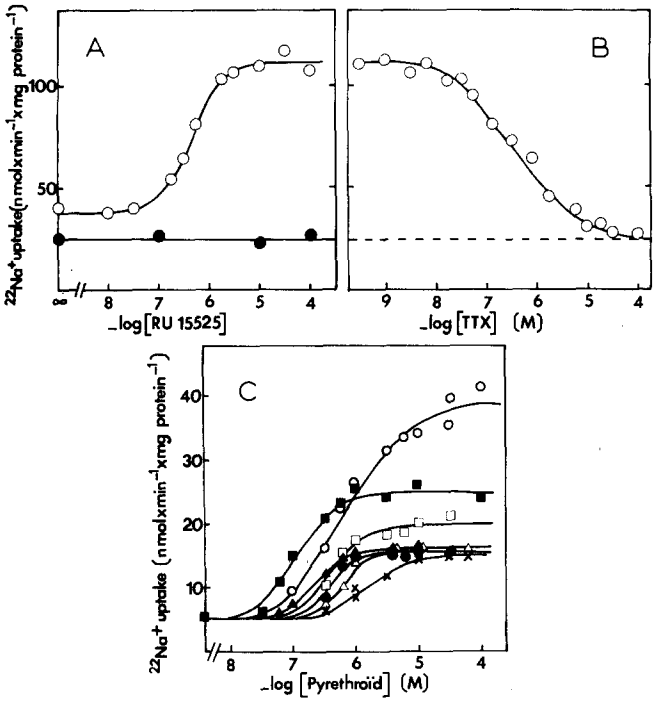


Fig. 5. The interaction of pyrethroids with the Na^+ channel of C9 cells. Synergism with sea anemone toxin II and inhibition by tetrodotoxin. The initial rates of $^{22}Na^+$ influx into C9 cells were measured at 37°C (A and B) or 5°C (C). (A) Dose-response curve for RU 15525 in the absence (●———●) or in the presence (○———○) of 10 μM sea anemone toxin II. (B) The effect of increasing concentrations of tetrodotoxin (TTX) on the stimulation of $^{22}Na^+$ influx by 10 μM RU 15525 plus 10 μM sea anemone toxin II. (C) Dose-response curves for various pyrethroid compounds in the presence of 10 μM sea anemone toxin II. Decis (○———○), RU 15525 (■———■), RU 22090 (●———●), RU 11484 (X———X), RU 12063 (△———△), RU 11679 (□———□) and RU 25475 (▲———▲).

selves to influence rates of $^{22}Na^+$ influx measured in the presence of ouabain. However, one observes again that pyrethroids interact in a synergistic manner with sea anemone toxin II to stimulate $^{22}Na^+$ influx. In the presence of 10 μM sea anemone toxin II, increasing concentrations of RU 15525 stimulate influx from 40 to 110 nmol $^{22}Na^+$/min per mg protein (Fig. 5A). The half-maximum effect is observed at 0.14 μM RU 15525. The stimulated $^{22}Na^+$ influx is fully inhibitable by tetrodotoxin (Fig. 5B), with a half-maximum inhibitory concentration of 0.47 μM. This value is close to that reported recently [22] for the apparent dissociation constant of the tetrodotoxin-receptor complex in the absence of pyrethroids. The low affinity of the Na^+ channel for tetrodotoxin in C9 cells is not changed by pyrethroids. The interaction of pyrethroids with the Na^+ channel has been reported to be temperature dependent [29,30], with increased sensitivity at low temperatures. Neuroblastoma cells are fragile and sustain cellular damage at low temperatures, whereas C9 cells are stable at temperatures between 5 and 15°C for at least 30 min. For this reason, C9 cells were used to investigate the activating effect of pyrethroids on $^{22}Na^+$ influx in the presence of sea anemone toxin II (10 μM) at 5°C. All pyrethroids tested stimulated $^{22}Na^+$ entry at this temperature

(Fig. 5C) with the most active compounds again being Decis and RU 15525. Although there are some differences between effects at 37 and 5°C, these differences are small. The main parameters describing the interaction of pyrethroids with the Na^+ channel of C9 cells at 5°C are the pyrethroid concentrations required for half-maximum effect ($K_{0.5}$). The $K_{0.5}$ values, determined from Fig. 5C, are 0.5 μM for Decis, 0.1 μM for RU 15525, 2.8 μM for RU 11484, 0.4 μM for RU 22090, 0.6 μM for RU 12063, 0.5 μM for RU 11679 and 0.16 μM for RU 25475. Pyrethroids form a class of compounds which differ from each other in their factor of stimulation of $^{22}Na^+$ influx in the presence of sea anemone toxin II, and by their apparent affinity for the Na^+ channel.

Electrophysiological analysis of pyrethroids effects on NIE115 neuroblastoma cells and C9 cells

Differentiated NIE115 neuroblastoma cells used in this study have resting potentials of −50 mV (±5 mV). Full-sized action potentials are elicited by brief depolarizing pulses of current after the membrane potential has been adjusted to a steady hyperpolarized level of −80 mV. The action potential can generally be decomposed into a fast spike of about 5 ms duration with an overshoot of 15—20 mV and a repolarizing phase with an exponential decay. The time constant of the decay varies between 5 and 50 ms depending upon the dimensions and the shape of the cell.

Among the pyrethroids which have been tested, only Decis, RU 15525 and RU 25475 have an effect on the electrical activity of NIE115 neuroblastoma cells. Their effects are very similar, particularly in that they act in the same range of concentration (greater than 0.1 μM). The main effect of these molecules is to prolong the action potential. The fast spike is generally followed by a second rising phase that is much slower than the initial one. From the peak value of this second phase, the membrane potential returns to its steady-state level with a typical time course consisting of an initial slow repolarizing phase followed by an exponential decay with a time constant of the order of 0.5 to 1 s (Fig. 6A). The fast spike is slightly affected by pyrethroids. The rising phase remains unchanged, and the repolarizing phase has a decreased rate of fall. In some experiments, the overshoot has an increased amplitude as shown in Fig. 6A. The resting potential is not significantly affected by pyrethroids even at high concentrations (10 μM).

Washing periods of more than 2 h with pyrethroid-free medium does not remove the pyrethroids' effects. Accordingly, from an electrophysiological point of view, it can be surmised that the pyrethroid effects are irreversible.

The pyrethroid effects can be inhibited by the application of 0.1 μM tetrodotoxin (Fig. 6B). Addition of the tetrodotoxin first causes a progressive decrease in the duration of the slow repolarizing phase and, second, a suppression of the spike.

Measurements of $^{22}Na^+$ influx demonstrated that pyrethroids act in synergy with sea anemone toxin II and with veratridine. Electrophysiological experiments confirm these properties (Fig. 7). The data shown are for Decis, however, similar results were obtained with RU 15525 and RU 25475; neither sea anemone toxin II (10 nM) nor Decis (0.1 μM) alter the action potential

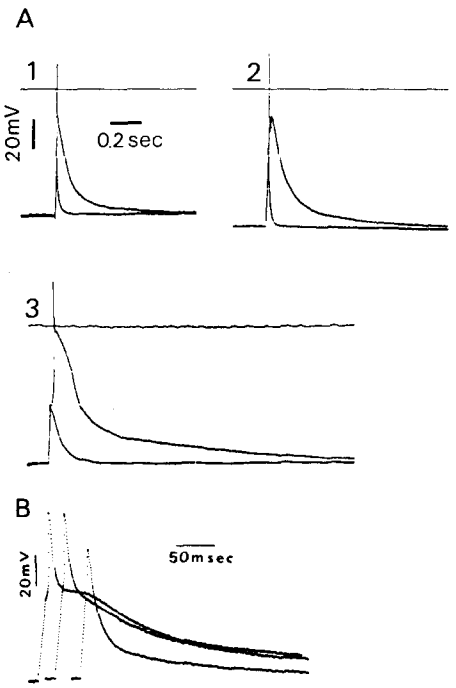


Fig. 6. (A) Effect of Decis concentration on the action potential of NIE115 neuroblastoma cells. (1) Control, stimulated action potential by a brief depolarizing pulse of current; the membrane potentials have been previously adjusted to a steady level of about —80 mV. (2) After a 30 min exposure to 1 μM Decis. (3) After a 30 min exposure to 10 μM Decis. In each record, the zero voltage line is indicated. (B) Reversal of Decis effects by tetrodotoxin. Left record: stimulated action potential from a NIE115 neuroblastoma cell treated with 1 μM Decis. Middle record: 2 min after the addition of 0.1 μM tetrodotoxin to the medium containing 1 μM Decis; note the decrease of the duration of the slow repolarizing phase. Right record: 5 min after the addition of 0.1 μM tetrodotoxin; note the decrease of the amplitude of the fast spike. The fast spike is suppressed after about 10 min exposure to 0.1 μM tetrodotoxin (not shown).

(Fig. 7A). However, a combination of the two at these concentrations gives rise to a plateau phase of approx. 100 ms duration. This plateau is characteristic of the action of sea anemone toxin II [31]. The synergy between Decis and veratridine is less marked. However, the effect of 10 μM Decis in the presence of 1 μM veratridine is more pronounced than the summation of the separate effects of the two toxins (Fig. 7B).

Despite their lack of electrophysiological effect on neuroblastoma cells, pyrethroids such as RU 11484, RU 22090, RU 12063 and RU 11679 can prevent the action of pyrethroids which do alter the electrical response of neuroblastoma cells (e.g., RU 15525, Decis and RU 25475). As shown in Fig. 8A, the application of 10 μM RU 11484 does not modify the action potential. Moreover, even 20 min after the addition of 1 μM RU 15525 to the preceding medium, the action potential remains unchanged. When the order of introduction of the two pyrethroids is reversed (Fig. 8B), the addition of RU 11484 does not counteract the previously demonstrated effect of RU 15525. RU 11484 was also inactive when tested in the presence of 10 nM sea anemone toxin II.

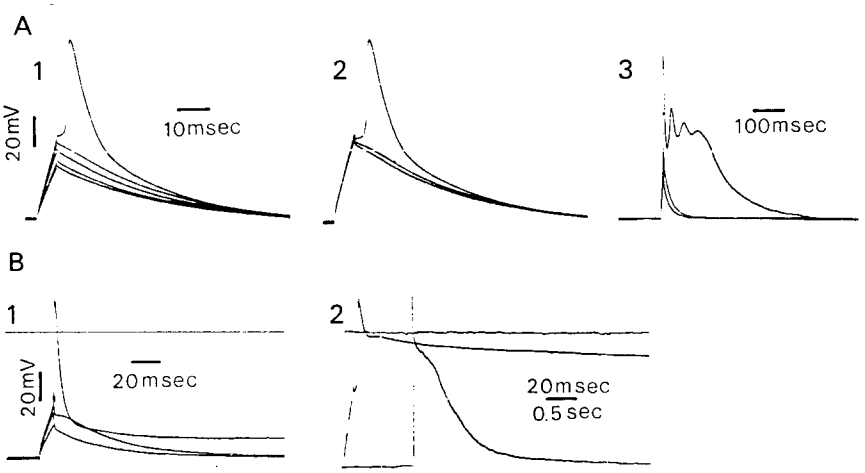


Fig. 7. (A) Synergy of action between sea anemone toxin II and Decis on NIE115 neuroblastoma cells. (1) Control action potential. (2) The action potential is not modified after a 20 min exposure to 10 nM sea anemone toxin II. (3) 15 min after the addition of 0.1 μM Decis to the medium containing 10 nM sea anemone toxin II. (B) Synergy of action between veratridine and Decis on NIE115 neuroblastoma cells. (1) Effect of 1 μM veratridine. (2) 10 min after addition of 10 μM Decis to the medium containing 1 μM veratridine. The zero voltage line is indicated in B.

Electrophysiological properties of the C9 cell line have been described in a previous work [22]. These cells have a resting potential of about −50 mV (Fig. 9). Application of 0.2 μM Decis induces a depolarization of the cell membrane of about 20 mV and the appearance of a spontaneous slow wave activity. The amplitude of these waves can reach 30 mV and their duration is about 5 s. Further application of 0.2 μM tetrodotoxin reduces the amplitude of these waves by about 50%. The slow wave activity is readily suppressed by 10 μM tetrodotoxin (Fig. 9).

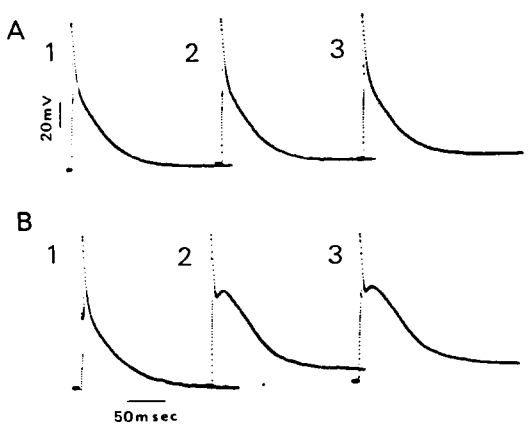


Fig. 8. Mutual effects of RU 15525 and RU 11484 on NIE115 neuroblastoma cells. (A) Protection of the RU 15525 effect by RU 11484. (1) Control action potential. (2) After a 15 min exposure to 10 μM RU 11484. (3) 20 min after the addition of 1 μM RU 15525 to the medium containing 10 μM RU 11484. (B) Non-displacement of RU 15525 effect by RU 11484. (1) Control action potential. (2) After a 10 min exposure to 1 μM RU 15525. (3) 20 min after the addition of 10 μM RU 11484 to the medium containing 1 μM RU 15525.

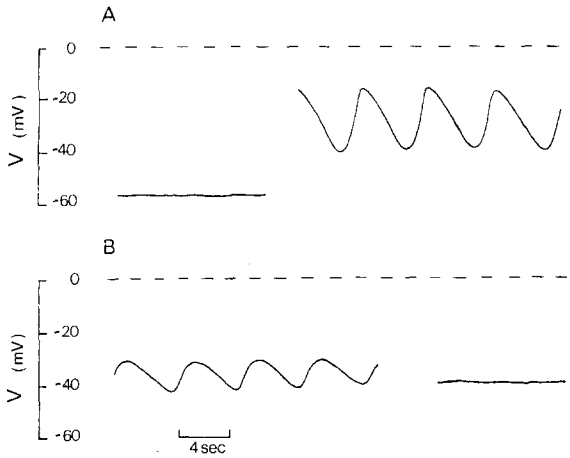


Fig. 9. (A) Effect of Decis on C9 cells. Left: control resting potential. Right: spontaneous slow-wave activity after a 15 min exposure to 0.2 μM Decis. (B) Blocking of Decis effect by tetrodotoxin. Left: 10 min after the addition of 0.2 μM tetrodotoxin to the medium containing 0.2 μM Decis. Right: 10 min after the addition of 1 μM tetrodotoxin to the medium containing 0.2 μM Decis.

Discussion

A variety of neurotoxins are known to be specific for the fast Na^+ channel. These include tetrodotoxin and saxitoxin, which bind at or near the selectivity filter of the channel, and veratridine, batrachotoxin, dihydrograyanotoxin II, sea anemone and scorpion toxins, which alter the functioning of the gating system.

Allethrin, one of the first synthetic pyrethroids, has been shown to interact with the fast Na^+ channel of the nerve membranes of invertebrates [8] and vertebrates [32]. Its main effect is to slow down the rate of inactivation of the Na^+ channel, thereby inducing a prolongation of the transient increase in sodium permeability during the action potential. More recently developed pyrethroids such as permethrin (RU 22090), cispermethrin or decamethrin (Decis) [33] have been shown to display a similar type of action [34,35].

The initial rate of $^{22}Na^+$ influx through the Na^+ channel of neuroblastoma cells is not appreciably affected by insecticides of the pyrethroid series when they are applied alone to the excitable membrane. However, when applied in conjunction with alkaloids (e.g., veratridine, batrachotoxin), dihydrograyanotoxin II, or polypeptide neurotoxins (e.g., sea anemone toxin II, scorpion toxin II), several pyrethroids stimulate $^{22}Na^+$ entry through the Na^+ channel. This stimulatory effect, like that induced by alkaloids or polypeptide neurotoxins alone is fully inhibited by tetrodotoxin, with an apparent dissociation constant for the tetrodotoxin-receptor complex of 1.6 nM (Fig. 4). It is this inhibition by tetrodotoxin which indicates that pyrethroids interact with the Na^+ channel of mammalian cells. As seen by the $^{22}Na^+$ flux experiments, half-maximum saturation of the pyrethroid receptor on the Na^+ channel occurs in the micromolar range for the most active pyrethroids, Decis and RU 15525. There are at least two steps in the action of pyrethroids. The molecule is

initially bound to the receptor, with a subsequent stabilization of an open state of the Na^+ channel which lasts longer than that of the unpoisoned Na^+ channel. The present work shows that there are pyrethroids which bind to the receptor structure in neuroblastoma cells, but which are unable to stimulate $^{22}Na^+$ entry, indicating an inability to carry out the second step of the toxic pyrethroid action. One of these 'inactive' pyrethroids, RU 11484, binds to its receptor with a reasonably high affinity (Fig. 3B). The inactive pyrethroids behave as antagonists for the active pyrethroids.

The synergism observed between pyrethroids and other toxic compounds known to be specific for the gating system of the fast Na^+ channel is not the first observation of this kind regarding the pharmacology of this channel. Scorpion and sea anemone toxins have been shown both by $^{22}Na^+$ flux experiments and by electrophysiological techniques to act in synergy with toxins such as veratridine or batrachotoxin [21,36—38]. The synergism observed between the effects of pyrethroids on the one hand, and the effects of sea anemone toxin II, scorpion toxin II, veratridine, batrachotoxin and dihydrograyanotoxin II on the other, indicate that the receptor binding component of pyrethroids is distinct from the receptors of the other toxins. The Na^+ channel comprises at least four different toxin binding sites: (i) one site for toxins which bind at or near the selectivity filter, e.g., tetrodotoxin and saxitoxin [39—41]; (ii) one site for the family of toxins including batrachotoxin, veratridine and dihydrograyanotoxin II [28]; (iii) at least one site for polypeptides toxins [42,43]; and (iiii) one site for pyrethroids.

Measurements of $^{22}Na^+$ flux are not sufficiently sensitive to demonstrate an interaction of pyrethroids with the excitable membrane of neuroblastoma cells in the absence of other toxic compounds such as sea anemone toxin II or veratridine. However, these interactions can be shown using electrophysiological techniques (Figs. 6—8). The electrophysiological data essentially confirm the results obtained in the $^{22}Na^+$ flux experiments. (i) The pyrethroids which are most active in stimulating $^{22}Na^+$ influx (Decis and RU 15525) are also the most active in producing an electrophysiological response. (ii) Pyrethroids have the effect expected for molecules which slow down the inactivation of the Na^+ channel (and thereby increase the quantity of $^{22}Na^+$ which enters the cell through the Na^+ channel per unit of time). That is, they give rise to a plateau phase following the fast component of the action potential. As expected, the electrophysiological effect of pyrethroids is inhibited by tetrodotoxin at concentrations which normally block action potentials. (iii) The stimulatory effect of active pyrethroids is enhanced in the presence of veratridine and sea anemone toxin II. For example, at concentration of sea anemone toxin II as low as 10 nM, the addition of only 0.1 μM Decis gives rise to a marked plateau phase in the action potential. These results also clearly confirm that the pyrethroid site is distinct from those of veratridine and sea anemone toxin II. (iv) Pyrethroids like RU 11484, which do not alter the action potential, do bind to the pyrethroid receptor and act as antagonists for active pyrethroids.

C9 cells used in this work were derived from a metastasis of a rat brain tumor [44]. The properties of interaction of pyrethroids with these cells are similar to those observed with neuroblastoma cells in the respect that pyre-

throids stimulate $^{22}Na^+$ influx and this stimulation is suppressed by tetrodotoxin. However, although these cells have a good resting potential of —50 mV, they are unable to generate action potentials upon electrical stimulation. The Na^+ channel in these cells is in a silent form; it cannot be activated electrically but has normal receptors for tetrodotoxin, veratridine and scorpion and sea anemone toxins [22]. The data presented in Figs. 5 and 9 show that pyrethroids also interact with these silent channels; furthermore, this interaction occurs at concentrations lower than those necessary to elicit action on neuroblastoma cells. These silent channels are activated chemically by neurotoxins like veratridine and sea anemone toxin II which slow down the inactivation kinetics of the channel. It is therefore not surprising that Decis, which also slows down inactivation kinetics [35], also activates these silent channels (Fig. 9). As expected, the spontaneous slow wave activity generated by Decis is fully inhibitable by tetrodotoxin.

In conclusion, pyrethroids are active on Na^+ channels which are present in mammalian neurones. These molecules are very interesting pharmacological tools to study the Na^+ channel, since they work at concentrations similar to those used with the potent toxin, batrachotoxin. Their action is quasi-irreversible, and they bind to a receptor site which is different from those of all other toxins known to alter the gating system of the Na^+ channel.

Acknowledgments

We thank Dr. Daly and Dr. Tokuyama for their very kind gift of batrachotoxin. Thanks are also due to Dr. Nakajima for a very generous gift of dihydrograyanotoxin II. The series of pyrethroids used in this work were kindly given to us by Dr. Carles and Hervé (Procida) who initially told us of the interest in these compounds. This work has benefited from the excellent technical assistance of N. Alenda and M.C. Lenoir. We thank Ms. Mary Murphy for a careful reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Institut de la Santé et de la Recherche Médicale, the Délégation Générale à la Recherche Scientifique et Technique and the Commissariat à l'Energie Atomique.

References

1 Lowenstein, O. (1942) Nature 150, 760—762
2 Welsh, J.H. and Gordon, H.T. (1947) J. Cell. Comp. Physiol. 30, 147—172
3 Yamasaki, T. and Ishii, T. (1952) J. Nippon Soc. Appl. Entomol. 7, 157—164
4 Lalonde, D.I.V. and Braun, A.W.A. (1954) Can. J. Zool. 32, 74—81
5 Burt, P.E. and Goodchild, R.E. (1971) Entomol. Exp. Appl. 14, 179—189
6 Camougis, G. and Davis, W.M. (1971) Pyrethrum Post 11, 7—14
7 Schallek, W. and Wiersma, G.A.G. (1948) J. Cell. Comp. Physiol. 31, 35—47
8 Narahashi, T. (1976) Pestic. Sci. 7, 267—272
9 Narahashi, T. (1971) Bull. W.H.O. 44, 337—345
10 Narahashi, T. (1962) J. Cell. Comp. Physiol. 59, 61—65
11 Narahashi, T. and Anderson, N.C. (1967) Toxicol. Appl. Pharmacol. 10, 529—547
12 Muramaya, K., Abbott, N.J., Narahashi, T. and Shapiro, B.I. (1972) Comp. Gen. Pharmacol. 3, 391—400
13 Griffin, C.S. (1973) Pyrethrum Post 12, 50—58
14 Bond, H., Mauger, K. and Defeo, J.J. (1973) Pyrethrum Post 12, 59—63

15 Barthel, W.F. (1974) in Pyrethrum, The Natural Insecticide (Casida, J.E., ed.), pp. 123—142, Academic Press, New York
16 Elliott, M., Janes, N.F., Kimmel, E.C. and Casida, J.E. (1972) J. Agric. Food Chem. 20, 300—313
17 Van den Bercken, J., Akkermans, L.M.A. and van der Zalm, J.M. (1973) Eur. J. Pharmacol. 21, 95—103
18 Akkermans, L.H.A., van den Bercken, J. and Versluijs-Helder, M. (1975) Pestic. Biochem. Physiol. 5, 451—459
19 Wouters, W., van den Bercken, J. and van Ginneken, A. (1977) Eur. J. Pharmacol. 43, 163—171
20 Evans, M.H. (1976) Pestic. Biochem. Physiol. 6, 547—552
21 Jacques, Y., Fosset, M. and Lazdunski, M. (1978) J. Biol. Chem. 253, 7383—7392
22 Romey, G., Jacques, Y., Schweitz, H., Fosset, M. and Lazdunski, M. (1979) Biochim. Biophys. Acta 556, 344—353
23 Beress, L., Beress, R. and Wunderer, G. (1975) FEBS Lett. 50, 311—314
24 Beress, L., Beress, R. and Wunderer, G. (1975) Toxicon 13, 359—367
25 De Barry, J., Fosset, M. and Lazdunski, M. (1977) Biochemistry 16, 3850—3855
26 Ulbricht, W. (1969) Ergeb. Physiol. Biol. Chem. Exp. Pharmakol. 61, 18—71
27 Ohta, H., Narahashi, T. and Keeler, R.F. (1973) J. Pharmacol. Exp. Ther. 184, 143—154
28 Catterall, W.A. (1975) Proc. Natl. Sci. U.S.A. 72, 1782—1786
29 Narahashi, T. (1971) in Advances in Insect Physiology (Beament, J.W.L., Treherne, J.E. and Wigglesworth, V.B., eds.), Vol. 8, pp. 1—93, Academic Press, New York
30 Wang, C.M., Narahashi, T. and Scuka, M. (1972) J. Pharmacol. Exp. Ther. 182, 442—453
31 Romey, G., Abita, J.P., Schweitz, H., Wunderer, G. and Lazdunski, M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4055—4059
32 Wouters, W. and van den Bercken (1978) Gen. Pharmacol. 9, 387—392
33 Elliott, M. (1977) in Synthetic Pyrethroids, ACS Symposium Series (Elliott, H., ed.), Vol. 42, American Chemical Society, Washington.
34 Vijverberg, H.P.M. and van den Bercken, J. (1979) Eur. J. Pharmacol. 58, 501—504
35 Duclohier, H. and Georgescauld, D. (1979) Comp. Biochem. Physiol. 62C, 217—223
36 Catterall, W.A., Ray, R. and Morrow, C.S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2682—2686
37 Bernard, P., Couraud, F. and Lissitzky, S. (1977) Biochem. Biophys. Res. Commun. 77, 782—788
38 Romey, G., Renaud, J.F., Fosset, M. and Lazdunski, M. (1980) J. Pharmacol. Exp. Ther. 213, in the press
39 Narahashi, T. (1974) Physiol. Rev. 54, 813—889
40 Ulbricht, W. (1977) Annu. Rev. Biophys. Bioeng. 6, 7—31
41 Ritchie, J.M. and Rogart, R.B. (1977) Rev. Physiol. Biochem. Pharmacol. 79, 1—50
42 Catterall, W.A. (1978) J. Biol. Chem. 253, 7393—7396
43 Vincent, J.P., Balerna, M., Barhanin, J., Fosset, M. and Lazdunski, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1646—1650
44 Stahn, R., Rose, S., Sanborn, S., West, G. and Herschman, H. (1975) Brain Res. 96, 287—298