Investigations of Excitability of Isolated and Non-isolated Neurons from the Terminal Ganglion of *Periplaneta americana* by Current/Voltage Clamp and Intracellular Perfusion

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Abstract. It was shown by means of current and voltage clamp measurements in combination with the intracellular perfusion method that the isolated motoneuron nerve cell bodies of the cockroach *Periplaneta americana* show overshoot action potentials. A regenerative Na current was found to be responsible for the excitability of these membranes; however, the results are not in agreement with a *de novo* synthesis of these channels.

Key words: Voltage/current clamp — Intracellular perfusion — Insect motoneuron — Na current — Induced excitability

Introduction

Many electrophysiological experiments have shown that the somata of insect motoneurons are not excitable under *in vivo* conditions (Hoyle 1970; Burrows 1977). Excitability means in that case spontaneous generation of action potentials or their evoking by injection of depolarizing current pulses. Nevertheless, Pitman et al. (1972) and Pitman (1975) established that in cell body membranes of motoneurons excitability can be induced by axotomy or administration of colchicine to the corresponding nerve. By means of intracellular measurements and with tetrodotoxin (TTX) it was shown (Pitman et al. 1972; Pitman 1975) that a regenerative sodium current is involved in the generation of action potentials. Continuing these efforts Goodman and Heitler (1979) investigated *in situ* the excitability and the ionic nature of action potentials of two identified neurons of the locust *Schistocerca nitens* and showed that the somatic membrane of DUMETi is excitable, whereas that of FETi is not. In agreement with the results of Pitman et al. (1972) and Pitman (1975) the somatic membrane of

Table 1. Composition of the extra- and intracellular solutions used (in mmol/l).

Solution	No	NaCl	KCl	$CaCl_2$	$MgCl_2$	Sucrose	Tris-Cl	KF	NaF	Tris-F
Extra-cellular	1	180	5	5	2	15	17.5	(50 TEA-Cl)		
	2	130	5	5	2	15	67.5			
	3	130	5	5	2	15	17.5			
	4		5	5	2	15	197.5			
Intra-cellular	5	10	180		2		20			
	6							180	10	25
	7						40	5	10	180

FETi also becomes excitable after axotomy or treatment with colchicine. Horridge and Burrows (1974), however, did not observe such a transformation of FETi after axotomy.

In the experiments carried out by Pitman et al. (1972) and Pitman (1975) as well as in those by Goodman and Heitler (1979) the sodium dependent action potentials occurred only a few days after axotomy or treatment with colchicine. Therefore Goodman and Heitler set up the hypothesis that excitability is caused by *de novo* synthesis of sodium channels and their incorporation into the plasmalemma.

Further progress was achieved by the experiments carried out by Suter and Usherwood (1985). These authors isolated motoneurons from the metathoracic ganglion of the locust *Schistocerca gregaria* and found, by intracellular recordings, a higher excitability.

The present paper addresses the phenomenon of induced excitability by means of current and voltage clamp recordings. It is shown that the induced excitability is caused by a fast sodium current, but these channels cannot be *de novo* synthesized after isolation.

Some of the results have been reported in a preliminary form (Gűndel et al. 1987).

Materials and Methods

The experiments were performed using adult male cockroaches of the species *Periplaneta americana L.* from our own breeding stock, 1 to 3 days after their last moult. The preparation of the terminal ganglion and the isolation of the cells was carried out according to Gundel et al. (1987). In the present work only cells with sodium currents were taken for the voltage clamp experiments.

The solutions used are given in Table 1. All the solutions were adjusted to pH = 7.3—7.4. In the experiments with TTX, 4-aminopyridine (4-AP) and cycloheximide the substances were dissol-

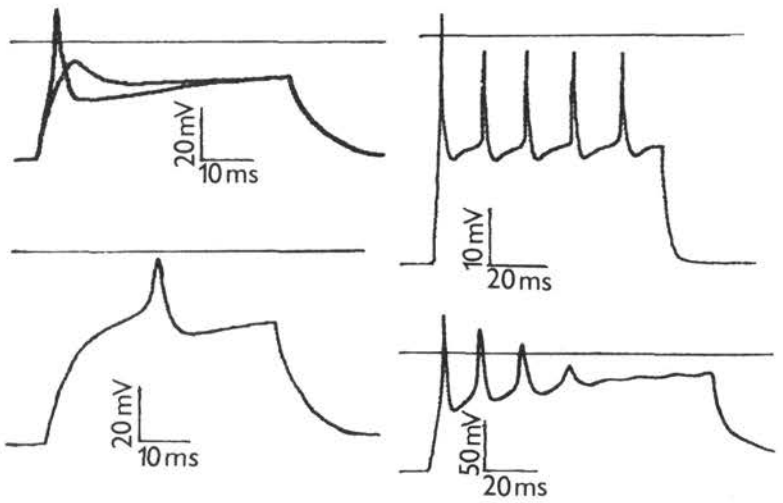

Fig. 1. Voltage courses generated by isolated neurons under current clamp conditions. A) *Upper left*: Full-sized action potential (curve *a*), eliminated by application of 10^{-6} mol/l TTX (curve *b*). The remaining spike vanished after perfusion with K-free solution. *Bottom left*: Graduated spikes depending on the intensity of the injected current. The other patterns of electrical activity are rarely observed: Repetitive activity (*upper right*) and burst-like discharges with decreasing amplitude (*bottom right*).

ved in solution 1. In the experiments with TEA-Cl 50 mmol/l Na ions were replaced by 50 mmol/l TEA-Cl in the external solution (solution 3) and solution 2 was used as the reference solution. The measurements were performed at room temperature.

The method of intracellular perfusion using polyethylene pipettes according to Kostyuk and Krishtal (1977) or using glass pipettes according to Lee et al. (1978) was employed.

In the current clamp measurements, a current produced by a constant current source was injected into the dialyzed cell producing a defined value of the membrane potential. Depolarizing current pulses (0.6 — 3 nA) were added to this holding current and generated action potentials. The action potentials were measured by an electrometer amplifier, recorded by a storage oscilloscope, photographed and analyzed.

The voltage clamp measurements were performed with an experimental set-up according to Osipchuk and Timin (1984). Recording and analysis of data were done as described above.

Results

Current clamp measurements of isolated neurons

Isolated neurons measured under current clamp conditions exhibited voltage courses shown in Fig. 1. An action potential with overshoot is shown in Fig. 1

(upper left, curve a). The spike amplitude for $n = 14$ cells was (61.8 ± 6.6) mV, the corresponding overshoot amounted to (20.4 ± 4.7) mV. Hyperpolarizing afterpotentials of (20.5 ± 7.7) mV were clearly visible. However, often only graduated spikes could be registered (Fig. 1; bottom left). The height of these spikes depended on the amplitude of the injected current pulse.

Other excitation patterns that rarely occur are repetitive activity (Fig. 1; upper right) and burst-like discharges with decreasing amplitude (Fig. 1; bottom right).

The administration of 10^{-6} mol/l TTX led to the elimination of the overshoot spike (Fig. 1, upper left, curve b). Therefore, one could expect the sodium ions to be responsible for the regenerative inward current. The remaining small spike vanished after perfusion with K-free internal solution. After this the response to an impulse was purely electrotonical. By reperfusion of the solutions it could be shown that these effects were reversible.

Voltage clamp measurements of isolated neurons

To provide direct evidence for currents involved in excitation, the same cells were investigated under voltage clamp conditions. Cells with overshoot potentials exhibited ion currents shown in Fig. 2*A*. These currents consisted of an outward and a fast inward component. The current voltage relationships for the inward and the stationary outward component are shown in Fig. 2*B*, curves a and b, respectively. The inward current could be entirely eliminated by the addition of 10^{-6} mol/l TTX or by the use of Na-free external solution (solution 4, Table1). Hence, this current is carried by Na ions.

The remaining outward current consisted of several components. The stationary outward current could be reduced to maximum 20% by the external administration of 50 mmol/l TEA (Fig. 2*B*, curve c). The fast transient outward current vanished at holding potentials lower than −45 mV or could be blocked reversibly by addition of 2 mmol/l 4-AP (to be published). The N-shaped range of the voltage current relationship of the outward current (Fig. 2*B*, curve b) at positive membrane potentials indicates the existence of a Ca-dependent K current.

Excitation of non-isolated cells

The aim of further investigations was to determine whether cells remaining in the tissue environment of the ganglion but incubated in an enzyme-containing solution are also excitable. Similar experiments are possible, because the cells are located at the periphery of the ganglion. Thus they can be sucked into the dialysis pore, perfused and investigated by current and/or voltage clamp with-

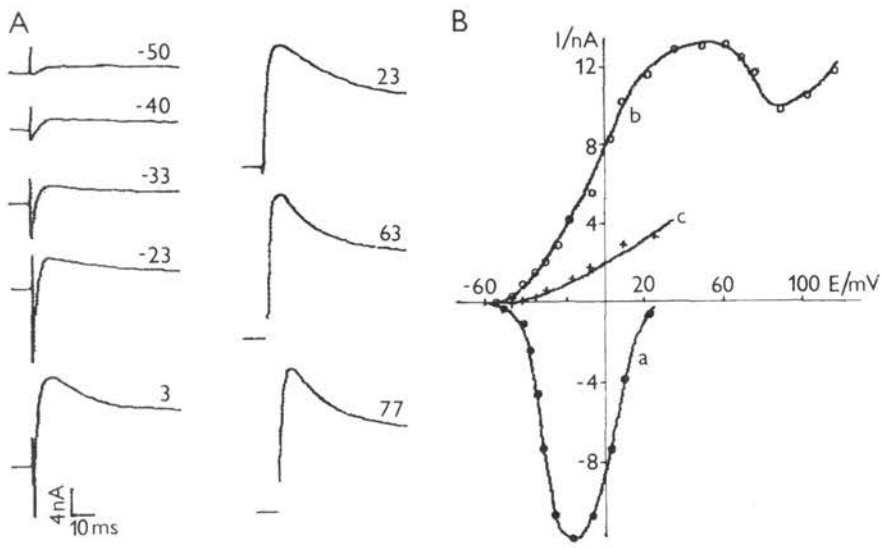

Fig. 2. Ionic currents of voltage-clamped isolated somata. *A*) Time courses of the total ionic currents exhibited by cells with overshoot action potentials. Corresponding membrane potentials (in mV) are given next to the curves. *B*) Current voltage relationship of the fast inward current (curve *a*) and the stationary outward current (curve *b*) of the currents in part *A*. The steep slope of the characteristic of the sodium current (curve *a*) at positive potentials is due to the overlap of the non-eliminated outward current. The N-shaped curve indicates the existence of a Ca dependent K current. Curve *c* shows the blocking action of 50 mmol/l TEA (equimolar for Na ions; solution 3; solution 2 used as the reference solution).

out previous isolation. Under these conditions the cells showed spikes as seen in Fig. 1 or ionic currents as in Fig. 2*A*.

In repeated experiments of the same design enzymatic treatment was omitted. In this case the interval between the start of the preparation and the electrophysiological experiment could be shortened to 15 min. No further shortening of the preparation time could be achieved because layers of glial cells prevented a fast development of an adequate leakage resistance. Nevertheless, even after this short time full-sized action potentials were observed. It is essential for the interpretation of these experiments that the number of excitable cells did not increase when the duration of measurements was prolonged.

If *de novo* synthesis of Na channels is responsible for induced excitability under *in vitro* conditions this excitability should also be preventable by a protein synthesis inhibitor. Therefore, the ganglion was prepared in Ringer solution containing 100 μg/ml cycloheximide. All other solutions used subsequently, including those used in measurements, contained the same amount of the

inhibitor substance. Na currents similar to those shown in Fig. 2*A* occurred in these experiments as well, even after incubation of the cells for more than 6 h. No synthesis of channel proteins occurred within this sufficiently long interval.

Discussion

An important result of the present work is the finding that freshly isolated insect motoneuron somata are also excitable. Hence, the intracellular perfusion method (Kostyuk and Krishtal 1977; Lee et al. 1978) can be employed in combination with the voltage and/or current clamp techniques to investigate this class of cells as well.

It has been known for long that axotomy results in substantial electrophysiological changes in nerve cell bodies (Eccles et al. 1958; Kuno and Llinas 1970). These changes are subject of numerous investigations because of their relevance to regenerative processes in the nervous system (Gordon et al. 1987). Assuming that the biological processes are uniform on the molecular level, the investigations into the consequences of axotomy would profit from the use of insect motoneurons for the following reasons: 1) The nerve cell bodies of mammalian neurons generate Na spikes even without axotomy. Because this is not the case in insect motoneurons the experimental situation is much simpler. 2) Unlike the mammalian nerve cell bodies insect motoneuron somata have no synapses. Therefore, synaptic processes can be ruled out as reasons for the changes occurring after axotomy.

Pitman et al. (1972) and Pitman (1975) as well as Goodman and Heitler (1979) showed that axotomy in the distal part of nerve processes leads to the generation of action potentials in the nerve cell bodies but only after at least two days. In contrast, the isolation procedure, which involves axotomy proximal to the soma, supplies cells which immediately generate spikes under current clamp conditions (Gündel et al. 1987). These observations confirm the results of Suter and Usherwood (1985) who also observed a stronger excitability of isolated somata from the metathoracic ganglion of *Schistocerca gregaria*. Differences were registered only with regard to the number of spontaneously active cells observed. While Suter and Usherwood (1985) saw approximately 25% of cells with spontaneous activity, such cells were only exceptional in our experiments (Fig. 1, upper right). However, these exceptional cases could also be the spontaneous active dorsal unpaired neurons (Kerkut et al. 1969), since no identification of cells in suspension is possible.

A possible explanation for the different numbers of spontaneously active cells can be different types of current clamp techniques used (microelectrodes, intracellular perfusion) or different kinds of isolation procedures employed:

Table 2. Characteristic parameters of action potentials (in mV) for somatic membranes of insect neurons.

Species	Ganglion	Cell type	Action potent.	Overshoot	After-potent.	Literature
Periplaneta americana	meta-thoracic	DUM	110	40	10—20	Crossman et al. (1971)
Periplaneta americana	terminal	DUM	81	26.5	14.5	Kerkut et al. (1969)
Periplaneta americana	terminal	DUM	70—100			Jego et al. (1970)
Schistocerca gregaria	meta-thoracic	non-identif.	20—70		20—30	Suter and Usherwood (1985)
Schistocerca gregaria	meta-thoracic	FETI	59		12	Goodman and Heitler (1979)
Periplaneta americana	terminal	non-identif.	62	21	20	This work

Suter and Usherwood (1985) isolated the neurons in a purely mechanical manner whereas in our experiments the cells were isolated by enzymatic and mechanical means (Gundel et al. 1987).

Table 2 shows some characteristic parameters of action potentials measured in insect neurons. Our own results included in the Table concern only action potentials with overshoots. The spike amplitudes registered in isolated neurons in our experiments are somewhat lower compared to those measured under *in vivo* conditions.

The administration of TTX eliminated the overshoot spike (Fig. 1, upper left). It can be inferred from this that the spike is caused by a regenerative Na current. Direct evidence for the regenerative Na current was provided by voltage clamp measurement. The inward current could be completely blocked by TTX or by Na-free external solution.

These measurements have raised the question whether the Na channels do exist permanently in the membrane, or whether they are synthesized *de novo* and incorporated into the plasmalemma.

Goodman and Heitler (1979) advocate the hypothesis of *de novo* synthesis of the channel proteins since excitability could be observed but after several days after the axotomy. This hypothesis is supported by the observation of the formation of perinuclear RNA rings in axotomized cells indicating an increased protein synthesis (Cohen and Jacklet 1965; Pitman et al. 1972).

However, our experiments showed that excitability is also present right at the beginning of the measurements, regardless of whether the somata remain in

the ganglion tissue or have been isolated. Neither could a systematic increase in the peak value of the Na current be recorded over longer intervals after the isolation. This would be the case if new channels were synthesized. In conclusion, our results contradict the suggestion of Goodman and Heitler (1979). Other hypotheses that might explain induced excitability will be tested in the future.

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