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ACTION OF THE STEROL-BINDING FORM OF FILIPIN ON THE LOBSTER AXON MEMBRANE

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SUMMARY

The present work demonstrates by electron microscopy and spectrofluorimetry the interaction of the sterol-binding form of filipin with the axolemma of lobster nerves. The results also indicate that the axolemma cholesterol accessible to filipin seems to play no specific role in the functioning of the tetrodotoxin receptors and sodium channels.

INTRODUCTION

The steroi-binding form of the neutral polyenic antibiotic filipin [1] was utilized to investigate (a) its interaction with preparations of axon membrane (axolemma) isolated from lobster nerves by electron microscopy and spectrofluorimetry, (b) its effect on the binding of tetrodotoxin to the axolemma and (c) its effect on the membrane potentials of intact axons and on the blocking action of tetrodotoxin. Tetrodotoxin has a high affinity for the axon membrane specifically blocking the sodium channels (for a review see ref. 2). Schroeder et al. [1] have pointed out that filipin in aqueous solution can occur in sterol-binding and non-binding forms, that a ratio of the absorbance peaks of the filipin molecule (A_{338}/A_{305}) of less than 1.6 is a consistent indicator of the presence of predominantly the sterol-binding form (active filipin) and that the stoichiometry of the filipin : cholesterol interaction is 1 : 1. Only filipin solutions with absorbance ratios of less than 1.6 were used in the present work.

The role on tetrodotoxin binding of axolemma components other than cholesterol has already been explored by enzymatic hydrolysis, revealing that the binding is diminished by phospholipase A [3, 4], after which proteases diminish it even further (ref. 3 and our unpublished results). The Triton-solubilized axolemma requires no previous treatment with phospholipase A [5]. The toxin receptor, therefore, appears to be a membrane lipoprotein or a membrane protein in a lipidic environment. Since the enzymes so far utilized to modify the receptors do not degrade cholesterol, it appears interesting to explore the role that this molecule may play in tetrodotoxin

binding and/or functioning of the sodium channels. The results, besides revealing the interaction of filipin with the axolemma of lobster nerves, indicate that cholesterol plays no functional role in the receptors and/or sodium channels.

EXPERIMENTAL METHODS

The active form of filipin was obtained from the so-called filipin complex (for a review see ref. 6) following the method of Schroeder et al. [1]. The filipin complex dissolved in lobster physiological solution was incubated at 50 °C for 2 h. The mM composition of the lobster physiological solution is: 465 NaCl, 10 KCl, 8 $MgCl_2$, 25 $CaCl_2$, 10 Tris · HCl (pH 7.5). The filipin solution was then kept in the dark and always used within 24 h. The absorbance ratio (A_{338}/A_{305}) of the solution was always measured before use and found to be less than 1.6.

Electron microscopy and fluorescence

The axolemma preparations utilized in these experiments were isolated from walking-leg nerves of living *Panulirus argus* lobsters following the method previously described in detail [7]. The total plasma membrane fraction obtained on centrifugation of nerve homogenate, in 0.33 M sucrose over 1.95 M sucrose, was separated into two membrane fractions by centrifugation over a linear gradient between 0.66 and 1.195 M sucrose. The lighter membrane fraction, banding at 1.072 g/cm^3, which has been identified as axolemma [7], was collected and washed twice with 5 mM Tris buffer (pH 7.4). The final axon membrane pellets were divided and then suspended, one half in lobster solution containing filipin and the other (control) in filipin-free solution. Then they were incubated for 30 min at 20–22 °C. Protein and cholesterol were determined by the methods of Lowry et al. [8] and Bowman and Wolf [9], respectively.

For the electron microscopy, samples treated with 25 μM filipin and their controls were negatively stained for 10 min with a 2 % solution of phosphotungstic acid at pH 7.2 and examined in a Siemens Elmiskop 101 electron microscope. The filipin/membrane cholesterol molar ratio in the suspensions was adjusted to 0.1, 0.5 and 1.0.

For the spectrofluorometric measurements, filipin solutions, with or without axolemma, as well as filipin-free control membrane suspensions, were excited at 335 nm, and the uncorrected emission spectra recorded between 400 and 600 nm using an Aminco-Bowman Fluorimeter (American Instruments, Co., Silver Springs, Md.).

Binding of [^3H]tetrodotoxin

The dose-binding curves of [^{3}H]tetrodotoxin to isolated axolemma were determined both in the presence of filipin (25 μM; filipin : cholesterol molar ratio, 1 : 1) and in its absence (controls). The method employed to assay [^{3}H]tetrodotoxin binding has been previously described in detail [7]. [^{3}H]Tetrodotoxin labelled by the method of Wilzbach [10] and purified according to the procedure of Hafemann [11] as modified by Benzer and Raftery [3] was used. The specific activity of the toxin was 35.4 Ci/mol. It had a radiochemical purity of 85 %. The experimental method eliminates the contribution of radioactive impurities to the [^{3}H]tetrodotoxin binding

measurements. Thus, in order to assay tetrodotoxin binding under a given set of conditions, duplicate samples of membrane were used, one of each pair serving to measure non-specific binding. Binding of [^{3}H]tetrodotoxin was calculated by subtracting the radioactivity (cpm/mg protein) present in pellets of membranes pre-incubated with an excess of nonradioactive tetrodotoxin (50 times that of the [^{3}H]tetrodotoxin) from that found in pellets of membranes incubated with [^{3}H]tetrodotoxin only.

Axon membrane potentials

Single axons from circumesophageal nerves of *P. argus* lobsters were used to study the effect of filipin on intact axons. The resting membrane potential, the magnitude of the action potential and the maximum rate of rise of the action potential were measured. The maximum rate of rise is linearly proportional to the membrane ionic currents under the present conditions, i.e. a large volume of solution and a constant conduction velocity [12]. The electrical potentials were measured before and at the end of a 60 min period of incubation in 40 μM filipin. Glass microelectrodes filled with 3 M KCl solution and having an electric resistance of about 10 MΩ were inserted into the axons to record the membrane potential. The microelectrode was connected to a high input impedance amplifier with a 30 kHz band width (NF-1, Bioelectric Instruments, Inc., Hasting-on-Hudson, N.Y.). The capacitance of the microelectrode was electronically compensated at the amplifier. A simple R-C circuit [12] with a 10 μs time constant, connected at the output of the amplifier, served as differentiator to determine the action potential rate of rise.

The effect of the same filipin treatment on the tetrodotoxin receptors was investigated. The axons were incubated for 30 min in lobster physiological solution containing 5 nM tetrodotoxin and then washed for 30 min in tetrodotoxin-free solution. In these axons, in which the irreversible tetrodotoxin-binding component was saturated [13], the effects of three different tetrodotoxin concentrations (1.0, 3.0 and 5.0 nM; 15 min each) were measured before and after a 60-min term of exposure to 40 μM filipin. The filipin treatment of the axons was preceded and followed by 15-min periods of washing with lobster physiological solutions free of filipin and of tetrodotoxin.

RESULTS AND DISCUSSION

Electron microscopy and fluorescence

Fig. 1 shows electron micrographs of fresh negatively stained suspensions of axolemma, one treated with 25 μM filipin and the other untreated, used as control. For the experiment shown in the figure, the amount of axolemma in the suspension was adjusted to give a filipin/cholesterol molar ratio of 1. The changes produced by active filipin on the axolemma resemble those produced by the antibiotic on other natural and artificial systems containing sterols [6, 14]. The extent of the modifications increases with the molar ratio. In the present experiments the ratios used were 0.1, 0.5 and 1.0.

Fig. 2 shows the fluorescence emission spectrum obtained from a 25 μM solution of filipin and that obtained after the addition of axolemma to obtain a filipin/cholesterol molar ratio of 0.2. The diminution of the fluorescence emission produced by the axolemma is similar to that caused by pure cholesterol [1].

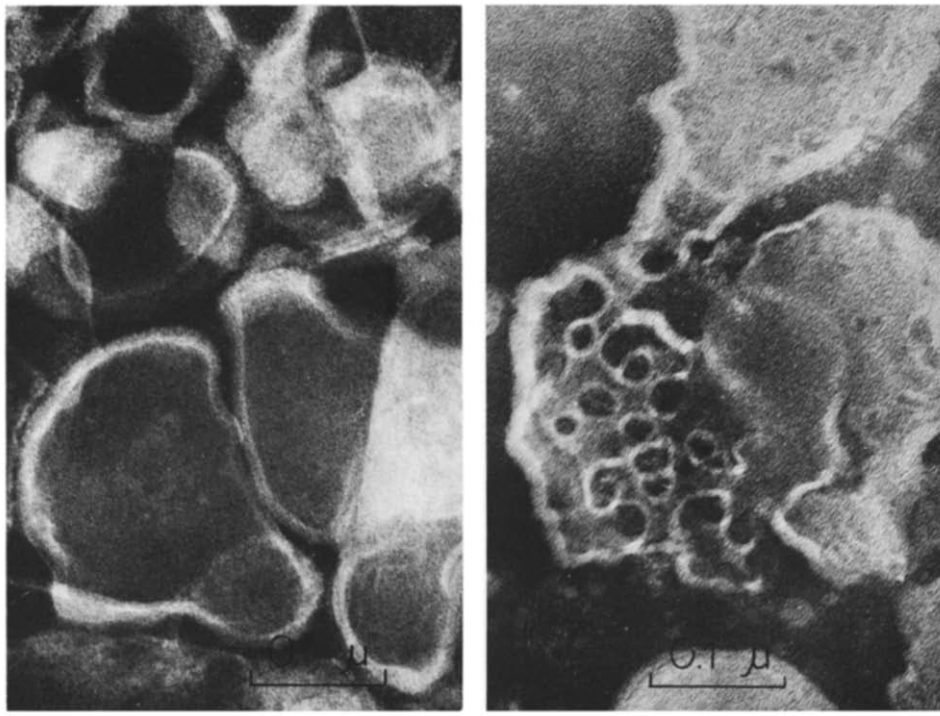

Fig. 1. Electron micrographs of fresh, negatively stained lobster axolemma preparations. (a) Control; (b) preparation treated with 25 μM filipin at a 1 : 1 filipin: membrane cholesterol molar ratio. The filipin-treated axolemma shows a honeycomb-like aspect produced by the presence of pits 150–260 Å in diameter, surrounded by a less electron-dense halo, 35–60 Å thick. The aspect of the pits at the edge of the membrane profile indicates that they are not penetrating holes.

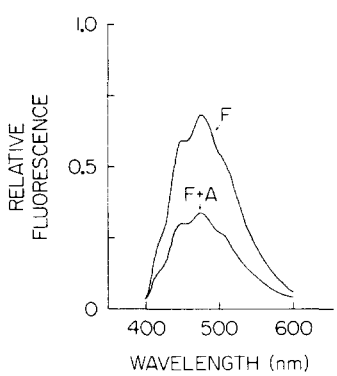

Fig. 2. Emission fluorescence spectra obtained from 25 μM filipin in lobster physiological solution (F), and from the same solution after adding axolemma to obtain a filipin/cholesterol molar ratio of 0.2 (F+A). The samples were excited at 335 nm.

Binding of [^{3}H]tetrodotoxin

After making certain that filipin interacts with the axolemma cholesterol, the dose-binding curves of [^{3}H]tetrodotoxin to three filipin-treated axon membrane prep-

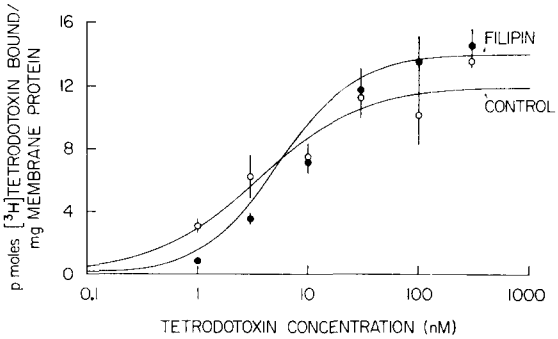

Fig. 3. Dose vs. binding of [^{3}H]tetrodotoxin to control (○) and filipin-treated (●) axolemma preparations in lobster physiological solution at pH 7.5. The filipin concentration was 25 μM, and the filipin : membrane cholesterol ratio was 1 : 1. Values plotted are the means $\pm$S.E.M. of three different preparations. The maximum tetrodotoxin binding, the dissociation constant and the molecularity of the reaction are for the controls 11.9$\pm$1.0 pmol/mg protein, 3.1$\pm$1.0 nM and 0.93, and for the filipin-treated experiments 14.0$\pm$1.1 pmol/mg of protein, 8.1$\pm$2.1 nM and 1.24. The correlation coefficients of the data to the lines are 0.926 and 0.977, respectively.

arations (25 μM filipin; filipin : membrane cholesterol molar ratio, 1 : 1) and their respective controls were determined. Fig. 3 shows the corresponding dose-binding curves. Each one of the sigmoid curves corresponds to the semilogarithmic plot of the rectangular hyperbola best fitting, as calculated by least-squares, the data obtained with the filipin-treated and the control preparations. The values of the maximum tetrodotoxin binding, the dissociation constant, the molecularity of the tetrodotoxin-receptor association and the correlation coefficient of the data to the curve, under both experimental conditions, are given in the legend of Fig. 3. Besides showing that the values for the control curve are closely similar to those previously reported for the lobster isolated axolemma [7, 15], the results indicate that filipin does not modify the

TABLE I

[^{3}H]TETRODOTOXIN BINDING FROM A 100 nM SOLUTION TO AXOLEMMA PREPARATIONS TREATED WITH FILIPIN

The protein content of the control and the filipin-treated membranes was measured directly. The initial amount of protein in both the control and the filipin-treated membrane suspensions was the same. The cholesterol content of the samples was unaffected by filipin. The binding of [^{3}H]tetrodotoxin is expressed in terms of the protein content of the respective final membrane pellet. The values are means $\pm$S.E.M.

Number of experiments	Membrane cholesterol (μmol/1 membrane suspension)	Filipin concentration (μM)	Relative amount membrane protein	Tetrodotoxin binding (pmol/mg protein)
6	250	0	1.00	9.5$\pm$0.7
6	250	25	1.03	9.6$\pm$0.7
5	50	0	1.00	9.1$\pm$1.0
5	50	25	0.86	11.7$\pm$0.7
6	25	0	1.00	9.5$\pm$1.6
6	25	25	0.69	15.7$\pm$2.6

binding kinetics of tetrodotoxin, thus suggesting that the cholesterol accessible to filipin is not directly involved in the process. Filipin seems to cause only a slight increment of the maximum tetrodotoxin binding. In an attempt to explain the nature of this increment, further measurements were made of the binding from a 100 nM [^{3}H]tetrodotoxin solution to six different axolemma suspensions treated with 25 μM filipin, and their respective controls. The amount of axolemma in the suspensions was adjusted to obtain filipin/cholesterol molar ratios of 0.1, 0.5 or 1.0. As shown in Table I, the increment of the binding caused by filipin seems to be directly proportional to the protein loss which is, in turn, a function of the filipin/cholesterol molar ratio. The increment may thus be only apparent, as it is possible that the protein removed is not involved in the binding.

Axon membrane potentials

Six axons were utilized to explore the action of filipin on the resting membrane potential, the magnitude of the action potential and its maximum rate of rise. As shown in Table II, no significant differences were observed between the results obtained in filipin-free lobster physiological solution and at the end of the 60 min period in 40 μM filipin. As explained in Experimental Methods, the maximum rate of rise of the action potential is linearly proportional to the membrane ionic currents which are mostly, but not only, sodium currents during the rising phase of the action potential.

After it was determined that filipin has no effect on the membrane potential at rest and during activity, a possible action of the antibiotic on the tetrodotoxin

TABLE II

AXON MEMBRANE POTENTIALS AND EFFECT OF TETRODOTOXIN ON FILIPIN-TREATED AXONS

(a) Membrane potentials and action potential maximum rate of rise before and at the end of a 60 min treatment period with 40 μM filipin solution. Temperature 20–22 °C. Values are means ±S.E.M.

Number of experiments	Control			Filipin		
	Resting potential	Action potential	Action potential rate of rise	Resting potential	Action potential	Action potential rate of rise
	mV	mV	V/s	mV	mV	V/s
6	78±1	104±2	1008±64	76±1	103±2	988±68

(b) Percentage decrease of the action potential maximum rate of rise caused by tetrodotoxin after a 60 min period with 40 μM filipin. Temperature 20–22 °C. Values are means ±S.E.M. The maximum rate of rise in the absence of tetrodotoxin for this group of axons is 1000±63 V/s for the control period, and 1013±74 V/s after filipin treatment.

Number of experiments	Percentage decrease of the maximum rate of rise of the action potential					
	Control period			After filipin treatment		
	Tetrodotoxin concentration			Tetrodotoxin concentration		
	1 nM	3 nM	5 nM	1 nM	3 nM	5 nM
6	12.1±1.8	32.7±2.8	49.4±4.9	9.2±2.3	27.8±2.9	43.7±3.9

receptors was investigated. The effect of three different tetrodotoxin concentrations (1.0, 3.0 and 5.0 nM) on the maximum rate of rise of the action potential, before and after exposure to 40 μM filipin for 60 min, was measured in six different axons. As shown in Table II, besides confirming that the antibiotic does not affect the maximum rate of rise, the results also reveal that filipin does not significantly modify the action of tetrodotoxin. Since the sodium conductance is not linearly related to the maximum rate of rise, as can be easily verified by solving numerically the Hodgkin and Huxley equations [16], no attempt was made to calculate from the changes caused by tetrodotoxin a precise value for its dissociation constant. Note, however, that 5 nM tetrodotoxin produces an almost 50 % diminution of the maximum rate of rise, before and after filipin treatment, suggesting that the dissociation constant in both conditions should be close to 5 nM.

CONCLUSION

The electron microscopic observations and the spectrofluorimetric measurements demonstrated the filipin-axolemma interaction. In spite of this, and of the removal of protein it produces, the [^{3}H]tetrodotoxin binding to the isolated axolemma, the axon membrane potentials and the blocking action of tetrodotoxin remain unaffected.

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