Eur Biophys J (1989) 16:351–356

European Biophysics Journal

Effects of toxin II from the scorpion *Androctonus australis Hector* on sodium current in neuroblastoma cells and their modulation by oleic acid

P. Jourdon [1]*, Y. Berwald-Netter [2], E. Houzet [2], F. Couraud [3], and J. M. Dubois [1]

[1] Laboratoire de Physiologie Comparée (UA CNRS 1121), Université Paris-Sud, Bât. 443, F-91405 Orsay Cedex, France
[2] Laboratoire de Biochimie Cellulaire, Collège de France, F-75231 Paris Cedex 05, France
[3] Laboratoire de Biochimie, Faculté de Médecine Secteur Nord, F-13326 Marseille Cedex 15, France

Received May 12, 1988/Accepted in revised form October 6, 1988

Abstract. The effects of toxin II (*AaH* II) isolated from the scorpion *Androctonus australis Hector* on sodium current in neuroblastoma X glioma NG 108-15 hybrid cells were analysed under patch clamp conditions in the whole cell configuration. *AaH* II (70 n*M*) induced a maintained sodium current, as well as increasing both fast and slow inactivation time constants and the amplitude of the peak current. This latter effect occurred *via* a shift of the activation-voltage curve towards negative voltage values by about 9 mV. Oleic acid (5 μ*M*), which had no effect on I_{Na} under control conditions, decreased the *AaH* II-induced maintained current. It also reversed, or prevented the increase of the peak current induced by *AaH* II. However, it neither prevented nor modified the *AaH* II-induced increase in inactivation time constants. The binding of the toxin to its specific site and the number of binding sites for *AaH* II were not significantly modified by oleic acid. The oleic acid-induced effects could not be related to the activation of protein kinase C since PMA, a potent activator of this enzyme, did not produce oleic acid-like effects. From these results, it is concluded that *AaH* II has several independent effects on sodium channels, some of which could be modulated by the lipid environment of sodium channels in the membrane.

Key words: Neuroblastoma cell, sodium current, alpha scorpion toxin, oleic acid, whole cell patch clamp

Introduction

In most excitable cells, α scorpion toxins partly remove and slow down sodium current inactivation (Meves et al. 1984; Benoit and Dubois 1987). The prevailing view of their mode of action is that they bind to a receptor site located at the outer face of the Na channel and specifically alter its inactivation mechanism, without modifying activation of the channel (Couraud et al. 1978; Catterall 1979; Watt and Simard 1984). However, in neuroblastoma cells, it has been recently shown that the toxin isolated from the scorpion *Leiurus quinquestriatus* not only slows the inactivation of Na current but also shifts the Na activation-voltage curve by 7 to 11 mV towards more negative voltages (Gonoi et al. 1984; Gonoi and Hille 1987). From these results, it seems that α scorpion toxins induce somewhat different effects depending on the type of cell under study. Such discrepancies may result from intrinsic differences in the properties of different Na channels (Gonoi et al. 1984; Gonoi and Hille 1987) or from differences in the lipid environment of the Na channels (Naumov 1983; Rack et al. 1986; Takenaka et al. 1987; Benoit and Dubois 1987). In order to test this latter hypothesis, we first analysed the effects of toxin II from the scorpion *Androctonus australis Hector* (*AaH* II) on Na current in neuroblastoma X glioma hybrid NG 108-15 cells under control conditions and then tried to modify the effects of *AaH* II by exogenous lipids. For this purpose it was necessary to test lipids which quickly incorporate into the membrane and do not alter sodium channel functioning in the absence of toxin. Oleic acid has been shown to incorporate rapidly into biological membranes (Giraud et al. 1981) and to have no effect on the action potential generated in NG 108-15 neuroblastoma cells (Love et al. 1985). For these reasons we used this fatty acid as a means of modifying the channel environment.

A preliminary report of these results has appeared elsewhere (Jourdon et al. 1986a).

Methods

NG 108-15 cells were grown in DMEM culture medium (Gibco) supplemented with fetal calf serum (5%) and HAT. To induce morphological differentiation,

* To whom offprint requests should be sent

the culture medium was supplemented with 2% dimethyl sulfoxide for 72 h, then replaced by medium without DMSO but containing 0.5 m*M* dibutyryl-cAMP (Jourdon et al. 1986 b). The voltage clamp was done by the suction pipette, giga-ohm seal whole cell recording technique (Hamill et al. 1981). The standard external solution had the following composition (m*M*): 140 NaCl; 5 KCl; 1 $CaCl_2$; 2 $MgCl_2$; 10 Hepes; 11 glucose; (pH 7.3). The composition of the medium contained in the pipette was (m*M*): 90 CsF, 50 CsCl, 10 Hepes (pH 7.3). The experiments were carried out at room temperature (18 °–20 °C). The series resistance was compensated. Stimuli were applied, from a holding potential of −80 mV, at a frequency of 1Hz. Resting Na inactivation was removed by 250 ms pre-pulses to −120 mV. The non-sodium maintained currents were subtracted from the total current. Samples of toxin II extracted from the venom of scorpion *Androctonus australis Hector* (Couraud et al. 1978) were kept at −18 °C and diluted, immediately before experiments, in external solution to give a final concentration of 70 n*M*. Oleic acid was added to the external medium in order to give a final concentration of 5×10^{-6} *M*. Its effects on sodium currents were tested at least 10 min after its addition in order to allow its incorporation into the membrane (Giraud et al. 1981). Phorbol 12-myristate 13-acetate (PMA) was used at final concentrations ranging between 8 and 32 n*M*. ^{125}I-*AaH* II scorpion toxin binding measurements were carried out in two incubation conditions: in sodium-free choline substituted medium (choline chloride 140 m*M*, KCl 5.4 m*M*, $CaCl_2$ 1.8 m*M*, $MgSO_4$ 0.8 m*M*, glucose 5.5 m*M*, HEPES 25 m*M* and BSA 1 g/l; pH 7.3 adjusted with *Tris*-base) or in the same buffer but with NaCl as the major component and TTX (10^{-6} *M*) added to prevent cell depolarization. The details of the binding procedures have been described (Berwald-Netter et al. 1981). Briefly, cells were incubated for 30 min at 37 °C with different concentrations of ^{125}I-labeled toxin, with or without unlabeled toxin (2×10^{-7} *M*), giving respectively the non specific and the total ^{125}I-toxin binding. Unbound toxin was then removed by three washes with the incubation medium. To test its effect on toxin binding, oleic acid was added to the incubation medium as for electrophysiological recordings (10 min, 5×10^{-6} *M*). The assays were performed in triplicates or in quadruplicates. Values are expressed as mean ± SEM. Statistical comparison were made using Student's *t*-tests.

Results

Before studying the effects of *AaH* II we first determined whether a portion of the voltage-activated Na sodium current remained during long imposed voltage steps, since a late sodium current has been described in a number of other preparations (see Patlak and Ortiz 1986). In order to test this hypothesis the total current was recorded during voltage pulses of various amplitudes, successively without and with 300 n*M* tetrodotoxin in the external medium. Under both conditions, the current was measured at peak time and at the end of 40 ms duration voltage pulses. From the experiment presented in Fig. 1 it appears that: 1) the leakage conductance is larger at positive than at negative voltages (see also Moolenaar and Spector 1978) and 2) a small fraction of the sodium current does not inactivate during long lasting depolarizations. In the example shown, the maintained sodium conductance relative to the peak Na conductance was 0.98%. In two other experiments, it was 0.72% and 2.14%. Thus in this respect, neuroblastoma cells are very similar to skeletal muscle and axons in which a small maintained Na current has been described (see Patlak and Ortiz 1986). In the following experiments, the zero Na current was determined either after blockade of Na channels by tetrodotoxin or from the current level at the end of long lasting depolarizations in the absence of scorpion toxin. In the latter case, an error of one to two percent in the amplitude of the peak current was introduced into the determination of zero Na current. We considered this error negligible compared to the effects of scorpion toxin (see below).

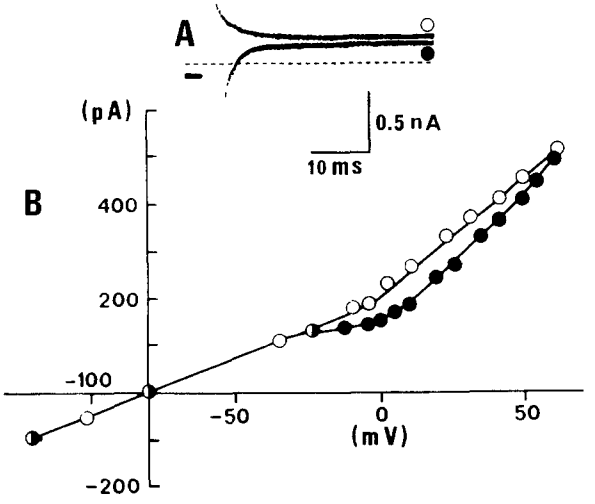

Fig. 1 A and B. Zero sodium current determination and evidence for a small maintained sodium current under control conditions. **A** Superimposed current traces recorded during depolarizations to 0 mV in absence (filled circles) and in presence (open circles) of TTX (3×10^{-7} *M*). Dotted line corresponds to the level of current at the holding potential (−80 mV). The peak of I_{Na} is out of scale, only the end of the inactivation phase of this current is observed. **B** Current-voltage relationships for the current measured at the end of 40 ms duration pulses, in absence (filled circles) and in presence (open circles) of TTX (3×10^{-7} *M*). The amplitude of this current corresponds to the difference between the current at −80 mV (zero current level) and the one at the end of each pulse

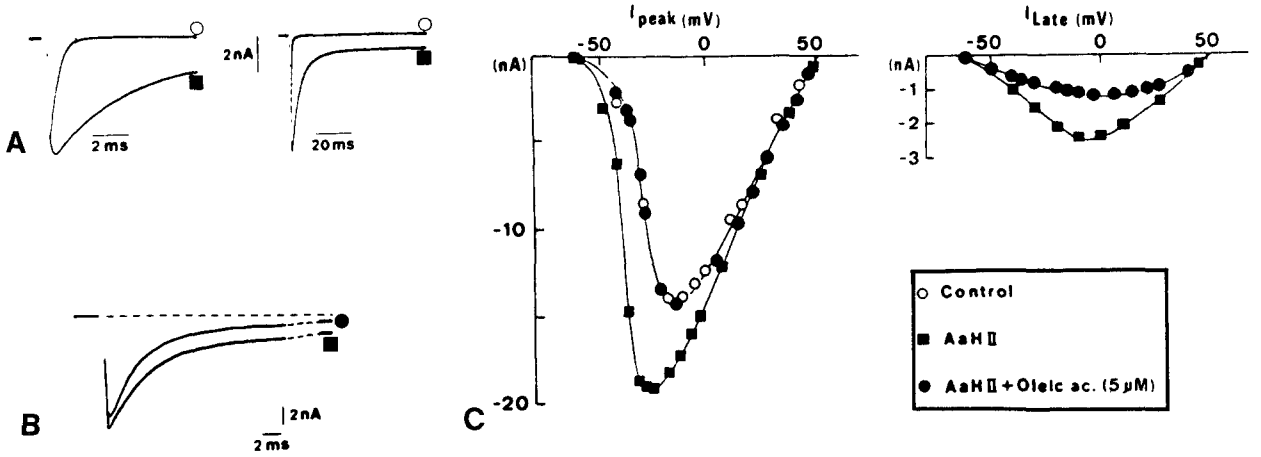

Fig. 2 A–C. Scorpion toxin-induced effects on I_{Na}. **A** Superimposed recordings of Na currents recorded during depolarizations to 0 mV from a holding potential of −80 mV, under control conditions (open circles) and in the presence of 70 n*M* *AaH* II (filled squares), at two different time scales. **B** Superimposed recordings of Na currents recorded during depolarizations to 0 mV (holding potential = −80 mV), in presence of 70 n*M* *AaH* II (filled squares) and 10 min after addition of 5×10^{-6} *M* oleic acid to *AaH* II containing medium (filled circles). Dotted line corresponds to zero current level. Notice the interruption of the recordings (40 ms, *dashed lines*). **C** Peak (*left*) and *AaH* II-induced maintained (*late*, *right*) Na current-voltage relationships recorded under control conditions (open circles), in the presence of 70 n*M* *AaH* II (filled squares) and after addition of oleic acid (5×10^{-6} *M*) to a *AaH* II containing medium (filled circles)

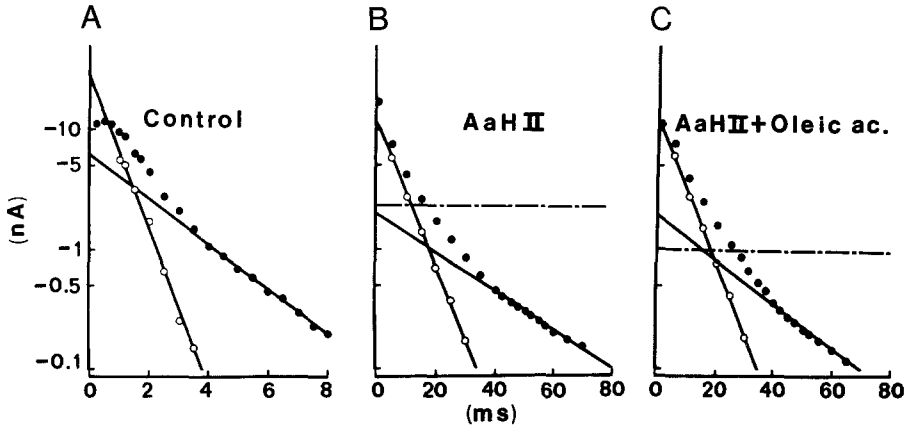

Fig. 3 A–C. Semi-logarithmic representations of the inactivation phase of the peak current recorded at 0 mV under control conditions (**A**), in the presence of 70 n*M* *AaH* II (**B**) and 10 min after adding 5×10^{-6} *M* oleic acid to a medium containing 70 n*M* *AaH* II (**C**). *Dotted lines* represent the maintained current induced by *AaH* II and extrapolated to the time of depolarization. *Filled circles* give the overall Na current (**A**) or overall Na current minus the maintained current (**B** and **C**) and *open circles* give the fast phase of inactivation after subtraction of the slow phase of inactivation (*continuous line* through *filled circles*). Time contants of fast and slow phases of current inactivation were respectively: 0.70 and 2.4 ms (control), 6.0 and 26 ms (*AaH* II), 6 and 24 ms (*AaH* II plus oleic acid)

Effects of AaH II on the sodium current

In the presence of toxin (70 n*M*), maintained and peak currents were increased and the inactivation of the peak current was slowed (Fig. 2 A). The amplitude of the maintained current, under these conditions, relative to that of the peak averaged 0.13 ± 0.01 ($n = 13$). In 13 cells depolarized to 0 mV, the amplitude of the peak current in the presence of toxin was 1.23 ± 0.03 times that of the peak current before the addition of *AaH* II. Under control conditions, the inactivation of the peak current at 0 mV could be described by the sum of two exponentials (Fig. 3 A) whose time constants ($\tau_F = 0.68 \pm 0.22$ ms and $\tau_S = 2.31 \pm 0.61$ ms, $n = 6$), were increased after addition of *AaH* II by a factor of 7.5 ± 1.6 and 10.6 ± 2.8 ($n = 5$), respectively (Fig. 3B). The increase in the peak sodium current induced by *AaH* II was due to a negative shift of the activation-voltage curve (Fig. 2C). The shift of the activation-voltage curve induced by the toxin (expressed as the shift of the half maximum peak conductance along voltage axis ($V_{gNa=0.5}$) was -9.1 ± 1.6 mV ($n = 8$). The peak current was not modified at positive voltages.

Modifications of the AaH II-induced effects by oleic acid

In the absence of toxin, oleic acid (5 μ*M*) did not significantly modify the sodium current. In contrast, when oleic acid was added to a toxin-containing medium, both peak and maintained currents were decreased (Fig. 2). Ten minutes after oleic acid application, the peak current at 0 mV, which was increased from 6.8 ± 0.9 nA ($n = 13$) under control conditions to 8.2 ± 1.0 nA ($n = 13$) in the presence of the toxin, had returned to its control value (6.9 ± 0.5 nA, $n = 9$) and the maintained current was decreased to $44 \pm 6\%$ ($n = 9$) of its value in presence of *AaH* II alone. Analysis of the peak current-voltage curve revealed that the effect of oleic acid on peak current, recorded in the presence of *AaH* II, was due to a positive shift of the curve (Fig. 2 C) with recovery of its control position along the voltage axis ($V_{gNa=0.5} = -25.0 \pm 1.03$ mV under control conditions and -27.0 ± 1.25 mV in presence of *AaH* II plus oleic acid, $n = 4$). In contrast to the effects on the amplitudes of peak and maintained currents, oleic acid did not modify the inactivation time constants of the Na peak current (Fig. 3 C). The ratio between the time constant values (*AaH* II plus oleic acid/*AaH* II) was $0.87 + 0.05$ ($n = 9$) for the fast time constant and 0.99 ± 0.05 ($n = 9$) for the slow one. Similar effects were observed when the toxin was added after oleic acid. Under these conditions, in three cells, the inactivation time constants were increased in the same proportions as in the absence of oleic acid, whereas the peak current was not increased and the voltage corresponding to half maximum peak conductance was shifted by only 1 to 3 mV towards negative values.

Binding of AaH II

The above results show that oleic acid alters the effects of the toxin. This could be due to a modification by oleic acid of the channel-toxin complex or/and to a decrease in the toxin binding. In order to test these possibilities, we carried out binding assays of ^{125}I labeled *AaH* II to *NG* 108-15 cells in the absence and in the presence of 5 μ*M* oleic acid. The results of a representative experiment are given in Fig. 4. In four independent assays the binding isotherms and the Scatchard plots of specific binding (inset Fig. 4) showed no or little difference in toxin binding values between control and oleic acid treated cultures. The mean dissociation constant values were $K_D = 0.96 \pm 0.26$ n*M* ($n = 4$) in the controls and $K_D = 1.14 \pm 0.27$ n*M* ($n = 4$) in presence of oleic acid. Likewise, the ratio between the B_{Max} values (oleic acid treatment/controls) was little modified: 1.13 ± 0.16 ($n = 4$). Similar results were obtained when the binding assays were performed in incubation medium containing choline or Na^+ (in the presence of 10^{-6} *M* TTX) as the major cation. Thus, under the experimental conditions of this study oleic acid did not affect the properties of scorpion toxin binding.

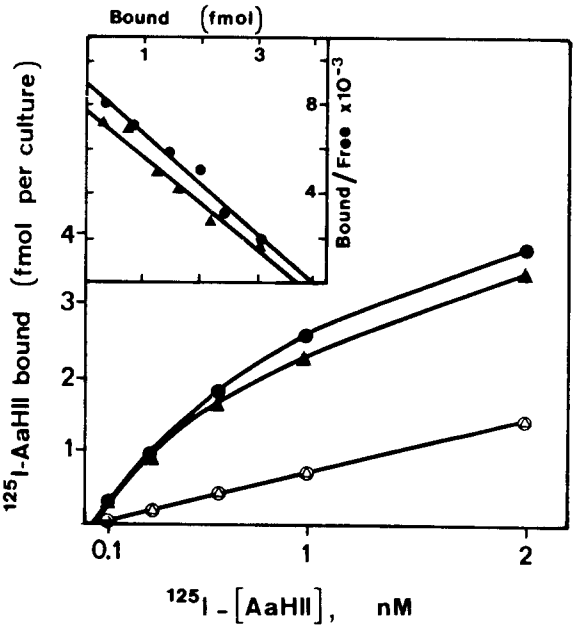

Fig. 4. Binding of ^{125}I-*AaH* II scorpion toxin to NG 108-15 cells. Replicate cultures were incubated with increasing concentrations of ^{125}I-*AaH* II with (open and filled triangles) or without (open and filled circles) 5 μ*M* oleic acid. Non specific binding (open triangles and open circles) was measured in the presence of 0.2 μ*M* of unlabeled *AaH* II. *Inset*: Scatchard plot of specific binding, calculated as the difference between the total binding and the corresponding non specific binding. Each point was determined in triplicate; the SEM values were only $3.6 \pm 1.6\%$ of the mean binding values and were thus smaller than the size of the symbols

Effects of phorbol 12-myristate 13-acetate on the sodium current

It has been shown that unsaturated fatty acids produce a direct activation of protein kinase C (Murakami and Routtenberg 1985). One might suggest that the observed effects of oleic acid are somehow related to this. In order to test this hypothesis, experiments were performed in the presence of PMA, which is known to stimulate protein kinase C. Under control conditions PMA, up to 32 n*M*, had no significant effects on the Na current voltage-curve within 5 min of its application. A slight reduction (about 5%) in the amplitude of the Na current was observed if PMA was applied for a longer period of time (8–10 min). In the presence of *AaH* II (70 n*M*) PMA neither significantly affected the *AaH* II-induced shift of the Na current activation-voltage curve ($V_{gNa=0.5} = -30.7 \pm +1.1$ mV in the presence of *AaH* II and 28.5 ± 1.6 mV in the presence of *AaH* II + PMA, $n = 3$), nor did it modify the inactivation time course of the Na current. Only a

slight reduction in peak and late current amplitudes was observed. Similar results were obtained when *AaH* II was added after PMA.

Discussion

The principal findings in this report are that toxin II of the scorpion *Androctonus australis Hector* (*AaH* II) has three different and independent effects on sodium current in neuroblastoma X glioma hybrid cells (NG 108-15): it increases the inactivation time constants of peak current, induces a late current and enhances the peak current amplitude *via* a negative shift of the current-voltage curve. These two latter effects could be reversed by external oleic acid which by itself has no effect on sodium current.

Comparison of the effects of AaH II in NG 108-15 cells with the effects of scorpion toxins in other preparations

The use of tetrodotoxin to block Na current reveals the existence of a small Na current that does not inactivate and which represents about 1% of the peak current. This maintained Na current is similar to the late or persistent Na currents previously described in squid giant axon, node of Ranvier and skeletal muscle fibre (see Patlak and Ortiz 1986). It could correspond either to Na channels which are temporarily in a non inactivatable form (Patlak and Ortiz 1986; Benoit and Dubois 1987) or be due to the existence of several inactivated states of Na channels (Schmidtmayer 1985). In the present work we show that in neuroblastoma cells *AaH* II causes an increase of this maintained Na current to about 13% of peak current amplitude. After subtracting the maintained Na current from the total Na current, the inactivation decay of the peak current can be described by the sum of two exponentials whose time constants are increased by the scorpion toxin. These results are similar to those previously described for scorpion toxins in nerve fibres (see Benoit and Dubois 1987). The large negative shift of the peak conductance-voltage curve induced by α scorpion toxins (Gonoi et al. 1984; present results Fig. 2) seems to be specific to neuroblastoma cells since it is not observed or is very small in the node of Ranvier (Benoit and Dubois 1987). According to Gonoi and Hille (1987) Na channels in neuroblastoma cells, in contrast to those of other preparations, could have sequential and irreversible transitions between closed, open and inactivated states. In the framework of this model, a slowing of inactivation should induce a negative shift of the peak conductance-voltage curve. While this model might take into account the effects of *AaH* II, it does not account for any interaction of toxin molecules with activation channel gates which might also occur. The dissociation of the effects of the toxin upon channel inactivation and activation evoked by oleic acid supports this latter explanation.

Modulation of AaH II effects by oleic acid

In previous reports (Naumov 1983; Benoit and Dubois 1987), it has been suggested that the effects of α scorpion toxins and other Na inactivation gate modifiers would not only be dependent on the binding of these agents to specific channel sites but also on channel lipid environment. This hypothesis is compatible with the observation that oleic acid evidently modulates some of the effects of *AaH* II on Na inward current in neuroblastoma cells. However, oleic acid is known to activate protein kinase C (Murakami and Routtenberg 1985), which has been shown to phosphorylate α subunits of Na channels (Costa and Catterall 1984). But PMA, a very potent activator of protein kinase C, failed to reproduce the effects observed in the presence of oleic acid. Although oleic acid had no effect upon the membrane potential or control Na current of either neuroblastoma cells (Love et al. 1985; present results) or node of Ranvier (Benoit, personal communication) we show that oleic acid modifies the response of neuroblastoma cells to *AaH* II. It had no effect upon *AaH* II modified Na currents in node of Ranvier (Benoit, personal communication). Giraud et al. (1981) showed that oleic acid altered the properties of Na/K-ATPase in red blood cells by increasing membrane fluidity and since membrane viscosity changes during the cell cycle and is different in normal and transformed cells (see DeLaat et al. 1977) it is tempting to think that the differential effets of *AaH* II in neuroblastoma cells and in node Ranvier could be related to different lipid environments of Na channels in these two preparations. That oleic acid modifies the effects of *AaH* II without altering its binding strongly suggests that at least a part of the effects of α scorpion toxins on Na channels (and likely of other Na inactivation gate modifiers) may be dependent on membrane lipids (Naumov 1983; Rack 1986; Benoit and Dubois 1987). The most likely explanation of the effects of *AaH* II on Na channels and their modulation by oleic acid is that the increase in inactivation time constants is dependent on the direct interaction between channels and toxin molecules that bind to specific channel sites (Couraud et al. 1978; Berwald Netter et al. 1981; 1983; present results Fig. 4), whereas the increase in maintained current and the shift of the conductance-voltage curve are dependent on interactions with membrane lipids as well. The observation that oleic acid reverses the *AaH* II-induced shift of the activation-voltage curve, whereas it does not modify the *AaH* II effects on inactivation time constants, is not in favor of the model proposed by Gonoi and Hille (1987). Fur-

ther experiments would be needed to determine precisely the mode of action of oleic acid.

As concluded for other membrane molecular systems (DeLaat et al. 1977; Giraud et al. 1981), the present results indicate that functional properties of the glycoprotein forming the action potential Na channel might be not only dependent on its composition and organization but also on its environment and especially the proportion and nature of membrane lipids (see Feller et al. 1985). Exploration of the effects of other exogenous lipids on Na channels properties and toxin effects could help to confirm this hypothesis.

Acknowledgements. We thank Dr. I. Findlay for critical reading of the manuscript and helpful discussions. This work was supported by grants from the Ministère de la Recherche et de la Technologie (85 C 1134), the Institut National de la Santé et de la Recherche Médicale (866 006) and the Direction des Recherches Etudes et Techniques (85.34.144).

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