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Effects of Fatty Acids on Membrane Currents in the Squid Giant Axon

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Summary. The effects of fatty acids on the ionic currents of the voltage-clamped squid giant axon were investigated using intracellular and extracellular application of the test substances. Fatty acids mainly suppress the Na current but have little effect on the K current. These effects are completely reversed after washing with control solution. The concentrations required to suppress the peak inward current by 50% and Hill number were determined for each fatty acid. ED_{50} decreased about $\frac{1}{3}$ for each increase of one carbon atom. The standard free energy was -3.05 kJ mole^{-1} for CH_2. The Hill number was 1.58 for 2-decenoic acid. The suppression effect of the fatty acids depends on the number of carbon atoms in the compounds and their chemical structure. Suppression of the Na current was clearly observed when the number of carbon atoms exceeded eight. When fatty acids of the same chain length were compared, 2-decenoic acid had strong inhibitory activity, but sebacic acid had no effect at all on the Na channel. The currents were fitted to equations similar to those proposed by Hodgkin and Huxley (*J. Physiol. (London)* **117**:500–544, 1952) and the changes in the parameters of these equations in the presence of fatty acids were calculated. The curve of the steady-state activation parameter (m_∞) for the Na current against membrane potential and the time constant of activation (τ_m) were shifted 20 mV in a depolarizing direction by the application of fatty acids. The time constant for inactivation (τ_h) was almost no change by application of the fatty acids. The time constant for activation (τ_n) of K current was shifted 20 mV in a depolarizing direction by the application of the fatty acids.

Key Words fatty acids · membrane currents · membrane excitation · 2-decenoic acid · lateral motion · squid axon

Introduction

According to the fluid mosaic model both proteins and lipids are free to diffuse in the cell membrane bilayer, implying a random organization of proteins and lipids (Singer & Nicolson, 1972). However, experimental evidence indicates that the lateral motion of most proteins is not determined primarily by free diffusion but is constrained by various mechanisms, such as interaction with cytoskeletal components. Karnovsky et al. (1982) present the concept that a lipid may be organized in domains which have functional significance and that proteins could order the packing of surrounding lipids by cooperative types of interactive force. The application of certain fatty acids to a biological membrane could be expected to profoundly affect its nature. A recent study of ours has shown that myrmicacin suppresses the excitability of the axon (Takenaka et al., 1981). Myrmicacin is a substance secreted by South American leaf-cutting ants and has been identified as 3-hydroxydecanoic acid (Schildknecht & Koob, 1971). The effects of octanoic acid on the Na current of the squid axon have been described by Elliott et al. (1984).

The experiments reported in this paper were designed to explore the effect of fatty acids on membrane currents and on the Hodgkin and Huxley parameter. The Na current records from experiments with perfused axons were analyzed by means of equations similar to those proposed by Hodgkin and Huxley (1952). In this way the effects of test substances can be described in terms of alteration in the steady-state parameters (m_∞, h_∞) and time constants (τ_m, τ_h, τ_n) used in the Hodgkin-Huxley formulation.

It is well known that under normal conditions the long-chain fatty acids predominate in mammals. However, medium-chain fatty acids (C8–C12) can appear after the β-oxidation of fatty acids. These fatty acids are increased by the disease of medium-chain acyl-CoA dehydrogenase deficiency. For these reasons we focused our work on these medium-chain fatty acids and analyzed their effect on excitable membranes. First we examined the unsaturated medium-chain fatty acids, because they are more water-soluble than saturated fatty acids, and then we examined the saturated fatty acids. We found that the number of the carbon chain was related to the inhibition effects of Na current. Next we compared the effects of fatty acids of the same chain length. We examined the position of -OH in

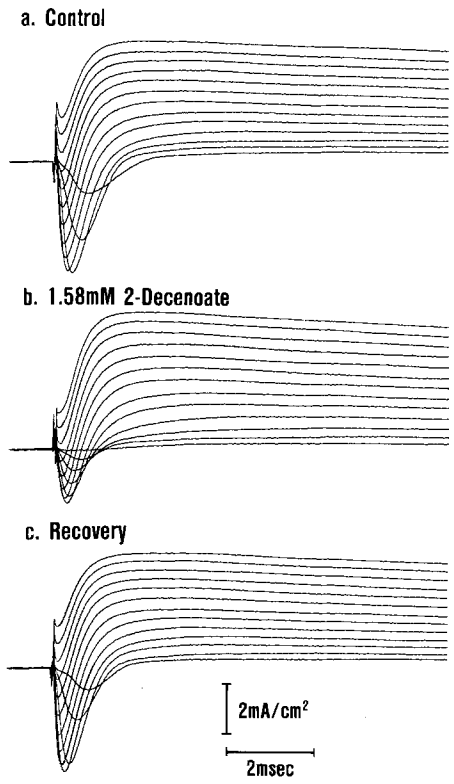


Fig. 1. Superimposed traces of the membrane currents in voltage-clamped squid axons, showing families of membrane currents associated with step depolarization before (*a*) and after (*b*) application of 2-decenoic acid internally, and after washing with fatty acid-free internal solution (*c*). The membrane was held at −70 mV and 10-msec pulses were applied which took the potential to a level varying between −40 and +80 mV in 10-mV steps

the fatty acids and found that the position of -OH was also related to the Na current inhibition. 10-hydroxydecanoate was also studied, but it had no effect on the Na current. Likewise, sebacic acid, which has carboxyl groups at both ends of the chain, had no effect on the Na current.

Materials and Methods

Experiments were carried out with squid giant axons dissected from the mantles of freshly killed *Doryteuthis bleekeri* obtained at the Misaki Marine Biological Station of Tokyo University. The axons were carefully cleaned before use, and were usually between 400 and 600 μm in diameter.

The composition of the external artificial seawater was (mM): 490 NaCl, 10 KCl, 50 $MgCl_2$, and 10 $CaCl_2$. It was buffered with 10 mM Tris-HCl (pH 8.0). When necessary, sodium currents were suppressed by the addition of 0.3 μM tetrodotoxin. The internal perfusing fluid normally used contained 300 mM K-glutamate, 50 mM NaF, 6 mM K-HEPES and sufficient glycerol to maintain osmolality. For analyzing the Na currents we added 15 mM tetraethylammonium fluoride to the internal perfusing solution. The pH of the solution was adjusted to 7.2. The fatty acid compounds were supplied by Tokyo Kasei Co., Japan. The fatty acids with the hydroxyl groups were provided by Dr. Yoshiro Miura, Dept. of Biochemistry, Teikyo University, and Yozo Iwanami, Dept. of Biology, Yokohama City University.

The method of internal perfusion was the same as that described by Takenaka and Yamagishi (1969). The perfused zone of the axon was 15 mm in length. The internal electrodes consisted of an 80-μm glass capillary for measuring the internal potential and a 50-μm platinum wire for sending currents. The glass capillary was filled with 0.6 M KCl in contact with a KCl-calomel electrode in the shank of the capillary. A 25-μm uncoated platinum wire ran through the capillary to reduce the high frequency impedance. The external electrodes were 0.6 M KCl agar in contact with a calomel electrode for membrane potential recording and an Ag-AgCl wire for current recording.

For the voltage-clamp-experiments, we followed the techniques described by Bezanilla, Rojas and Taylor (1970). In order to reduce the error caused by the resistance in series with the membrane we employed a feedback system with a positive loop permitting potential compensation (Hodgkin, Huxley & Katz, 1952; Bezanilla et al., 1970). The data from voltage-clamp currents were stored in a digital oscilloscope (Nicolet model 2090). Sampling points were 20 μsec apart and calculated with an NEC PC-8801. These data were analyzed by the nonlinear least squares method, either the damping Gaus Newton method or the Symplex method (Nelder & Mead, 1965; Haydon & Kimura, 1981, Yamaoka et al., 1981).

The temperature at which the experiments were carried out was 5.0 ± 1.0 °C for analyzing the voltage-clamp currents and 15.0 ± 1.5 °C for the other experiments.

Results

Effects of 2-Decenoic Acid on the Membrane Character

When 1 mM of 2-decenoic acid was applied externally to the intact axon bathed in artificial seawater, the action potential gradually decreased in amplitude and was eventually blocked completely 15 to 30 min after application. Washing with fatty acid-free artificial seawater caused a complete recovery of the action potential. The membrane potential was usually depolarized 2 to 3 mV by the application of 2-decenoate, but the membrane resistance was little affected by external application of this substance. Internal application of 2-decenoate caused the same effect as the external application, but the time course was much faster, and block of the action potential occurred 2 to 3 min after application. The rate of recovery after washing was fairly rapid and complete recovery occurred. Figure 1 illustrates the membrane currents before and after internal application of 1.58 mM 2-decenoate. The top set of curves (*a*) shows a family of membrane currents associated with step depolarization before application of 2-decenoate. The second set of curves (*b*)

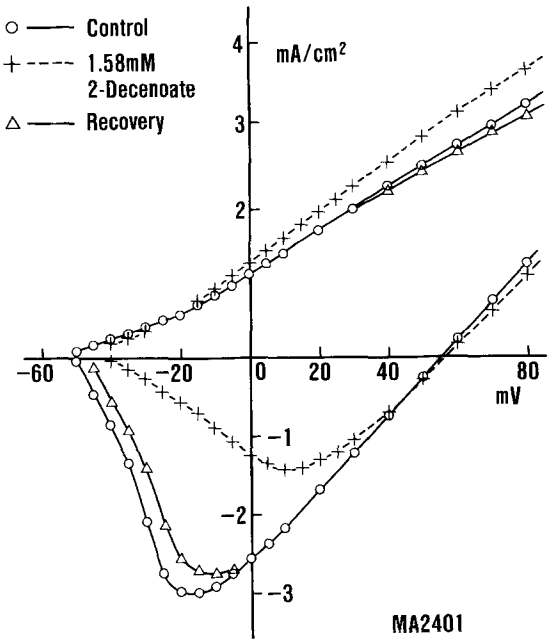


Fig. 2. Current-voltage relation for peak inward current and steady-state outward current before and during application of 2-decenoic acid, and after washing with fatty acid-free internal solution

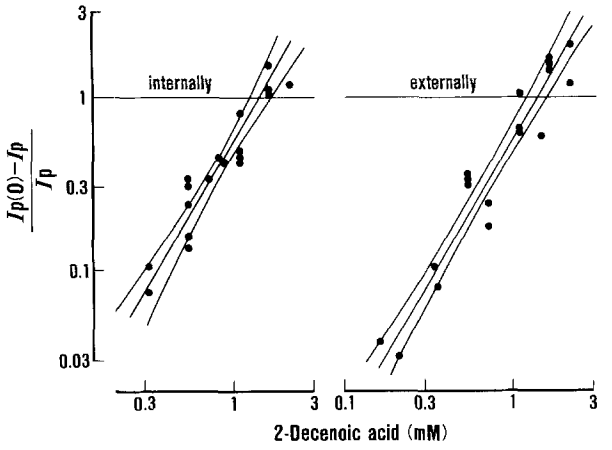


Fig. 3. Natural variation of Hill plot for the inhibitory action of 2-decenoic acid. Abscissa: concentration of 2-decenoic acid in logarithmic scale. Ordinate: the relative value of suppression to control. I_p: the peak Na current during application. $I_p(0)$: peak Na current in the control. The linear regression lines show the 95% confidence interval

shows changes after internal application of 2-decenoate. The inward Na currents were suppressed, but the outward K currents were little affected by application of this drug. Note that the time to peak transient current changes during blockage. The third set (*c*) is the family of currents after washing, showing recovery from the drug effects. External application of 2-decenoic acid exerts a very similar effect on the membrane current. A shift of the peak Na current was also observed in both internal and external application of 2-decenoate. These results imply that the potential-dependent kinetic parameters of the Na current also shift along the voltage axis.

The peak Na current and steady-state outward K currents before and after internal application of 2-decenoate are plotted as a function of membrane potential in Fig. 2. It is clearly seen that 2-decenoate blocks inward Na current but has virtually no effect on the K current. Complete recovery also occurred after washing with fatty acid-free internal solution. External application of 2-decenoate also caused the same effects. 2-decenoate blockade of Na channels is accompanied by a shift of the current-voltage curve towards more positive membrane potentials, but the application induced no changes in the reversal potential of the Na current at +55 mV.

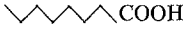
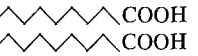
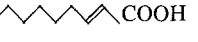
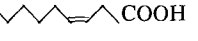
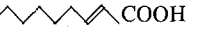
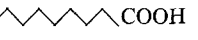
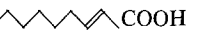
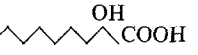
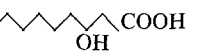
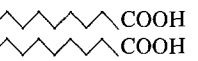
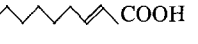
Dose-Response Relation for 2-Decenoate

The relation between the suppression of the Na current and the concentration of 2-decenoate was studied (*see* Fig. 3). The concentration that caused 50% suppression of the maximal Na current (ED_{50}) was 1.37 mM for external application and 1.43 mM for internal application. Analyses were also extended to Hill's plot where the ordinate represents the responses with reference to the suppression relative to the control and the abscissa shows the concentration of 2-decenoate in logarithmic scale. Linear regression analysis was done and a 95% confidence interval was calculated to establish natural variation. A slope equal to 1.58 was obtained in both cases, indicating that the stoichiometry of the blocking action of 2-decenoate was not one-to-one.

Effects of Other Fatty Acids

The suppression of Na currents by other fatty acids was compared, and the results are summarized in Fig. 4 and the Table. Fatty acids with less than 7 carbon atoms had little effect on either the Na or the K currents. Obvious suppression was observed only when the number of carbon atoms exceeded 8. The ED_{50} decreased about $\frac{1}{3}$ for each increase of one carbon atom. The standard free-energy change of transfer was 3.05 kJ mole^{-1} for CH_2. Fatty acids with more than 13 carbon atoms are almost insoluble in artificial seawater. The inhibition of Na currents by the fatty acids could be divided into three types. One was a completely reversible type in

Table. Effects of fatty acids on the inhibition of Na current[a]

Fatty acid		ED_{50}	n_H	N
Internally applied:				
Saturated				
Caprylate		16 (9–30)	1.6 (1.13.3)	5
Pelargonate		4.3	2.9	3
Caprate		1.1 (0.8–1.5)	2.8 (1.9–6.4)	6
Undecanoate		0.41	2.2	3
Laurate		0.32 (0.22–0.47)	2.0 (1.3–4.1)	5
Tridecanoate		0.36	2.1	3
Unsaturated				
2-Decenoate		1.43 (1.24–1.65)	1.66 (1.39–2.06)	18
3-Decenoate		1.2 (0.8–1.9)	1.6 (1.2–2.7)	5
2-Undecenoate		0.46 (0.41–0.51)	2.3 (2.0–2.8)	6
10-Undecenoate		1.04 (0.80–1.36)	2.6 (1.9–4.2)	6
2-Tridecenoate		0.20	5	3
OH-				
2-Hydroxydecanoate		0.76 (0.66–0.88)	1.85 (1.36–1.89)	12
3-Hydroxydecanoate		9.6	0.9	4
2-Hydroxyundecanoate		0.40	2.8	3
3-Hydroxyundecanoate		0.46 (0.39–0.55)	2.0 (1.6–2.6)	7
Externally applied:				
Pelargonate		3.4	2.0	3
Caprate		1.7		
2-Decenoate		1.37 (1.20–1.57)	1.67 (1.49–1.90)	19
3-Hydroxydecanoate		6.1	1.5	2

[a] In order from above: the saturated fatty acids (internal application), the unsaturated fatty acids (internal application), the fatty acids with a hydroxyl group (internal application), and the fatty acids (external application). The inhibitory effects of fatty acids on Na current depend on the number of carbon atoms in the compounds and its chemical structures. 95% intervals are given in parentheses. N shows the number of experiments.

which mainly Na current was suppressed with little depolarization. This type was represented by the fatty acids with less than 10 carbon atoms, such as 2-decenoic acid, capric acid, etc. The second type was represented by undecanoic acid, lauric acid, etc. These acids suppressed the Na current and gradually depolarized the resting membrane potential, and their effects were irreversible. In the third type of inhibition suppression of both Na and K currents was observed, in addition to depolarization. It was completely irreversible and the membrane was destroyed. Typical substances with this type of inhibition were palmitic acid, linoleic acid, linolenic acid, etc. These irreversible effects may be caused by micelles. When we compared the effects of fatty acids of the same chain length, 2-decenoate and 3-decenoate had about the same effects as caprate. In other words, saturated fatty acids and unsaturated fatty acids have almost the same effects on the suppression of the Na current. The position of -OH in the fatty acids was related to the Na current inhibition. 2-hydroxydecanoate had about the same effect as caprate, but 3-hydroxydecanoate was weaker than caprate and 10-hydroxydecanoate had no effect on the Na current. Sebacic acid, which has carboxyl groups at both ends of the chain, had no effect on the Na current or the resting potential. The same tendency toward inhibition of

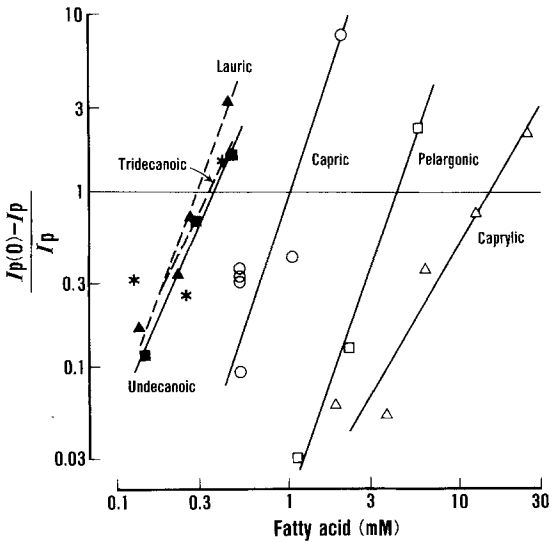


Fig. 4. Dose-response relation for inhibition of Na current by fatty acids. I_p: the peak Na current during application. $I_p(0)$: peak Na current in the control

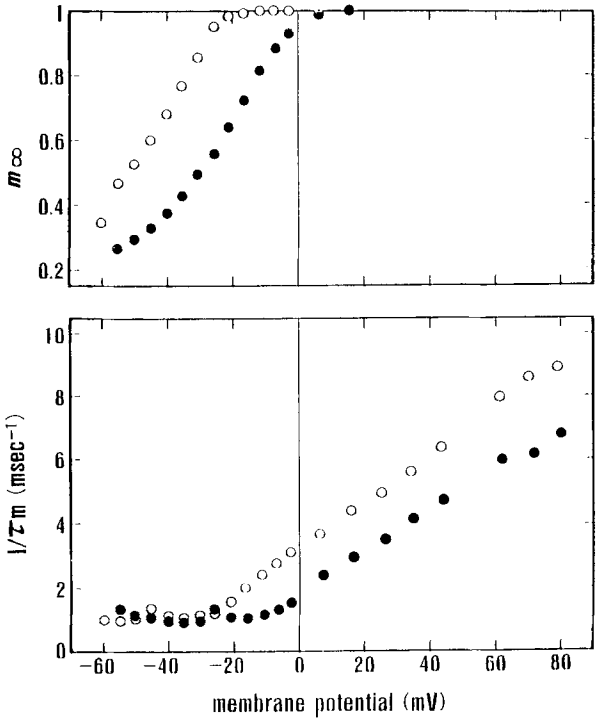


Fig. 5. Effects of internal application of 1.54 mM 2-decenoate on the steady-state activation (m_∞) and activation time constant (τ_m) of the Na current. White dots: control records; black dots: test records. Abscissa shows the membrane potential

the Na current was observed when these compounds were applied extracellularly. Therefore, the suppression of the Na current depends on the number of carbon atoms in the compound and its chemical structure.

Hodgkin-Huxley Parameters

The membrane currents were analyzed as in the work of Haydon and Urban (1983*a*) according to an equation derived from the relationships of Hodgkin and Huxley (1952):

$$I_{Na} = I'_{Na}[1 - \exp(-t/\tau_m)]^3[h_\infty(1 - \exp(-t/\tau_h)) + \exp(-t/\tau_h)]. \quad (1)$$

The data for an axon analyzed with the Hodgkin-Huxley parameters are shown in Figs. 5 and 6. There is a depolarizing shift of m_∞ and $1/\tau_m$ of about 20 mV. The inactivation parameters h_∞ and $1/\tau_h$ are little affected. The major contributors to the suppression of the peak Na current I_p are the positive m_∞ and $1/\tau_m$ shift. Records of K currents before and after exposure of the axon to 1.58 mM 2-decenoate were analyzed by the following equation of Hodgkin and Huxley (1952):

$$I_K = I'_K[1 - \exp(-t/\tau_n)]^4. \quad (2)$$

There is a depolarizing shift of $1/\tau_n$ of about 20 mV (Fig. 7).

Discussion

Fatty Acids and the Membrane Currents

The suppression of the Na current depends on the number of carbon atoms and the chemical structure of fatty acids. Fatty acids with less than 7 carbon atoms have little effect on the Na current. The ED_{50} increased about 3 times for each decrease of one carbon atom in the chain. The chain-length dependence suggests that a hydrophobic interaction is an important factor. Assuming that the ED_{50} can be equated with the dissociation constant, the standard free energy change per mole per methylene group for the fatty acids-channel interaction can be calculated. The value was -3.05 kJ mole^{-1} for CH_2 for the series of compounds examined, suggesting that a hydrophobic interaction is indeed involved in the channel suppression. Requena and Haydon (1985) showed that the standard free energy of adsorption per CH_2 from the aqueous phase to the lipid is -2.92 kJ · mol^{-1} for the phospholipid system and -3.10 kJ · mol^{-1} for the phospholipid-cholesterol system of eggs. Kamaya, Kaneshitna and Ueda (1981) also showed that the increase in the absolute value of the standard free energy change with the

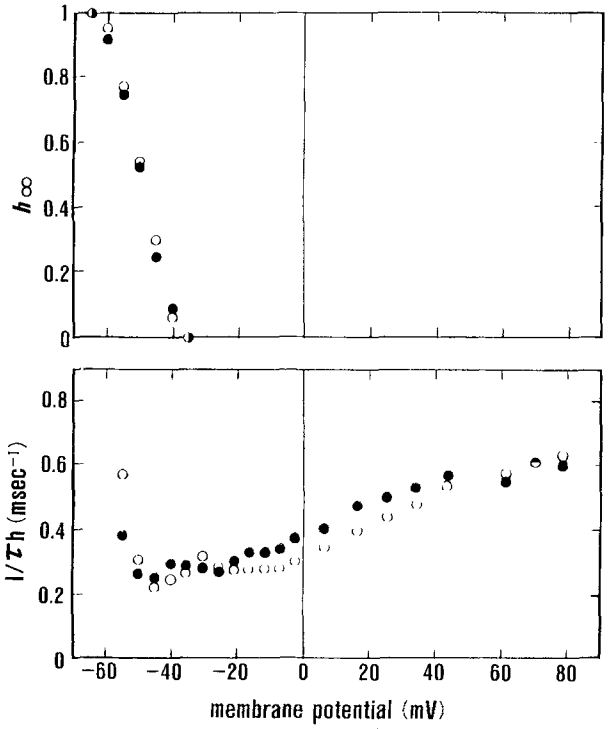


Fig. 6. Effects of internal application of 1.54 mM 2-decenoate on the inactivation (h_∞) and inactivation time constant (τ_h) of the Na current. White dots: control records; black dots: test records. Abscissa shows the membrane potential

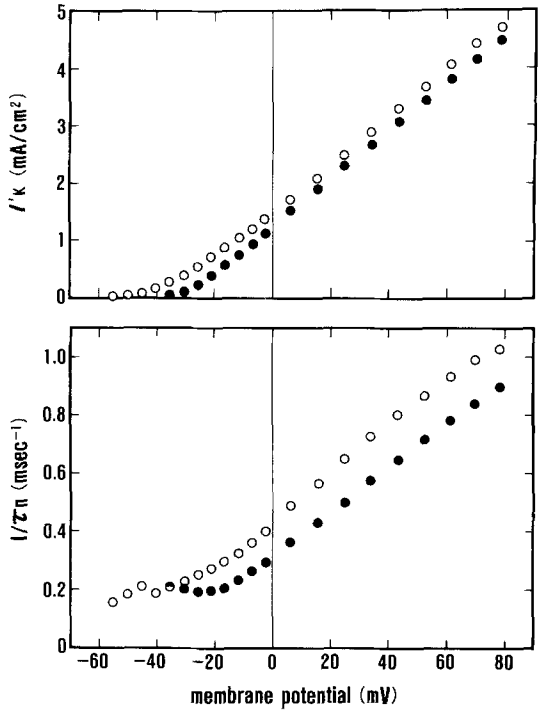


Fig. 7. Effects of 1.54 mM 2-decenoate on the I_k and $1/\tau_n$. White dots: control records; black dots: test records. Abscissa shows the membrane potential

increase of the carbon chain length of the *n*-alcohols is -3.43 kJ · mol^{-1} per CH_2 for dipalmitoylphosphatidylcholine. Yeh (1982) summarized the data and obtained the value of 600 calories · mole^{-1} per CH_2 for tetramethylammonium and tetramethylammonium derivatives. These data indicate that the perturbation of the lipid bilayer structure by the longer-chain fatty acids differs in important respects from that caused by the short-chain fatty acids. Another possibility is that interactions directly with membrane proteins are involved.

When the effects of fatty acids having the same chain length were compared, we found that inhibition depended on the chemical structure. Sebacic acid had no effect on the Na current. 10-hydroxy decanoic acid also had no effect on the Na current. We might surmise that the fatty acids got into the lipid layer of the membrane and caused the suppression of the Na current, whereas sebacic acid and 10-hydroxydecanoic acid, having carboxyl or hydroxyl groups in the ω position, were not able to get into the membrane and therefore had no effect on the Na current. Both internal and external application of the drugs inhibited the current, but internal application was more effective than external application. One reason for this may be that the external side of the membrane is covered with a thick Schwann sheath which impedes drug permeation, and another reason may be that the reacting point of the fatty acids is located near the internal surface of the membrane. As we discuss later, the fatty acids might affect a gate, and this gate might be located near the internal surface of the membrane (Armstrong, Bezanilla & Rojas, 1973; Narahashi & Seyama, 1974). Elliot et al. (1984) also observed the long time course caused by external application of octanoic acid and suggested that this acid acts at the internal surface.

Hodgkin-Huxley Parameters and Membrane Fluidity

The negative peak in the peak current-voltage relation measured by the voltage-clamp method was shifted towards the positive side by the application of fatty acids. These effects are different from the effects of tetrodotoxin, because tetrodotoxin causes no shift of the negative peak on the peak current-voltage relation (Narahashi, 1974). These effects are also different from those of local anesthetics, which cause no shift of the negative peak (Nara-

hashi & Frazier, 1971). On the other hand, these effects are similar to those of octyltrimethylammonium (Elliott et al., 1984) and alcohols (Haydon & Urban, 1983*b*).

The steady-state activation (m_∞) curve and $1/\tau_m$ show a shift in the depolarizing direction. The physical origin of the shift in the steady-state activation curve is not yet clear. Some change in the steady-state properties of the membrane must be considered and obvious candidates are the surface free energy or surface tension, and the dipole potential at the surface. We have found that fatty acids increase membrane fluidity (Takenaka, Horie & Kawasaki, 1983). The lipid lateral motion of the membrane complex was increased about 40% by the application of 2-decenoic acid, whereas valeric acid caused much smaller changes in the lateral motion of the membrane lipids. These results correspond to the electrophysiological phenomena in the axon membrane. 2-decenoic acid, having 10 carbon atoms, inhibits the Na current and shifts m_∞ and τ_m, whereas valeric acid, having 5 carbon atoms, does not inhibit the Na current. We might suppose that 2-decenoic acid gets into the lipid layer of the membrane and increases the lateral motion of the membrane, which causes perturbation of the membrane lipids and the Na channel lipoproteins.

The Role of Lipids in Excitability

How to explain the mechanism of this block of the Na channel by the fatty acids? Na channels in the nerve membranes are supposed to be surrounded by lipids. The lipid environment of the Na channel might play an important role in the functioning of the channel. In our experiments, the fatty acids having more than 8 carbon atoms showed a strong inhibitory effect on the Na channels. Usually, palmitic acid and stearic acid are the main fatty acids in the membrane lipids, but oleic acid and linoleic acid are also observed in 10 to 20% of the membrane lipids. Both fatty acids have a double bond at position 9. Oleic acid has a *cis* double bond at position 9 and linoleic acid has the double bond at positions 9 and 12. The role of the *cis* double bond in the unsaturated fatty acids is important. It lowers the transition temperature of the membrane, thereby enhancing the fluidity and disorder within the membrane. The *cis* double bond in position 9 is significant in the membrane (Haines, 1979), but there is no explanation of its meaning yet. This double bond creates a kink in a large fraction of membrane fatty acid chains enhancing the separation of two regions in the membrane. We have designated these regions the "stiff" and the "plastic" (disordered) regions. The stiff region is between the head group and the position 9 double bond. This region is in a regular array of *trans* conformations and the double bond enhances the rigidity of the membrane in this region because the *cis* double bond at position 8 will tend to maintain these *trans* conformations utilizing pi-pi interactions between adjacent chains at position 9. The plastic region is distal to the 9 carbon and has a greater degree of freedom and gauche conformations. The double bonds in the polyunsaturated fatty acids distal to carbon 9 facilitate *trans* bilayer pi-pi interactions enhancing compaction of the bilayer during the electrostriction. These Na channel proteins are usually amphilic: they have hydrophobic regions that interact with the hydrophobic tails of the lipid molecules in the interior of the bilayer and hydrophilic regions that are exposed to water on both sides of the membrane. The hydrophobicity is increased by the covalent attachment of fatty acid chains that help anchor these proteins in the bilayer. Gate *m* might be located in the hydrophobic region which corresponds to the plastic region. It appears that the fatty acids will be adsorbed with their long axes perpendicular to the membrane surface. The fatty acids increase the disorderliness in the plastic region of the membrane. This causes the perturbation of gate *m* and suppresses the function of *m* and as a result the Na channel is suppressed.

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