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Effects of Arachidonic Acid and the Other Long-Chain Fatty Acids on the Membrane Currents in the Squid Giant Axon

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Summary. The effects of arachidonic acid and some other long-chain fatty acids on the ionic currents of the voltage-clamped squid giant axon were investigated using intracellular application of the test substances. The effects of these acids, which are usually insoluble in solution, were examined by using α-cyclodextrin as a solvent. α-cyclodextrin itself had no effect on the excitable membrane. Arachidonic acid mainly suppresses the Na current but has little effect on the K current. These effects are completely reversed after washing with control solution. The concentration required to suppress the peak inward current by 50% (ED_{50}) was 0.18 mM, which was 10 times larger than that of medium-chain fatty acids like 2-decenoic acid. The Hill number was 1.5 for arachidonic acid, which is almost the same value as for medium-chain fatty acids. This means that the mechanisms of the inhibition are similar in both long- and medium-chain fatty acids. When the long-chain fatty acids were compared, the efficacy of suppression of Na current was about the same value for arachidonic acid, docosatetraenoic acid and docosahexaenoic acid. The suppression effects of linoleic acid and linolenic acid on Na currents were one-third of that of arachidonic acid. Oleic acid had a small suppression effect and stearic acid had almost no effect on the Na current. The currents were fitted to equations similar to those proposed by Hodgkin and Huxley (Hodgkin, A.L., Huxley, A.F. (1952) *J. Physiol (London)* **117**:500–544) and the change 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 10 mV in a depolarizing direction by the application of fatty acids. The time constant for inactivation (τ_h) has almost unaffected by application of these fatty acids.

Key Words arachidonic acid · long-chain fatty acids · membrane currents · Na channel · squid axon · membrane excitation

Introduction

Recently we have shown that medium-chain fatty acids inhibit the Na current in the excitable membrane (Takenaka, Horie & Hori, 1987). These fatty acids mainly suppress the Na current reversibly but have little effect on the K current. The membrane potential and the resistance are almost unaffected by these fatty acids. The lateral diffusion of the membrane lipid is greatly increased by these fatty acids (Takenaka et al., 1981).

It is necessary to expand this middle-chain fatty acid study to the long-chain fatty acids, because the living excitable membrane mainly contains long-chain fatty acids. The lipid composition of membranes varies dramatically among different cells or organelles and different sides or monolayers of the same membrane contain different lipid species. The main phospholipids of the membrane are phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl inositol, and sphingomyelin. Each lipid series exhibits a characteristic fatty acid composition. In glycerol-based phospholipids, it is usual to find a saturated fatty acid esterified at the 1-position of the glycerol backbone, and an unsaturated fatty acid at the 2-position. Phosphatidyl ethanolamine and phosphatidyl serine are most unsaturated fatty acids than other phospholipids. The fatty acids contained in the phospholipids of the membrane are palmitic acid, stearic acid, oleic acid, linoleic acid, arachidonic acid, adrenic acid, cervonic acid, and nervonic acid. These fatty acids are involved in the synthesis of prostaglandin and some of them are converted into prostaglandin. Arachidonic acid especially is important for biological membranes (Cullis & Hope, 1985). It is important to study the effects of these long-chain fatty acids on the membrane, but it is difficult to discover these effects because of the insolubility of these fatty acids in saline solution; they make micelles in solution. Recently, Singh and Kishimoto (1983) found that α-cyclodextrin helps to dissolve these acids in solution. Therefore, we planned to observe the effects of these long-chain fatty acids on the membrane current using α-cyclodextrin.

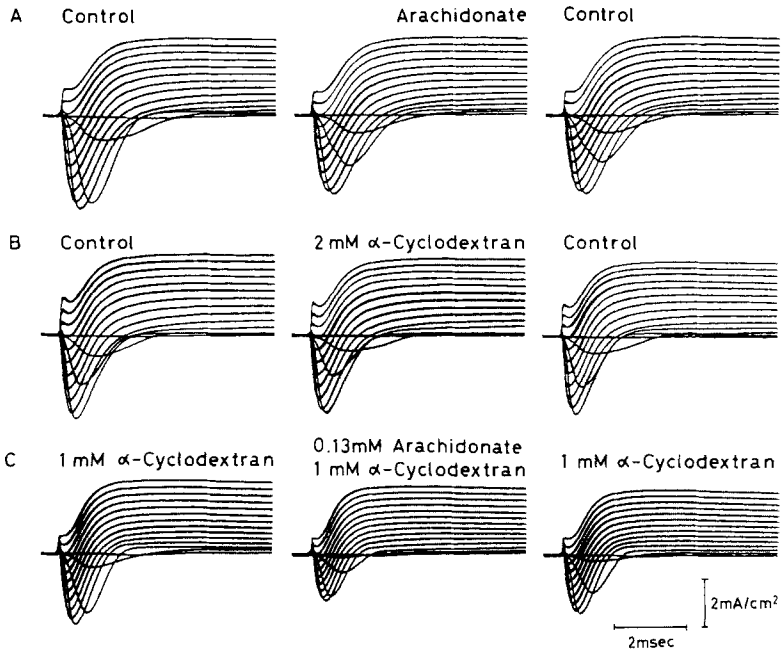


Fig. 1. Superimposed traces of the membrane currents in voltage-clamped squid axons, showing families of membrane currents associated with step depolarization before and after application of drugs internally, and after washing with drug-free internal solution. Line *A* shows the application of arachidonic acid alone and the equivalent of 0.64 mM applied internally. Line *B* shows the effect of α-cyclodextrin. Line *C* shows the effects of arachidonic acid with α-cyclodextrin. The membrane was held at −70 mV and 10-msec pulses were applied, which took the potential to a level varying between −50 and −10 mV in 5-mV steps and between −10 and +10 mV in 10-mV steps

Materials and Methods

Giant axons obtained from squid *Doryteuthis bleekeri*, available at the Misaki Marine Biological Station of Tokyo University, were used for these experiments. 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 490 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, and 10 mM $CaCl_2$. 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 to block the K current. The pH of the solution was adjusted to 7.2. The fatty acid compounds were supplied by Tokyo Kasei Co., Japan, and by Keizou Waku, School of Pharmacology, Teikyo 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 coated with platinum black 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 NaCl agar in contact with a calomel electrode for membrane-potential recording and a platinum electrode 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, using 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 Arachidonate on the Membrane Currents

When 0.13 mM of arachidonic acid with α-cyclodextrin was applied internally to the perfused axon, the action potential decreased in amplitude and was eventually blocked completely 2–3 min after application. Washing with arachidonic-free internal solution caused a complete recovery of the action potential. The membrane potential was almost unaffected by the application of arachidonic acid.

Figure 1 shows the effects of arachidonate on the membrane currents after internal application. Line *A* illustrates the internal effects of arachidonate without α-cyclodextrin. This drug has little effect on the membrane currents, since it is only slightly soluble in the internal solution. α-cyclodextrin at a concentration of 1–8 mM helps to dissolve the acid in the internal solution. The solubility of arachidonic acid increased almost linearly on in-

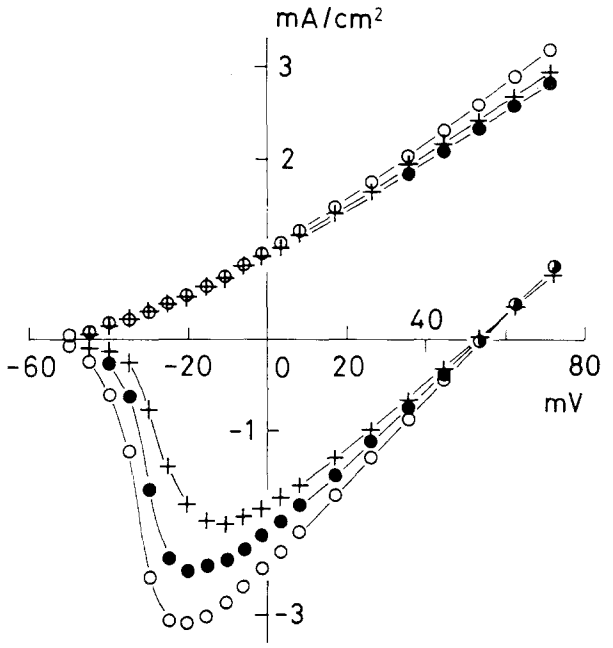


Fig. 2. Current-voltage relation for peak inward current and steady-state outward current before (○) and during (X) application of 0.13 mM arachidonic acid with α-cyclodextrin and after (●) washing with fatty acid-free internal solution

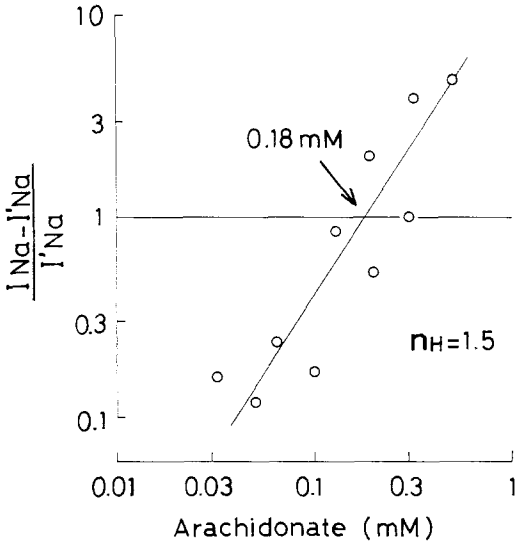


Fig. 3. Hill plot for the inhibitory action of arachidonic acid. Abscissa: concentration of arachidonic acid in logarithmic scale. Qrdinate: the relative value of suppression to control. I_{Na}: the peak Na current during application. I'_{Na}: peak Na current in the control

creasing the α-cyclodextrin concentration up to 2.5 mM before it leveled off. Without α-cyclodextrin, only a small percentage of the arachidonic acid was in solution. The solubility increased greatly on adding 1.0 mM α-cyclodextrin, and nearly all the acid was in solution at 1.5 mM of α-cyclodextrin. α-cyclodextrin itself has no effect on the membrane currents, as is shown in Fig. 1*B*. The effects of arachidonate with 1 mM α-cyclodextrin are illustrated in Fig. 1*C*. The first set of curves (*C*) shows a family of membrane currents associated with step depolarization before application of arachidonic acid. The second set of curves (*C*) shows changes after internal application of arachidonate with α-cyclodextrin. The inward Na currents were suppressed, but the outward K currents were little affected by application of this drug. The third set (*C*) is the family of currents after washing with the internal solution containing 1 mM α-cyclodextrin, showing recovery from the arachidonate effects. The time to peak transient current changes during blockage. Almost no shift of the peak Na current was observed in this case. These results imply that the potential-dependent kinetic parameters of the Na currents do not shift along the voltage axis. External application of arachidonate with α-cyclodextrin was impossible because of the insolubility of this drug in artificial seawater.

The peak Na current and steady-state outward K currents before and after internal application of arachidonate with α-cyclodextrin were plotted as a function of membrane potential in Fig. 2. It is clearly seen that arachidonate suppresses inward Na current but has virtually no effect on the K current. Recovery was observed after washing with arachidonate-free internal solution. There is a 10-mV shift of the current-voltage curves towards more positive membrane potential, but this shift is very small compared with the effect of 2-decenoate (Takenaka et al., 1987). The reversal potential of the Na currents was at +55 mV and the application of this drug induced no changes in the reversal potential.

Dose-Response Relation for Arachidonate

The relation between the suppression of the Na current and the concentration of arachidonate is shown in Fig. 3. The concentration that caused 50% suppression of the maximal Na current (ED_{50}) was 0.18 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 arachidonate on logarithmic scale. A slope equal to 1.5 was obtained, indicating that the stoichiometry of the blocking action of arachidonate was not one to one.

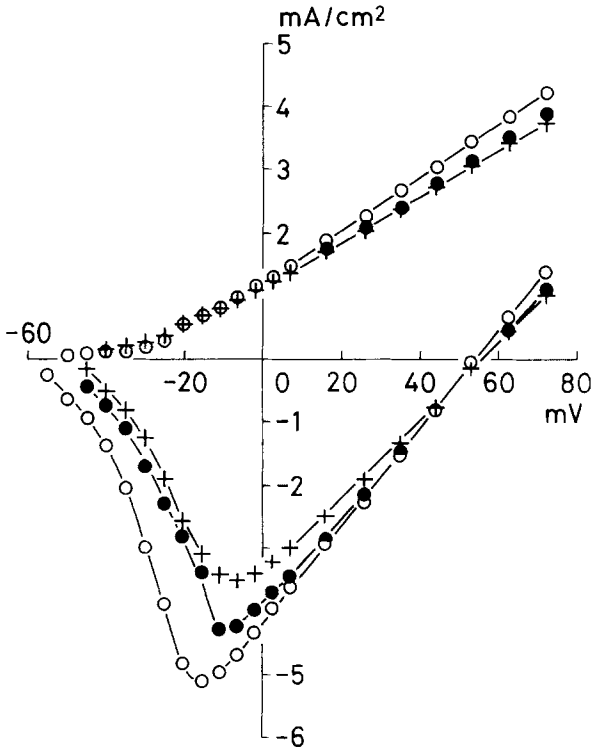
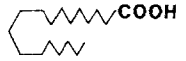
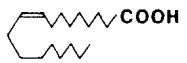
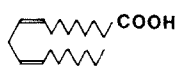
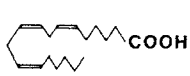
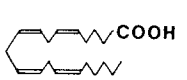
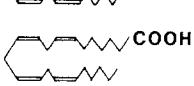
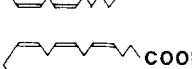


Fig. 4. Current-voltage relation for peak inward current and steady-state outward current before (○) and after (X) application of docosatetraenoic acid with α-cyclodextrin and after washing with fatty acid-free internal solution (●)

Table. Effects of the long-chain fatty acids on the inhibition of Na current at a 0.1-mM drug concentration

Acid (ratio)		Inhibition of Na current(%) 0.1-mM drug	N
Stearic acid (18:0)		0	4
Oleic acid (18:1)		3.7	6
Linoleic acid (18:2)		8.3	5
Linolenic acid (18:3)		8.9	5
Arachidonic acid (20:4)		29.3	6
Docosatetraenoic acid (22:4)		30.1	6
Docosahexaenoic acid (22:6)		28.7	6

The results show the inhibition ratio to the control (the mean values).

Effects of Other Long-Chain Fatty Acids

The suppression of Na currents by other long-chain fatty acids was compared, and the results are illustrated in Fig. 4 and the table. Figure 4 shows the effect of docosatetraenoic acid with α-cyclodextrin on the membrane currents. The peak Na current and steady-state outward K current before and after internal application of this drug were plotted as a function of membrane potential. It can be seen that docosatetraenoic acid, like arachidonic acid, suppresses inward Na current, but has virtually no effect on the K current. Recovery was also observed after washing with docosatetraenoic acid-free internal solution. A shift of the current-voltage curves towards a more positive side was observed and the value of the shift was 8.0 mV. The reversal potential of the Na currents was 53 mV and the application of this drug induced no changes in the reversal potential. During application of this drug, the membrane potential stayed at the same value and the membrane resistance showed no change. The table compares the inhibition effects of the long-chain fatty acids in the same concentration of 0.1 mM with 1.0 mM α-cyclodextrin. The values of the table show the inhibition ratio of the Na currents compared with the control. Oleic acid decreased the Na current to the value of 3.7% and linoleic acid had about the same effects as linolenic acid. On the other hand, arachidonic acid, docosatetraenoic acid and docosahexaenoic acid decreased Na currents about 30% from the control value. The effect of these fatty acids on the membrane currents were reversible. The membrane potential and the membrane resistance were not changed by these long-chain fatty acids. Stearic acid formed micelles and a turbid solution without α-cyclodextrin. When we used this turbid solution, both membrane currents and resistance decreased and membrane depolarization occurred. This was completely irreversible and the membrane was destroyed.

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

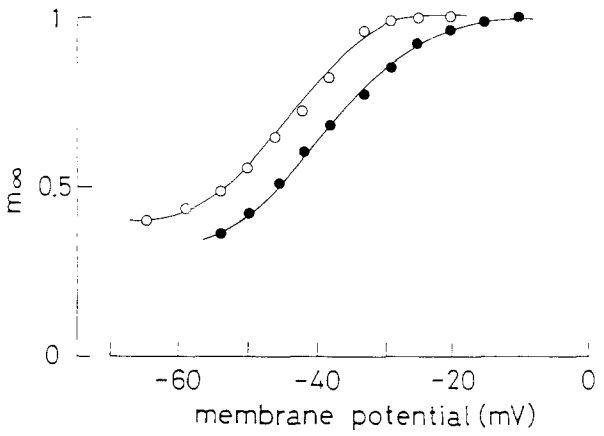


Fig. 5. Effects of internal application of arachidonic acid on the steady-state activation (m_∞) of the Na current. White dots: control records; black dots: test records. The abscissa shows the membrane potential

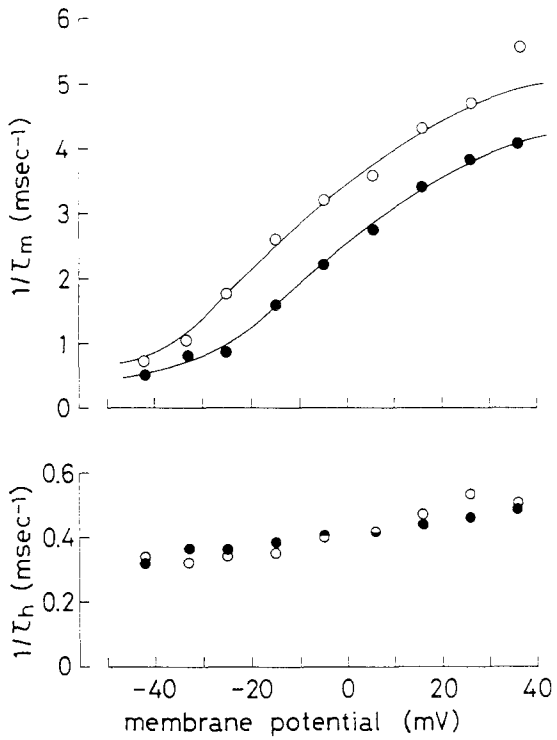


Fig. 6. Effects of internal application of arachidonic acid on the activation time constant (τ_m) and inactivation time constant (τ_h) of the Na current. White dots: control records; black dots: test records. The abscissa shows the membrane potential

and Huxley (1952)

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

where $I'_{Na} = \bar{g}_{Na} m_\infty^3 (E - E_{Na})$. This equation applies to the case that $m_0 = 0$ and $h_0 = 1$. 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 7–10 mV. The inactivation parameters h_∞ and $1/\tau_h$ are little affected (Fig. 5). The major contributions 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 0.13 mM arachidonate were analyzed by the following equation of Hodgkin and Huxley (1952)

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

where $I'_K = \bar{g}_K n_\infty^4 (E - E_K)$ and $n_0 = 0$ is supposed. There is no depolarizing shift of $1/\tau_n$.

Discussion

Solubility of Long-Chain Fatty Acid

The biological membrane is composed of phospholipids, which contain the long-chain fatty acids. Among these fatty acids, arachidonic acid is especially important because of the arachidonic cascade concerned with prostaglandin. These long-chain fatty acids are usually insoluble in saline solution and they form micelles in solution. α-cyclodextrin helps them to dissolve in solution (Singh & Kishimoto, 1983) and α-cyclodextrin itself has no electrophysiological effects on the excitable membrane. This method of dissolving the long-chain fatty acids in solution with α-cyclodextrin becomes a good tool to study the effects of the long-chain fatty acids.

Long-Chain Fatty Acids and Medium-Chain Fatty Acids

Long-chain fatty acids mainly suppress the Na current reversibly and have little effect on the K current. These effects are almost similar to those of medium-chain fatty acids (Takenaka et al., 1987). If we compare long-chain fatty acids with medium-chain fatty acids, the following points can be observed. The resting potential is almost unaffected or slightly hyperpolarized by the application of long-chain fatty acids, but medium-chain fatty acids produced a 2–3 mV depolarization in the resting potential. A shift of the Na current peak was observed in the application of medium-chain fatty acids, but it was negligible for long-chain fatty acids. The shift of the current-voltage curve towards more positive membrane potentials was 30–35 mV in the case of

medium-chain fatty acids and 10 mV for long-chain fatty acids, but the reversal potentials had the same value of +55 mV in both cases. The concentration that caused a 50% suppression of maximal Na current (ED_{50}) was 1.43 mM for medium-chain fatty acids and 0.18 mM for arachidonic acid. The effect of long-chain fatty acids was about 10 times more potent than that of medium-chain fatty acids. The Hill number was 1.58 for medium-chain fatty acids and 1.5 for arachidonic acid. This result means that the stoichiometry of the blocking action of long-chain fatty acids is the same as that of medium-chain fatty acids. In conclusion, the mechanism of the long-chain fatty acid effect on the excitable membrane is the same as that of the medium-chain fatty acids. The main difference of the effect on the Na current is that the effect of the long-chain fatty acids is much stronger than that of the medium-chain fatty acids.

Hodgkin-Huxley Parameter of Long-Chain Fatty Acids

The voltage-clamp experiments showed that the negative peak in the peak current-voltage relation was shifted towards the positive side by the application of fatty acids. This effect is similar to that of octyltrimethylammonium (Elliott, Haydon & Hendry, 1984) and alcohols (Haydon & Urban, 1983*b*,*c*; Kamaya, Kaneshita & Ueda, 1981). On the other hand, it is different from the effect of tetrodotoxin and of anesthetics, which cause no shift of the negative peak in the peak current-voltage relation (Narahashi, 1974; Narahashi & Frazier, 1971; Yeh, 1982).

The steady-state activation (m_∞) curve and $1/\tau_m$ show a shift in the depolarizing direction. The physiological meaning of this shift is not yet clear but 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. The lipid lateral motion of the membrane complex was increased enormously by the application of medium-chain fatty acids (Takenaka, Horie & Kawasaki, 1983; Takenaka, et al., 1986). The suppression mechanism of the long-chain fatty acids might be the same as that of the medium-chain fatty acids (Takenaka et al., 1987). In a previous paper (Takenaka et al., 1987), we separated the lipid layer of the membrane into two layers, a stiff and a plastic region. The plastic region is connected with the function of the Na channel. Fatty acids get into the lipid layer of the membrane and especially cause perturbation of the plastic region, which results in the suppression of the Na channel. Arachidonic acid has four double bonds at position 5, 8, 11, 14. Docosatetraenoic acid also has four double bonds and docosahexaenoic acid has six double bonds, which have the same effect on the Na channel suppression as that of arachidonic acid. The Na-channel proteins have hydrophobic and hydrophilic regions and the hydrophobic regions interact with the hydrophobic tails of the lipid molecules in the plastic region. Gate *m* may be located in the hydrophobic region, which corresponds to the plastic region. The fatty acids will get into the lipid layer with their long axes perpendicular to the membrane surface and their moveable tail, which comes from a double bond, and causes the disorder in the plastic region. This causes the perturbation of gate *m* and suppresses the *m* function. On the other hand, the gate of the K channel has no relation with the plastic region, so it is not affected by these fatty acids.

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