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Effects of local anesthetics on the *Chara* plasmalemma

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The effects of lidocaine, tetracaine, procaine and bupivacaine (< 1000 μM) on the *Chara corallina* internodal cell were studied. These local anesthetics depolarized the membrane at rest, while they affected the rising phase and the peak level of action potential not appreciably. Instead, they prolonged the time course of the falling phase of action potential as slowly as the repolarization was imperfect, even after enough lapse beyond the refractory period. Consequently, an action potential appeared to enhance the degree of depolarization at rest. Such a depolarization with stimulus/excitation was named use-dependent depolarization, while the depolarization without excitation, the resting one. The order of the potency of the use-dependent depolarization almost coincided with that of the nerve-blocking potency. During depolarization the change in membrane conductance was not simple. However, the conductance–voltage (G_m–V_m) relationship curve in the presence of local anesthetic suggested that depolarization was due to, not only the decrease in the electrogenic H^+-pump, but also the increase in the diffusion conductance.

Introduction

The most striking action of local anesthetics (LAs) is the blocking of nerve excitation without affecting the resting potential appreciably [1]. In the node of Ranvier from frog nerve fibers, however, the membrane hyperpolarizes in the presence of LA [2]. The nerve blocking action of LAs increases with frequency of stimulus (use-dependent block; [3–6]). In some cell membranes, other than nerves, LAs increase the diffusion conductance [7–9]. Reports suggest that LAs inhibit active ionic transport [8,10–12] and that they affect various phosphorylation-related enzyme activities by acting as antagonists of calmodulin [13–15].

The resting potential of the excitable *Chara* internodal cell is usually more negative than −200 mV. This large negative potential is due to the electrogenic H^+-pump [16,17]. The pump electromotive force, E_p, is more negative than −200 to −250 mV, while the diffusion potential, E_d, is −100 to −140 mV [18,19]. E_d is mainly dominated by K^+ ions [20,21]. The resting potential is the weighted average of E_p and E_d, where the weights are the conductances of the pump and the diffusion channels, i.e., G_p and G_d, respectively. The sum of G_p and G_d is the membrane conductance, G_m ($= G_p + G_d$). The pump activity can be to some extent characterized by the patterns of both the current–voltage (I–V_m) and the conductance–voltage (G_m–V_m) relationships. The former relationship shows a deformed S-shape and the latter one a bell-shape having a conductance peak (G_t) at around −200 mV [22]. In the presence of a pump inhibitor such as DES (diethylstilbestrol) or DCCD (dicyclohexylcarbodiimide) the G_m–V_m relationship decays to the G_d–V_m relationship losing the above bell-shape pattern and decreasing G_t [18,19].

The excitation of the *Chara* cell is due to a series of opening events of three different diffusion channels. (1) Opening of the Ca^{2+} channel which increases cytoplasmic Ca^{2+} concentration [23–25]. (2) Opening of the Ca^{2+}-activated Cl^- channel which depolarizes the membrane largely toward the equilibrium potential of Cl^- ions (+60 to +90 mV) [24,26–28]. The patch-clamp study on the Ca^{2+}-activated Cl^- channel [29] supports strongly that calmodulin should be involved in the opening of this channel [30–33]. (3) Opening of the

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Abbreviations: c-APW, control artificial pond water; LA(s), local anesthetic(s); DCCD, dicyclohexylcarbodiimide.

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voltage-dependent K^+ channel [20,21]. The peak of action potential is usually determined by the ratio of the K^+ conductance to the Cl^- conductance. In addition to closing of the three diffusion channels, G_p contributes to the large repolarization beyond the diffusion potential [34].

If LAs block the diffusion channel alone in the *Chara* plasmalemma, they should hyperpolarize the plasmalemma decreasing G_m ($= G_p + G_d$). If LAs block the H^+-pump alone, they should depolarize the plasmalemma decreasing G_m. If LAs inhibit the activity of calmodulin existing in this cell [35], they will not only depress the excitability by inhibiting the opening of the excitable Ca^{2+}-activated Cl^- channel [29–34], but also depolarize the plasmalemma by affecting the electrogenic H^+-pump [30–33]. These were our preliminary questions on the LA effect on the *Chara* plasmalemma. We report that LAs depolarize the membrane decreasing G_p.

Materials and Methods

Chara corallina, solutions, and electrical measurements

Single internodal cells of *Chara corallina*, 4–5 cm in length and 600–1000 μm in diameter, were used. They were kept in artificial pond water (c-APW) under constant light (about 2000 lux) at about 20°C for 2 days. The ionic composition of c-APW were: 0.5 mM KCl, 0.2 mM NaCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. The pH of c-APW was adjusted to 7.0 with 5.0 mM Tes (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer. Test solutions of different LA concentrations below 1000 μM were prepared. Below 1000 μM all tested LAs did not alter the pH of c-APW. The test concentration of tetracaine, however, was usually limited to 500 μM, because 1000 μM tetracaine depolarized the membrane within a few minutes and decreased the rate of the cytoplasmic streaming [36]. These effects were markedly blocked by raising the Ca^{2+} concentration to 2.0 mM. Therefore, in the case of 1000 μM tetracaine, the internode was kept in 2.0 mM Ca-APW. The ionic compositions were the same as those of c-APW, except 2.0 mM $CaCl_2$, and the experiment was carried out in the same solution (cf. Fig. 2).

The internodal cell was placed on the measuring vessel with three pools. The microelectrode was impaled at the middle pool of 4 mm in length. A pair of Ag-AgCl-Pt electrodes [37] in the two side pools supplied current externally [16]. The cell parts which were partitioned by a bank 12 mm in length between the measuring and each side pool were electrically insulated with a 4 mm wide air gap in addition to silicon grease.

The electrical arrangements for voltage- or current-clamping and data collection have been described previously [38]. Briefly, the current-voltage (I–V_m) relationship was obtained by changing V_m at a rate of 100 mV/60 s under voltage-clamp conditions. This rate yielded almost the steady-state I–V_m relationship. A train of square voltage pulses (-10 to -20 mV in amplitude and 20–40 ms in duration) was superimposed on the above V_m change. The ratio of the steady-state current to each square voltage pulse gave us the instantaneous chord conductance. G_m was determined by compensating the series resistance of about 0.5 to 1.2 kΩ cm^2 which had been measured for individual experiments by step currents.

The temperature ranged between 18 and 24°C. It was kept constant within ± 0.5 C° of the desired level during any individual experiment.

Drugs

Sources of drugs were as follows: tetracaine-HCl, Kyorin Chemicals; lidocaine-HCl and bupivacaine-HCl, Fujisawa Chemicals; and procaine-HCl and DCCD, Wako Chemicals. DCCD, 100 mM in 99.6% methanol, was stocked at 5°C. Just before use, APW containing 50–100 μM DCCD was prepared by adding 1.0–2.0 ml of DCCD stock solution to 2.0 liter of c-APW. The effect of 0.1% methanol on the *Chara* plasmalemma was negligible.

All drug tests were carried out 3.5–4.0 h after microelectrode penetration. This time was unavoidably necessary to prevent a hyperpolarization beyond the prior resting potential, when the LA-treated internode was washed with c-APW. The internodal cell was externally perfused with either c-APW or a test solution. When c-APW was replaced with a test solution, the LA concentration in the measuring pool reached the desired test value in 10 s. The internode was washed with c-APW after each LA test. The period of washout was 2–3-times longer than that of LA exposure.

Results

LA-induced depolarization

Fig. 1 shows changes in V_m and G_m caused by tetracaine at concentrations between 10 and 500 μM. This experiment was carried out at 0.1 mM Ca^{2+}. The internode was exposed to a different concentration for 5 min and washed with c-APW for 10 min. Below 30 μM, tetracaine did not change V_m markedly, while above 50 μM the membrane depolarized with increase in concentration. At 300 and 500 μM, the membrane depolarized by 50–80 mV. The membrane was almost completely repolarized with washout. The time course of repolarization, however, became longer when the tetracaine concentration had been above 300 μM. G_m decreased slightly during the LA-induced depolarization and recovered with washout. The pattern of the G_m recovery was sometimes sluggish compared with that of V_m.

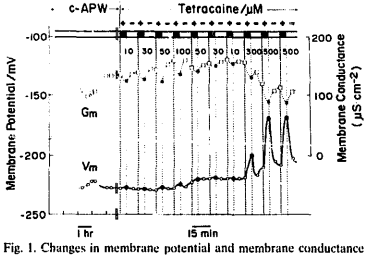

Fig. 1. Changes in membrane potential and membrane conductance during treatment and washout of tetracaine. The internode was exposed to different concentrations of tetracaine (+) for 5 min and washed with c-APW (−) for 10 min. The series of test experiments began about 4.5 h after penetration. V_m (○ and ●), membrane potential; G_m (□ and ■), membrane conductance. Open symbols (○ and □), in the absence of LA; filled symbols (● and ■), in the presence of LA. Numerals above the G_m trace are concentrations in μM of tetracaine.

To test effects of tetracaine at a higher concentration range, it was necessary to raise the Ca^{2+} concentration (see Materials and Methods). The dose-response curve shown in Fig. 2 was obtained at 2.0 mM Ca^{2+}. The tetracaine concentration was successively increased from 0 to 1,000 μM and the internode was exposed to each LA concentration for 10 min. Both V_m and G_m at 10 min of each exposure were plotted against LA concentration (Fig. 2). The dose-response curve for V_m was fundamentally sigmoidal. The concentration, K_m, which caused half the maximum depolarization was about 500 μM. In spite of the large

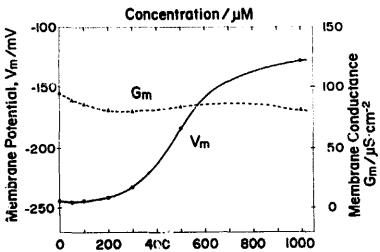

Fig. 2. Dose-response curves for V_m (filled circles) and G_m (filled triangles). Tetracaine concentration was varied successively up to 1000 μM. The exposure time for each concentration was 10 min. V_m and G_m at 10 min of exposure are plotted in the figure. The sample shown in this figure differed from that shown in Fig. 1. This experiment was carried out at 2.0 mM Ca^{2+} concentration.

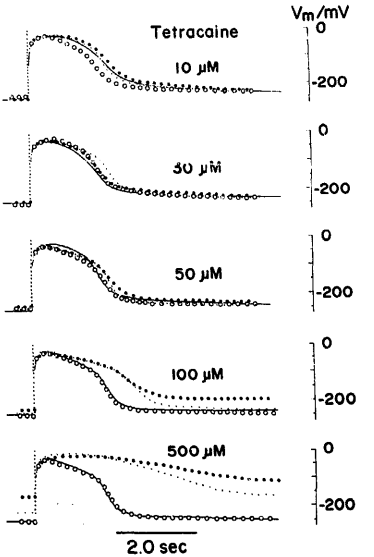

Fig. 3. Effects of LA on action potential. Action potentials at different concentrations of tetracaine are shown. The internode was exposed to each concentration for 30 min and washed with c-APW for about 100 min. Action potentials at 5 min before application of LA (real lines; controls), at 10 min (small dots) and 20 min (filled circles) after application of LA, and at about 90–95 min after the wash (open circles) are shown. The last action potential (open circles) was the control for the following LA application as well as the recovery test.

membrane depolarization, the change in G_m of this internode was small showing a tendency to decrease at high concentrations.

Fig. 3 shows action potentials before (real lines), during (small dots and filled circles), and after (open circles) treatment with different concentrations of tetracaine. During each exposure to tetracaine the internode was stimulated twice with an interpulse interval of 10 min. As shown in the figure, tetracaine did affect the rising phase as well as the peak level of action potential not appreciably. This means that the interpulse interval of 10 min was enough long over the refractory period. Alternatively, tetracaine slowed the later time course of the falling phase, which became marked at high concentrations beyond 100 μM. Be-

sides, the membrane did not seem to repolarize perfectly and the action potential seemed to enhance a depolarization. Note that the resting potential just before the second action potential (filled circles) is less negative than that just before the first one (small dots). These effects of tetracaine on the *Chara* action potential were almost removed with washout (open circles). Similar effects on action potential were found also by LAs other than tetracaine.

The difference between depolarizations with and without stimulus/excitation is clearly shown in Figs. 4A, 4B and 4C. Fig. 4A shows the membrane depolarizations caused by 100 μM tetracaine, where the internode was exposed to tetracaine for 25 min. The upper panel shows the time course of the V_m change and the lower one shows that of the G_m change. In the experiment shown by curve I_r the internode was not stimulated, while in the experiment shown by curve I_e, the internode was stimulated twice at the times marked by S.

Before the first stimulus the time course of the V_m change curve I_e was almost the same as that of the V_m change curve I_r. The time course of repolarization phase was so slow that V_m was still far from the prior level even 10 min after stimulus (curve I_e). The second stimulus further depolarized the membrane. The change in V_m is 5 mV at most for curve I_r (from −248 to −243 mV), while it is 18 mV at least for curve I_e (from −241 to −223 mV). Thus, we name such a depolarization enhanced by a stimulus a 'use-dependent' depolarization (curve I_e) and the depolarization for curve I_r the 'resting' depolarization.

However, the change in G_m was not marked for curve I_r, while it was marked for curve I_e after the second stimulus. The overall change in G_m was only 0.2 μS/cm^2 (from 252.1 to 252.3 via 258.7 μS/cm^2) for curve I_r, while it was a reduction of 118.4 μS/cm^2 (from 279.4 to 161.0 μS/cm^2) for curve I_e.

Fig. 4B shows lidocaine-induced depolarizations and Fig. 4C bupivacaine-induced ones. In both figures

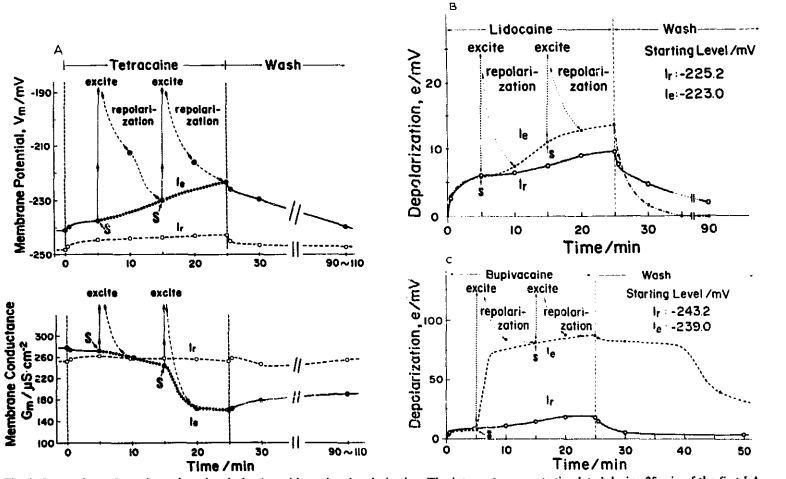

Fig. 4. Comparison of use-dependent depolarization with resting depolarization. The internode was not stimulated during 25 min of the first LA exposure (resting depolarization; curve I_r). After washout the internode was again exposed to LA for 25 min. During this second exposure a step potential change to $V_m = -100$ mV was applied twice for 20 s at the times marked by S (use-dependent depolarization; curve I_e). The main time course of the cell excitation is omitted from the figure and curve I_e (dots) is an imaginary line. (A) 100 μM tetracaine. Upper panel is the time courses of the V_m changes and lower one is those of the G_m changes. (B) 500 μM lidocaine. (C) 1000 μM bupivacaine. In (B) and (C) the depolarization is exhibited as the deviation (e) of V_m from the starting level which was the V_m just before each LA application.

TABLE I

Relative depolarization extent [a] caused by various LAs (resting depolarization)

A.D., Average of depolarization; S.D., standard deviation.

Local anesthetic	Concentration of local anesthetic (μM)								Parameters for the Hill function [a]	
	10	30	50	100	200	300	500	1000	K_m (μM)	A_m (relative)
Tetracaine										
A.D.	0.011	0.000	0.060	0.256	0.600	0.810	1.000 [a]		190	1.14
S.D.	0.019	0.034	0.018	0.212	0.100	0.390	0.190			
Number	4	4	3	11	3	9	5			
Lidocaine										
A.D.	0.000	0.054	0.021	0.067	0.163	0.182	0.323	0.521	215	0.38
S.D.		0.017		0.042	0.067	0.074	0.157	0.109		
Number	1	3	1	9	8	10	9	5		
Bupivacaine										
A.D.				0.064	0.249	0.190	0.232	0.720	200	0.22
S.D.				0.072	0.162	0.124	0.258	0.413		
Number				5	3	8	7	5		
Procaine										
A.D.				0.011	0.075	0.125	0.107	0.192	335	0.21
S.D.				0.011	0.075	0.063	0.021			
Number				2	2	6	2	1		

[a] The average depolarization (e) was normalized by using the value, $e_0 = 46.8$ mV, which is the average of the resting depolarizations caused by 500 μM tetracaine. The steepness factor of the Hill function was 2, that is, $A = e/e_0 = A_m \cdot (C/K_m)^2/(1+(C/K_m)^2)$, where A and C are the extent of depolarization and the LA concentration, and A_m and K_m are the maximum value of depolarization and the concentration which depolarizes the membrane by half the maximum.

curves I_r are resting depolarizations and curves I_c use-dependent depolarizations. The lidocaine-induced use-dependent depolarization was rather moderate, while the bupivacaine-induced one quite marked. This marked depolarization occurred at concentrations higher than 500 μM. Procaine also depolarized the

TABLE II

Relative depolarization extent [a] caused by various LAs (use-dependent depolarization)

A.D., Average of depolarization; S.D., standard deviation.

Local anesthetic	Concentration of local anesthetic (μM)								Parameters for the Hill function	
	10	30	50	100	200	300	500	1000	K_m (μM)	A_m (relative)
Tetracaine										
A.D.	0.096	0.128	0.481	0.848		1.652	2.058		132	2.15
S.D.	0.118	0.064	0.139	0.549		0.598	0.291			
Number	2	2	2	7		3	4			
Lidocaine										
A.D.				0.192		0.812	1.321	1.218	189	1.34
S.D.				0.220		0.492	0.647	0.579		
Number				5		5	6	3		
Bupivacaine										
A.D.				0.267	0.737	0.591	1.068	1.963		
S.D.				0.032	0.417	0.176	0.684	0.409		
Number				2	2	3	2	6		
Procaine										
A.D.						0.299		0.449		
S.D.						0.021				
Number						2		1		

[a] The standard value for the normalization of averaged depolarization (e) was the same value as used in Table I, i.e., 46.8 mV ($= e_0$). The steepness factor of the Hill function was 2. A_m and K_m have the same meaning as used in Table I.

membrane at rest and prolonged the repolarization phase of action potential. Even at 1000 μM, however, procaine-induced resting and use-dependent depolarizations were not as large as those of other LAs (cf. Table I and II).

In Figs. 4A, 4B and 4C, the time courses of resting depolarization (curves I_r) are very similar among different LAs. Each time course may have at least two phases, i.e., a fast phase having a time constant of about a few tenths of a sec and a slow phase. The latter depolarization phase appeared 5 to 15 min after each LA application, which is marked in curve I_r for lidocaine (Fig. 4B). Thus, the dose–response curve shown in Fig. 2, which was obtained by varying the tetracaine concentration successively, should be considered to be a mixture of effects of both concentration and exposure time.

The dose-response curves which would be obtained by direct replacements of c-APW with different LA concentrations are exhibited in Fig. 5. In order to obtain these curves, we first selected data having a resting potential between -220 and -260 mV in c-APW. Secondly, among them we selected data showing V_m recovery more than 80% of its change during washout. Then, in the selected data, the difference (e)

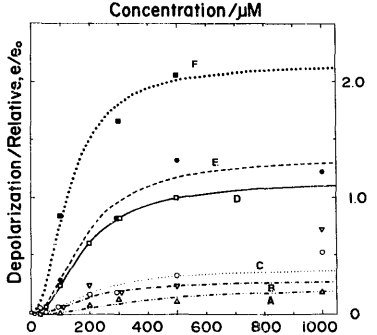

Fig. 5. Dose–response curves for V_m (averages of depolarizations). The averages of LA-induced depolarizations in Tables I and II are plotted against various LA concentrations. Curves A to D are the resting depolarization curves: A, procaine; B, bupivacaine; C, lidocaine; D, tetracaine. Curves E and F are the use-dependent depolarization curves for lidocaine and tetracaine, respectively. The relative ratio of 1.0 corresponds to 46.8 mV which was the average (e_0) of depolarizations caused by tetracaine of 500 μM. All curves were generated from the equation: $A = e/e_0 A_m\ (C/K_m)^2/(1+(C/K_m)^2)$, where A_m is the maximum value of the relative ratio, K_m is the half value of the concentration at which the depolarization is half of the maximum, and C the LA concentration.

in the resting potential between immediately before and at 5 min after each LA application was compared with the standard value (e_0), which was the averaged resting depolarization in the presence of 500 μM tetracaine. The e_0 value was 46.8 ± 8.8 mV (five samples). Thus, the extent of resting depolarization, or the potency to depolarize the membrane at rest, was expressed by the ratio e/e_0. Similarly, the extent of use-dependent depolarization was defined as follows; the internode was stimulated 2–4-times during each LA exposure of 25 to 100 min. The difference in V_m between immediately before each LA application and the beginning of each washout was compared with e_0. The extent ($= e/e_0$) of depolarization is summarized in Tables I (resting depolarization) and II (use-dependent depolarization). The average value of depolarization was plotted against the concentration (Fig. 5). In Fig. 5 each dose-response curve for V_m was stimulated by adopting the Hill function having a steepness factor of 2. The K_m value for resting depolarization was 190 μM for tetracaine (curve D), 215 μM for lidocaine (curve C), 200 μM for bupivacaine (curve B), and 335 μM for procaine (curve A). The maximum $e/e_0\ (= A_m)$ of resting depolarization was 1.14 for tetracaine, 0.38 for lidocaine, 0.22 for bupivacaine, and 0.21 for procaine.

The A_m value of use-dependent depolarization was much larger than that of resting depolarization; A_m was 2.15 for tetracaine (curve F) and 1.34 for lidocaine (curve E). The K_m value of use-dependent depolarization was smaller than that of resting depolarization; K_m was 132 μM for tetracaine and 189 μM for lidocaine. The bupivacaine-induced use-dependent depolarization was marked at concentrations higher than 500 μM as shown in Fig. 4C. As described already, the procaine-induced use-dependent depolarization was small.

Tetracaine and lidocaine decrease pump conductance and increase diffusion conductance

The I–V_m relationship curve A (upper panel) and the G_m–V_m relationship curve A (lower panel) in Fig. 6A are the controls. These were obtained in c-APW about 270 min after microelectrode penetration, which corresponded to the time 10 min before application of 500 μM lidocaine. The resting potential was -211 mV, and G_m at rest was 138 μS/cm^2. The I–V_m relationship forms an S-shape and the G_m–V_m relationship a bell-shape. These relations characterize the property of the electrogenic H^+-pump. The peak of G_m is about 145 μS/cm^2 at about -190 mV. In this paper V_m, at which V_m is the peak (G_t), is expressed by V_t. Four successive stimuli (at intervals of 5 min) depolarized the membrane to -108 mV in about 25 min. In this membrane state both I– and G_m–V_m relationships curves B were obtained. In curve B (upper panel) the

currents increased markedly by depolarization, while they decreased by hyperpolarization. Thus, rectification was marked. Correspondingly, G_m markedly decreased with hyperpolarization (curve B in lower panel). The G_m value was 250 μS/cm^2 at rest (= −108 mV), 75 μS/cm^2 at −150 mV, and 70 μS.cm^2 at −250 mV. Note that the bell-shape in the G_m–V_m relationship disappears and that G_m appears to be larger than that of the control, if V_m is more negative than −270 mV. Then, the internode was washed with c-APW for 180 min, during which the membrane repolarized to −185 mV. Though the recovery of the S-shape in the I–V_m relationship was not complete (curve C in upper panel), it should be noted that the bell-shaped G_m–V_m relationship having G_t (about 90 μS/cm^2) at around −190 mV (= V_t) appeared again (curve C in lower panel). This V_t was almost the same voltage as that of the control (curve A). Note also that in the large negative V_m range the G_m values in curve C are much smaller than those in curve B.

Both I– and G_m–V_m curves A in Fig. 6B were obtained in c-APW, while curves B in the presence of 100 μM tetracaine, and curves C to E in the presence of 75 μM DCCD. Both control curves A are similar to those shown in Fig. 6A. The resting potential was −248 mV, G_m at rest 180 μS/cm^2, and G_t was 265 μS/cm^2 at −190 mV (= V_t). The exposure to tetracaine for 20 min depolarized the membrane by 10 mV (V_m = −237 mV) and shifted the I–V_m relationship almost in parallel with that of the control. G_m at rest increased to 200 μS/cm^2, while G_t decreased to 240 μS/cm^2 almost without changing V_t (= −190 mV). G_m increased slightly in the large negative V_m range with tetracaine, while it decreased in the depolarized range. The bell-shaped pattern in the G_m–V_m relationship remained even with exposure to tetracaine.

Tetracaine was replaced with 75 μM DCCD (curves C, D, and E). During the DCCD-induced depolarization the S-shaped pattern in the I–V_m relationship disappeared and G_t decreased successively without

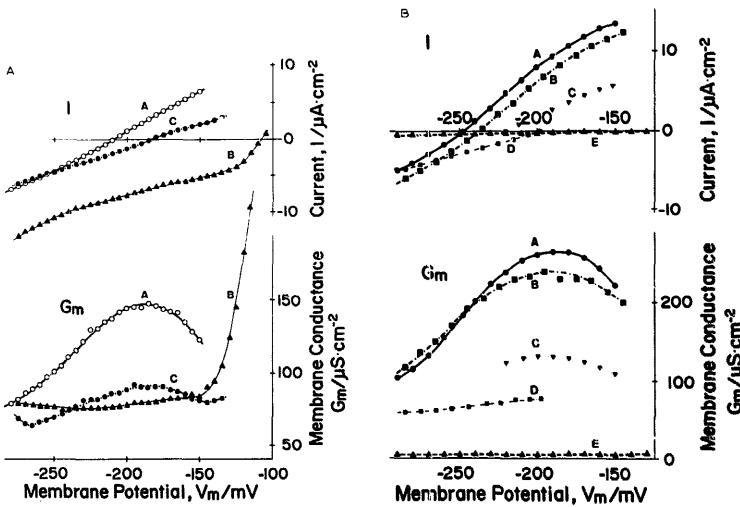

Fig. 6. Current–voltage (I–V_m) and conductance–voltage (G_m–V_m) relationships in the presence of LA. (A). 500 μM lidocaine. The control curves A were obtained about 10 min before LA application, while curves B were obtained about 30 min after LA exposure. During the exposure the internode was stimulated 4-times at interpulse interval of 5 min, which depolarized the membrane from −211 to −108 mV. Then the internode was washed with c-APW for 180 min. In this state curves C were obtained. (B). 100 μM tetracaine and 75 μM DCCD. Curves A were the controls in c-APW. Curves B were obtained about 20 min after LA application. Soon after recording curves B 100 μM tetracaine was replaced with 75 μM DCCD. Curves C, D, and E were obtained at about 30, 45, and 60 min after DCCD application.

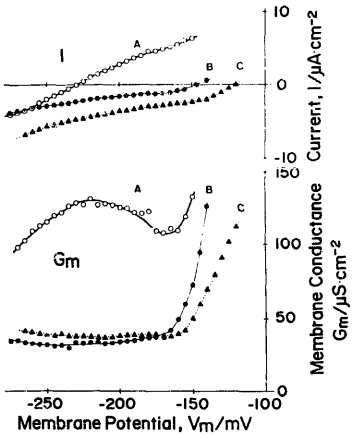

Fig. 7. I–V_m and G_m–V_m relationships in the presence of LA after DCCD application. Curves A were the controls. Curves B were obtained about 80 min after 75 μM DCCD treatment. Then, DCCD was replaced with 500 μM lidocaine and about 30 min after this LA exposure curves C were recorded.

changing the V_t level from its control. Together with the decrease of G_t, G_m in the large negative V_m range decreased. Finally, V_m was stable at -130 mV, and the C_m values in the V_m range more negative than -160 mV were very small (about 5 μS/cm^2).

Both drugs, i.e., LA and pump inhibitor DCCD, caused a G_t decrease and a loss of the bell-shaped pattern in the G_m–V_m relationship (Figs. 6). These suggested a decrease in G_p and a loss of its voltage dependence. On the other hand, G_m (about 70 μS/cm^2) at large hyperpolarization (Fig. 6A) was larger in the presence of LA than that (5 μS/cm^2) in the presence of DCCD (Fig. 6B). The difference in G_m between 70 and 5 μS/cm^2 suggested that LA might increase G_d.

We investigated the LA-induced G_d increase more directly by changing our experimental procedure. In the experiment of Fig. 7, after having recorded the control I– and G_m–V_m curves A, 75 μM DCCD was first applied for 80 min, when the resting potential was -147 mV and G_m at rest was 85 μS/cm^2. In this membrane state both I– and G_m–V_m curves B were obtained. Both characteristic patterns observed in the control curves A, i.e. S-shape in the I–V_m and bell-shape in the G_m–V_m relationships, were lost in curves B, and the G_m in the presence of DCCD was 50 to 30 μS/cm^2 in the V_m range more negative than -160 mV. These are typical for the *Chara* plasmalemma treated by DCCD and considered to be the property of the diffusion channel [18,19]. The replacement of DCCD with 500 μM lidocaine further depolarized the membrane by 27 mV. In this state curves C were obtained. At the same time, G_m at rest increased by 25 μS/cm^2. The G_m in the V_m range more negative than -170 mV was larger in curve C than in curve B by about 7 μS/cm^2. Lidocaine was replaced again with 75 μM DCCD. The membrane repolarized to -130 mV in 60 min, where both I– and G_m–V_m relationships curves were similar to curves B (data not shown).

Discussion

All tested LAs depolarized the membrane at rest and slowed the later time course in the falling phase of action potential (cf. Figs. 1, 2 and 3). Besides, even 10 min after stimulus, which is a sufficiently long interpulse interval beyond the refractory period, the membrane remained at a more depolarized level. Consequently, excitation enhanced the degree of depolarization at rest (Figs. 4). By analogy with the use-dependent block in nerve excitation, we named the LA-induced depolarization with stimulus/excitation the use-dependent depolarization and that without excitation the resting depolarization. These were our preliminary, but clear, results of the effects of LAs on *Chara*. However, the changes in the rising phase and the peak of action potential were not marked (Fig. 3). This observation suggested that the LA action on the *Chara* excitation differs from the action of calmodulin-antagonists which depress excitability greatly [33–37].

The falling phase during action potential is prolonged if the time constant of closing of one or more of the three diffusion channels, i.e., Ca^{2+}, Ca^{2+}-activated Cl^-, and voltage-dependent K^+ channels, is increased by LA (see Introduction). However, in the internode which was largely depolarized to the diffusion potential (-100 to -140 mV) by LA, the duration of action potential was not always prolonged (in preparing). The prolongation caused by LAs was characterized by a loss of the potency for large repolarization beyond the diffusion potential (Fig. 3). Besides, after excitation the G_m decrease was marked (Fig. 4A). Thus, the loss of the potency for large repolarization is well supposed to be due to a loss of the contribution of G_p to the repolarization, i.e., a G_p decrease.

Both the use-dependent depolarization and the order of the use-dependent depolarization potency are involved in the questions on both pathway and binding site of LAs. Compared to the degree of the use-dependent depolarization, the changes in both rising phase and peak of action potential were generally small (Fig.

3). Besides, as described already, the duration of action potential elicited at the diffusion potential level was almost comparable to that of control action potential in the absence of LAs. These suggest that the diffusion channels may be the pathway of LAs, but not necessarily their binding site. On the other hand, as in the case of the nerve-blocking potency of LAs [39,40], the order of the use-dependent depolarization potency (tetracaine ≒ bupivacaine > lidocaine > procaine; Table II) almost coincides with that of the solubilities of LAs to lipid [41,42]. This supports that the lipid solubility of LAs is primarily important, but not completely that lipid is the binding site.

The order of the resting depolarization potency of LAs was tetracaine > lidocaine = bupivacaine > procaine (Fig. 5 and Table I). This order differed from the order of the use-dependent depolarization potency. Such a difference in the order of the depolarization potency of LAs between with and without stimulus may be due to a change in lipid state of the membrane such as phase transition or phase separation; for instance, the lipid state changes depending on Ca^{2+} concentration [43–45]. In the *Chara* cell either during action potential or just before its initiation, a decrease in the Ca^{2+} density at the membrane surface is expected [46]. Consequently, a change in the order of the depolarization potency of LAs is expected between resting and excited states. Besides, LAs may be more lipophilic at a lower Ca^{2+} density during action potential than at a higher Ca^{2+} density in the resting state. In fact, a high Ca^{2+} concentration depressed the depolarization potency of LA; the K_m value (500 μm) for the tetracaine-induced resting depolarization at the Ca^{2+} concentration of 2.0 mM (Fig. 2) was much larger than that (190 μM) at 0.1 mM Ca^{2+} (Fig. 5). Anyway, these Ca^{2+} effects on the LA depolarization potency should be further tested.

The time course of resting depolarization had at least two phases; that is, fast and slow phases. The former had a time constant of the order of a few tenths of a second. The latter appeared after about 5 to 15 min of LA application (Figs. 4). The former time course resembles that of the cytoplasmic pH_i increase in *Neurospora* caused by procaine [47], where the tested concentration range was about 10 times higher than that described in the present paper. According to the kinetics of the electrogenic H^+-pump [18,19], elevation of pH_i will depolarize the membrane, decrease G_m at rest, and shift V_t to a less negative potential [48,49]. As exhibited in the present paper, G_m at rest did not always decrease and V_t did not shift during LA-induced depolarization. The change in pH_i may not always be the cause of the fast phase of LA-induced depolarization.

During LA exposure, G_m increased in some tests, and it decreased in the other tests even at the same concentration. Whether LA decreased G_m or increased it seemed in some cases to relate with our experimental procedure such as LA exposure time, number of repeat of LA application or number of stimuli. However, it seemed to depend on individual internode. Such an ambiguous G_m change could be to some extent understood by studying the change in the G_m–V_m relationship. The control G_m–V_m relationship showed a bell-shape having a conductance peak (G_t) at around −200 mV. As shown in Fig. 7, DCCD which inhibited the H^+-pump depolarized the membrane to the diffusion potential about −100 to −140 mV). In this state the G_m–V_m relationship is considered to correspond almost to the voltage dependence of the diffusion conductance, G_d–V_m relationship [18,19]. The G_d values were 20 to 40 $\mu S/cm^2$ in the V_m range more negative than −150 mV. These G_d values are within the acceptable range of G_d (18 to 20 $\mu S/cm^2$) determined from the data of flux measurement [50]. During DCCD poisoning, G_t decreased and the G_m–V_m relationship finally lost its bell-shape as shown in curve B in Fig. 7. These were also the case for the G_t change caused by LAs. That is, LAs decreased G_p. With LA, however, the G_m value (50 to 100 $\mu S/cm^2$) at the foot of the bell-shape was generally 2–3-times larger than that (20 to 40 $\mu S/cm^2$) in the presence of DCCD (cf. Figs. 6A and 7). This suggests that LA increased G_d by affecting the diffusion channel.

According to Belton and Van Netten [7], procaine at high concentrations (50 to 100 mM) depolarizes the *Nitella flexilis* plasmalemma increasing G_m. They supposed that the depolarization was due to the increase in the potassium conductance, G_K. In our present results the LA application after DCCD treatment further depolarized the membrane by 5 to 25 mV, but did not always increase G_d greatly in the examined whole V_m range (Fig. 7). Under our present experimental conditions G_K increase alone is not sufficient to cause such a depolarization of the membrane. The increase in channel conductance such as the Cl^- channel might to some extent contribute to the LA-induced depolarization. The DCCD application after LA treatment depolarized the membrane to the diffusion potential, but G_m (5 $\mu S/cm^2$) was extremely low (Fig. 6B). The action of LA on the diffusion channel should be further studied.

A partial recovery of the bell-shaped G_m–V_m relationship (Fig. 6A) can be partly explained by the dual action of LAs on the G_m change in the *Chara* plasmalemma, G_p decrease and G_d increase. The reappearance of the bell-shape and the decrease in G_m at large negative V_m suggests a partial recovery of G_p and that of G_d, respectively. Such a dual action of LAs on G_m resembles somewhat the conclusions concerning LA actions on oxidative phosphorylation in rat liver mitochondria [51]. LA inhibits ATP synthesis by caus-

ing decoupling, a slip of the H^+-pump, which suggests the decrease in the pump conductance. On the other hand, LA causes uncoupling in the coexistence of hydrophobic anions, which suggests an increase in the H^+ diffusion leak.

The present experiments were carried out at pH 7.0 and the data suggested that the lipid solubility of LAs is of primary importance. Then, the LA-induced depolarizations will increase in the pH range higher than 7.0, because the pK_a values of LAs tested are 7.8 to 8.9 [41,42] and the concentration of the more lipophilic, i.e., uncharged, form of LA increases with the increase in pH. We are studying effects of pH on the LA-induced depolarizations at different Ca^{2+} concentrations.

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