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Modification of the Voltage Dependent Na^+ Conductance by External pH in the Dinoflagellate *Noctiluca miliaris*

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ABSTRACT—Effects of the external pH on the voltage-dependent Na conductance in the marine dinoflagellate *Noctiluca miliaris* were examined under voltage-clamp conditions. The depolarizing Na spike (positive spike) in the tentacle regulating potentials became attenuated when the external pH was lowered from 8 (normal value) to less than 5. Under voltage-clamp conditions, a depolarization-activated transient inward current corresponding to the positive spike decreased with lowering the external pH to less than 5, and entirely disappeared at pH 3. Threshold for the inward current shifted towards the depolarizing direction while the reversal potential of the current shifted towards the hyperpolarizing direction with lowering the external pH. Rate of increase in the inward current reduced whereas rate of decrease did not change at low pH. Lowering of the external pH did not affect the steady state inactivation of the inward current. It is concluded that the external pH controls appearance of the positive spike through modification of activation kinetics and selectivity of the voltage-dependent Na conductance responsible for the spike.

INTRODUCTION

The marine dinoflagellate *Noctiluca miliaris* exhibits spontaneous membrane potential changes controlling extension-flexion movements of the food-gathering tentacle. The potential changes are termed tentacle regulating potentials (TRPs) (Hisada, 1957; Eckert and Sibaoka, 1967; Sibaoka and Eckert, 1967; Oami *et al.*, 1988; Oami and Naitoh, 1989). The TRPs consist of a depolarizing (positive) spike followed by a plateau potential and a subsequent hyperpolarizing (negative) spike. The positive spike is produced by regenerative activation of the depolarization-dependent Na conductance and the negative spike by the hyperpolarization-dependent Cl conductance (Oami *et al.*, 1988, 1995a). The positive spike is attenuated in artificial sea water with normal Ca^{2+} concentration. However, it becomes conspicuous when the external Ca^{2+} concentration is lowered (Oami *et al.*, 1988). External Ca^{2+} controls appearance of the positive spike through modification of the inactivation kinetics of the voltage-dependent Na channels (Oami *et al.*, 1995b).

In this study, we found that the external pH affects appearance of the positive spike. Among unicellular organisms, voltage dependent Na channel responsible for the Na action potential has been found only in *Noctiluca*. To understand characteristics and ionic modification of this Na channel, we examined effects of the external pH on the Na^+ conductance responsible for the depolarizing action potential. The results indicate that the external pH modifies the voltage- and the time-dependency of the activation process of the Na^+ conductance responsible for the positive spike. Some of these results have been verbally presented elsewhere (Koike and Oami, 1994).

MATERIALS AND METHODS

Specimens of a marine dinoflagellate *Noctiluca miliaris* were collected at Oarai (Ibaraki, Japan) and were cultured as previously described (Oami *et al.*, 1988). Specimens of medium size (500-600 μm) obtained from the stationary growth phase-culture were used in the experiments.

Membrane potential responses were recorded after the methods described by Oami *et al.* (1988). A specimen of *Noctiluca* was held at the tip of a holding pipette (inner diameter; 100 μm) by slightly reducing the hydrostatic pressure inside the pipette. A glass capillary microelectrode filled with 3M KCl (10-30 MΩ) was inserted into the flotation vacuole of the specimen. A reference electrode (polyethylene tube filled with 3% agar-ASW) was placed in the experimental vessel about 3 mm distant from the specimen. These electrodes were connected to a high-input resistance ($> 3 \times 10^{11}$ Ω) preamplifier through Ag-AgCl wire.

Voltage clamp experiments were performed by inserting two microelectrodes filled with 3 M KCl (<10 MΩ), one for voltage-sensing, the other for current delivery, into the specimen and by employing a conventional feedback circuit (Oami *et al.*, 1995a). A step change in the membrane potential was 90% complete within 500 μs. Membrane current responses were recorded on a thermal array recorder (Graphtec Inc., WR7700) together with the membrane potential responses.

The steady-state inactivation of the inward current was examined by employing a two step pulse protocol. Membrane potential was first held at -60 mV which approximated the potential level just before generation of the positive spike, then changed to various potential

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levels for 700 ms (pre-pulse). After this pre-pulse, membrane potential was changed to a fixed level of -20 mV (final depolarization). Amplitude of the transient inward current produced in response to the final depolarization was measured and plotted against the pre-pulse potential. Amplitude of the current was expressed as a value relative to that of the maximum inward current obtained when the pre-pulse potential was more negative than -80 mV.

Normal artificial sea water (ASW) consisted of 500 mM NaCl, 10 mM KCl, 20 mM $MgCl_2$, 30 mM $MgSO_4$, 10 mM $CaCl_2$ and 5 mM Tris-HCl buffer, adjusted to pH 8.0. In the present experiments, Ca^{2+}-deprived ASW was used as the control solution since the positive spike was most conspicuous in this medium (Oami *et al.*, 1988). About 10 μM Ca^{2+} was contaminated in Ca^{2+}-deprived ASW. The external pH was changed by replacing the external medium (flow rate; about 5 ml/min) in the experimental vessel (volume; 1 ml). Tris-HCl buffer was used in a pH range from 7 to 9 and citric acid (5 mM) from 6 to 3.

All experiments were performed at room temperature ranging from 20 to 23°C.

RESULTS

Effects of the external pH on the tentacle regulating potentials

Effects of the external pH on the tentacle regulating potentials (TRPs) were examined under the Ca^{2+}-deprived conditions. Figure 1Aa shows representative trace of the TRPs recorded in the control solution (pH 8). The TRPs consisted of a depolarizing spike (positive spike; PS), a following plateau potential (slow depolarization; SD) and a hyperpolarizing spike (negative spike; NS). Figure 1Ab shows TRPs recorded when the external pH was lowered to 4. In this condition, the positive spike became attenuated.

Figure 1B shows traces of the positive spikes in ASWs with different pH values recorded with an expanded time scale. Changes in the external pH between 10 to 6 had no effect on the positive spike (Fig. 1Ba; pH 8). However, the peak level of the positive spike shifted toward the hyperpolarizing direction and the time for the spike to reach its peak became longer when the external pH was lowered to less than 5. At the same time, the threshold for the positive spike shifted toward the depolarizing direction (Fig. 1Bb-d). When the external pH was lowered to 3, the membrane potential level was kept constant at about -20 mV and the positive spike did not appear (Fig. 1Be). Effects of the external pH on the positive spike were reversible in the range examined (pH 10-3).

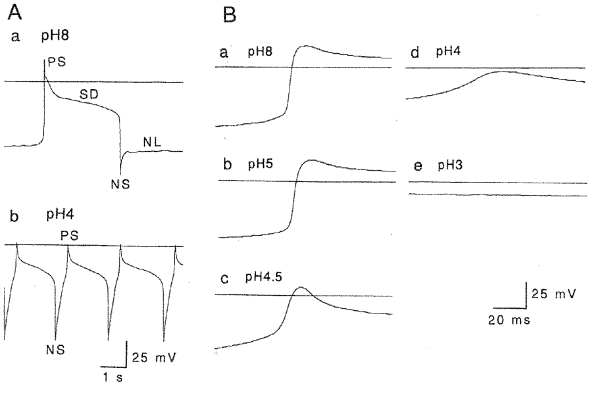

Fig. 1. A, The tentacle regulating potentials exhibited by specimens of *Noctiluca miliaris* immersed in Ca^{2+}-deprived ASWs with two different pH values (a, pH 8; b, pH 4). PS, positive spike; SD, slow depolarization; NS, negative spike; NL, negative DC level. B, Faster sweep recordings of the positive spikes of the tentacle regulating potentials obtained in Ca^{2+}-deprived ASWs with different pH values (a, pH 8; b, pH 5; c, pH 4.5; d, pH 4; e, pH 3).

Membrane current responses under voltage clamp conditions

To see effects of the external pH on the positive spike more quantitatively, we examined the membrane current responses exhibited by specimens of *Noctiluca* under voltage clamp conditions. Figure 2 shows representative traces of the membrane current responses obtained from a single specimen immersed in the control solution (Ca^{2+} deficient ASW, at pH 8). The specimen produced a transient inward current in response to a membrane step depolarization from a holding potential level of -80 mV to potential levels more positive than -50 mV (Fig. 2Ab-d). The inward current corresponds to the positive spike in the TRPs (Oami *et al.*, 1995a). With increasing the step depolarization beyond 0 mV, the specimen produced a delayed outward current following the inward current (Fig. 2Ae-h).

Figure 2B shows current-voltage (I-V) relationships for the peak transient inward current (open circles), the peak outward current (open triangles) and the current value 200 ms after the onset of the step change in the membrane potential (filled circles). Characteristics of these I-V relationships were consistent with those obtained in the earlier studies (Oami *et al.*, 1995a, b).

Effects of the external pH on the I-V relationship of the transient inward current

We next examined effects of the external pH on the

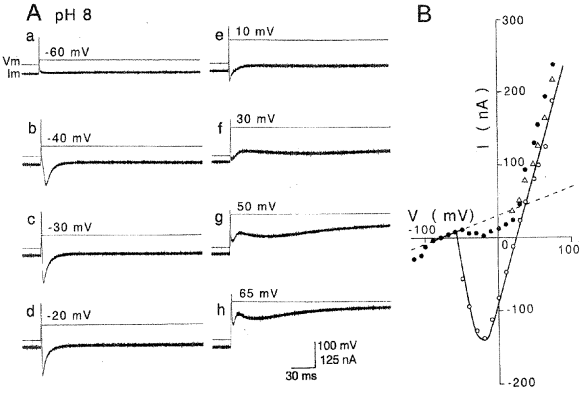

Fig. 2. A, Membrane current responses exhibited by a single specimen of *Noctiluca* to a step depolarization of the membrane to various potential levels under voltage-clamp conditions (A). Upper trace in each pair of recordings represents membrane potential (Vm) and lower trace membrane current (Im). Membrane potential had been kept at -80 mV (holding potential) and then changed abruptly to a potential level indicated on the Vm trace. B, I-V relationships for the membrane current responses. Open circles, peak value of the inward currents. Open triangles, peak value of the delayed outward currents. Filled circles, membrane currents 200 ms after the onset of step changes in the membrane potential. Dashed line indicates expected leakage current.

depolarization-induced transient inward current. Figure 3 shows four representative I-V relationships for the peak inward current, the peak transient outward current and the current value 200 ms after the onset of the step change in the membrane potential, obtained with four different specimens immersed in Ca^{2+}-deprived ASWs with different pH values (A, 5; B, 4.5; C, 4; D, 3). Changes in the external pH in a range from 10 to 6 scarcely affected the I-V relationships for the transient inward current. When the external pH was lowered from 8 to less than 5, the peak value of the transient inward current decreased and the threshold for the current shifted toward the depolarizing direction (Fig. 3A-C). The specimen did not produce a transient inward current in response to a membrane step depolarization, when the external pH was lowered to 3 (Fig. 3D).

Figure 4 shows the threshold (filled circles) and the reversal potential (open circles) of the transient inward current plotted as a function of the external pH. The threshold was little affected by changes in the external pH between 10-5. It shifted toward the depolarizing direction with lowering the external pH to less than 5. The shift was about 30 mV when the external pH was changed from 5 to 4. Contrary to the threshold, the reversal potential shifted toward the hyperpolarizing direction with lowering the external pH below 5. The shift was about 15 mV when the pH was changed from 5 to 4.

Effects of the external pH on the time course of the transient inward current

To see effects of the external pH on the time course of the transient inward current, the inward currents evoked by membrane step depolarization from a holding potential of -80 mV to -20 mV were recorded in Ca^{2+}-deprived ASWs with different pH values. This potential level is sufficient for activation of the transient inward current but below the threshold of the delayed outward current (see Fig. 2). Representative traces of the currents were shown in Fig. 5 (A, pH 10; B, pH 8; C, pH 5; D, pH 4.5). Lowering of the external pH below 5 reduced the rate of increase in the inward current so that the time to peak became longer.

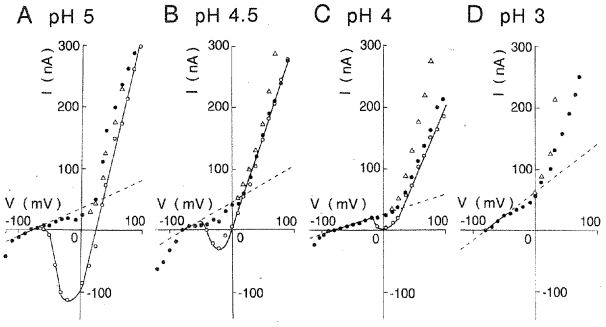

Fig. 3. I-V relationships for the membrane currents induced by step changes in the membrane potential in specimens of *Noctiluca miliaris* immersed in Ca^{2+}-deprived ASWs with different pH values (A, pH 5; B, pH 4.5; C, pH 4; D, pH 3). Symbols are identical to those shown in Fig. 2B.

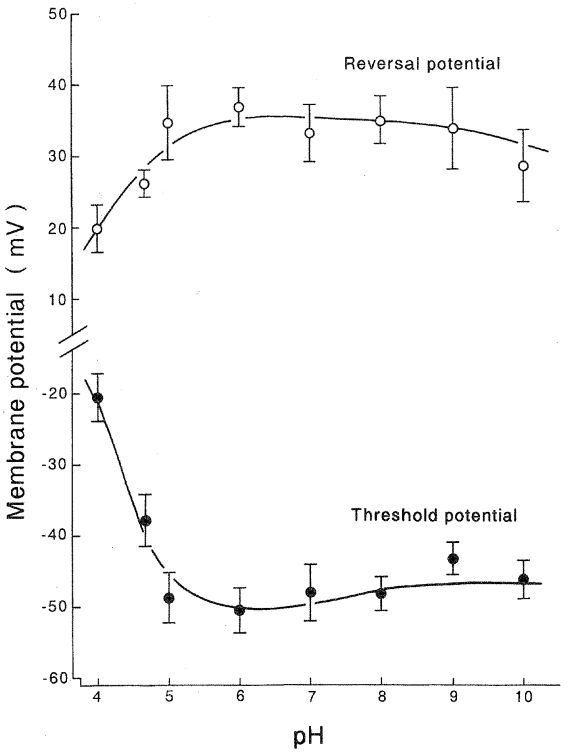

Fig. 4. Threshold (open circles) and reversal potential (closed circles) for the depolarization-induced transient inward current (ordinate) plotted against the external pH value (abscissa). Mean and its standard error of 4-6 measurements with different specimens are shown.

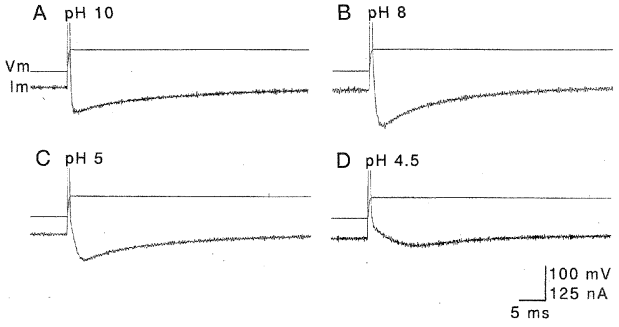

Fig. 5. Time course of the depolarization-activated inward current recorded in Ca^{2+}-deprived ASWs with different pH values (A, pH 10; B, pH 8; C, pH 5; D, pH 4.5). The inward currents were evoked by membrane step depolarization from -80 mV to -20 mV. Upper trace in each pair of recordings shows membrane potential (Vm) and lower trace membrane current (Im).

Figure 6 shows measurements of the time from the onset of the step depolarization to the peak of the currents. As shown in the figure, the time to peak was constant in the pH range from 10 to 6. It became longer with lowering the external pH below 5.

We next examined the decreasing phase of the inward current. Figure 7A shows the time course of the logarithm of the inward current measured at pH 8 and pH 4.5. The inward currents were evoked by membrane step depolarization from

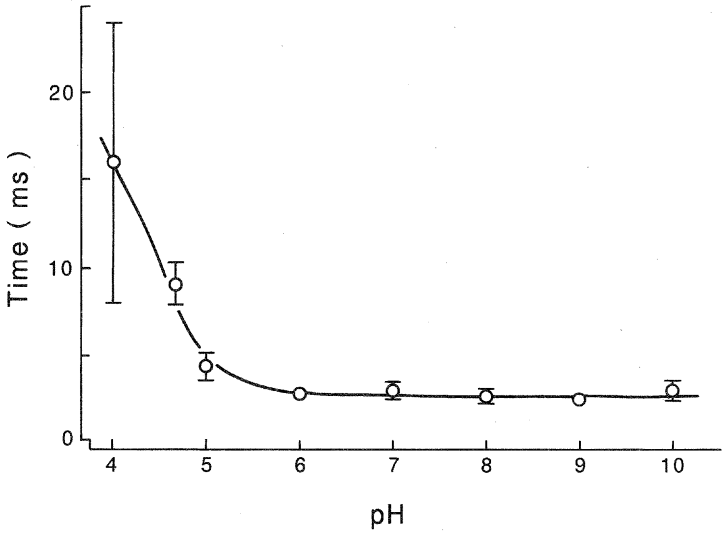

Fig. 6. Measurements of duration in the increasing phase of the depolarization-induced inward current. Time from the onset of the membrane depolarization to the peak of the inward current were measured at various pH and plotted against the external pH value. Mean and its standard error of 4-6 measurements with different specimens are shown.

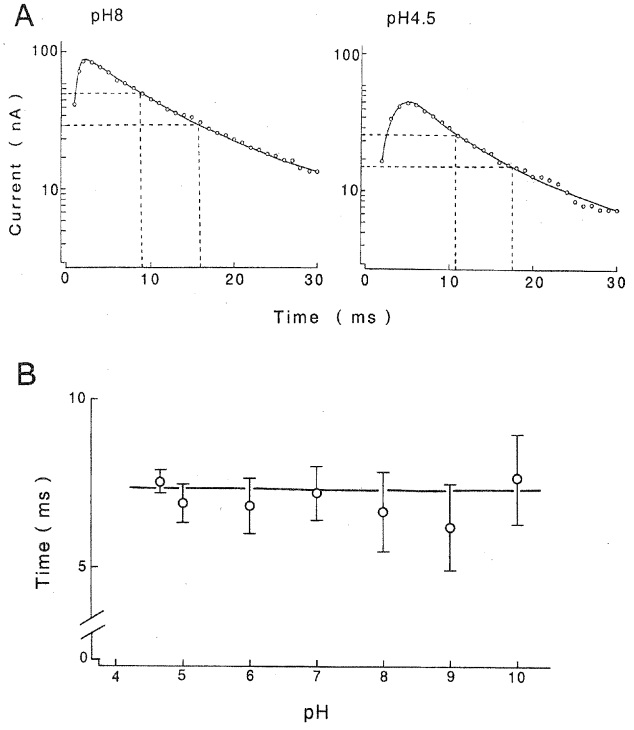

Fig. 7. A, Plots of the logarithm of the depolarization-induced inward current at pH 8 and at pH 4.5. Broken lines indicate the time when the current declined half to its maximum and 1/4 of its maximum. B, Rate of decrease in the depolarization-induced inward current measured in Ca^{2+}-deprived ASWs with different pH values. Rate of decrease was expressed as the duration of the current from half maximal value to 1/4 of maximal value in the decreasing phase. Mean and its standard error of 4-6 measurements with different specimens are shown.

a holding potential of -80 mV to -20 mV. The decreasing phase of the plots was not linear but slightly concave in both traces. The amplitude of the current obtained at pH 4.5 was smaller than that obtained at pH 8. However, the approximate slope of the plots in its decreasing phase was almost identical in both recordings.

To compare the rate of decrease in the inward currents, the duration of current from half maximal value to 1/4 of maximal value in the decreasing phase was measured with varying the external pH in Fig. 7B. The rate of decrease in the inward current did not consistently change with changing the external pH.

Effects of the external pH on the steady-state inactivation of the inward current

Effects of the external pH on the steady-state inactivation of the inward current were examined by employing a two step pulse protocol. Figure 8A shows a comparison of steady-state inactivation curves obtained at pH 8 and pH 4.5. The shape and voltage dependency of the steady-state inactivation curves were similar at both pH. Inactivation of the inward current took place when the pre-pulse potential was more positive than -70 mV and the current was completely inactivated at potentials more positive than -30 mV.

Figure 8B shows the effects of external pH on the voltage dependency of the steady state inactivation. The pre-pulse potential at which the inward current became half of its maximum was measured in Ca^{2+}-deprived ASWs with varying pH values and plotted against the pH. The half inactivation potential was not affected by changes in the external pH in the range examined (4-10).

DISCUSSION

In response to lowering of the external pH, the peak of the depolarizing spike of the tentacle regulating potentials decreased (Fig. 1). Under voltage-clamp conditions, the amplitude of the depolarization-activated transient inward

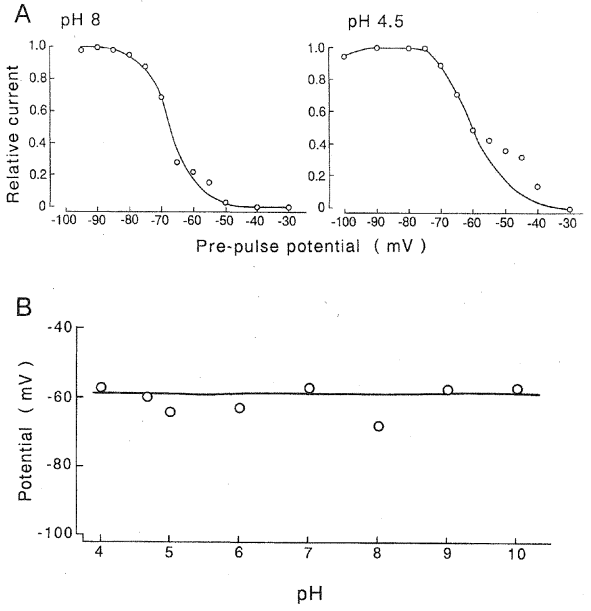

Fig. 8. A, Representative steady state inactivation curves of the depolarization-induced inward current obtained by a two-step pulse protocol at pH 8 and at pH 4.5. B, Half inactivation potential of the depolarization-induced inward current measured at various pH values. Each point represents a single measurement.

current decreased when the external pH was lowered (Fig. 3). Because the inward current corresponds to the positive spike in a non-clamped specimen (Oami *et al.*, 1995a), the decrease in the peak value of the positive spike at lower pH values is caused by the decrease in the inward current.

The decrease in the transient inward current is attributable to either a decrease in the conductance responsible for the current and/or a decrease in driving force for the current. The present findings suggest that both mechanisms are involved in the decrease. The shift in the threshold (Fig. 4) and the change in the rate of increase of the transient current (Figs. 5, 6) indicate that the external pH modifies the activation process of the Na^+ conductance. The reduced rate of activation results in a decrease in the amplitude of the inward current since inactivation of the conductance took place before it is sufficiently activated. Therefore, the Na^+ conductance does not increase enough to produce a noticeable positive spike at low pH.

The positive shift in the threshold (Fig. 4) indicates a positive shift in voltage dependency of the activation process of the Na^+ conductance and is equivalent to the reduced degree of depolarization for the activation process of the conductance during the step depolarization. As shown in Fig. 2, the rate of increase in the transient inward current was slower when the degree of the step depolarization was smaller. Therefore, it is presumable that the positive shift in the threshold of the Na^+ conductance at low external pH results in a reduced rate of activation of the conductance. The reduced rate of activation might be also involved in the decrease in the inward current as mentioned before.

The rate of decrease in the transient current and the steady state inactivation were not affected by changes in the external pH (Figs. 7, 8). These facts indicate that the external pH does not modify the inactivation properties of the Na^+ conductance in the range examined.

The shift in the reversal potential directly affects the peak value of the spike. The decrease in the peak value of the positive spike in the non-clamped specimen is attributable to a negative shift of the reversal potential of the transient inward current (Fig. 4).

Lowering of the external pH brought about a shift in the equilibrium potential for H^+ towards the depolarizing direction. However, the direction of the shift in the reversal potential for the inward current is opposite to that of the equilibrium potential for H^+. Therefore, the shift of the reversal potential is most probably caused by a change in the ionic selectivity of the Na^+ conductance.

Mechanisms underlying the pH-modification of the Na^+ conductance in *Noctiluca* are not clear at present. However, it should be noted that the negative charges involved in the carboxylic residues of the side chain of amino acids, glutamate or aspartate, are neutralized in an acidic pH range (pK' is 4.25 for γ-carboxylic residue of glutamate and 3.86 for β-carboxylic residue of aspartate). These pH dependencies are comparable to those of changes in the Na^+ conductance found in the present study. Since charges of the channel molecules are assumed to play important roles in regulation of channel function, charge neutralization of carboxylic residues in the side chain of glutamate or aspartate in the channel protein might be involved in the pH-modification of the Na^+ conductance in *Noctiluca*.

It has been reported that the external pH has several effects on the ionic channels such as a modifier of the gating (Shrager, 1974; Campbell and Hahin, 1984), a blocker (Woodhull, 1973; Hagiwara *et al.*, 1978; Begenisich and Danko, 1983) and a modifier of the surface charges involved in the voltage sensor (Hille, 1968; Krafte and Kass, 1988; Zhang and Siegelbaum, 1991; Klockner and Isenberg, 1994). The present findings offer an example of pH-modification of the kinetics of the voltage-dependent ion conductance. In contrast to the present findings in *Noctiluca*, modification of K channel gating in crayfish axons is attributed to protonation of a histidine group at low pH (Shrager, 1974). In frog skeletal muscle, lowering of external pH reduces the rate of gating charge movement in the Na channel. However, the gating charge is not affected by change in external pH (Campbell and Hahin, 1984). The site of the action of H^+ ions in the Na channel of *Noctiluca* should be further examined.

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