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Intracellular Binding of Cationized Ferritin Prolongs the Time Course of Sodium Channel Inactivation in Squid Giant Axons

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Summary. Cationized ferritin (CF) applied intracellularly in squid giant axons bound to the negatively charged sites on the cytoplasmic surface of the axolemma. Under the electron microscope, the distribution of CF was found to be dense and uniform over the axolemmal surface. However, the effect of CF on the membrane excitability was highly specific, the major effect being a prolonging of the inactivation time course of the sodium channel without altering the properties of the potassium channel. The binding of CF did not alter the surface potential related to the membrane excitability. When CF was present intracellularly, the time course of the inactivation was characterized by two time constants (slow and fast). The slow component increased with an increase in CF binding and its time constant had a unique value (26 msec) irrespective of the duration of perfusion and concentration of CF. The concentration of CF at which the half-maximum response occurred was about 150 nM. Poly-L-glutamate, charged negatively at neutral pH, removed CF from the axolemma and counteracted the CF effect on the sodium channel, although this poly-acid *per se* did not affect the membrane excitability. Our results indicate that CF binds electrostatically to the inactivation site of the sodium channel but does not affect the voltage sensor, which is supposed to be located deep in the channel.

Key Words sodium channel · inactivation · cationized ferritin · cytoplasmic surface · surface charge · anionic sites

Introduction

It is widely accepted that under physiological conditions the excitable membrane and the ionic channels of axons are negatively charged. Modification or screening of the charged sites affects the membrane excitability, as shown by experiments involving changes in internal ionic strength [5, 20], external divalent-cation concentration [8, 10, 12, 14–17, 21], external pH [3, 9, 16, 17, 21] and internal pH [4, 29]. We have directly visualized the anionic sites on the cytoplasmic surface of the axolemma by electron microscopy employing cationized ferritin (CF) as a marker [18]. CF, a polycationic derivative of ferritin, is a useful tool for visualizing anionic sites such as the sialic acid of glycoproteins and gangliosides, the carboxyl group of proteins, and phosphates of membrane phospholipids [7, 24, 25].

When applied intracellularly, CF bound densely to the whole inner surface of the axolemma. The fixed charges of the inner surface are considered to be neutralized or reversed by CF binding. Usually, the change in surface charges of excitable membranes affects the voltage dependence of several parameters related to the membrane excitability, as shown by the experiments mentioned above. However, the effect of CF binding on the membrane excitability is very unique in the sense that no shift in the voltage dependence of these parameters occurs. We report here the detailed effects of CF binding on the ionic channel, and discuss the properties and distribution of the anionic sites on the excitable membrane and the ionic channel.

Materials and Methods

Giant axons of squid, *Doryteuthis bleekeri*, obtained at Ine, Kyoto, Japan, were used throughout. The diameters of axons used were between 350 and 650 μm. The axoplasm was squeezed out by passing a rubber-covered roller over the axon in a series of sweeps [2], and the axon was then mounted in a Lucite® chamber filled with artificial seawater. The internal perfusion was performed by using inlet and outlet cannulae made of glass [19, 27]. The inlet cannula (about 150 μm in diameter) was inserted into the squeezed region of the axon from one cut end. The axon was perfused with internal solutions supplied through this cannula at a hydrostatic pressure of about 25 cm H_2O. The outlet cannula (about 400 μm in diameter) was inserted into the perfused axon from the other cut end. The length of the perfused zone was about 20 mm.

The internal potential was measured with a glass pipette electrode (about 120 μm in diameter) filled with 3 M KCl-agar in

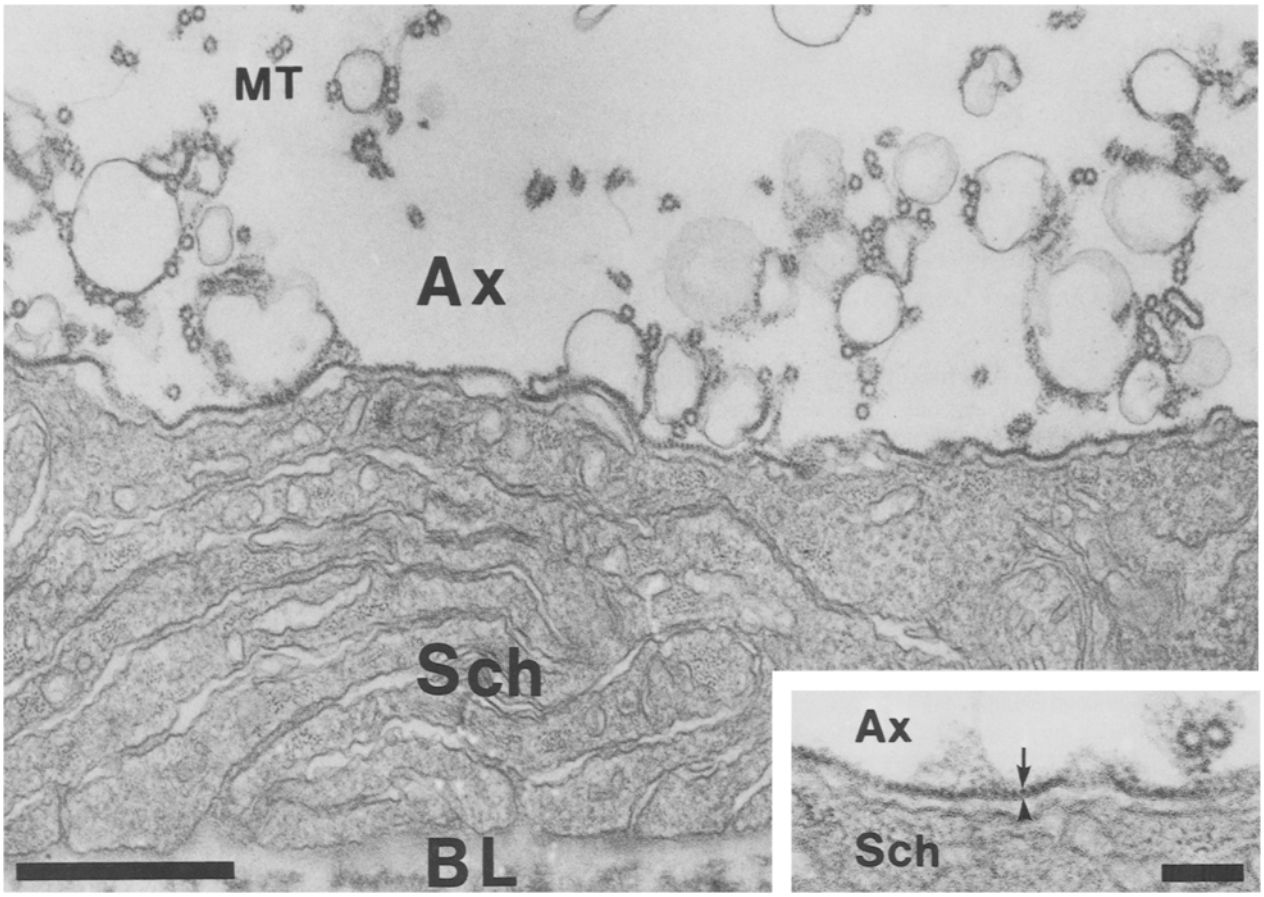

Fig. 1. Electron micrograph of a transverse section of a squid giant axon perfused intracellularly with 0.5 mg/ml CF. 58,000× (inset; 108,000×). The entire cytoplasmic surface of the axolemma is densely labeled with CF molecules. CF also binds to microtubules. *Ax:* axonal lumen, *Sch:* Schwann cells, *BL:* basal lamina, *MT:* microtubules. The arrowhead indicates the axolemma and the arrow indicates CF molecules. The bar indicates 0.5 μm (inset: 0.1 μm)

contact with a Ag-AgCl wire. The external reference electrode was a glass capillary filled with an agar gel of artificial seawater and connected to a coil of Ag-AgCl wire through a 3 M KCl solution. The internal current-supplying electrode was a piece of platinized platinum wire (70 μm in diameter). Both the recording electrode and the current-supplying electrode were introduced into the axon through the outlet cannula. The membrane current was measured with coiled Ag-AgCl wire electrodes (7 mm in width) arranged in a guard system. The voltage clamp was performed by the conventional method.

The compositions of artificial seawater were (in mM): 510 NaCl, 5 KCl, 50 $CaCl_2$ in ASW-1 and 435 NaCl, 5 KCl, 100 $CaCl_2$ in ASW-2. The pH of these solutions was adjusted to 8.0 by adding 12.7 mM HEPES. A solution containing 250 mM KF, 6.8 mM NaCl, 6.9% (by volume) glycerol and 7 mM HEPES (pH 7.4) was used as a standard internal solution (control). Cationized ferritin (CF; Miles-Yeda) was added to the standard solution at a concentration of 0.01 to 1 mg/ml. When the potassium current or the sodium current was measured separately, 0.5 μM tetrodotoxin (TTX) or 10 mM tetraethylammonium (TEA) was added to the external or the internal solution, respectively. Native ferritin (NF; Pentex-Miles) and poly-L-glutamate (PLG; Miles-Yeda) were added directly to solutions, as necessary. The external bathing solution was circulated by a peristaltic pump and the temperature of the solution was kept at 10 ± 0.5°C using thermoelectric modules.

At the beginning of the internal perfusion, a solution containing 0.1 mg/ml pronase (Calbiochem) was used for 2 to 4 min to remove the axoplasm remaining near the cytoplasmic surface of the axolemma [26]. Such a brief pronase-treatment did not significantly affect the membrane excitability, but enabled CF to gain direct access to the axolemmal surface.

After electrophysiological measurements, axons were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1% OsO_4 in the same buffer. The specimens were block-stained with 1% uranyl acetate, dehydrated with a graded series of ethanols, and embedded in Araldite or Epon 812. Ultrathin sections were prepared, then stained with uranyl acetate and lead citrate, and observed under a JEOL 100CX or Philips EM 400 electron microscope.

Results

CF binding and its Effect on the Membrane Excitability

CF applied intracellularly bound to various negatively charged sites on the cytoplasmic surface of the axolemma and to organelles such as microtu-

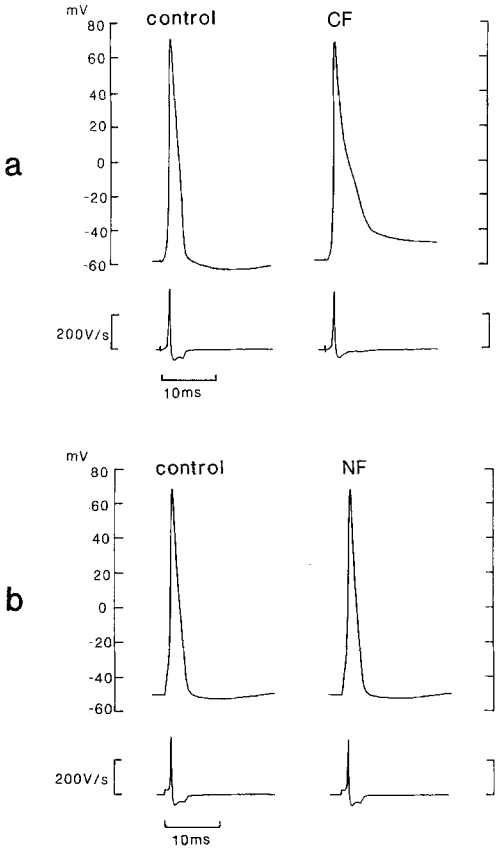

Fig. 2. Effects of CF and NF on the action potential and its derivative. Axons were perfused intracellularly with 0.5 mg/ml CF (*a*) or 0.5 mg/ml NF (*b*) for 10 min. The external solution was ASW-2

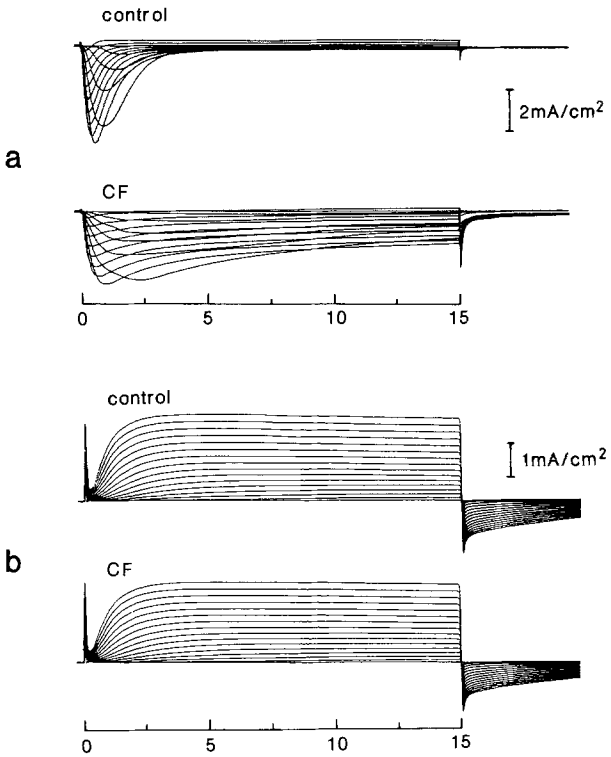

Fig. 3. Effects of CF on the membrane currents. *a*. Effect of CF (0.5 mg/ml) on the sodium current. The external solution was ASW-1 and the internal solution contained 10 mM TEA. The holding potential was −80 mV. Leakage currents were subtracted. *b*. Effect of CF (0.5 mg/ml) on the potassium current. The external solution was ASW-1 containing 0.5 μM TTX. The holding potential was −60 mV. Each curve was obtained by step depolarizations (10 mV increment) from the holding potential. Currents were measured after 10-min perfusion with 0.5 mg/ml CF

bules and endoplasmic reticulum (Fig. 1). The mode of CF binding was described in the previous report [18]. The entire cytoplasmic surface of the axolemma was thoroughly labeled with CF molecules. However, effects of CF on the membrane excitability were very specific. As shown in Fig. 2*a*, the duration of the action potential was prolonged by perfusion with a solution containing 0.5 mg/ml CF, while the amplitude of the action potential, the resting potential, the membrane resistance and the maximum rate of rise were practically unaffected by the CF perfusion. Washing with the control solution for 30 min failed to remove CF from the axolemma and to reverse electrophysiological effects.

Anionic native ferritin did not bind to axonal components [18] and did not affect the membrane excitability (Fig. 2*b*). When the axon was not initially treated with pronase (*see* Materials and Methods), perfusion with a CF-solution did not affect the membrane excitability. In that case, it was shown by microscopy that CF bound only to the surface of the axoplasmic layer remaining on the axolemma. External application of 1 mg/ml CF also had no effect on the membrane excitability.

Effects on the Membrane Current

Figure 3*a* shows the effect of CF on the inward sodium current during internal perfusion with a solution containing 10 mM TEA. When CF was applied intracellularly, the peak of the inward current was slightly lower and the decay was clearly lengthened. The outward potassium current, however, measured with ASW-1 containing 0.5 μM TTX, was practically unaffected by the CF-perfusion (Fig. 3*b*). Figures 4*a* and 4*b* illustrate *I-V* relations obtained from the data shown in Figs. 3*a* and 3*b*, respectively. The maximum sodium current was decreased by 5 to 25% by CF binding. The reversal potential of the sodium current was unaffected by CF binding.

There was no voltage shift in the *I-V* relations due to CF binding, in spite of the large amount of positive charges bound to the cytoplasmic surface of the axolemma (Fig. 1), probably neutralizing its surface charges. The voltage dependence of the

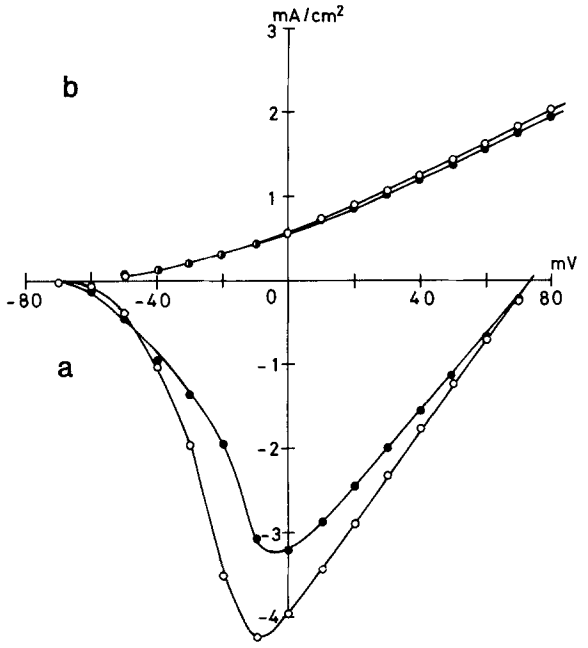

Fig. 4. The *I-V* relations of the peak sodium current (*a*) and the steady potassium current (*b*) obtained from data shown in Figs. 3*a* and 3*b*, respectively. Open circles indicate the control. Closed circles indicate the CF-treated axon

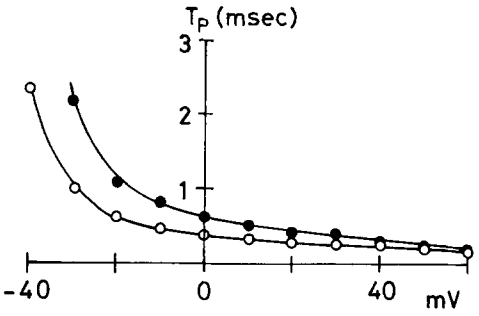

Fig. 5. Effects of CF on the voltage dependence of the time-to-peak of the inward current. Open circles indicate the control, closed circles indicate the CF-treated axon

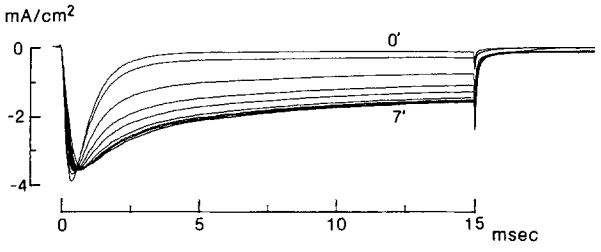

Fig. 6. Changes in sodium current with the time of perfusion with 0.5 mg/ml CF. Currents were measured at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 7 min after the onset of CF perfusion. The external solution was ASW-1. The holding potential was −80 mV. Each current was obtained by step depolarization to 0 mV

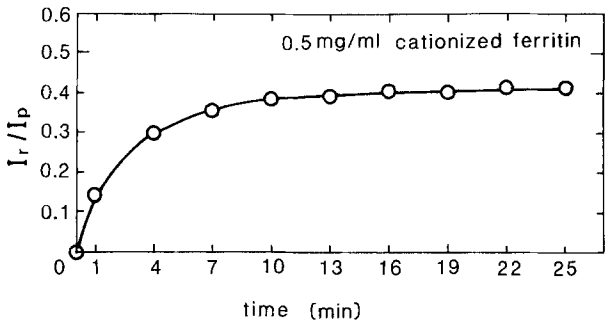

Fig. 7. Time course of the action of 0.5 mg/ml CF. The ordinate is the ratio of the residual current at the end of the step pulse to the peak inward current (I_r/I_p), indicating the extent of inactivation. The external solution was ASW-2

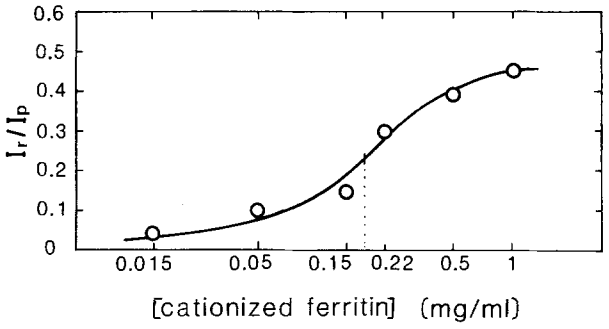

Fig. 8. Dose dependence of the CF action determined by measuring I_r/I_p at 10 min after the onset of CF perfusion. The external solution was ASW-2. The vertical broken line indicates the half-effective dose (0.17 mg/ml)

time-to-peak of the inward current (T_p) is shown in Fig. 5. T_p was prolonged by CF binding and its voltage dependence shifted towards the depolarizing direction. However, this shift is not due to the charge effect of CF, because the binding of positive charges to the internal surface is expected to shift the voltage dependence towards the hyperpolarizing direction. The voltage dependence of the steady-state inactivation (h_∞) did not change at all upon CF binding.

Effects on the Inactivation of the Sodium Channel

From the above-mentioned results, we can conclude that CF binding only affects the sodium channel, and especially its inactivation process. In the following, the effects of CF on the inactivation of the sodium channel measured in TEA-treated axons are described.

Figure 6 illustrates the sodium inward currents before (0′) and after CF perfusion obtained by step

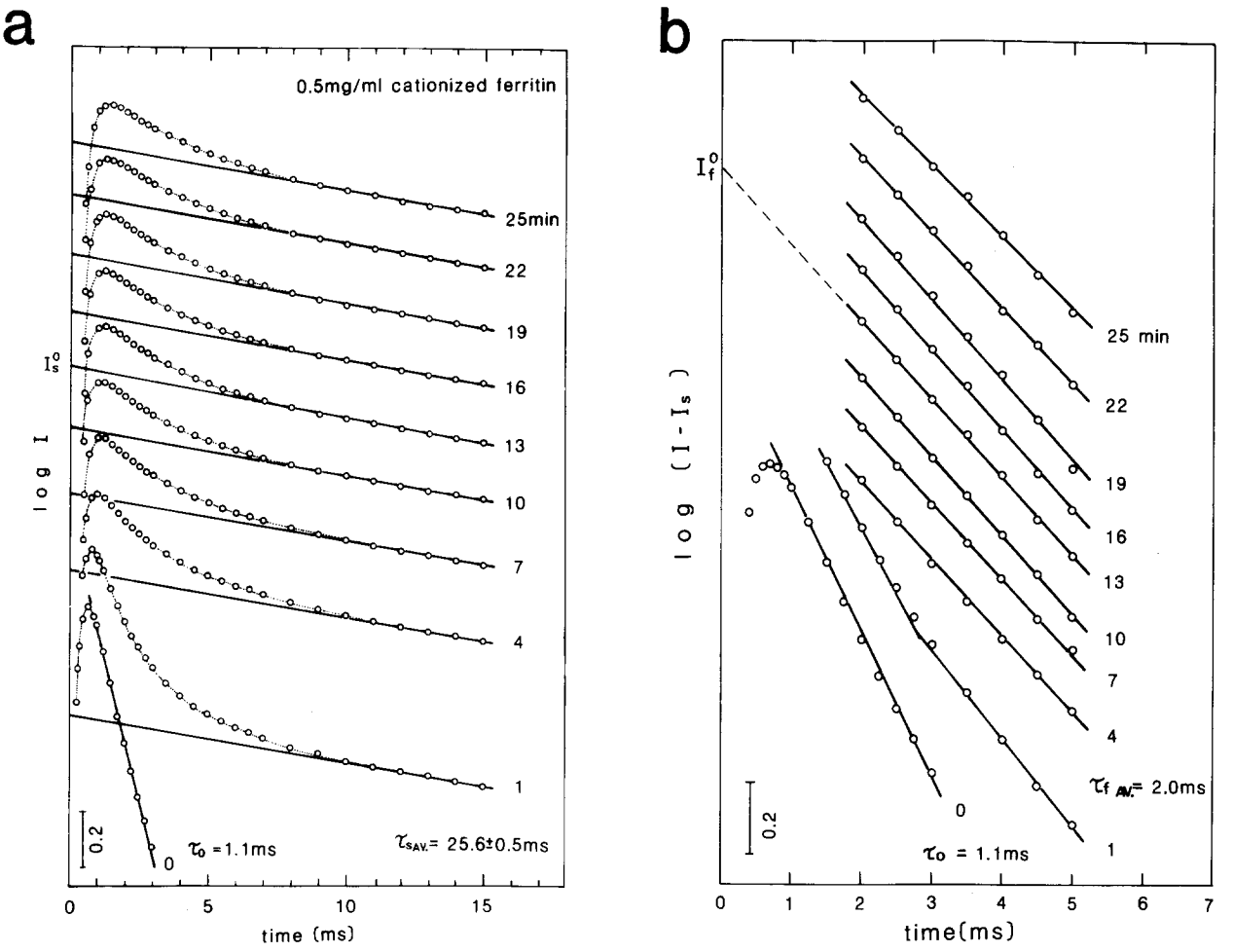

Fig. 9. Changes in the slow and fast component of the sodium inactivation with the time of perfusion with CF. *a*. Logarithmic plots of a series of inward sodium currents measured at 0 to 25 min after the onset of 0.5 mg/ml CF perfusion. Each curve is shifted vertically for the sake of clarity. The holding potential was −60 mV. Currents were measured by step depolarization to 0 mV. The external solution was ASW-2. Straight lines at 1 to 25 min show the slow component of the inactivation (I_s). *b*. Logarithmic plots of the fast component obtained by subtracting the slow component from the original current ($I_f = I - I_s$)

depolarizations to 0 mV from the holding potential of −80 mV. The decay of the inward current became slow immediately after the onset of CF perfusion and then changed gradually for about 7 min.

The ratio of the residual current at the end of the step pulse (I_r) to the peak inward current (I_p) was taken as an indication of the effect on the inactivation. With a solution containing 10 mM TEA and 250 mM KF, I_r measured with a potential pulse to 0 mV was negligibly small, although with a larger depolarizing pulse a small potassium current appeared (*see* Fig. 3*a*). Figure 7 shows the time course of the effect of 0.5 mg/ml CF represented by the I_r/I_p ratio. The effect of CF was saturated after 7 to 10 min. Figure 8 shows a dose-response curve for the I_r/I_p ratio measured 10 min after CF application. The dose of CF at which the half-maximum response occurred was 0.17 mg/ml in this figure.

Analysis of the Inactivation Time Course

Figure 9*a* shows a logarithmic plot of a series of the inward sodium currents measured 0 to 25 min after the onset of perfusion with 0.5 mg/ml CF. The curves are arranged with arbitrary shifts in the vertical direction. When CF was absent, the current decay had a single component (control component) with a time constant (τ_0) of 1.1 msec. When CF was applied internally, a slow component of the decay appeared within 1 min. Exponential curve-fitting using the current values at 10 to 15 msec, indicated that, once it had appeared, the time constant of the slow component (τ_s) did not change with the duration of perfusion. The average value of τ_s obtained at various perfusion times was 25.6 ± 0.5 (SD) msec. In Fig. 9*a*, the straight lines were drawn using this averaged τ_s. Figure 9*b* shows a logarithmic plot of

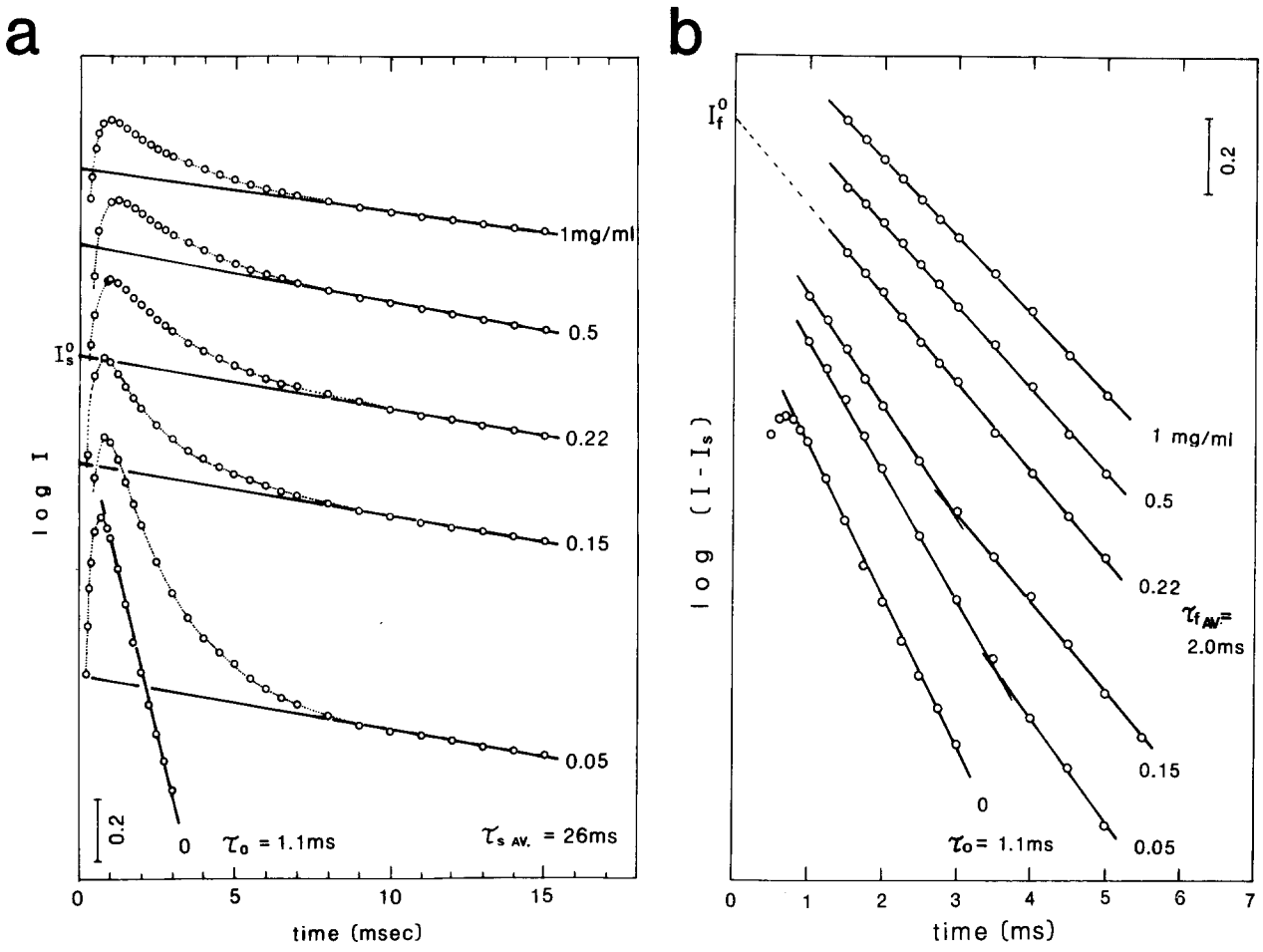

Fig. 10. Changes in the slow and fast component of the sodium inactivation with concentration of CF. *a*. Logarithmic plots of sodium currents measured at various concentrations of CF. Each curve is shifted vertically for the sake of clarity. The holding potential was −60 mV. Currents were measured by step depolarization to 0 mV. The external solution was ASW-2. Straight lines for 0.05 to 1 mg/ml show the slow component of the inactivation (I_s). The slow component (I_s^0) was determined from the intercept obtained by extrapolation of the line to the ordinate. *b*. Logarithmic plots of the fast component obtained by subtracting the slow component from the original current ($I_f = I - I_s$)

the fast component of the currents, obtained by subtracting the value of the slow component from that of the original current (i.e., $I_f = I - I_s$). The time constants of the fast component (τ_f) at times later than 4 min after the onset of perfusion were very similar, giving an averaged τ_f value of 2.0 msec. The curve at 1 min after the onset of perfusion seems to consist of two components; the fast component (τ_f), mentioned above, and the control component (τ_0).

Figure 10*a* shows a logarithmic plot of the inward sodium currents measured 10 min after the application of various concentrations of CF. The slow component of the decay appeared even at 0.05 mg/ml, and increased with concentration. The averaged value of its time constant was 26 msec (the same as in Fig. 9*a*). Figure 10*b* shows the fast component, obtained by subtracting the values of the slow component from those of the original current. The time constant of the fast component obtained at doses higher than 0.22 mg/ml was similar in each case, giving an averaged τ_f value of 2.0 msec. The curves obtained at concentrations of 0.15 and 0.05 mg/ml seemed to consist of the fast component (τ_f) and the control component (τ_0) again.

The current was represented by the expression, $I = I_f + I_s = I_f^0 \exp(-t/\tau_f) + I_s^0 \exp(-t/\tau_s)$, where I_f^0 and I_s^0 were initial currents of the fast and slow components, respectively, obtained by extrapolation to zero time as shown in Figs. 9 and 10. At low concentrations of CF and during early stages of CF perfusion, this expression was not correct, since I_f showed two decay time constants (τ_f and τ_0). However, with the present data it is difficult to divide I_f into two components without ambiguity. So, in those cases, I_f^0 was estimated by assuming that the current decay had a single time constant. The slow

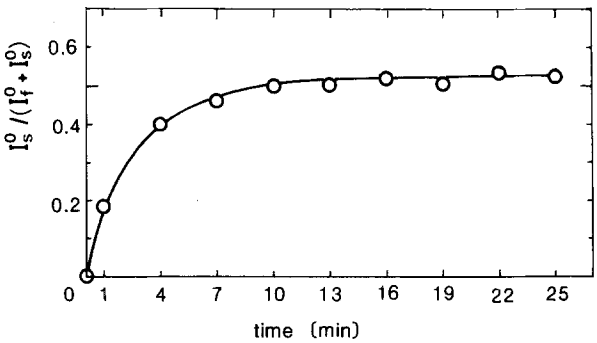

Fig. 11. Time course of the effect of 0.5 mg/ml CF obtained from Fig. 9. The ordinate shows the fraction of slow component $I_s^0/(I_f^0 + I_s^0)$. The action of CF reached a steady level after 7 to 10 min, when the value was about 0.5

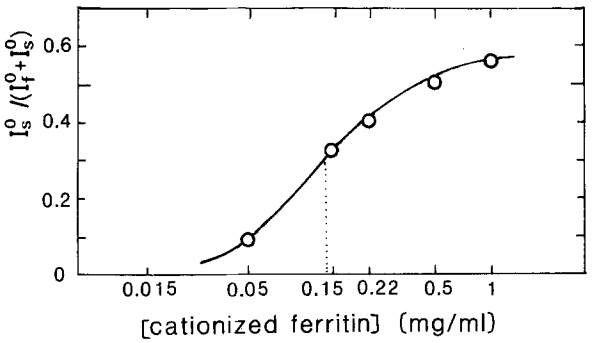

Fig. 12. Dose dependence of the CF-action expressed in terms of the fraction of slow component, obtained from Fig. 10. The vertical broken line indicates the half-effective dose (0.13 mg/ml = 150 nM). The fraction of slow component reached a maximum of 0.5 to 0.6

component (I_s^0) increased both with perfusion time and with concentration of CF, whereas the fast component (I_f^0) decreased. It was assumed that the slow component was due to the current through sodium channels directly modified by the binding of CF, while the fast component was due to the current through those which were indirectly affected by CF without being bound to. The fraction of the slow component was calculated to be $I_s^0/(I_f^0 + I_s^0)$. Figure 11 shows the time course of the increase of this fraction after the onset of 0.5 mg/ml CF perfusion. Figure 12 shows the dose dependence of this fraction. In both Fig. 11 and Fig. 12 the maximum value obtained was 0.5 to 0.6. The concentration of CF giving the half-maximum response was 0.13 mg/ml, or 150 nM based on a molecular weight of CF of about 840,000 (the protein shell; 445,000) [6].

The time constant of the slow component (τ_s) depended on the concentration of external calcium. The averaged τ_s values were 26 msec in ASW-2 (100 mM $CaCl_2$) and 50 msec in ASW-1 (50 mM $CaCl_2$). Changes in sodium concentration between 510 mM (ASW-1) to 435 mM (ASW-2) did not affect the time constant.

Counteracting effect of PLG Perfusion

The binding of CF to the axolemma and the effect of CF on the sodium channel inactivation process could not be eliminated by extensive washing of the axon with the control solution. However, the effect could be eliminated by internal perfusion of 0.1 mg/ml PLG. PLG itself scarcely affected the excitability unless axons were previously perfused with CF. After the PLG-perfusion of CF-treated axons, the slow component of the decay of the sodium current disappeared and the time constant of the fast component returned to the control value.

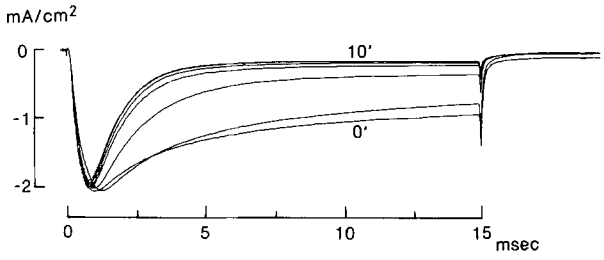

Fig. 13. Recovery of the inactivation time course induced by perfusion with 0.1 mg/ml PLG. The axon was initially perfused with CF. Currents were measured at 0, 0.5, 1, 2, 3, 5, 7 and 10 min after the onset of PLG perfusion. The external solution was ASW-1. Each current was measured by step depolarization to 0 mV from the holding potential of −80 mV

Electron micrographs of PLG-treated axons showed that some CF molecules were removed completely and that others were all detached from the axolemmal surface by some distance.

Discussion

Our results indicate that there exist negatively charged sites on the cytoplasmic surface of the axolemma and on the inactivation site of the sodium channel. The fact that negatively charged native ferritin (NF) and poly-L-glutamate (PLG) do not bind or affect the excitability indicates the absence of positively charged sites accessible to these large molecules.

There have been many reports indicating that the surface charge or surface potential of the axon membrane affects the membrane excitability, especially its voltage dependence. Changes in pH [3, 9, 16, 17, 21] and concentration of divalent cations [8, 10, 12, 14–17, 21] in the external solution, and

changes in pH [4, 29] and ionic strength [5, 20] of the internal solution induce shifts of the voltage dependence of the Na channel and/or the K channel. These voltage shifts have been explained in terms of the change or screening of the surface charge (or surface potential). CF has about 200 positive charges per molecule at physiological pH [7] and has been used as a label of the surface negative charge in electron microscope studies [7, 24, 25]. Since CF neutralizes or reverses the surface charge, it might be expected that voltage dependences of certain parameters of the membrane excitability would shift along the voltage axis. However, in the present work, practically no voltage shift due to binding of positive charges was observed. Based on the estimation of the charge density [17] and the experiment of the charge modification of the phospholipid headgroup [11], it was suggested that only the charges near to or on the channel are related to the membrane excitability. Our CF binding experiments clearly show that almost all the surface charges distributed on the membrane surface are unrelated to the membrane excitability, but that only those located on the channel can contribute to the surface potential which is effectively perceived by the channel. The CF molecule is about 13 nm in diameter [6]. Therefore, CF cannot affect the charge in the channel, but can bind to the inactivation site supposedly exposed on the surface of the channel [1]. When the axon was treated with pronase for a long time (10 to 30 min), and thus the inactivation process was removed, CF was able to affect the voltage gate and to suppress the excitability (*unpublished data*).

The effect of CF on the membrane excitability is very specific and is restricted to the sodium channel, while the binding of CF observed by electron microscopy distributed throughout the whole cytoplasmic surface. The specific effect of CF is the prolongation of the time course of the sodium channel inactivation. The findings that the half-effective dose is of submicromolar order and that the time constant τ_s has a unique value under various conditions indicate that CF binds to the sodium channel inactivation site in a specific manner. In other words, CF can be regarded as a 'toxin' which acts intracellularly on the sodium channel inactivation site. These properties of CF are similar to those of scorpion toxin [22] or chloramine-T [28] applied externally, or N-bromoacetamide (NBA) [23] applied internally. However, CF is unique because its effect and binding are reversible by the negatively charged molecule, PLG, which counteracts the CF effect on the sodium inactivation by removing CF from the binding sites. It is probable that the binding of CF is simply due to the electrostatic force between CF and anionic sites of the channel.

There are minor effects of CF binding on the inward sodium current. The current decreased slightly and its activation kinetics slowed down or shifted towards the depolarizing direction. This direction of the shift was opposite to that expected from the binding of positive charges to the internal surface. These effects were also found in the axon internally perfused with certain divalent and trivalent cations (*unpublished data*).

External application of CF does not affect the membrane excitability. A plausible explanation is that the CF molecule cannot reach the external surface of the squid axon membrane because of the surrounding Schwann cells and connective tissues. However, this was also observed in neuroblastoma × glioma hybrid cells (NG108-15) which develop typical sodium spikes after 7 days in culture with dibutyryl cyclic-AMP [13] (*unpublished data*). Therefore, it is also possible that CF is completely unable to act on the extracellular site of the sodium channel.

The sodium inactivation process of axons perfused with CF showed slow and fast components. The slow component increased and the fast component decreased with increasing perfusion time and concentration of CF. The slow component may reflect the inactivation of the CF-bound sodium channel. A problem concerning the fast component is that its time constant (τ_f) is clearly different from the control time constant (τ_0). Both (τ_f, τ_0) appear simultaneously at low concentrations of CF (Fig. 10) or during the early stages of CF perfusion (Fig. 9). The fast component may reflect the inactivation of the sodium channel indirectly affected by CF. Another problem is that the fraction of the slow component ($I_s^0/(I_f^0 + I_s^0)$) reached only 0.5 to 0.6 in all cases (Fig. 11 and Fig. 12). This indicates that about half of the population of Na channels in the axon are directly accessible to CF but that the remaining half are only indirectly accessible. At the present, the manner of the CF binding cannot be fully explained. Further studies of the interactions of CF with the sodium channel may provide useful information in elucidating the structure and the function of the sodium channel.

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