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ALTERATION OF BIREFRINGENCE SIGNALS FROM SQUID GIANT AXONS BY INTRACELLULAR PERFUSION WITH PROTEASE SOLUTION

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SUMMARY

The optical signal, arising from a transient birefringence change associated with excitation, was recorded from a squid giant axon together with the membrane potential change, and the effect of removal of the axoplasm on the optical signal was examined. In an unperfused axon, repetitive stimulation at a frequency of about 100 Hz produced two kinds of optical response. The initial response had a brief, spike-like time course and was elicited by each stimulating pulse. The delayed response had a slow time course and the sign of decreased light intensity, and summated with repetitive stimulation. Most of the axoplasm was removed from interior of the axon by intracellular perfusion with solutions containing pronase at a concentration of 0.1 mg/ml. The delayed response could selectively be eliminated by perfusion with a pronase-containing solution for 2–8 min. The result was interpreted as showing that the delayed birefringence signal originates from axoplasm when its gel structure was transiently disturbed by an increased Ca^{2+} influx associated with excitation. When perfusion was further continued the duration of the action potential started increasing and often a prominent after-depolarization appeared. At this stage the initial optical response was again followed by a large slow signal with the sign of increased light intensity. This reversed delayed response was tentatively assumed to originate from the membrane with some remaining axoplasm, but its cause is still not understood.

INTRODUCTION

Cohen, et al. [1] found that the birefringence of the squid giant axon changed slightly when the axon was excited. The time course of the birefringence signal closely resembled that of the action potential recorded intracellularly, although usually the signal at the later phase was accentuated in comparison with the after-hyperpolarization of the simultaneously recorded action potential [2]. A voltage-clamp pulse of relatively short duration produced an approximately rectangular birefringence signal, supporting the hypothesis that the optical effect was primarily a potential-dependent phenomenon [3]. When the pulse duration was prolonged, however, more complicated optical responses were revealed. The response pattern

changed gradually and irreversibly after dissection. Each of the response patterns was described by a number of time constants which varied from 30 μs to 20 ms [4].

Recently we found another slow birefringence change, which was produced when a series of repetitive stimuli was applied to the squid giant axon [5]. The slow time course suggested a hypothesis that the signal is produced from a change in the orderly structure within the axoplasm. The main purpose of the present paper is to examine the hypothesis with the use of intracellular perfusion. The axoplasm was enzymatically removed from the axon and the resultant change of the pattern of optical response was examined. It was found that the slow optical signal disappeared selectively after a brief perfusion with a dilute protease solution, but a new slow optical signal with reversed polarity appeared after prolonged perfusion with the same solution, or after brief perfusion with a concentrated protease solution.

METHODS

Giant axons

Squid (*Doryteuthis bleekeri*) were obtained from the Iné Fishery Plant, Kyoto Prefecture, and the Misaki Fishery Market, Kanagawa Prefecture. Giant axons of Iné squid were dissected locally and transported to the laboratory in Tokyo. Experiments started 7–14 h after dissection. Misaki squid were kept alive in a special aquarium in the Electrotechnical Laboratory in Tokyo, and were kindly supplied to us by Dr. G. Matsumoto. Experiments with Misaki squid could be performed on fresh axons. The design of the experimental chamber was similar to the one employed by Tasaki and his collaborators for the intracellular perfusion [6]. The polarizer was made from a piece of Polaroid film (HN 22) and placed below the base of the experimental cham-

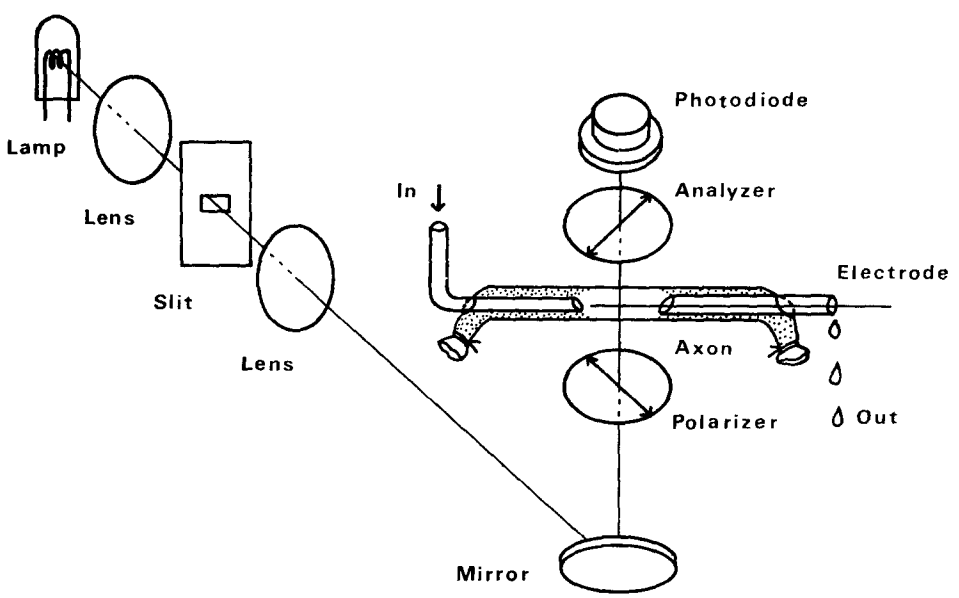

Fig. 1. A diagram for the optical arrangement of the experiment.

ber, with its transmission axis at 45 ° to the longitudinal direction of the axon. The analyzer was also made from the same material and mounted in front of the optical detector, a photodiode (United Detector, 1221). The transmission axis of the analyzer was usually set crossed with that of the polarizer. The analyzer-photodiode assembly was mounted on a steel beam which was held horizontally by two brass pillars, each screwed onto a magnetic base fixed on the experimental table. The assembly was placed on the top of the axon during recording of the optical signal, but it could be removed when necessary and could later be reinstalled to the same position. A diagram of the optical set-up is shown in Fig. 1. A white light from a 150 watt tungsten lamp was employed as the light source. Infrared radiation was suppressed by a red-suppression filter. The image of the filament was focused with a condenser on a slit with an adjustable width. The image of the slit was in turn focused on the axon with another condenser. The beam was split by a thinly coated mirror, which deflected the major part of the light by 90 ° to illuminate the giant axon from below. The minor part went through the mirror to illuminate the reference photodiode. The difference of the outputs of two photodiodes was amplified 3200 times and fed to a signal-averaging computer and at the same time displayed on one of the double beams of a cathode-ray oscilloscope. The optical signal was accumulated 500 or 1000 times on the averaging computer to improve the signal-to-noise ratio. The intracellular electrode was a piece of 50 μm platinum wire which was inserted into the axon through the outlet pipette for the intracellular perfusion. Action potentials were displayed on the second beam of the cathode-ray oscilloscope. External medium was natural sea water. The solutions for the intracellular perfusion were mixtures of 0.6 M KF/0.3 M K_2HPO_4/0.6 M KH_2PO_4 and 12 % glycerol. A standard solution contained 0.4 M K^+ and 0.36 M F^-, with some phosphate ions to adjust the pH and with glycerol to maintain tonicity. The pH of the solutions was always maintained between 7.2 and 7.4. The protease employed was pronase (Kaken Chemical Co.); the concentration was 0.1–0.5 mg per ml of perfusing fluid. To mark the enzyme solution, chlorphenol red (0.1 mg/ml) was also added to the perfusing fluid containing the enzyme. When we switched from the enzyme-containing perfusing fluid to the standard solution, we usually added to the latter solution a trypsin inhibitor from hens egg white (Boehringer, 15444) at a concentration of 0.2 mg/ml. An in vitro experiment showed that the inhibitor at this concentration reduced the initial rate of digestion of bovine serum albumine by pronase to a value less than one-tenth of the control. Experiments were performed at a room temperature of 15–21 °C.

RESULTS

The delayed optical response

When a train of stimuli consisting of five or six shocks was delivered at an end of the axon, an optical signal as shown in Fig. 2A was observed from almost all unperfused squid giant axons [5]. The signal consisted of rapid optical spikes which corresponded to each of the action potentials in response to an individual stimulus, and a slow component which always took the direction of decreased light intensity. We call the initial rapid optical spikes the initial responses, and the slow component the delayed response. With an optical analysis it is possible to show that the major part of the delayed response is due to the slow decrease in birefringence of the giant axon

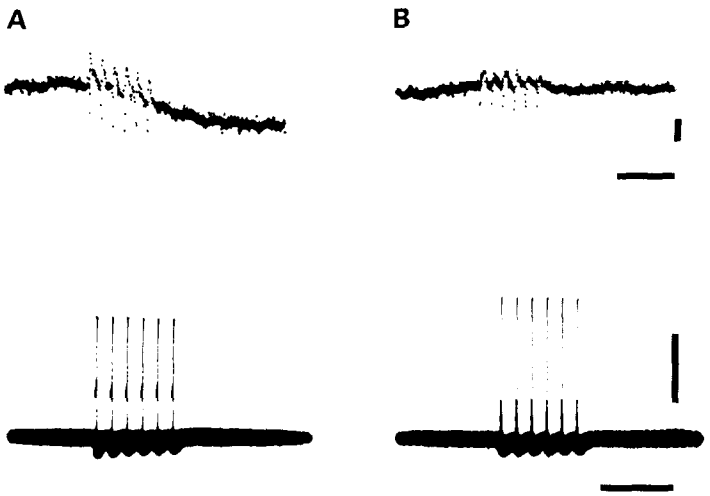

Fig. 2. The birefringence signal and the membrane potential change of a squid giant axon in response to a train of six stimulating electric shocks. Upper beams show birefringence signals. Vertical bar indicates $2 \cdot 10^{-6}$ of the background light intensity. Horizontal bar indicates 50 ms. Lower beams show membrane potential changes. Vertical bar, 50 mV. Horizontal bar, 50 ms. Records in (A) were obtained before perfusion. Records in (B) were obtained after 2 min perfusion with an enzyme-containing solution (0.1 mg/ml) at 20 °C.

[5]. The time course of the initial response was similar to that of the action potential except that the phase of increased light intensity is disproportionally large in comparison with the amplitude of after-hyperpolarization of the action potential [4]. The delayed response did not apparently have any counterpart in the membrane potential, as long as the recording was made with a usual amplification to observe the action potential. A description of a small, long-lasting potential change will be given later.

Disappearance of the delayed response with protease perfusion

Fig. 2B, lower trace, shows an electrical signal in response to the same pattern of stimulation after perfusing the axon with a dilute (0.1 mg/ml) pronase-containing solution for about 2 min. An increase in amplitude of the spike was produced because the perfusing fluid contained fluoride ions [7]. In other respects the shape of the action potential remained similar to that before perfusion, except that the after-hyperpolarization became somewhat shorter when examined on an expanded time base. No prolongation of the spike was recognized, which should have appeared if the period of enzyme perfusion were more prolonged [8–10].

A major change in the optical response was an almost complete disappearance of the delayed optical response (Fig. 2B, upper trace). In comparison, the change in the initial optical responses was minor; a small decrease in amplitude, especially at the later phase with increased light intensity, was usually observed, but detailed observations using an expanded time base were not performed. The initial responses never disappeared even by prolonged perfusion with the protease solution as long as the excitability was maintained.

In this experiment the control signal was taken from the axon before

separation of the perfusion pipettes. Therefore, it is possible that the perfusion itself, rather than removal of axoplasm by enzyme digestion, was responsible for a change in the optical signals. To examine the possibility we tried several experiments in which the axon was perfused with the standard solution containing no enzyme. In two successful experiments we confirmed that a perfusion with a protease-free solution did not eliminate the slow signal (Fig. 5A). Unexpectedly, however, the slow signal did not disappear even after switching the perfusing fluid to the enzyme-containing solution. The reason for this phenomenon is not entirely clear. It is possible that during the period of perfusion most free Ca^{2+} was removed from the axoplasm after combining with fluoride to form insoluble CaF_2, and a later administration of pronase was inhibited by the lack of Ca^{2+}. It is known that pronase is a mixture of several proteolytic enzymes and some of them are inhibited by the removal of Ca^{2+} [11]. However, in several in vitro experiments we found that pronase in KF solution digested both bovine serum albumin and gelatin at approximately the same rate as did pronase in KCl solution. On the other hand, we also found that when gelated gelatin layer was first superposed with an enzyme-free KF solution a visible boundary was formed between the gel and the solution, and when the superposed solution was later replaced with the enzyme-containing KF solution the rate of fall of the boundary was much slower than the rate of fall in a similar experiment employing KCl instead of KF. These experiments suggest another possible explanation, that when we perfused the axon with enzyme-free KF solution, the surface of the axoplasm was influenced by KF so that later penetration of the enzyme was retarded.

In most axons the resting birefringence was also decreased by enzyme perfusion [12]. The time course of the decrease in background light intensity was exponential with a time constant of approx. 5 min. The amount of decrease was variable among axons; in some of them we did not observe any decrease, while in others the final value was close to 50 % of the control.

The disappearance of the slow signal was observed in axons which were perfused with 0.1 mg/ml pronase solution for a period of between 2 and 8 min. When the period of enzyme perfusion was less than 2 min, we saw some slow signal remaining.

Effect of prolonged perfusion with enzyme-containing solution

When perfusion with the enzyme-containing fluid was continued for more than about 10 min, the rate of fall of the membrane potential from the peak of the spike gradually decreased and finally a long-lasting plateau was produced following the spike [8–10]. The corresponding optical signal was illustrated in Fig. 3. The signals shown in Fig. 3, A and B, were recorded from an axon which had been perfused with an enzyme-containing solution (0.1 mg/ml) for about 13 min. The direction of the initial optical responses did not change, but the delayed optical response appeared as a slow change with the direction of increased light intensity. The size of the reversed delayed response was often very large; with successive five or six stimuli it easily attained a value of approx. 10^{-5} times the background light intensity. As shown in Fig. 3B, the record of an optical signal in response to a single shock showed a second phase which was remarkably prolonged. The reversed delayed response could also be obtained by perfusion with a concentrated pronase solution for a short time. The optical and electrical signals shown in Fig. 3C were recorded from an axon perfused with a solution containing the enzyme at a concentration of 0.5 mg/ml for only about 3 min.

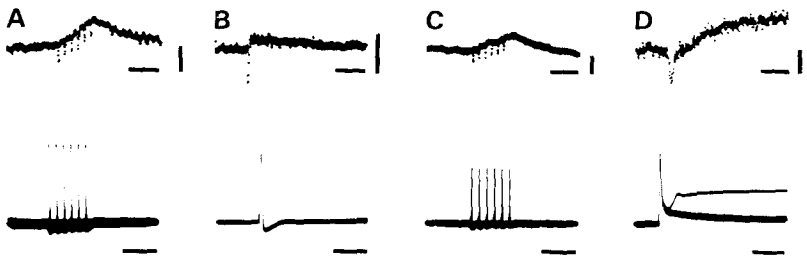

Fig. 3. Reversed delayed optical signals (upper traces) and membrane potential changes (lower traces). (A), time of enzyme perfusion, 12.5 min. Enzyme concentration, 0.1 mg/ml. Horizontal bars, 50 ms. Vertical bar, 10^{-5} of the background light intensity. Spike height in the lower trace, 104 mV at 19 °C. (B), records from the same axon under the same conditions as in (A), except that the stimulation was a single shock. Upper horizontal bar, 50 ms. Vertical bar, $5 \cdot 10^{-6}$. Lower horizontal bar, 10 ms. Spike height in lower trace, 100 mV. (C), records from another axon perfused with a solution containing 0.5 mg/ml of the enzyme for about 3 min. Horizontal bars, 50 ms. Vertical bar, $5 \cdot 10^{-6}$. Spike height on the lower trace, 104 mV at 21 °C. (D), records from an axon which had been perfused with a solution containing 0.1 mg/ml of the enzyme for 30 min. Inside medium contained 100 mg ion K^+. Lower trace shows a superimposed record of action potentials; usually the spike was followed by an after-depolarization but once in about 30 stimuli the spike was followed by a long-lasting plateau. Spike height was 97 mV. The lower horizontal bar, 20 ms. The upper trace shows the optical signal; responses to 500 stimuli were averaged including those associated with action potentials with plateau. Horizontal bar, 100 ms. Vertical bar, $5 \cdot 10^{-6}$ at 19 °C.

As Takenaka and Yamagishi [8] showed, an axon which is perfused with an enzyme-containing solution suffers changes in electrophysiological properties of the membrane; even when the shape of the action potential is barely influenced, the action potential shows a definite tendency to be followed by a plateau when the ionic strength of the perfusing fluid is reduced. The records shown in Fig. 3D were from an axon perfused with an enzyme-containing fluid for about 30 min. The action potential was only slightly prolonged under perfusion with 400 mM K^+ solution (see Fig. 4, C and D). But when the potassium concentration was reduced to 100 mM, the action potential showed a pronounced after-depolarization, which sometimes jumped up to form a long-lasting plateau. The corresponding optical signal showed an after-response with the direction of increased light intensity (Fig. 3D, upper trace), which, with repetitive stimulation, formed a conspicuous reversed delayed optical response (not shown).

Because the appearance of the reversed delayed response was so unexpected to us, we performed several experiments to exclude possible sources of the signal other than the birefringence change of the axon. Fig. 4A shows an example of the reversed delayed optical signal from an axon which was perfused with the protease solution for 30 min (the same axon as that shown in Fig. 3D). The shape of the action potential did not change very much (Fig. 4C), although a hump appeared at its falling phase (Fig. 4D). A quarter-wave plate was placed between the axon and the analyzer, and the slow axis of the plate was set perpendicular to the longitudinal axis of the axon. The signal reversed its sign with this procedure (Fig. 4B), indicating that the major part of the signal originated from an increase in axon birefringence. In a separate experiment, we set the direction of the analyzer either parallel with, or perpendicular to, the longitudinal direction of the axon (no quarter-wave plate employed). The resultant

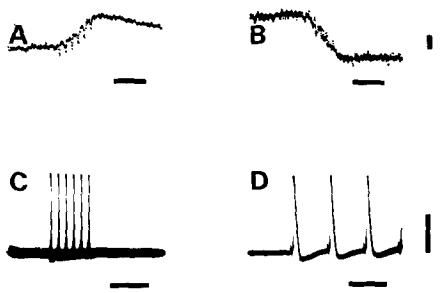

Fig. 4. Modification of the reversed delayed birefringence signal with the use of an optical compensator. The records were taken from the same axon as that shown in Fig. 3D. Enzyme concentration, 0.1 mg/ml. Time of enzyme perfusion, 30 min. (A), an optical signal in response to five successive stimuli. Horizontal bar, 50 ms. (B), the response was reversed by an insertion of a mica quarter-wave plate (546 mμ) between the axon and the analyzer with its slow axis directed perpendicularly to the longitudinal direction of the axon. Horizontal bar, 50 ms. The vertical bar in (B) indicates 10^{-5} of the background light intensity without the compensator, and applicable to both (A) and (B). (C), action potentials. Horizontal bar, 50 ms. (D), action potentials on an expanded time base. Horizontal bar, 10 ms. The vertical bar in (D) indicates 50 mV, applicable to both (C) and (D) at 19 °C.

signals should indicate the light intensity changes due to the turbidity changes of the axon [5]. The experiments showed that the turbidity changes were so small that they barely influenced the light signal obtained under the cross-polar conditions.

Long-lasting after-depolarization

A detailed examination of the membrane potential indicated the existence of two types of slow changes when repetitive stimulation was applied to the axon. One of them was a progressive decrease in amplitude of the after-hyperpolarization. Another was a slight long-lasting depolarization after the train of spikes. These two effects

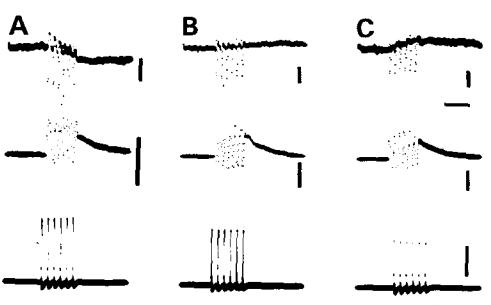

Fig. 5. Birefringence signals from three different axons and simultaneously recorded membrane potential change with high and low amplifications. The top traces are optical signals. Vertical bars, $5 \cdot 10^{-6}$. Horizontal bar, 50 ms. The middle traces are membrane potential changes in high amplification; signals were accumulated 40 or 50 times in the averaging computer. Vertical bars indicate 3 mV. The lower traces are membrane potential changes in low amplification. Vertical bar, 50 mV. Horizontal bar, 50 ms, applicable to both middle and lower traces. (A), signals from an axon perfused with the standard perfusion fluid containing no enzyme at 19 °C. (B), signals from an axon perfused with an enzyme-containing perfusion fluid for about 5 min. Enzyme concentration, 0.1 mg/ml at 20 °C. (C), signals from an axon perfused with an enzyme-containing perfusion fluid for about 12 min. Enzyme concentration, 0.1 mg/ml at 20 °C.

were described in detail by Frankenhaeuser and Hodgkin [13], and explained as being due to an accumulation of potassium ions just outside the excitable membrane. In our own records (Fig. 5) depolarization after the train of spikes was of the order of several millivolts and decayed with a time constant of about 50 ms; these are generally in agreement with those reported by Frankenhaeuser and Hodgkin. The long-lasting after-depolarization could also be observed in axons perfused with the protease solution (Fig. 5, B and C). No consistent influence of enzyme perfusion on the long-lasting depolarization was recognized. It is unlikely that the long-lasting after-depolarization, described above, is the cause of the delayed optical signals, because its amplitude is small and its time course is different from that of the delayed optical signals.

DISCUSSION

When an axon was intracellularly perfused with a dilute enzyme solution for a limited period, the delayed birefringence signal disappeared while the initial birefringence signal still remained with little changes in shape and magnitude, as reported by Sato et al. [12]. The finding is consistent with the hypothesis that the delayed birefringence signal originates from axoplasm, which undergoes a transient structural change during repetitive stimulation. The production of the delayed signal can be interpreted in the following manner. During excitation the rates of interdiffusion of cation across the membrane are greatly increased, and the ionic composition of the medium near the membrane significantly changed especially after repetitive stimulation. Hodgkin and Keynes [14] and Tasaki et al. [15] showed that extra influx of Ca^{2+} during excitation is of the order of 10^{-14} mol/cm^2 per impulse. With repetitive stimulation at a frequency of 100 Hz the protoplasm is invaded by an extra influx of Ca^{2+} at a mean rate of 10^{-12} mol/cm^2 per s. If one uses a one-dimensional solution of the diffusion equation (see Eqn 3.18 in Crank, ref. 16), and adopts 10^{-5} cm^2/s as an approximate diffusion coefficient for Ca^{2+} [17], one obtains a value of approximately 10^{-7} M as the Ca^{2+} concentration at the inside boundary of the membrane when the repetitive stimulation is applied for 50 ms. Hodgkin and Keynes [14] showed that the spreading of the radioactive Ca^{2+} in the axoplasm can be described by adopting an effective diffusion coefficient of $6 \cdot 10^{-7}$ cm^2/s. With this value, a similar calculation yields a value of $4 \cdot 10^{-7}$ M as the Ca^{2+} concentration at the inside boundary of the membrane. Recently Haga et al. [18] reported that Ca^{2+} at a concentration of $5 \cdot 10^{-7}$ M can reduce flow birefringence of a solution of the microtubule protein, tubulin, by approximately 5 % of the control. Therefore, it seems possible that invaded Ca^{2+} are responsible for producing the delayed birefringence signal by interfering polymerized tublin in the axoplasm. As an alternative to the above hypothesis, it is also possible to assume that the protease attacks some fraction of the membrane and changes its properties in such a way that the production of the delayed birefringence signal is somehow inhibited. If one further assumes that the attacked fraction of the membrane has no bearing on the production of the action potential and the initial birefringence signal, the alternative explanation is also consistent with the experiment, although we feel that the assumptions are arbitrary. At present we see no way of testing the alternatives with the experiment.

The reversal of the delayed birefringence signal by prolonged perfusion with an

enzyme solution is a remarkable phenomenon for which we do not have a definite explanation at present. Although the major part of the axoplasm (which will be called the endoplasm) must have been removed with this procedure [19, 20], a recent electron-microscopical examination revealed that some proteinic structure (which will be called the ectoplasm) remains inside the axonal membrane even after prolonged digestion with pronase (Tasaki, Metuzals and Inoue, personal communication). Therefore the site of occurrence of this phenomenon has still to be sought either in the membrane, or in the ectoplasm, or in the Schwann cells. The last possibility cannot be easily disregarded either, especially because of the experiments performed by Villegas [21], who showed that following repetitive stimulation of the axon the Schwann cell membrane undergoes long-lasting hyperpolarization. It is also known that after perfusion with protease-containing solutions several morphological changes take place in the cytoplasm of the Schwann cells [19], possibly because of a change in ionic environment of the Schwann cells caused by the permeability change of the axonal membrane [22]. Nevertheless, we are inclined to think that the site of occurrence is within the membrane-ectoplasm system, because when we see the reversed signal we also recognize some change in electrophysiological properties of the membrane. We often see a hump at the falling phase, which tends to develop to the long-lasting plateau, especially when the intracellular ionic strength is reduced [8, 10]. The voltage-clamp experiment yielded evidence that such changes in electrophysiological properties were the result of destruction of sodium conductance inactivation [22, 23]. We would therefore like to assume that the reversed delayed optical signal is a result of the conformational change in the membrane-ectoplasm system associated with the long-lasting excitation process, although at present the above assumption remains uncertain.

In the present work, the length of the perfusion zone was limited (7–13 mm), and the possibility exists that the local current between perfused and unperfused zones made some influence on the electrophysiological and optical signals which were recorded at the center of the perfusion zone. We believe, however, that the influence was slight, if any, because the difference in resting potentials between perfused and unperfused zones was in the order of only several millivolts.

Our reversed delayed birefringence signal is probably correlated with a similar observation in absorbance change obtained from a stained squid giant axon by Tasaki and Warashina [24]. They found that the direction of the absorbance change in a stained giant axon was reversed when the action potential was prolonged by intracellular injection of tetraethylammonium ions. Further experiments are needed to elucidate chemical species and properties of microstructures that are responsible for producing these reversed changes in anisotropy and absorbance.

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