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MODIFICATION OF OPTICAL RESPONSES ASSOCIATED WITH THE ACTION POTENTIAL OF LOBSTER GIANT AXONS

MICHAEL W. KAPLAN* and MELVIN P. KLEIN

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, Calif. 94720 (U.S.A.)

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SUMMARY

The sources of optical retardation changes and light scattering changes occurring during the action potential propagation of lobster giant axons have been investigated. A technique has been developed for resolving the total transmitted-light intensity change into a retardation change component, dI_r, and a forward direction light scattering change, dI_s.

Trypsin, pronase, neuraminidase and hyaluronidase all reduce the magnitude of dI_r without diminishing the action potential, probably by cleaving charged saccharides. Dithiothreitol has no effect. This suggests that glycoproteins and hyaluronic acid polymers at the surface of the axon are involved in the optical responses, either by being passively realigned or by contributing to compression and expansion forces as the membrane electric field changes.

Large dI_s responses are generated by trypsin and pronase treatment. The modifying effects of these proteases may be due to modification of the membrane or to increases in the refractive index of the medium surrounding the axon, since similar large dI_s responses are produced by increasing the refractive index with sucrose.

Since large reductions in dI_r can be produced without concurrent reductions in the action potential, a significant portion of the optical retardation responses cannot be attributable to structural changes that are causally related to membrane ionic permeability changes during the action potential.

INTRODUCTION

The experiments described in this paper were designed to identify the molecular species involved in the extremely small transient changes in optical retardation and light scattering that occur in the region of the axonal plasma membrane during action potential propagation. Using signal-averaging computers, it is possible to resolve these optical transients, which originate in the region of the plasma membrane.

* Current address: Department of Anatomy, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19174, U.S.A.

Current and potential-dependent light scattering transients have been observed at various angles in crab and lobster nerve trunks, and in squid giant axons [1–5]. Potential-dependent optical retardation changes have also been detected [1, 3, 6, 7]. These optical changes, particularly the retardation changes, have been cited as evidence for membrane structural transitions that cause the conductance changes during the action potential [3, 8].

MATERIALS AND METHODS

Nerve optical retardation changes are measured by positioning the axon, on the stage of a polarizing microscope, with its long axis oriented at a 45° angle from the polarizer and analyzer (crossed polarizer) axes. Stimulating the axon causes a transient decrease in intensity of the light emerging from the analyzer in the region near the plasma membrane [6]. In lobster giant axons this intensity change is due to a change in θ caused by a decrease in birefringence, and to changes in optical parameters not related to the birefringence change, such as light scattering. The static light intensity emerging from the analyzer that originates near the axon boundary is given by

$$I = I_0 k \left(\sin \frac{\theta}{2}\right)^2 \tag{1}$$

where I_0 is the intensity of the light incident upon the polarizer, k is an isotropic transmission coefficient representing losses due to absorption, light scattering, reflection, etc., and θ is the phase retardation angle.

$$\theta = \frac{2\pi d}{\lambda} (n_\perp - n_\parallel) \tag{2}$$

where d is the mean path length through the material, λ is the wavelength of the light, $n_\perp$ and $n_\parallel$ are the refractive indices of the annular region near the plasma membrane perpendicular and parallel to the nerve axis. Differentiating equation (1)

$$\mathrm{d}I = I_0 \left(\sin \frac{\theta}{2}\right)^2 \left(\frac{\delta I}{\delta k}\right) \mathrm{d}k + I_0 k \left(\sin \frac{\theta}{2}\right) \left(\cos \frac{\theta}{2}\right) \mathrm{d}\theta \tag{3}$$

or, in abbreviated form,

$$\mathrm{d}I = \mathrm{d}I_\mathrm{s} \pm \mathrm{d}I_\mathrm{r} \tag{4}$$

where $\mathrm{d}I_\mathrm{s}$ is the intensity change due to changes in forward direction light scattering, reflectance, etc. (hereafter referred to as the light scattering component), and $\mathrm{d}I_\mathrm{r}$ is the magnitude of the intensity change due to changes in retardation. Depending upon the sign of θ, $\mathrm{d}I_\mathrm{r}$ can be positive or negative. Also, the maximum change in I for a given change in θ will be achieved when $\theta = \pm\pi/2$. These facts led to the inclusion of a quarter-wave plate between the polarizer and analyzer in order to maximize the response, and to make the separation of $\mathrm{d}I_\mathrm{s}$ and $\mathrm{d}I_\mathrm{r}$ possible. With the quarter-wave plate oriented such that the total θ is maximally positive, the intensity change observed during the action potential is

$$\mathrm{d}I_1 = \mathrm{d}I_\mathrm{s} - \mathrm{d}I_\mathrm{r} \tag{5}$$

Rotating the quarter-wave plate 90° reverses the sign of θ. Since θ and $d\theta$ are small compared to $\pi/2$, dI_r has the same magnitude but opposite sign for the two orientations. In the second orientation,

$$dI_2 = dI_s + dI_r \tag{6}$$

Subtracting dI_1 from dI_2 yields the pure retardation component since

$$dI_r = \tfrac{1}{2}(dI_2 - dI_1) \tag{7}$$

Adding dI_1 to dI_2 yields the pure scattering component since

$$dI_s = \tfrac{1}{2}(dI_2 + dI_1) \tag{8}$$

This technique for resolving the two intensity change components was employed in the experiments to be described.

The experimental apparatus is diagrammed in Fig. 1. The basic functions of the electronic equipment were generating and monitoring the action potential, measuring the light intensity changes, and signal-averaging the results of these measurements in order to resolve the very small responses. A transient recorder was used to store and digitize the amplified output of the photodiode. Signal averaging was accomplished by adding several thousand responses in a memory unit. A preset counter and appropriate control logic determined the number of responses added. Results from different measurements could be added or subtracted by the computer as

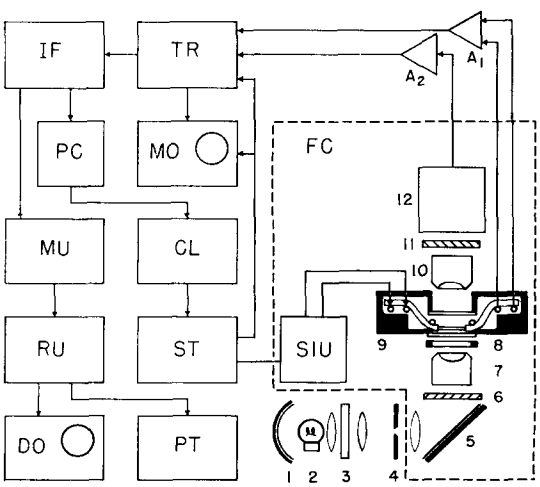

Fig. 1. Experimental apparatus designed to stimulate axon and monitor action potential, measure light intensity changes caused by axon depolarization, signal-average the results, and process the final data to resolve the optical retardation component and the forward light scattering component from the total intensity change. 1 spherical mirror; 2, 200 W Quartzline lamp; 3, infrared filter; 4, slit; 5, mirror; 6, polarizer; 7, condenser; 8, quarter-wave plate; 9, experiment/perfusion chamber showing nerve in position with isolated giant axon in light path; 10, objective lens; 11, analyzer; 12, photodiode-detector and preamplifier assembly; A_1, action potential amplifier; A_2, optical signal amplifier; TR, transient recorder; IF, interface; MO, monitor oscilloscope; PC, preset counter; CL, control logic; ST, stimulator; SIU, stimulus isolation unit; MU, memory, unit; RU, readout unit; DO, display oscilloscope PT, paper tape punch; FC, Faraday cage.

required. Action potentials were generated and recorded externally using two pairs of platinum wire electrodes.

Identification of the molecular moities participating in the structural changes responsible for the optical transients in lobster (*Homarus americanus*) giant axons was attempted using the following approach. Using freshly dissected circumesophageal connective nerves with a 5 mm portion of the giant axon isolated, control measurement of dI_s and dI_r were made with Dalton's lobster saline as the bathing medium [10]. Specific modifying reagents dissolved in saline were then perfused into the sealed measurement chamber for appropriate periods. After flushing the chamber with fresh saline solution, dI_s and dI_r were remeasured. The second set of optical changes were then compared with the controls, and any significant differences were interpreted to mean that the moiety being perturbed by the reagent has some role in producing the optical transients.

All reagents were used as purchased without further purification. Trypsin (EC 3.4.4.4, grade A, from bovine pancreas, crystalline, 10^4 BAEE units/mg), pronase (grade B, *Streptomyces griseus*, 45000 PUK units/gm), and dithiothreitol (grade A) were purchased from Calbiochem. Neuraminidase (EC 3.2.1.18, purified, type V, *Clostridium perfringens*, 0.08 units/mg using *N*-acetylneuraminyllactose as substrate), and hyaluronidase (EC 3.2.1.35, type III, ovine testes, 680 NF units/mg) were purchased from Sigma Chemical Company.

All measurements and modifying-reagent incubations were done at room temperature (20.5–23.0 °C).

RESULTS

Controls

Transient decreases in optical retardation and in forward direction light scattering occur in a layer at the surface of lobster giant axons as a result of action potential propagation. The initial retardation decrease appears to have a time course closely resembling the development of the action potental. The recovery process is more complex, however, and its rate appears to depend inversely upon the thickness of the sheath surrounding the axon. This suggests that the recovery phase of dI_r has a sheath-related component. The magnitude of dI_s is occasionally as much as 50 % of the magnitude of dI_r, but usually it is closer to 20 %. No correlation of the magnitude of the scattering component with any obvious morphological characteristics of the axon could be made.

Proteases

Exposure to trypsin or pronase causes the surface of lobster giant axons to become granular and deformed [11, 12]. The resistance to microelectrode penetration decreases. However, gentle digestion produces no significant effects upon the action potential or resting potential of either lobster or squid giant axons. It is unlikely that these proteases penetrate to the internal side of the plasma membrane, since internal perfusion of squid giant axons with proteases promptly abolishes the action potential [13, 14].

Axons exposed to solutions of 0.20–0.25 mg/ml trypsin or pronase in saline solution for 10–15 min usually show a 30–50 % decrease in the magnitude of dI_r.

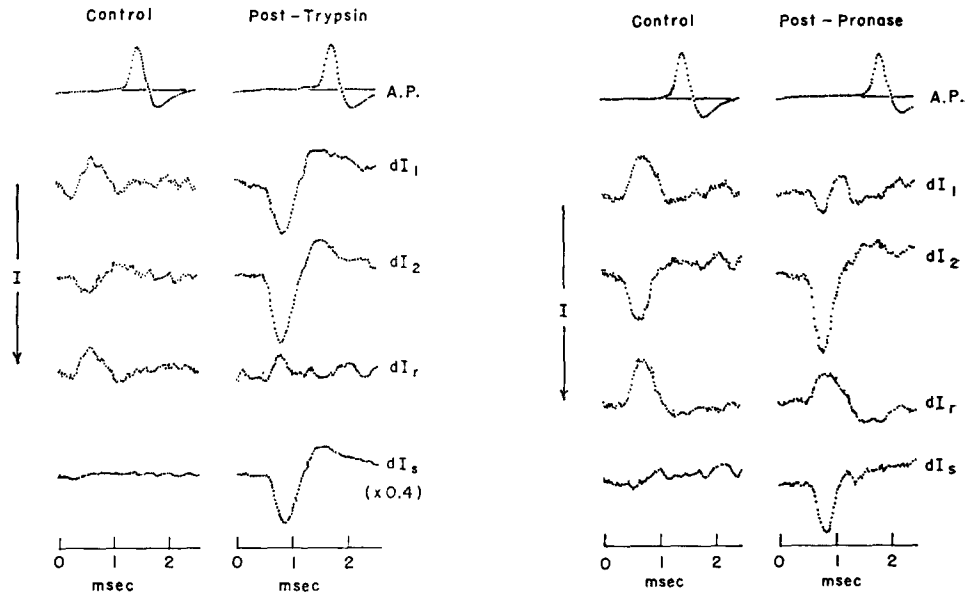

Fig. 2. Modification of the optical transients by a 10-min incubation in 0.25 mg/ml trypsin/saline solution. Control experiment traces on the left; post-trypsin traces on the right. A.P., action potential traces; dI_1, total light intensity transient for initial quarter-wave plate orientation; dI_2, total light intensity transient with the quarter-wave plate rotated 90°; dI_r, optical retardation component of the intensity change; dI_s, forward light scattering component of the intensity change (reduced by a scale factor of 0.4). Optical traces are the signal-averaged result of 3000 sweeps. A downward deflection represents a light transmission increase.

Fig. 3. Modification of the optical transients by a 15-min incubation in 0.25 mg/ml pronase/saline solution. Control experiment traces on the left; post-pronase traces on the right. A.P., action potential traces; dI_1, total light intensity transient for initial quarter-wave plate orientation; dI_2, total light intensity transient with the quarter-wave plate rotated 90°; dI_r, optical retardation component of the intensity change; dI_s, forward light scattering component of the intensity change. Optical traces are the signal-averaged result of 3000 sweeps. A downward deflection represents a light transmission increase.

The effect on dI_s was somewhat unpredictable. In almost all cases dI_s became much larger, typically showing a three-fold increase in magnitude, and often exceeding dI_r. Somewhat surprisingly, the direction of the response reversed to a transient intensity increase in some experiments. It is possible that this directional variabillity is associated with a lack of control over the tension applied to the axon [15].

Data from a typical trypsin treatment ase shown in Fig. 2. For this axon, dI_r was reduced slightly by incubation in 0.25 mg/ml trypsin/saline for 10 min. However, a very large biphasic dI_s was generated. It should be noted that the time courses of dI_r and dI_s are significantly different, particularly in their recovery phases.

The results of a typical pronase incubation are shown in Fig. 3. For this axon a 15 min treatment with 0.25 mg/ml pronase/saline reduced dI_r by approx. 30 %. A large dI_s signal representing an increase in light transmission was generated.

Neuraminidase

Treating lobster giant axons with neuraminidase causes no visually detectable

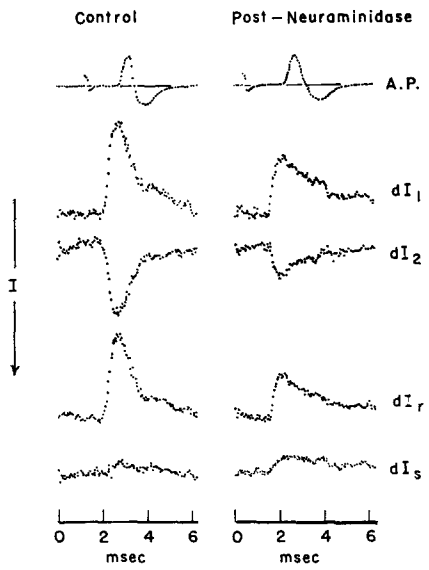

Fig. 4. Modification of the optical transients by a 30-min incubation in 0.20 mg/ml neuraminidase/saline solution. Control experiment traces on the left; post-neuraminidase traces on the right. A.P., action potential traces; dI_1, total light intensity transient for initial quarter-wave plate orientation; dI_2, total light intensity transient with the quarter-wave plate rotated 90°; dI_r, optical retardation component of the intensity change; dI_s, forward light scattering component of the intensity change. Optical traces are the signal-averaged result of 3000 sweeps. A downward deflection represents a light transmission increase.

changes in the morphology of the fiber. No changes are induced in the shape or magnitude of the action potential. However, a pronounced decrease in the refractory period is produced.

Incubation in 0.20 mg/ml neuraminidase/saline solution for periods of 30–60 min produces marked reductions in the magnitude of the dI_r response, usually by 40–50 %. In contrast with the protease results, no concurrent changes are produced in the scattering response.

A typical neuraminidase incubation experiment is shown in Fig. 4. This axon demonstrated a 50 % reduction of dI_r after a 30 min incubation in 0.20 mg/ml neuraminidase/saline. It is also apparent that dI_s was not significantly perturbed.

There is some ambiguity in interpreting the neuraminidase results. Warren [16] analyzed several non-neural lobster tissues for sialic acid residues and concluded that the sialic acid content of these tissues is no greater than 10 % of the equivalent vertebrate tissues. If lobsters do not have sialic acids, how can the reduction of dI_r and the decrease in refractory period after neuraminidase treatment be accounted for? The stability of the action potential argues against significant phospholipase contamination [12]. Protease contamination is unlikely, since prolonged incubation in neuraminidase solution produced none of the changes in sheath morphology and in dI_s seen with protease treatment. It is conceivable that the neuraminidase used is cleaving charged saccharides other than those which would be detected by Warren's assay, or that it binds to the axon in a non-specific manner where it interferes with the tran-

sitions that generate dI_r. Finally, it is possible that lobster axons do contain small amounts of sialic acid, and that the neuraminidase acts upon its normal substrate in these experiments.

Hyaluronidase

The effect of hyaluronidase upon dI_r is similar to that of neuraminidase. No appreciable morphological changes are produced, and action potentials are unaffected by hyaluronidase incubation.

In most experiments, a 1 h exposure to a 2.0 mg/ml hyaluronidase saline solution reduces dI_r by approx. 40 %. The dI_s component is usually not altered, and the kinds of changes produced by proteases are not observed.

An example of hyaluronidase treatment is shown in Fig. 5. The isolated dI_r response has been reduced significantly, but the small dI_s component is unchanged by the incubation.

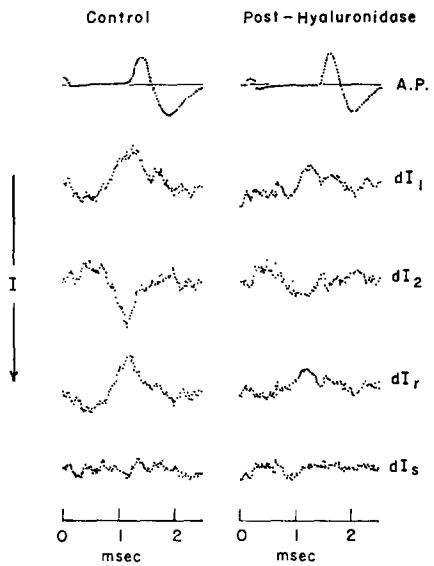

Fig. 5. Modification of the optical transients by a 60-min incubation in 2.0 mg/ml hyaluronidase/saline solution. Control experiment traces on the left; post-hyaluronidase traces on the right. A.P., action potential traces; dI_1, total light intensity transient for initial quarter-wave plate orientation; dI_2, total light intensity transient with the quarter-wave plate rotated 90°; dI_r, optical retardation component of the intensity change; dI_s, forward light scattering component of the intensity change. Optical traces are the signal-averaged result of 5000 sweeps. A downward deflection represents a light transmission increase.

Dithiothreitol

Treating lobster giant axons with dithiothreitol modifies the toxic effects of batrachotoxin, indicating that membrane surface proteins are altered [17]. However, dithiothreitol produces no significant changes in dI_r, and changes produced in dI_s are small compared to the changes caused by proteases.

Sucrose

It is possible that the changes in dI_s induced by trypsin and pronase might be caused by an increase in the refractive index of the immersion medium as products of hydrolysis are released into solution near the plasma membrane. This possibility was tested by raising the immersion medium refractive index using 30% sucrose/Ringer ($n = 1.38$). The morphological and optical signal changes produced by sucrose are not consistent, but it is possible to produce increases in the magnitude of dI_s, for both increasing and decreasing light transmission, similar to what is seen with proteases.

DISCUSSION

Changes are induced in the optical retardation and forward light scattering responses of lobster giant axons when the fiber is treated with agents that modify specific constituents of the membrane system. From the results of these modifications, mechanisms explaining the causes of the optical changes may be postulated, and some insight into the dynamic nature of the plasma membrane and adjacent regions can be gained. Cohen, et al. [7] have stated that Kerr effect dipole reorientations can account for the magnitudes of the retardation changes observed. They argue against a simple dipole reorientation mechanism, however, because the 40 μs time constant for the birefringence relaxation is much longer than the rotational relaxation of proteins in solution. It is probably more appropriate to compare the relaxation rate to those of other membrane bound proteins. For example, rhodopsin in situ has a 20 μs rotational time constant [18], a value reasonable close to the squid birefringence relaxation time constant. Cohen, et al. [7] additionally state that membrane compression and expansion effects can also be large enough to account for the changes in retardation. The changes in dI_r and dI_s reported here could be due to changes in the compessibility of the membrane, or, more likely, to changes in the surface charges and consequent changes in electrostatic forces due to the membrane potential. The experiments of this study suggest that charges associated with saccharides of glycoproteins (and perhaps glycolipids) are involved in the mechanism generating dI_r. Treating axons with trypsin and pronase cleaves polypeptides to which the saccharides are bound [19]. This could reduce dipole moments of molecules associated with such residues, or reduce compression/expansion forces because of a reduction in surface charge density. Neuraminidase produces changes consistent with the hypothesis that charged saccharides are involved. Hyaluronidase breaks up the charged hyaluronic acid polymer matrix that exists in the intercellular space of the axon sheath [20], rendering potential dependent transitions less co-operative. There are at least three conceivable mechanisms which could account for the observed modifications of dI_s. Axial stretching causing realignment of mobile membrane constituents may result as the structural integrity of the membrane and sheath are reduced by the proteases. Alternatively, products of protease lysis may increase the refractive index of the surrounding medium, causing scattering increases. Measurements using Dextran [1] and sucrose-containing medium tend to support this explanation. Since neuraminidase produces no appreciable changes in dI_s, the charge on the membrane apparently does not directly affect the scattering component of the light intensity change. Proteases, on the other hand, which generate molecular fragments to be dispersed into the sur-

rounding medium, often produce dramatic changes in dI_s. Finally, ionic collisions with the hydrocarbon chain moieties of the axon and sheath membranes may produce localized high turbulence regions that generate some of the light scattering effects seen in our experiments, similar to those seen in liquid crystals [21]. Enzymatic attack on membrane structures may alter the degree of turbulence for a given electric field change, thereby producing changes in dI_s.

The fact that large modifications of the scattering signal may be induced without concommitant large changes in the retardation response indicates that the two responses probably arise from different sources. Supporting this conclusion is the fact that after enzymatic modification, the retardation peak sometimes occurs significantly before the scattering peak.

Because the action potential is the same before and after treatment with the modifying agents used in this study, it may be concluded that a major portion of the optical responses being monitored is due to a passive reorganization of the membrane and adjacent regions caused by the action potential. It implies that the assumption that the optical changes are reflecting structural transitions which determine the action potential may not be justified [3, 8, 22–24]. However, the existence of a small component of the optical responses associated with the mechanism determining membrane ionic permeability changes during the action potential cannot be ruled out on the basis of these experiments, since the retardation change was never completely eliminated by the modifying agents.

ACKNOWLEDGMENTS

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