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

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Running title: MODIFICATION OF AXON OPTICAL RESPONSES

SUMMARY

The sources of optical retardation changes and light scattering changes occurring during the action potential depolarization 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 molecular transitions that generate 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. Neuraminidase, hyaluronidase and dithiothreitol have much less effect on the scattering response. The modifying effects of the proteases may be due 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.

Because their time courses are often markedly different, dI_r and dI_s appear to have different origins. Since large reductions in dI_r can be produced without concurrent reductions in the action potential, a significant portion of the optical retardation response cannot be attributed to structural changes, if any, that are causally related to membrane ionic permeability changes during depolarization.

INTRODUCTION

The experiments described in this paper were designed to identify the molecular species involved in the transient decrease in optical retardation that occurs in the region of the axonal plasma membrane during action potential depolarization. Using the relative phase retardation and scattering of visible energy photons as probes of the kinetics of membrane structure changes has the advantage that such photons interact with the membrane system in a non-destructive manner, and that certain of these interactions may be detected without inducing perturbations in the material by fixation, probe intercalation, etc. Axons have a thin, annular layer in the region of the plasma membrane that exhibits the properties of a uniaxial birefringent material having a radial optic axis [1]. The birefringence of this layer can result from two types of structural inhomogeneities. Intrinsic birefringence occurs in substances having ordered arrays of molecules which are anisotropic in the polarizabilities of their chemical bonds and thus in their dielectric constants. Form birefringence occurs when particles or layers of a substance having one dielectric constant are arrayed in a substance having a different dielectric constant. Straightforward electrostatic boundary conditions have been employed to derive expressions for the birefringence of rodlets in a matrix and for the birefringence of stacked layers [2]. The membrane system defining the external surface of an axon may owe its net birefringence to both form and intrinsic birefringence components.

With the advent of signal-averaging computers it became possible to elucidate the extremely small optical changes temporally associated with single action potentials. Light scattering transients were observed at

various angles in crab and lobster nerve trunks and in squid giant axons [1,3-6]. Changes dependent upon the membrane potential and the integrated membrane current were measured during action potential depolarizations and during voltage-clamp depolarizations and hyperpolarizations. Small potential dependent optical retardation changes were also detected [1,4,7,8]. These transients were found to be localized in a region close to the plasma membrane, and were attributed to structural changes in either the plasma membrane itself or in systems closely associated with it. Such optical changes, particularly the retardation changes, have been cited as evidence for cooperative membrane structural transitions that may be responsible for the conductance changes that occur during the action potential [4,9]. It has been assumed that some constituent of the plasma membrane is responsible for the optical transients, since this membrane is known to be the permeability barrier for ionic currents.

MATERIALS AND METHODS

Nerve optical retardation can be observed 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. For such a geometry the linearly polarized electric field vector of the light incident upon the nerve may be thought of as being composed of two mutually perpendicular, coherent components of equal magnitude whose relative phases are shifted as the axon is traversed because of the difference in refractive index for the two component polarizations. The emergent ellipitically polarized resultant formed by these two components is then incident upon the analyzer, which transmits only the component of the

resultant along the minor axis of the ellipse. A general equation describing the intensity of the light emerging from such a system was derived by Fresnel [10]. For the geometry just described, the equation simplifies to

$$I = I_0 k \sin^2 \theta/2 \qquad (1)$$

where I is the intensity of light emerging from the analyzer, I_0 is the initial intensity of the light incident upon the polarizer, k is a transmission coefficient representing absorption by the polarizer and analyzer, losses due to light scattering, reflection, etc., and θ is phase retardation angle. For the annular region near the plasma membrane, θ is given by

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

where d is the mean path length through the material, λ is the wavelength of the light, $n_{\parallel}$ and $n_\perp$ are the refractive indices parallel and perpendicular to the nerve axis.

Stimulating an axon positioned in a polarizing microscope as described above causes a transient decrease in intensity of the light emerging from the analyzer. Cohen et al. [7] have shown that this change is localized in a region near the plasma membrane. This intensity change may be due to a change in θ caused by a decrease in the birefringence, $(n_\perp - n_{\parallel})$, or to a change in optical parameters not related to the retardation change. Differentiating the Fresnel equation yields

$$dI = I_0 (\sin^2\theta/2)(\frac{\partial I}{\partial k}) dk + I_0 k (\sin \theta/2)(\cos \theta/2) d\theta. \qquad (3)$$

In abbreviated form this may be written as

$$dI = dI_s \pm dI_r \qquad (4)$$

where dI_s is the intensity change due to changes in forward direction light scattering, reflectance, etc. (hereafter referred to as the light

scattering component), and dI_r is the magnitude of the intensity change due to changes in retardation. Note that for a given $d\theta$, dI_r will be positive or negative depending upon the sign of θ, the total static retardation angle of the elements in the optic path. Also, the maximum $\frac{\partial I}{\partial \theta}$ will be achieved when the static θ is $\pi/2$. Both of these facts suggest the inclusion of a quarter-wave plate ($\theta = \pi/2$) between the polarizer and analyzer in order to maximize the response and to make possible the separation of dI_s and dI_r. If the quarter-wave plate is initially oriented with its axes parallel to the principal axes of the axon, and such that the net θ is positive, the intensity change observed during the axon depolarization is given by

$$dI_1 = dI_s - dI_r \tag{5}$$

Rotating the quarter-wave plate 90° reverses the sign of θ. Since the retardation of the nerve is very small compared to that of the quarter-wave plate, $\frac{\partial I}{\partial \theta}$ has the same magnitude but is of opposite sign for the two orientations. Since $d\theta$ is very small compared to $\pi/2$, dI_r has the same magnitude but opposite sign for the two orientations. In the second orientation,

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

If dI_1 is subtracted from dI_2, the pure retardation component is resolved since

$$dI_s = 1/2\ (dI_2 + dI_1) \tag{7}$$

Similarly, addition of the two results yields dI_s since

$$dI_s = 1/2\ (dI_2 - dI_1) \tag{8}$$

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. Light collected from a Quartzline lamp and its reflected image was focused on a slit after passing through an infrared absorbing filter. The light was polarized using a dichroic film polarizer, and the slit imaged on the axon by an inverted 10X objective lens used as a condenser. A rotatable quarter-wave plate was positioned just below the axon. Light emerging from the axon (oriented with its long axis at a 45° angle from the polarization axis) was collected by a second 10X objective lens (N.A. = 0.20), and passed through an analyzer (a second dichroic film polarizer crossed with respect to the initial polarizer). The intensity of the beam emerging from the analyzer was measured using a PIN photodiode.

(Fig. 1 near here)

The three basic functions of the electronic equipment were monitoring the action potential, measuring the light intensity changes caused by the axon depolarization, and signal averaging the results of these measurements in order to resolve the very small responses. A stimulator was used as the master clock for the system. At a rate of 3-10 times per second it provided a trigger pulse that started the measurement cycle, and, after a predetermined delay, it generated an excitation pulse to two stimulating Pt wire electrodes via an isolation unit. The action potential was measured as the biphasic voltage difference between two distal Pt wire recording electrodes using a high gain differential amplifier. The action potential was continuously monitored on an oscilloscope and with an audio monitor system. Light transmission was measured as the photocurrent output of the photodiode. Preamplification and impedance reduction was accomplished with an operational amplifier. A high gain, variable bandpass

amplifier was used for the final amplification stage. The experimental apparatus associated with the microscope was housed inside an electrostatic shield. A signal averaging system was constructed using a transient recorder to initially store and digitize each 2.56 msec or 6.40 msec sweep (128 points/sweep). After each sweep the data were transferred into one memory quadrant of a multi-channel scaler (MCS) memory unit using a custom-built interface. A preset counter and appropriate control logic circuit determined the number of sweeps averaged for a given experiment, usually several thousand. Using a memory readout unit and internal MCS circuitry, the results from different experiments stored in different memory quadrants could be added or subtracted. Data were displayed on an oscilloscope for photographic recording, and stored on paper tape for further reference and processing.

Identification of the molecular moieties participating in the structural changes responsible for the optical transients was attempted using the following approach. Signal averaged control measurements of dI_r and dI_s were made using a freshly dissected lobster (Homarus americanus) circumesophageal connective giant axon (100-120 μm diameter) bathing in Dalton's lobster saline solution [11]. Specific modifying reagents in saline were then perfused into the sealed measurement chamber for appropriate periods. After flushing the chamber with fresh saline solution, dI_r and dI_s were remeasured. The second set of optical changes were then compared with the control results, and any significant differences seen were interpreted to mean that the moeity being perturbed by the reagent had some role in producing the optical transients. The action potential was continuously monitored and recorded before and after reagent treatment.

All reagents were used as purchased without further purification. Trypsin (EC 3.4.4.4, grade A, bovine pancrease, 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 NAN-lactose as substrate), and hyaluronidase (EC 3.2.1.35, type III, ovine testes, 680 NF units/mg) were purchased from Sigma Chemical Company. Deuterium oxide (99.8% ^2H_2O) was purchased from International Chemical and Nuclear Corp. Sucrose (reagent grade) was purchased from Mallinckrodt Chemical Works. 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 freshly dissected lobster circumesophageal connective giant axons as a result of action potential depolarization of the plasma membrane. The retardation decrease, dI_r, appears to have a time course closely resembling the development of the action potential. The recovery process is more complex, however, and appears to depend upon the morphology of the sheath surrounding the axon. Slow recoveries, having time constants up to about five times the action potential duration, are often observed for axons having thin (less than 5 μm) sheaths. Axons with thicker sheaths (7-10 μm) usually demonstrate a fast, often biphasic, recovery for dI_r. This may imply that the fast recovery signal is dependent upon a separate, sheath-related component. In contrast to the results of Cohen et al.[5,6] using squid giant axons, the forward light scattering decrease, dI_s, is

often a significant fraction of the total optical transient seen in fresh lobster giant axons. The magnitude of dI_s is occasionally as much as 50% of the magnitude of dI_r, although usually it is closer to 20%. No correlation of the magnitude of the scattering component with any obvious morphological characteristics of the axon can be made. It is possible that differences in tension on the axon may be a contributing factor in the variability of dI_s, since tension control was only approximate in the experiments reported here. The nerves were mounted in the experiment chamber and stretched to approximately their in vivo lengths, but no further control was deemed practical.

Trypsin. Trypsin hydrolyzes peptide bonds on the carboxyl side of arginine and lysine. When applied to cell surfaces it attacks proteins and glycoproteins. It is therefore to be expected that proteins on external membrane surfaces will be disrupted, and that some of the charge associated with carbohydrate residues bound to glycoproteins will be cleaved as a result of trypsin treatment.

The effects of external trypsin application upon nerve fibers were first detailed by Tobias [12,13]. The morphological features on the surface of lobster giant axons become granular and deformed. The resistance to microelectrode penetration becomes much less. Susceptibility to stretching damage becomes much greater. Gentle trypsin digestion produces no significant effects upon the action potential or resting potential of either lobster or squid giant axons. It is unlikely that trypsin penetrates to the internal side of the plasma membrane, since internal perfusion of squid giant axons with proteases, including trypsin, promptly abolishes the action potential [14,15]. However, changes in the

ratio of chloride to potassium permeabilities for crayfish giant axons upon trypsin application suggest that proteases do reach the outside surface of the plasma membrane [16].

Axons exposed to solutions of 0.20-0.25 mg/ml trypsin in lobster saline for 10-15 min usually show a 30-50% decrease in the magnitude of dI_r, the optical retardation response. The effect on the forward direction scattering response, dI_s, was somewhat unpredictable. In almost all cases dI_s became much larger, typically showing a threefold increase in magnitude, and often exceeding dI_r. Somewhat surprisingly, the direction of the response occasionally reverses to a transient intensity increase. As previously mentioned, the variability shown in post-trypsin dI_s responses may be associated with the lack of control over the tension applied to the axon. A slight decrease in the conduction velocity is the only change noticed in the action potential subsequent to the mild trypsin digestions employed.

(Fig. 2 near here)

Data from a typical trypsin treatment experiment are shown in Fig. 2. For this particular 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 appeared to be significantly different, particularly in their recovery phases.

Pronase. Pronase is a non-specific endopeptidase, composed of a mixture of proteases. It hydrolyzes peptide bonds of proteins and glycoproteins at all available exposed sites. The effects of external pronase application are not well documented. As with trypsin, internal application

of pronase abolishes squid giant axon action potentials within minutes [17]. It also produces similar morphological alterations of the axon sheath. Like trypsin, pronase may be expected to remove charges associated with glycoprotein saccharides from the membrane surface.

Perturbations of dI_r and dI_s resulting from pronase incubation are similar to the perturbations resulting from trypsin incubation. In most cases dI_r is reduced, although not as much as with trypsin incubation. There is also a wide variation in the alteration of the magnitude and direction of dI_s produced by pronase digestion. As with trypsin, the only noticeable change produced in the action potential is a slight decrease in the conduction velocity.

(Fig. 3 hear here)

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

<u>Neuraminidase</u>. Neuraminidase cleaves neuraminic acid and sialic acid (N-acetyl neuraminic acid) residues from the oligosaccharide side chains of glycoproteins and glycolipids by hydrolyzing terminal α-2,6 links between the residues and adjacent 2-acetamido-2-deoxy-D-galactose residues. These neuraminic acid residues are negatively charged at physiological pH's. Treating lobster giant axons with neuraminidase causes no visually detectable changes in the morphology of the fiber. No changes are seen in the shape or magnitude of the biphasically recorded action potential. However, a slight reduction in the conduction velocity is apparent, and a pronounced decrease in the refractory period is produced as a consequence of neuraminidase treatment.

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

(Fig. 4 near here)

A typical neuraminidase incubation experiment is shown in Fig. 4. This axon demonstrated the typical 50% reduction of dI_r observed 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 [18] subjected several non-neural lobster tissues to acid hydrolysis and analyzed for liberated sialic acid residues. He concluded that the sialic acid content of these tissues could be 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 pronounced decrease in refractory period upon neuraminidase treatment be accounted for? Commercial neuraminidases sometimes contain phospholipases, but the stability of the action potential argues against significant phospholipase contamination since minimal phospholipase exposure destroys the membrane resistance and action potential [13]. Protease contamination is unlikely, since prolonged incubation in the neuraminidase solution produces none of the changes in sheath morphology and in dI_s seen with protease treatment. It is conceivable that the neuraminidase used is capable of cleaving charged saccharides other than those which would be detected by Warren's assay. It is also possible that the neuraminidase binds to the axon plasma membrane in a non-specific manner such that it interferes

with the motion of the species that generate dI_r. This seems unlikely to produce the reduction in the refractory period, however. Finally, it is possible that lobster axons do contain very small amounts of sialic acid, and that the neuraminidase acts upon its normal substrate in these experiments.

Hyaluronidase. Hyaluronidase hydrolyzes the polymeric bonding of long-chain hyaluronic acid polymers. As with neuraminidase, no appreciable morphological changes are induced in lobster giant axons when this enzyme is applied. No literature detailing electrophysiological effects of hyaluronidase treatment is available. Action potentials recorded in this study appear to be the same before and after hyaluronidase incubation, except for a slight decrease in the conduction velocity in some cases.

The effect of hyaluronidase upon dI_r is similar to that of neuraminidase. In most experiments, a 1-h incubation in a 2.0 mg/ml hyaluronidase saline solution reduces dI_r by approximately 40%. The dI_s component is usually not altered, and the kinds of effects produced by proteases are not observed.

(Fig. 5 near here)

An example of the effect of hyaluronidase treatment is shown in Fig. 5. The isolated dI_r response has been reduced significantly by hyaluronidase. Only very small forward scattering responses, dI_s, were found for this axon before and after incubation.

Deuterium oxide. Any of several effects might be expected when axons are immersed in saline solution made using deuterium oxide ($[^2H_2O]$) instead of water. The action potential should be affected by the change

in viscosity, since ionic mobility reductions produce effective decreases in the conductivities of the membrane and external medium. Frictional forces, hydrogen bond forces, and hydrophobic bond forces involved in any membrane structural transition might also be affected. The lower refractive index should affect any form birefringence contribution to the retardation response, and it might also perturb the scattering response.

Garby and Nordqvist [19] found that the conduction velocity of myelinated frog sciatic nerve decreases by 20% upon immersion in a 99.9% [^2H_2O] saline solution. The effect is reversible if the nerve is returned to a normal [H_2O] saline solution. The velocity change was attributed to the lowered ionic mobilities and to modifications of unspecified chemical reactions related to the action potential. Subsequent measurements by Spyropoulos and Ezzy [20] confirm the conduction velocity decrease for toad sciatic nerve and squid giant axon upon immersion in 99.9% [^2H_2O] saline solution. However, a 10% reduction in the height of the action potential is noticed, and there is an increase in the duration of its ascending and descending phases. The threshold potential also increases. All of the effects are reversible. The conduction velocity decrease was attributed to the increased threshold potential, and it was speculated that [^2H_2O] perturbs some excitation mechanism requiring a proton transfer.

Tasaki et al. [4] report that immersing crab walking leg nerve in a 50% [^2H_2O] produces no modification of the action potential related optical responses.

In our studies, the most dramatic consequence of using [^2H_2O] saline solution as the bathing medium for lobster giant axons is that

they shrink to a smaller diameter. The degree of shrinkage is greatest for the highest [^2H_2O] concentrations, a 99.8% [^2H_2O] solution causing the nerve to constrict to only 60% of its normal diameter. An irregularly wrinkled surface is produced as a result. The process is very rapid, requiring less than 1 min to reach the new equilibrium diameter. The most reasonable explanation is that dialysis of the axoplasmic water occurs as a consequence of the difference in membrane permeabilities for [H_2O] and [^2H_2O] [21]. The shrinkage of the axon may also account for much of the conduction velocity decrease observed. Thus it may not be necessary to invoke ionic mobility changes as the causitive mechanism, although the conduction velocity observations of this study were not done with sufficient care to rule it out.

A 25% (by volume) [^2H_2O] solution causes only minor shrinkage, so the majority of the axons were tested using such a solution. The effects of [^2H_2O] immersion upon dI_r is unpredictable, half of the axons showing an increase and half showing a decrease. This inconsistency probably can be attributed to the variability in the degree to which the fibers shrink and in the amount of resulting surface wrinkling. The dI_s response is similarly variable, probably for the same reason. Reimmersing the axon in [H_2O] saline solution produces rapid morphological recovery to the original diameter. The conduction velocity also increases as the axon expands, but never seems to reach its initial value. This may indicate some irreversible modification of the membrane, or it may mean that not all of the [^2H_2O] is removed. On the other hand, a significant part of the modification of the optical responses appears to be irreversible. Returning the axon to [H_2O] saline solution does not vitiate the alterations produced by [^2H_2O], even after complete morphological restoration.

Dithiothreitol. Dithiothreitol (DTT) reduces disulfide bonds. Tertiary structures of proteins cross-linked with disulfide bonds can be modified by DTT reduction. The dipole moments of such proteins, if any, could be modified by DTT if the reaction repositions the charges associated with the protein.

Albuquerque et al. [22] found that treating lobster giant axons with a 20 mM DTT solution for 10 min produces only negligible changes in the resting potential, but 10-15 mV reductions of the action potential. Axon surface proteins are modified by such treatment. The toxic effects of batrachotoxin are significantly reduced if the axon is pre-treated with DTT, indicating that the binding sites for the toxin have sulfhydryl groups that are altered by reduction.

DTT has no significant effect upon dI_r. Changes are produced in dI_s, but they are small compared to the changes produced by proteolytic enzymes. No morphological effects of DTT treatment are apparent.

Sucrose. It is possible that the retardation response decrease and the light scattering signal increase induced by trypsin and pronase might be caused by an increase in the refractive index of the bathing medium as products of the hydrolysis are released into solution. To test this possibility, the effect of raising the immersion medium refractive index by adding sucrose was measured. Adding sucrose also increases the viscosity and osmolarity of the solution, causing changes in axon morphology, action potential shape, and conduction velocity.

The morphological and optical signal changes produced by sucrose are not consistent, but it is possible to produce large light scattering responses similar to those seen upon protease digestion. Increases in

the magnitude of dI_s are seen for both increasing and decreasing light transmission, similar to what is seen with the proteases. As previously speculated, this variability might be caused by differences in the tension applied to the axon. The large scattering signals are much more apparent when 30% sucrose (n = 1.381) is used than when 15% sucrose (n = 1.356) is used. Since morphological alterations are also more apparent at the higher concentration, their role as the source of the change in dI_s cannot be ruled out. Similar comments can be made about the effect of sucrose upon the retardation signal. For one axon tested, no change in dI_r was produced at either of the concentrations used. The dialysis of this axon was also minimal. For other axons the shrinkage was more significant, especially in 30% sucrose, and the retardation response showed concurrent reductions. It seems likely that the reduction in dI_r is related to the morphological alterations rather than to the increased refractive index.

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 physically and/or chemically 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 surface and adjacent regions can be gained.

Modifications of the optical retardation responses may reasonably be attributed to one or more of the following mechanisms:

i) The dipole moments of various mobile species might be modified,

either by changing the amount of charge on the dipoles or by causing conformational changes that alter the relative positions of the charges. Reduction of the dipole moments of such mobile species may diminish their reorientation as the electric field reverses during the action potential.

ii) The extent to which the membrane may be compressed or expanded during the action potential might be affected, either by changing the compressibility of the structure, or by changing the surface charge density and/or composition.

iii) Products of enzymatic hydrolysis might alter the refractive index of the aqueous medium, thereby changing the form birefringence contribution to dI_r (and the scattering contribution to dI_s).

iv) Axial tension and stretching force changes might be generated and thereby affect the ability of dipoles to reorient. This mechanism is most likely to be operative in the protease digestion experiments, since the supporting tissues in the sheath are partially digested away, allowing the axon to stretch somewhat. However, signal reductions seen upon treatment with neuraminidase and hyaluronidase probably cannot be accounted for by this mechanism, since no gross morphological alterations are induced, and stretching does not appear to be a factor in these cases.

Cohen et al. [8] have stated that Kerr effect dipole reorientations can account for the magnitudes of retardation changes observed. They argue against a simple reorientation mechanism, however, because the 40 μsec dipole relaxation time constant measured using voltage-clamped squid axons is much longer than the relaxation times observed for proteins in solution. They invoke the slower process of conformational changes involving the breaking and reforming of non-covalent bonds as a

more likely mechanism. However, it may not be valid to assume that the dipole relaxation times of proteins in solution can be compared to the relaxation times of macromolecules imbedded in a lipid and/or saccharide matrix. For example, Cone [23] found a 20 μsec rotational relaxation time constant for rhodopsin in situ, a value reasonably close to the squid birefringence relaxation time constant. Cohen et al. [8] additionally state that membrane compression and expansion effects can also be of sufficient magnitude to account for the changes in retardation. The changes in dI_r and dI_s reported here could be due to changes in the compressibility of the membrane, or to changes in the surface charges and consequent changes in electrostatic forces due to the membrane potential. Since membranes are probably lipid bilayers with protein imbedded in them to varying degrees, it seems unlikely that the modifications to the membrane caused by the proteases and hyaluronidase could cause an appreciable compressibility change over most of the lipid phase. The reduction of expansion and compression effects by surface change modification is a reasonable hypothesis, however. The fact that protein-free lipid bilayers can be induced to give retardation changes comparable to those seen in natural membranes lends support to this supposition [24]. On the other hand, the compressibilities of protein-free lipid bilayers and natural membranes may be very different, so that a comparison of the two systems may not be valid.

Whatever the mechanism producing dI_r, the experiments of this study suggest that charges associated with saccharide residues of surface glycoproteins (and perhaps glycolipids) are involved in part of the process. Treating axons with trypsin and pronase cleaves polypeptides to which the

saccharides are attached. This could reduce dipole moments of glycoproteins and proteins 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 cleaved, reducing dipole reorientations and/or compression effects. In addition, the charged hyaluronic acid matrix that exists in the intercellular space of the axon sheath [25] appears to undergo a reorientation or compression chages as the electric field reverses direction during the action potential. Hyaluronidase breaks up the polymer matrix so that such transitions are rendered less cooperative, thereby reducing the net birefringence change. Morphological alterations of axons immersed in [^2H_2O] saline solution makes conclusions regarding the resulting changes in hydrogen-bond and hydrophobic bond energies impossible. Dithiothreitol has little effect upon the magnitude of dI_r, indicating that protein conformation changes induced in axonal membrane proteins by the reduction of disulfide bonds, if any, are not critical in determining the extent of the observed me brane reorganization. Changes in dI_r produced by sucrose-containing solutions seem to be related to morphological perturbations rather than to changes in a form birefringence component.

The modifications of the retardation response of squid giant axons during hyperpolarizing pulses reported by Cohen et al. [8] should not necessarily be considered as resulting from the same mechanisms investigated by this study. They used tetrodotoxin, high external calcium concentrations, terbium and lanthanum to produce what they call the "state 2" response. The reduction of the fast responses may be related to the reductions produced by the hydrolytic enzymes of our investigation, but

the slow response and the "rebound" response have time constants much longer than the 1 msec duration of the action potential. It therefore seems unlikely that alterations of the two slower effects are being monitored in the experiments presented here. It should be noted that no corrections for scattering were made in the detection of the "state 2" response, so it may be that the changes seen are scattering effects rather than retardation effects. Our results indicate that such corrections are essential for other types of modifying agents when applied to lobster axons.

Alterations induced in dI_s by the various modifying agents are quite variable, even within a given series of experiments using the same reagents. It is therefore very difficult to formulate a consistent model of what causes the sometimes large changes in the light scattering response, especially in the cases of the proteases. Two mechanisms can be postulated, both of which are likely to be applicable. Axial stretching may result as the structural integrity of the membrane and sheath system is reduced by the proteases. This would be consistent with the findings of Tobias [26] that the direction and magnitude of the long-term scattering change of nerve trunks depends upon the tension applied. Stretching may cause critical realignments of mobile membrane constituents which allow a much greater light scattering response to occur after enzymatic digestion. A second explanation might be that products of protease lysis may increase the refractive index of the surrounding medium, thereby causing scattering increases comparable to those seen by Cohen _et al._ [1] when they used Dextran to increase the immersion medium dielectric constant. Our results using sucrose-containing medium tend to support the second explanation. Large scattering changes, both increases and decreases, are produced with

sucrose solutions, similar to those produced by proteolytic modification. Apparently the charge on the membrane does not directly affect the scattering response as long as the action potential remains normal, since neuraminidase produces no appreciable changes in dI_s. Proteases, on the other hand, which undoubtedly generate large particles to be dispersed into the surrounding medium, often produce dramatic scattering changes.

Dynamic scattering effects are well known to experimenters in the field of liquid crystals. Since the multiple lipid bilayer system of the plasma membrane and surrounding Schwann cell membranes comprise a system similar to a smectic phase liquid crystal, the observations of light scattering changes of liquid crystals under various conditions may have some bearing upon the results of this study. For example, a nematic liquid crystal composed of anisylidene-p-aminophenylacetate (APAPA) is transparent in its unperturbed state when mixed with an ionic species. Upon application of an electric field, however, ionic collisions with the aligned APAPA molecules create local regions of turbulence that are light scattering, causing the liquid crystal to become opaque [27]. A similar role may be postulated for the ionic currents in and near the plasma membrane during the action potential. Ionic collisions with the hydrocarbon chain phase of the membranes may produce localized high turbulence regions that generate portions of the light scattering effects seen in our experiments. Enzymatic modification of membrane structures may alter the degree of turbulence for a given electric field change, since the relative positions and probabilities of collisions of membrane constituents might be changed.

The fact that large modifications of the scattering signal may be

induced without concommitant large changes in the retardation signal 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. Different time courses for the two types of signals with respect to the action potential were also reported by Cohen et al. [1] using microelectrode penetration of squid axons near the site of tight transmission. The kinetics of the two types of responses are also markedly different.

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 membrane electrical depolarization. The existence of a small component of the optical responses associated directly with the gating mechanisms controlling the membrane ionic permeabilities, and thereby causally related to 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.

Tasaki et al. [4] cite the retardation and scattering changes as evidence supporting Tasaki's action potential model involving cooperative membrane transitions and non-specific permeability changes [28]. The fact that the optical responses can be appreciably modified without changing the action potential implies that the assumption that they are reflecting structural transitions which determine the action potential may not be justified. Models involving dipole reorientations of the phospholipid moiety [9,29,30,31] are neither supported nor contradicted

by this investigation, since modifying agents affecting the lipid phase invariably also affect the action potential. For comparison purposes, our experiments require that no changes be produced in the action potential. Consequently, no useful lipid modification experiments were performed.

In conclusion, the original objective of identifying the molecular constituents producing the optical retardation change during axon depolarization has been at least partially realized. Passive realignments of glycoproteins and hyaluronic acid polymers appear to be involved. Glycolipids and proteins may also be implicated. A major fraction of the retardation change does not appear to be reflecting mechanisms directly related to the ionic permeability changes associated with axon membrane excitation. The somewhat surprising light scattering results are more difficult to interpret, since the results are not entirely consistent. However, refractive index changes in the extracellular space seem to be involved.

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FIGURE LEGENDS

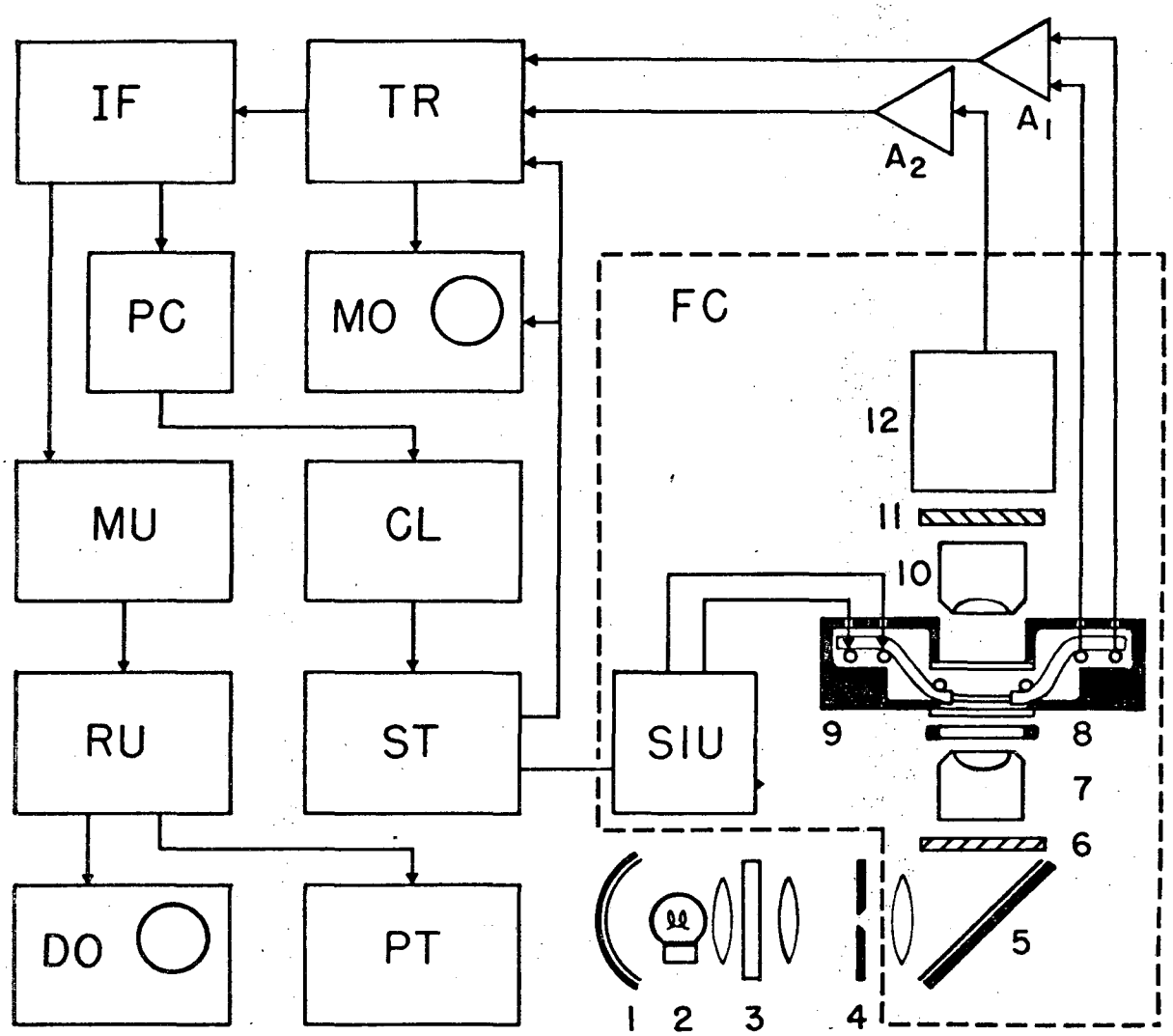
Fig. 1. Experimental apparatus designed to stimulate axon and monitor action potential, measure light intensity changes caused by axon depolarization, signal-average the results, and process the final data to resolve the optical retardation component and the forward light scattering component from the total light intensity change. 1-spherical mirror, 2-200W 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.

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 3,000 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 3,000 sweeps. A downward deflection represents a light transmission increase.

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 3,000 sweeps. A downward deflection represents a light transmission increase.

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 5,000 sweeps. A downward deflection represents a light transmission increase.



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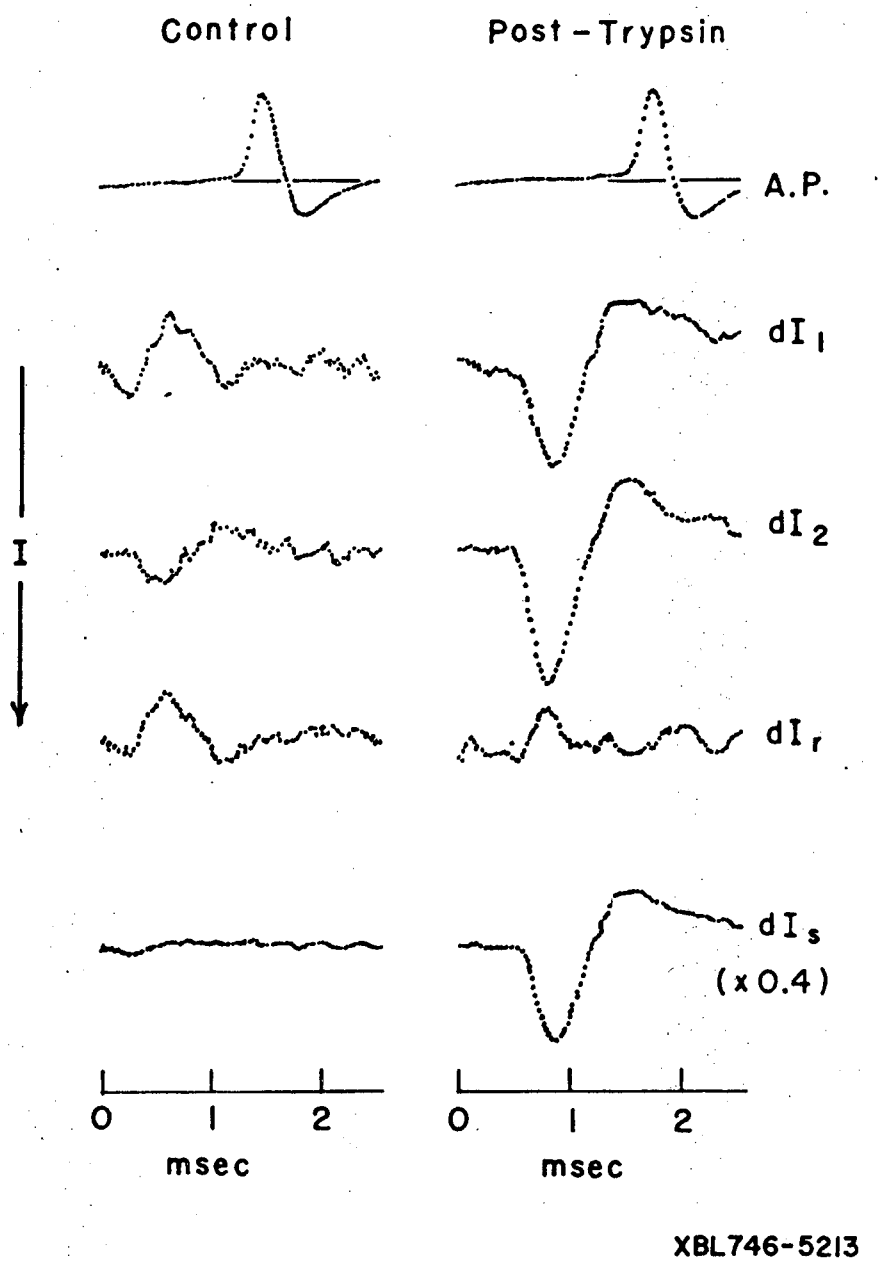

Control
Post-Trypsin
A.P.
dI1
dI2
dIr
dIs
(x0.4)
I
0
1
2
msec
0
1
2
msec
XBL746-5213

Fig. 2.

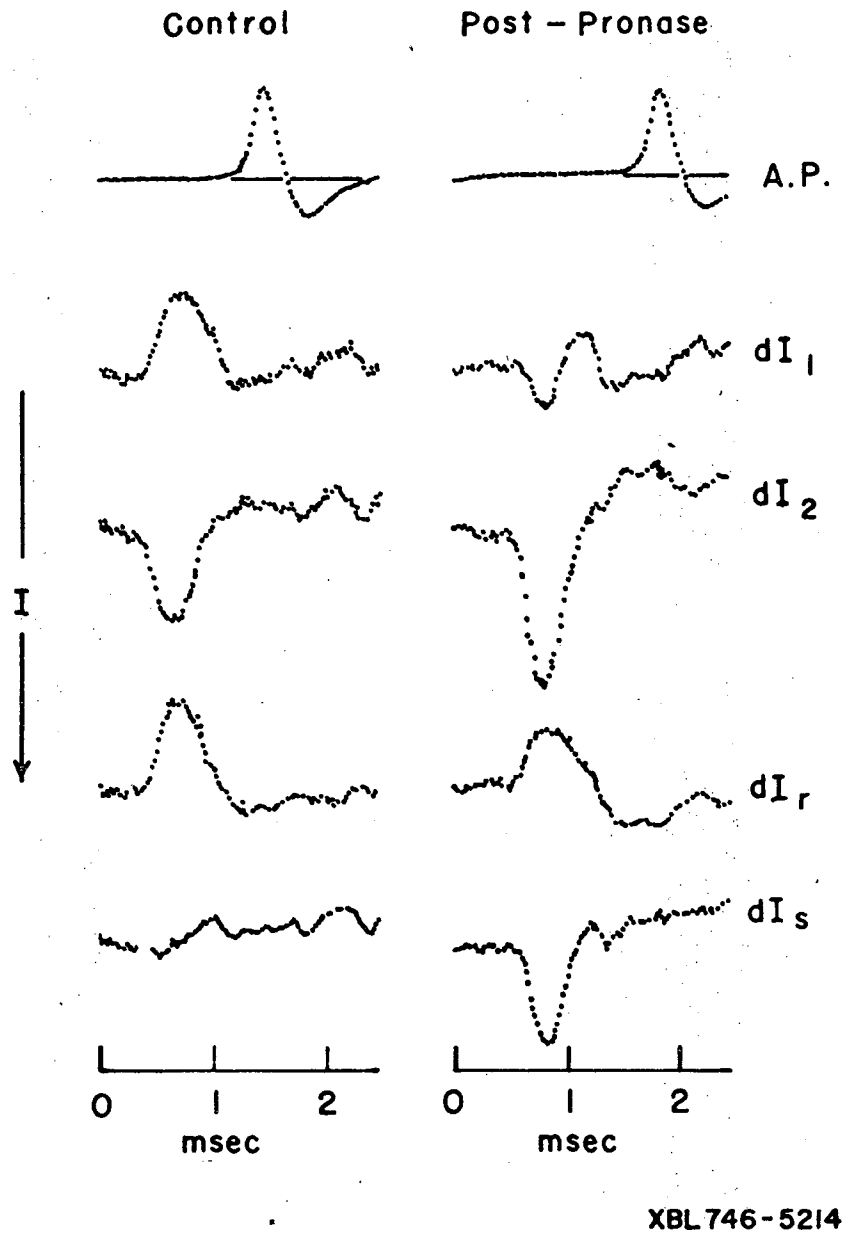
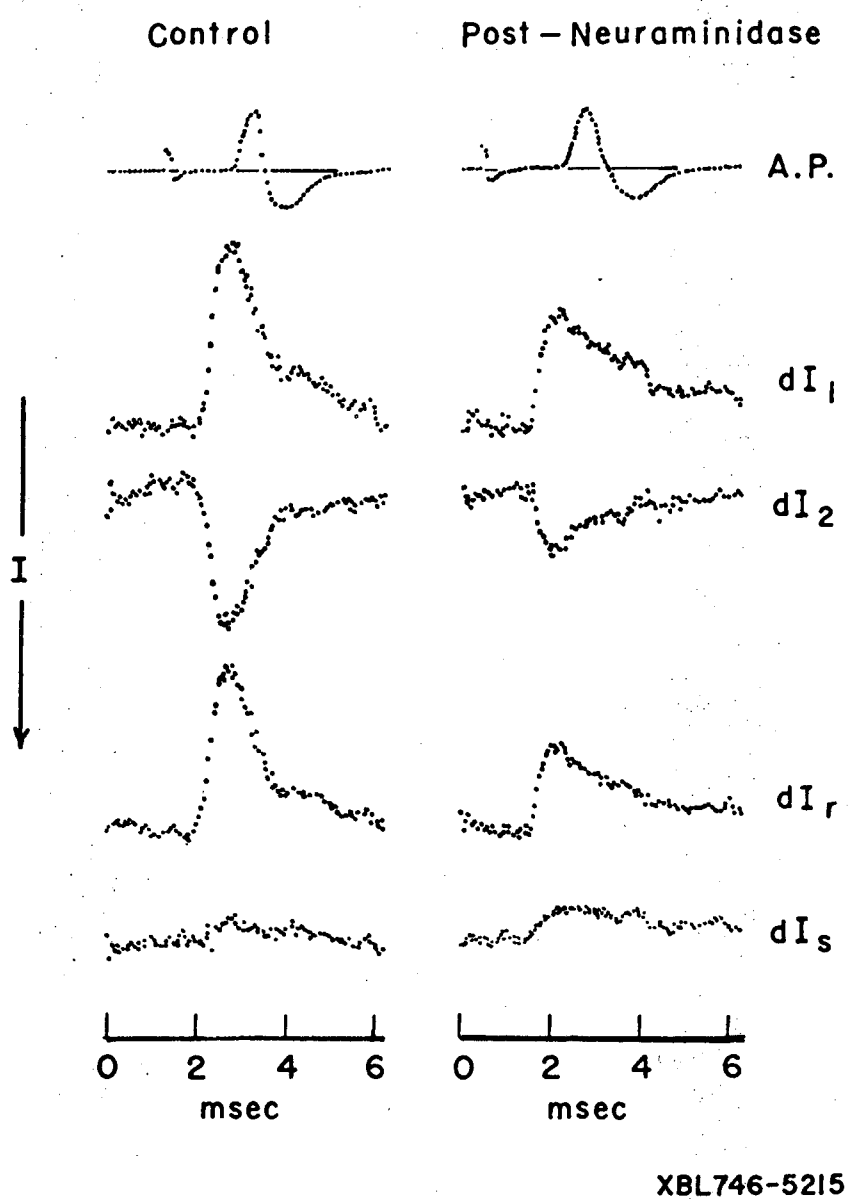

Control
Post – Pronase
A.P.
dI 1
dI 2
dI r
dI s
I
0
1
2
msec
0
1
2
msec
XBL 746-5214

Fig. 3.


Control
Post – Neuraminidase
A.P.
dI1
dI2
I
dIr
dIs
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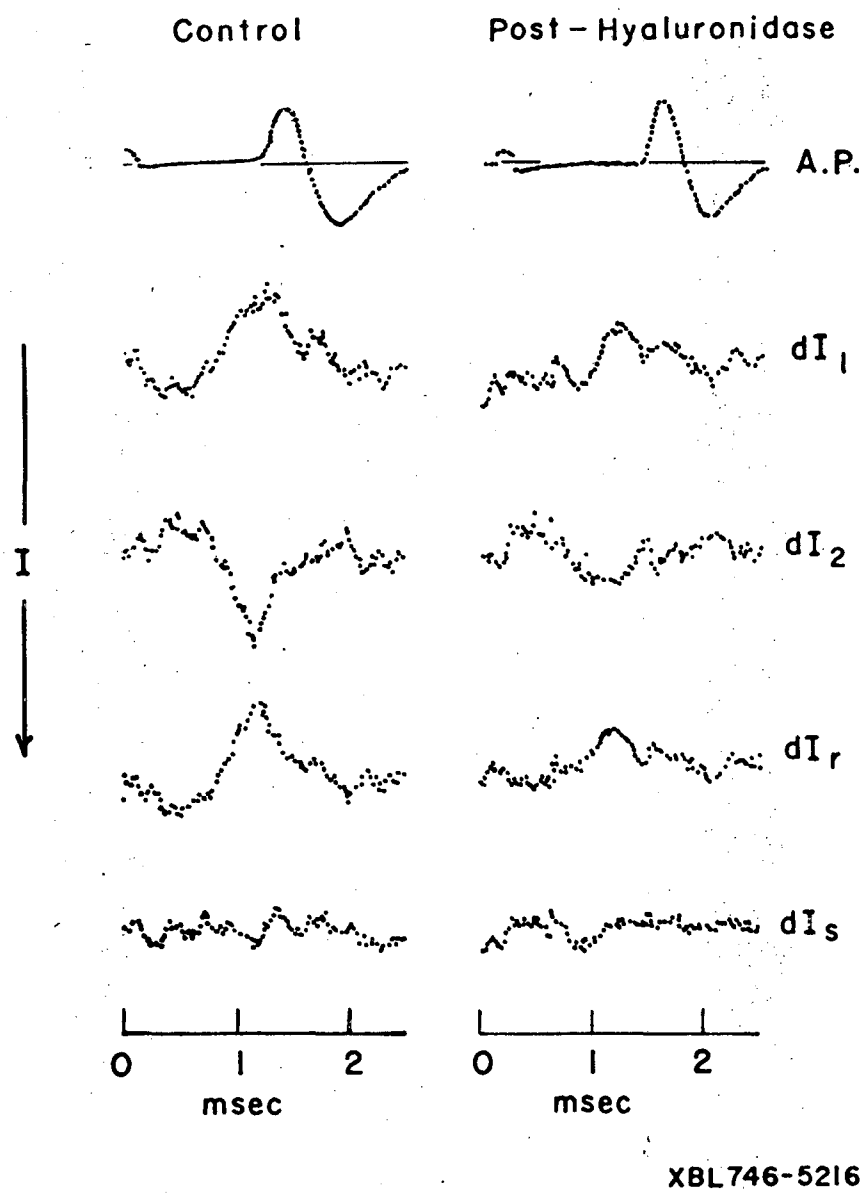
2
4
6
msec
0
2
4
6
msec
XBL746-5215

Fig. 4.


Control
Post – Hyaluronidase
A.P.
dI l
dI 2
dI r
dI s
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1
2
msec
0
1
2
msec
XBL746-5216

Fig. 5.

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