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LIGHT SCATTERING SPECTROSCOPY OF THE SQUID AXON MEMBRANE

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SUMMARY

Light scattering studies on the giant squid axon were done using the technique of optical mixing spectroscopy. This experimental approach is based on the use of laser light to detect the fluctuations of membrane macromolecules which are associated with conductance fluctuations. The light scattering spectra were similar to the Lorentzian-like behavior of conductance fluctuations, possibly reflecting an underlying conformational change in the specific membrane sites responsible for the potassium ion conductance. The amplitude of the spectra measured, increased when the membrane was depolarized and decreased on hyperpolarization. The spectra were fit to the sum of two terms, a ($1/f$ component and a simple Lorentzian term. Spectra from deteriorating axons did not show sensitivity to membrane potential changes. It is shown theoretically that fluctuations due to the voltage-dependent variable, n, of the Hodgkin-Huxley formalism are identical to the voltage fluctuations. The derived power spectrum is that of a second order system, capable of showing resonance peaking only if the voltage dependence of the potassium rate constants is included in the analysis. The lack of resonance peaking in the observed light scattering spectra, indicates that the data are best described by a damped second order system.

INTRODUCTION

The kinetic properties of nerve membranes have been investigated with relaxation methods [1], notably the voltage clamp technique [2], and with the laser-induced temperature jump method [3]. These experiments provided data which have led to quantitative formulations of the macroscopic properties of the membrane conductance [4, 5]. An additional test of these kinetic models lies in a quantitative investigation of the spontaneous fluctuations of the membrane conductance [6, 7]. The fluctuations provide natural perturbations which can be analyzed to give a microscopic or stochastic description of the membrane conductance. The comparison of the microscopic and macroscopic formulations imposes additional constraints on the kinetic mechanisms underlying the permeability changes which occur during the nerve impulse.

The experimental approach described in this paper is based on the use of monochromatic, polarized and coherent light to detect the fluctuations of membrane macromolecules which are presumed to be associated with conductance fluctuations. The illuminated, fluctuating molecules in the membrane volume element, which are associated with conductance fluctuations, can be expected to show kinetic behavior of a specific nature and are therefore distinguishable from the large number of other membrane sites. The determination of the specific kinetic properties of scattering elements is obtained by measurement of the frequency distribution of the scattered intensity, using a technique know as optical mixing spectroscopy [8, 9]. In principle, these measurements can provide data on the dynamic properties of the scattering elements such as translational and rotational motion as well as reaction rate constants [10].

The light scattering experiments on the squid axon membrane described here show that the spectra observed are similar to the Lorentzian-like behavior of voltage fluctuations [11], possibly reflecting an underlying conformation change in the specific membrane sites responsible for the K^+ conductance. The amplitude of the photocurrent power spectra measured increased when the membrane was depolarized and decreased on hyperpolarization. The shape of the photocurrent power spectra was relatively insensitive to the scattering angle.

METHODS

All experiments were done on single giant axons dissected from the hindmost stellar nerve of live squid, *Loligo pealei*. The axons were thoroughly dissected from all small nerve fibers and loose connective tissue. Single fibers of 400–800 μm were cannulated at one end by a 300 μm pipette and mounted vertically in an optical cuvette, containing filtered sea water. A double electrode assembly was inserted vertically through the cannula into the axon until the tip of the electrode was 1 mm above the focal point of the incident light beam. The electrode assembly consisted of a 150–200 μm pipette on the side of which was attached a 50 μm platinized platinum wire. The pipette was filled with 500 mM KCl solution in contact with an Ag/AgCl electrode which was connected to the input of an operational amplifier in a unity gain follower mode. The potentials were read directly from an oscilloscope or a standard voltmeter. The membrane potential was altered by passing a constant current through a 1 MΩ resistor in series with the platinum wire. All of the optical measurements were made under steady state conditions of constant current and temperature which was maintained at 14–16 °C by circulating distilled water through a water jacketed optical cuvette.

The optical system with an axon in cross-section is illustrated in Fig. 1. With the exception of the Spectra Physics 133 laser, the optical components were mounted on two optical rails. The polarized incident light was perpendicular to the plane of the scattering angle. The scattering angle was changed by rotating the rail containing the photomultiplier tube about the cuvette. In order to reduce artifacts arising from building vibrations, the optical apparatus was placed in a light free box mounted on composition stone slabs on which were placed numerous sacks of sand and lead bricks for a total weight of about 600 lbs. The slabs were cushioned by two inflated inner tire tubes which were mounted on a table whose legs were resting in individual cans of sand.

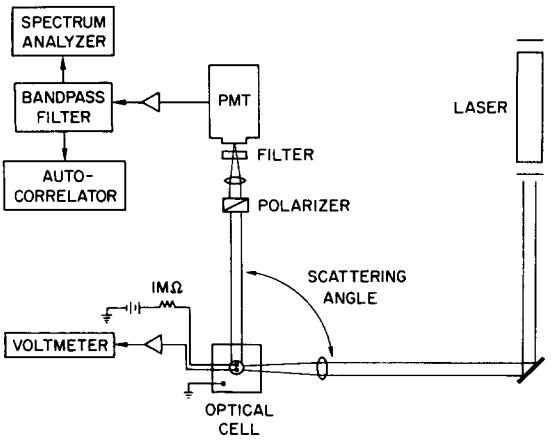

Fig. 1. Diagram of light scattering apparatus. The active surface of the photomultiplier tube (PMT) was 55 cm from the optical cuvette. A narrow band filter (Oriel) centered at 6331 Å preceded the PMT and a ×5 microscope objective focused the scattered light on the photocathode surface. The incident light was focused on the axon with a ×3 microscope objective.

A conventional photomultiplier tube (PMT) (RCA 931A) was used. The anode current was measured with Teledyne-Philbrick Nexus 1019 operational amplifier. The output of the current amplifier was analyzed with a 400-point on line spectrum analyzer (SAICOR, Model 52, Hauppauge, New York) or a 400-point autocorrelator (SAICOR, Model 42). Some data were recorded on magnetic tape with a Hewlett Packard Model 3960A FM tape recorder, which had a flat frequency response to 5 kHz.

The operation of the optical mixing spectrometer was checked by measuring the correlation function of polystyrene latex spheres (Dow Chemical Co.) suspended in distilled water [9]. The measured correlation function for 0.087 μm diameter spheres illustrated in Fig. 2 shows an exponential form with a time constant of about 0.2 ms. Latex spheres of 0.5 μm diameter showed a 5-fold increase in the correlation time. These time constants are consistent with those theoretically predicted for uniform spheres undergoing Brownian motion [12].

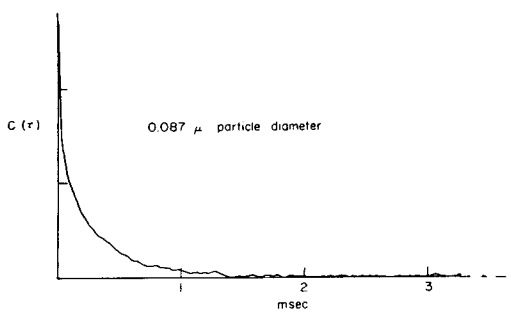

Fig. 2. Correlation function for polystyrene latex 0.087 μm diameter spheres. Abscissa, time in ms; ordinate, correlation function in arbitrary units.

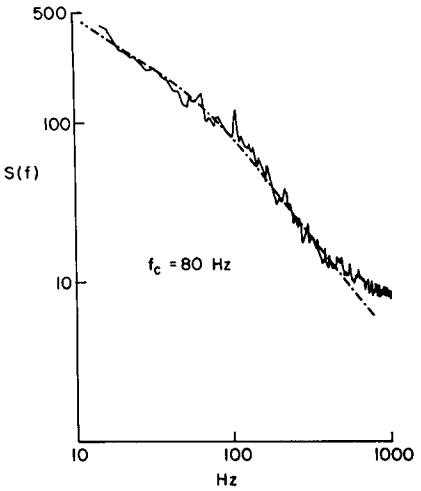

Fig. 3. Power spectrum of the photo-current from a hyperpolarized axon. This spectrum was measured at a 90° scattering angle from an axon hyperpolarized to −79 mV. The axon had an action potential of 100 mV and resting potential of −53 mV. The dashed line represents the expression $(3700/f)+[112/(1+(f/80)^2)]$ where f is the frequency in Hz. Abscissa, frequency in Hz; ordinate, photocurrent power in arbitrary units per unit bandwith. In this and subsequent graphs the resolution of the spectrum was 2.5 Hz.

The signal to noise ratio was determined as described by Cummins and Swinney [9]. A d.c. light source with the same average intensity as the scattered light showed a constant power level for the photocurrent in the frequency range 0–5 kHz. The shot noise spectrum of the laser source was demonstrated by scattering light from a suspended teflon tube of 500 μm diameter in sea water. It can be seen from Fig. 3 that the signal to noise ratio is of the order of 50 : 1 where the noise is taken to be the power at 1 kHz.

ANALYSIS

The output current, $I(t)$, of the photomultiplier tube is proportional to the intensity of the scattered light falling on the surface of the photocathode, that is $I(t) = \beta|\hat{E}(t)|^2$ where $\hat{E}(t)$ is the total electric field at the detector and β is a proportionality constant. The photocurrent fluctuation can be characterized by the correlation function for $I(t)$, $R_I(\tau) = \langle I(t)I(t+\tau) \rangle$. The spectrum of the random process associated with $I(t)$ is given by the Wiener-Khintchine theorem which states that the spectrum, $S_I(f)$ is the Fourier transform of the correlation function, $R_I(\tau)$, where f is the frequency. Assuming Gaussian statistics for the optical field and an exponential correlation function the frequency dependent portion of the power spectrum of the photocurrent is a Lorentzian function [8] of the form, $K[1+(f/f_c)^2]^{-1}$ where K is constant and f_c is the corner frequency.

The method of analysis chosen for the data obtained in these experiments rests on the assumption that in the range 0–5 kHz, the spectral components of the photocurrent signal are related to the processes causing the conductance fluctuations. Following the approach of Fishman [11] for the electrical noise studies, it is assumed

that in addition to a shot noise term, the photocurrent spectrum consists of two components, a ($1/f$ term, and a single Lorentzian term.The comparison of the theoretical and experimental curves in Fig. 3 show that this type of analysis provides a good description of the observed data.

RESULTS

Axons were mounted in the light scattering apparatus and their spectra measured as a function of the scattering angle. Apart from amplitude changes there was little variation in the shape of the power spectra measured. These results suggest that if there is a diffusional component in the scattered light, its amplitude is small. Analysis of the power spectrum as described in the Analysis section showed no angular dependence for the extracted Lorentzian curve. Analysis of the correlation function as sum of two exponentials also showed no angular dependence for either time constant.

The dependence of the optical spectrum on the membrane potential was investigated in 24 axons. The spectra showed a consistent potential dependence only in those cases where action potentials of near 100 mV were measured. It appears that the sensitivity of the optical response to potential changes is more dependent on the state of the axon membrane than excitability itself. The data illustrated in Fig. 4 show that a depolarization of the membrane leads to an increased amplitude in the power spectra for frequencies below 1 kHz. This result is also angular independent. The slightly different amplitudes of records a and c corresponding to nearly the same potential are indicative of the variability in these measurements, but do not detract from the general finding of a potential-dependent response.

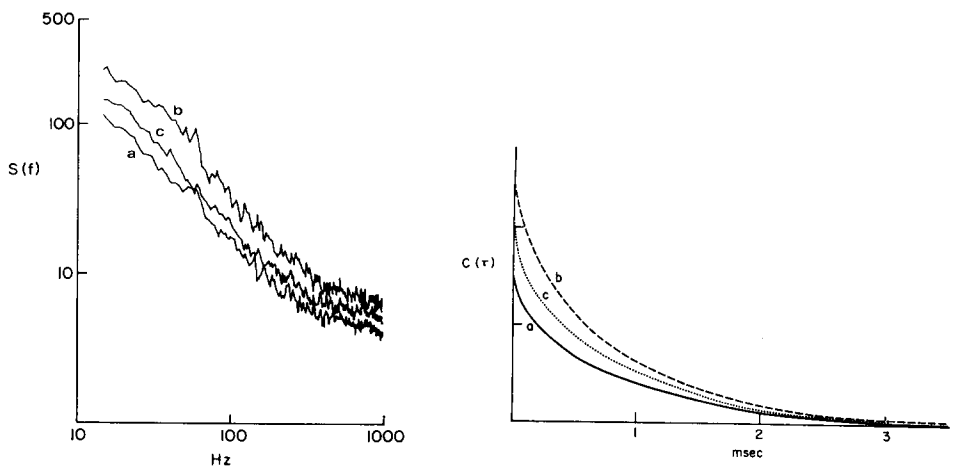

Fig. 4. Power spectra of light scattering signal at different membrane potentials. Curve a, was taken at the rest potential of −54 mV, curve b, at −35 mV, and curve c, at −55 mV. The scattering angle was 27.5°. Abscissa, frequency in Hz; ordinate, photocurrent power per Hz in arbitrary units.

Fig. 5. Correlation function of light scattering signal at different membrane potentials. The curves shown are smoothed tracings of the original correlation functions. Curves a, b and c are from the same data as in Fig. 4. Abscissa, time in ms; ordinate, correlation function in arbitrary units.

An alternative way to present this data is illustrated in Fig. 5, which shows the same data as Fig. 4 but expressed as correlation functions. The finding in Fig. 4 that the amplitude of the correlation function also increased with membrane depolarization suggests that the effect on the power spectra of the altered potential is in the Lorentzian or $1/f$ term and not an increase in the shot noise component of the total power spectrum. An increased level of shot noise would not increase the amplitude of the correlation function for times greater than zero, but would increase the power amplitude at all frequencies measured.

Experiments to determine the spectral content of depolarized light were done by placing an analyzer in front of the photodetector. The spectrum analysis of the completely depolarized signal showed a constant response to 5 kHz with a photocurrent power amplitude below the signal from polarized scattered light measured at 5 kHz. These data indicate that the principle scattering elements investigated were symmetrical for kinetic phenomena in the frequency range of 0–1 kHz.

DISCUSSION

The principle barrier to understanding the light scattering data for squid axons is the identification of the scattering elements. Even with a thoroughly cleaned and perfused fiber, the axonal membrane is completely enclosed with a Schwann cell layer. The carefully done experiments of Cohen et al. [13, 14], demonstrate the difficulty in extracting conductance related signals from such a complex structure. These studies were confined to frequency integrated intensities which provide information on the structural properties of the scattering elements. The advantage of optical mixing spectroscopy is in the frequency dispersion of the scattered intensity which provides additional data on dynamic properties, such as translational and rotational motion as well as reaction rate constants. Because of this additional frequency dependent information, the optical mixing methods applied to membrane systems are likely to be more sensitive for macromolecular events than frequency integrated scattering. The components of the spectrum which are potential sensitive thus have a unique character for a particular stationary state condition.

There are two arguments which support the hypothesis that the optical signals measured in these experiments originate at the axon membrane; the frequency range of the scattered light is the same as the membrane conductance or potential fluctuations, and the optical spectra are membrane potential dependent. In this regard it should be noted that all of the spectra were measured near the resting membrane potential, a condition for which the major part of the potential fall from the inside to the outside of the axon is across the axon membrane rather than an additional series resistance element such as the Schwann cell.

The potential dependence of the light scattering signal is subject to two interpretations: the observed response is due to fluctuation in either (1) the local electric field of the membrane or (2) the membrane scattering elements which are responsible for the conductance fluctuations. The experiments of Cohen et al. [13, 14] suggest the first interpretation. If this is correct, then the optical spectra should be similar in shape to the voltage noise spectra measured on squid axons. The similarity of these two spectra is illustrated in Fig. 6.

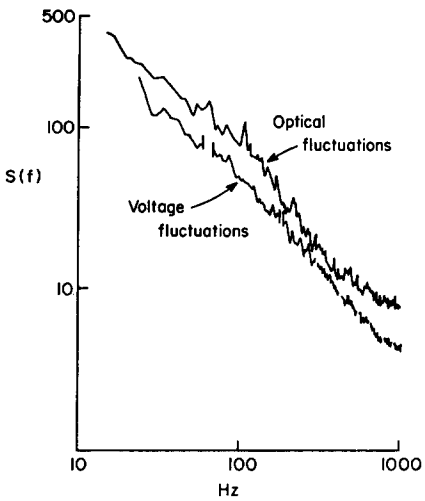

Fig. 6. Comparison of optical and voltage fluctuations. The power spectra are in different arbitrary units for the two curves. The optical signal is identical to Fig. 3. The voltage fluctuations were measured from a 350 μm axon under sucrose gap conditions which caused a hyperpolarization of 22 mV. The sucrose gap noise measurements were made in collaboration with Dr H. Fishman and Dr D. Poussart. Abscissa, frequency in Hz; ordinate, membrane potential and photocurrent power per Hz.

The voltage fluctuation spectra appear to be of at least three distinct types (Fishman, H., Poussart, D. and Moore, L., unpublished): (1) $1/f$ with a minimum at the potassium equilibrium potential [15], (2) a Lorentzian with a potential dependent corner frequency [11], and (3) a second or higher order system showing potential sensitive resonance peaks. The optical fluctuations show behavior of type (1) and type (2) but do not show resonance peaking. In order to quantitatively evaluate the possibility that the optical spectrum reflects voltage fluctuations it is useful to use the Hodgkin-Huxley formalism [4], to predict the voltage fluctuations. Considering only the potassium conductance and membrane capacitance, the total current, I_t, is given by

$$I_t = C_m \frac{dV}{dt} + \bar{G}_K n^4 (V - V_K) \tag{1}$$

where C_m is the membrane capacitance, $\bar{G}_K$ is the maximum potassium conductance, V is the membrane potential, V_K is the potassium equilibrium potential, and n is a voltage dependent variable which varies between 0 and 1. Following Hodgkin and Huxley [4] equation (1) can be linearized and the fluctuation of I_t expressed as

$$\delta I_t = C_m \frac{d\delta V}{dt} + \bar{G}_K n_\infty^4 \delta V + 4\bar{G}_K n_\infty^3 (V - V_K)\delta n \tag{2}$$

where n_∞ is the steady state value of n and

$$\frac{dn}{dt} = \alpha_n(1-n) - \beta_n n \tag{3}$$

It follows from equation (3) that

$$\frac{d\delta n}{dt} = -(\alpha_n+\beta_n)\delta n + \left(\frac{\partial \alpha_n}{\partial V}\right)\delta V + n_\infty \left[\left(\frac{\partial \alpha_n}{\partial V}\right) + \left(\frac{\partial \beta_n}{\partial V}\right)\right]\delta V \tag{4}$$

Defining

$$\frac{1}{R_K} = \bar{G}_K n_\infty^4, \qquad \omega_c = \frac{1}{R_K C_m}, \qquad \omega_n = \alpha_n+\beta_n,$$

and

$$\theta_n = 4(V-V_K)\left[\frac{1}{n_\infty}\left(\frac{\partial \alpha_n}{\partial V}\right) - \left(\frac{\partial \alpha_n}{\partial V}\right) - \left(\frac{\partial \beta_n}{\partial V}\right)\right]$$

these equations lead to the expression,

$$\frac{d^2\delta V}{dt^2} + (\omega_n+\omega_c)\frac{d\delta V}{dt} + \omega_c(\omega_n+\theta_n)\delta V = \omega_c \omega_n R_K \delta I_t$$

Assuming that δI_t is a forcing function with a power spectrum equal to κ, the spectrum of δV becomes [16],

$$S_{\delta V}(\omega) = \frac{\omega_c \omega_n R_K \kappa}{[\omega^2-\omega_c(\omega_n+\theta_n)]^2+(\omega_n+\omega_c)^2\omega^2}$$

where $\omega = 2\pi f$.

The condition for resonance is

$$(\omega_n+\omega_c)^2 < 4\omega_c(\omega_n+\theta_n)$$

or

$$(\omega_n-\omega_c)^2 < 4\omega_c \theta_n$$

Note that if $\theta_n = 0$, the inequality cannot hold and no resonance in the spectrum would be observed. In other words the interaction of the potassium system with the membrane capacitance to show the resonance typical of a second order system is a consequence of the voltage dependence of the rate constants. Identical coupling occurs whether one observes δV or δn under the above constant current conditions. Therefore, a probe of the n process would fluctuate in identical manner to a membrane potential probe. A difference in spectral content between these two types of membrane probes would occur only under voltage clamp conditions. Thus it is not possible to distinguish between the two proposed interpretations for the optical spectra.

If the potassium conductance or the membrane potential are described by a second order system there would be certain conditions where it would be difficult experimentally to distinguish the observed power spectra over a limited frequency range from a single Lorentzian curve. Therefore, it is not surprising that the electrical noise [11] or the optical fluctuation data of Fig. 3 can be approximated by a single Lorentzian curve.

The presence of a ($1/f$ component in both the optical and voltage fluctuations probably reflects a relaxation spectrum of multiple elementary steps, which are not resolvable by conventional kinetic methods.

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