Changes in Extrinsic Fluorescence in Squid Axons during Voltage-Clamp

Abstract. *Fluorescence changes in squid axons were examined after staining with rhodamine B, pyronin B, or 8-anilinonaphthalene-1-sulfonate by intracellular application. Gradual changes in fluorescence were detected during both hyperpolarizing and depolarizing voltage-clamp pulses. Abrupt changes were often observed at the onset and at the end of voltage-clamp. Possible sources of artifact in optical measurements of this type and some implications of the findings are discussed.*

Studies of optical properties of nerves have recently been undertaken to provide a means of investigating structural changes occurring in the axon membrane during nerve excitation. Changes in such properties as light scattering, birefringence, and extrinsic fluorescence have been observed by various authors (*1, 2*). It appears possible that one of these three properties, extrinsic fluorescence, offers direct means of investigating molecular events occurring in the nerve membrane during nerve excitation and conduction.

The quantum yield of fluorescence of various dyes is strongly dependent on the properties of the medium in which the dye molecules are immersed (see, for example, *3*). It is well known that slight changes in the polarity (and other properties) of the microenvironment of fluorescent dye molecules often bring about large changes in the amount of radiationless loss of energy by the photo-excited molecules. Recent studies of changes in intensity of fluorescence with a number of dyes applied to nerve membrane have shown that indeed well-detectable changes in the fluorescence are present during the process of nerve excitation (*2, 4, 5*). Since the currently accepted theory of nerve excitation is based mainly on the analysis of experimental data obtained under voltage-clamp (*6*), it seemed worthwhile to study optical changes under these electrophysiological conditions. The present work is concerned with the detection of fluorescence changes in artificially stained squid giant axons under voltage-clamp.

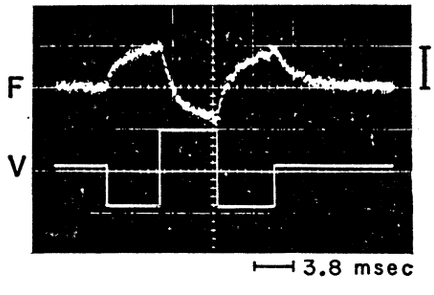

Fig. 1. Changes in the intensity of fluorescence recorded from a squid giant axon with intracellularly injected rhodamine B (about 0.2 mg/ml) produced by hyperpolarizing and depolarizing voltage-clamp. The top trace marked *F* represents the fluorescence intensity averaged over about 3000 trials. The lower trace (*V*) shows the time course of the clamping pulse (−50, +40, and −50 mv). The temperature of the axon was kept at 6°C. The bandwidth of the recording apparatus was from 0.8 to 1000 hertz. Vertical marker: 2.7×10^{-5} times the background fluorescence intensity.

All measurements were performed on giant axons of the squid *Loligo vulgaris*, available in Camogli, Italy. After extensive cleaning of a 22-mm middle region, the axons were mounted horizontally in a chamber made of black Lucite. Transparent Lucite was used in a narrow region underneath the axon to permit illumination during perfusion and insertion of the internal electrodes. The extracellular medium in almost all the experiments reported below was natural seawater. The axons were stained internally either by injection of small volumes of a potassium phosphate–glycerol solution (400 meq of K^+ per liter) containing 1 mg of dye per milliliter or by internal perfusion of the 22-mm middle portion of the axon with a potassium phosphate–glycerol solution containing the dye at the desired final intracellular concentration (between 0.05 and 0.1 mg/ml). Three dyes, rhodamine B, pyronin B, and 8-anilinonaphthalene-1-sulfonate (ANS) were used. These dyes are known to give rise to relatively large optical signals during nerve excitation (*5*). The perfusion technique used is described elsewhere (*7*). The stained axons were impaled with a double electrode assembly for space-clamping the 22-mm middle portion and for recording internal potentials. After impalement, the electrodes were disconnected from the manipulator and fixed to the chamber, which could now be moved to its final position in the optical setup.

A 100-watt, quartz-iodine incandescent lamp was used in conjunction with a narrow band interference filter as a source of exciting light. By the use of a spherical lens and a cylindrical lens, an image of the lamp filament was formed whereby the entire portion of the axon under study was uniformly illuminated. The fluorescent light (at least 20 nm longer in wavelength than the exciting light) was detected at right angles to the incident light with a photomultiplier (RCA 4463) through a cutoff filter. The output of the photomultiplier was fed, through a Bak electrometer (Electronics for Life Sciences) and an amplifier with variable gain and bandwidth (Tektronix type 122), to an average computer (Nuclear Data-801 Enhancetron 1024) which was used to increase the signal-to-noise ratio. The internal and external electrodes were connected to a voltage-clamp setup which has been described elsewhere (see *8*).

Figure 1 shows a record obtained from an axon containing approximately 0.1 mg of rhodamine B per

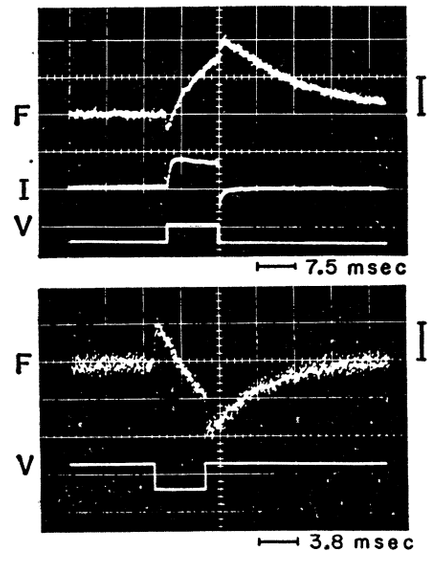

Fig. 2. Changes in fluorescence intensity recorded from an axon intracellularly perfused with pyronin B (0.05 mg/ml). (Top) The optical signal, averaged over about 400 trials (*F*), and the electric current (*I*) associated with a depolarizing voltage-clamp pulse of 100 mv in amplitude (*V*). Temperature: 11.5°C. Bandwidth of the recording apparatus: 0.8 to 1000 hertz. Vertical marker 1.4×10^{-4} times the background fluorescence intensity. (Bottom) Optical signal (*F*) averaged over about 6000 trials produced by a hyperpolarizing voltage-clamp pulse of 40 mv in amplitude (*V*) recorded with higher time resolution. Temperature: 10°C. Bandwidth of the recording apparatus: 0.8 to 10,000 hertz. Vertical marker: 2×10^{-5} times the background fluorescence intensity.

milliliter. The stained axon was subject to a series of voltage-clamp pulses each consisting of a 5-msec, 50-mv hyperpolarizing pulse, followed by a 5-msec, 40-mv depolarizing pulse and next by a 5-msec, 50-mv hyperpolarizing pulse. The fluorescence intensity measured was averaged over 3000 runs. It is seen that subsequent to a voltage step applied to the nerve membrane, the intensity of the fluorescence changed gradually. There was an increase in fluorescence in response to hyperpolarization and a decrease in response to depolarization. The change in fluorescence was of the order of 2.5×10^{-5} times the background intensity, and the time constant of the (roughly exponential) variation was about 1.5 msec. Apart from the sign and the exact magnitude, the time courses of the fluorescence changes for positive and for negative pulses are very similar. The magnitude of the change was found to vary with the amplitude of the clamping pulse.

In axons stained with pyronin B, the changes in the intensity of fluorescence associated with depolarization or hyperpolarization under voltage-clamp were of the type shown in Fig. 2. In response to hyperpolarizing voltage pulses, there was an abrupt increase followed by a slow decrease in fluorescence intensity. By applying pulses of long duration (up to 50 msec) it was possible to establish that the intensity of the fluorescence reaches roughly exponentially an approximately steady level with a time constant of about 10 msec at 10°C. The time resolution of the electronics apparatus used (0.8 hertz to 10 khertz bandwidth, 32 μsec shortest sampling interval) appeared to be the major factor determining the rapidity of the early, abrupt change which is seen merely as a sudden jump of the optical trace at the onset of the voltage-clamp (Fig. 2, bottom). The late, slow change in fluorescence from pyronin B has a sign opposite to that observed with rhodamine B. The amplitudes of both the abrupt and the slow changes were found to vary roughly linearly with the amplitude of the applied voltage pulse. A reversal of the sign of the applied voltage yielded an optical signal of the reversed sign. The amplitude of the optical signals observed was of the order of 10^{-5} to 10^{-4} times the background fluorescence intensity.

The following further observations were made with pyronin B. (i) Replacement of the natural seawater in the extracellular medium with a calcium chloride–glycerol isotonic solution (100 mmole of $CaCl_2$ per liter) did not alter the shape of the optical signal; it merely decreased the amplitude of the early phase slightly and increased the amplitude of the late phase by a factor of about 3. (ii) Replacement of natural seawater with a KCl solution (600 mmole/liter) eliminated the early phase of the optical signal, decreased the amplitude of the late phase by a factor of about 3, and decreased the time constant from 10 to about 1 msec.

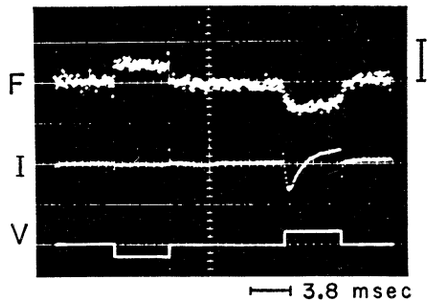

Fig. 3. Changes in fluorescence intensity (F) recorded from a squid axon carrying intracellularly injected ANS (about 0.1 mg/ml) produced by a hyperpolarizing or a depolarizing clamping pulse of 50 mv amplitude (V). A record of the membrane currents (I) is also shown. The optical signal was obtained by averaging over 30,000 trials in the period of about 40 minutes. Temperature: 9°C. Bandwidth of recording apparatus: 8 to 1000 hertz. Vertical marker: 2.9×10^{-5} times the background fluorescence intensity.

The time courses of the fluorescence changes observed in axons stained with ANS are shown in Fig. 3. The onset of a hyperpolarizing clamping pulse was associated with a rapid increase in the intensity of the fluorescence, which remained constant during the clamping period; the intensity fell rapidly to the original value at the end of the clamping pulse. In the range of hyperpolarization between 30 and 80 mv, the magnitude of the optical change increased with increasing amplitude of the clamping pulse. The time courses of the fluorescence changes observed with depolarizing clamping pulses were not simple mirror images of those obtained with hyperpolarizing pulses. The intensity of fluorescence was found to decrease gradually following the onset of the clamping pulse. The fluorescence intensity changed gradually also at the end of the depolarizing clamping pulse. Following the initial gradual fall, there was often a slight recovery in the fluorescence intensity during maintained voltage-clamping (Fig. 3). The time interval between the onset of the clamping pulse and the appearance of a minimum in the light intensity was about 1 msec for 50-mv depolarization at 10°C. In the range of depolarization between 30 and 80 mv, the rapidity of the decrease in fluorescence appeared to increase with the amplitude of the clamping pulses. The magnitude of the changes observed with depolarizing clamping pulses was about 50 percent larger than those obtained with hyperpolarizing pulses of the same amplitude. In most cases signals were of the order of 10^{-5} times the background intensity. Very slow changes in fluorescence intensity (of the type observed with rhodamine B or pyronin B) were never observed in axons stained with ANS.

The possibility of recording artifacts in the experiments of the type described above is great, and control experiments were important parts of the present investigation. The following control experiments were performed.

1) With the present experimental apparatus, no signal in the optical channel could be observed from unstained axons. This finding rules out the existence of electric artifacts in our records. It also indicates that the observed optical signals do not derive from changes in the light-scattering properties of the axon.

2) No optical signal from a stained axon could be detected when the secondary (cutoff) filter was placed between the primary filter and the axon. This rules out the possibility that these optical signals are directly related to light-scattering changes in stained axons.

3) One source of possible artifact is the electrolysis which takes place on the surface of the internal current electrode carrying strong currents during voltage-clamp. The possibility that such electrolysis may change the fluorescence property of the dye was ruled out by observing similar optical signals during voltage-clamp without using internal electrodes. This was accomplished by using a different chamber in which a 5-mm stained portion of the axon could be separated by means of two 7-mm grooves filled with Vaseline from the lateral ends which were immersed in KCl solution (600 mmole/liter). The accuracy of the voltage-clamp under these conditions is not very satisfactory. However, the optical signals obtained with hyperpolarizing clamping pulses had roughly the same magnitude and time courses as those observed with internal clamping elec-

trodes. This fact is considered as indicating that no effect due to electrolysis is present in our records.

4) The effects of impurities in the ANS preparation used (Eastman Organic) were ruled out by experiments demonstrating no difference in the optical signals when recrystallized samples were used. Artifacts arising from possible impurities in the rhodamine B and pyronin B preparations (Allied Chemicals) were not ruled out.

Attempts to interpret the experimental results described above are severely hampered by the fact that the physicochemical factors which influence the quantum yield of fluorescence in rhodamine B and pyronin B are not well known. The slow and progressive change in fluorescence seen in Figs. 1 and 2 suggests that an electrophoretic effect (accumulation or depletion of the dye and/or salts in and near the membrane) may be present. Various ions in the axon interior are expected to affect the rate (and the direction) of migration of the large dye molecules during voltage-clamp. Furthermore, changes in concentration of these ions can affect the conformation of the macromolecules (proteins and phospholipids) which in turn may influence the quantum yield of fluorescence. (Note that fluorescence of pyronin B in aqueous solution can be strongly quenched by addition of various macromolecules to the solution.)

In the case of ANS, it appears reasonable to assume that a finite portion of the fluorescent light from the axon in the resting state derives from the dye molecules located in the hydrophobic layer (*9*) in the axon membrane. Since the increase in the membrane conductance is considered to be associated with an increase in the water content of the major diffusion barrier in the axon membrane, a decrease in fluorescence during nerve excitation is expected (*5*). In order to explain the increase in fluorescence associated with a hyperpolarizing voltage pulse, one might postulate a change in the thickness of the hydrophobic layer during hyperpolarization. Further experimental and theoretical studies are needed to understand the physicochemical nature of the findings described in this report.

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Susceptibility to an Avian Leukosis-Sarcoma Virus: Close Association with an Erythrocyte Isoantigen

Abstract. *A dominant gene for susceptibility to early steps of cellular infection by subgroup B avian leukosis-sarcoma viruses is associated with the presence of an erythrocyte isoantigen. This gene may control both an isoantigen and a cell membrane receptor for an oncogenic virus.*

Host cell susceptibility to the early events of infection by subgroups A and B avian leukosis-sarcoma viruses is controlled in each case by a dominant allele at one of two independent, autosomal loci (*1*). Dominance of susceptibility indicates that the presence of a specific cell-membrane receptor substance is required for virus infection (*2*). It has been suggested that this receptor may also be antigenic and thus be detectable by immunologic methods (*1*). We now present evidence for a close association between the occurrence of an erythrocyte isoantigen and susceptibility to subgroup B leukosis-sarcoma viruses.

Individual chickens of inbred RPRL line 100, known to be segregating at the loci controlling susceptibility to viral subgroups A and B, are assigned genotypes each generation by test mating with a known double-recessive line (RPRL line 7, subline 2) (*1*). Progeny embryos are tested for susceptibility by inoculation of Rous sarcoma virus (RSV) of subgroups A [BH-RSV (RAV-1)] and B [BH-RSV(RAV-2)] on the chorioallantoic membrane (CAM) (*1, 3*).

Erythrocytes from a group of these birds were tested for agglutination with a large panel of independently prepared isoantiserums specific for antigens controlled by 11 blood group systems (*4*)—A, B, C, D, E, H, I, J, K, L, and P—and with an isoantiserum containing a new fraction of agglutinins, designated R1 (*5*). Among the birds tested, segregation was disclosed for the previously established I, K, and L systems and for the new R system. The distribution of blood group classes for these four systems among the parents classified for susceptibility (eight or more embryos surveyed for each of the virus subgroups) is presented in Table 1. Inspection of these data shows

Table 1. The distribution of blood group classes (BGC) for four segregating systems among parents independently assigned susceptibility genotypes for the subgroups A and B leukosis-sarcoma viruses. Each bird was mated to a homozygous resistant line and assigned a genotype based on at least eight embryos for each subgroup assayed on the chorioallantoic membrane. The lower case letter (*a* or *b*) represents the locus concerned with the virus subgroup. The *r* and *s* superscripts represent resistance and susceptibility, respectively. The results signify the number of birds assigned to each class.

BGC*	Susceptibility genotypes (No.)					
	Subgroup A			Subgroup B		
	a^sa^s	a^sa^r	a^ra^r	b^sb^s	b^sb^r	b^rb^r
I^2I^2	2	4	4	4	9	3
I^2I^s	0	6	6	12	6	2
I^sI^s	0	1	1	3	1	0
K^2—	2	7	6	16	8	2
kk	0	4	5	3	8	3
L^1L^1	1	5	5	9	4	0
L^1L^2	1	5	4	8	8	4
L^2L^2	0	1	2	2	4	1
R^1—	2	10	9	19	16	0
rr	0	1	2	0	0	5

* The upper-case letter represents the blood group locus (or system) and the numbered superscript the specificity of the corresponding cell surface antigen. For the K and R systems only K2 and R1 reagents, respectively, were available; thus, two genotypes in each system are indistinguishable, for example, R1-positive birds are either R^1R^1 or R^1r and are designated R^1—.