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Changes in Extrinsic Fluorescence Intensity of the Electroplax Membrane During Electrical Excitation

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Summary. The fluorescent dye 1-anilinonapthalene-8-sulfonic acid (ANS) has been used as a probe of changes in membrane conformation accompanying excitation of the electroplax of *Electrophorus electricus*. ANS binds reversibly to the excitable membrane at rest. During generation of an action potential, an increase in ANS-fluorescence intensity is observed which resembles but does not strictly follow the membrane potential. Experiments using the current-clamp technique have demonstrated a linear relationship between the change in membrane potential and the change in ANS fluorescence intensity. The change in fluorescence intensity is not a consequence of binding to membrane sites of increased affinity nor of an electrophoretic concentration of ANS molecules at the membrane surface.

It is not known whether the change in fluorescence intensity is due to a change in quantum yield of bound ANS or to an increase in the amount of bound ANS. In either case, the change in fluorescence intensity may be interpreted as a change in membrane conformation.

Although it is clear that an action potential results from selective alterations of membrane permeability (*see* [8]), the molecular mechanism of these permeability changes is largely unknown. Several theories have been proposed linking conductance changes to alterations in membrane structure, but none has been thoroughly tested experimentally [1, 2, 7, 9, 10, 21]. One assumption present in several of these theories is that electrical excitation is associated with a cooperative structural transition of the macromolecular elements involved in the selective translocation of ions. In order to distin-

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guish between the various theories, an extensive examination of membrane structure during the excitation process is required.

Several laboratories have begun to follow, in parallel with electrical potential and conductance, various physicochemical parameters which might reveal changes in membrane conformation. Cohen and his associates [4, 5] have demonstrated changes in the intensity of light scattering and of birefringence in various preparations, including the eel electroplax. Their results suggested a potential dependent reorientation of polar membrane components during excitation.

Tasaki and his collaborators [6, 22, 23] have detected changes of extrinsic fluorescence intensity in nerves labelled by various fluorochromes. The amplitude and the sign of the observed transient changes vary with respect to both the dye and the biological preparation. In none of these cases has a precise correlation between the time course and amplitude of the fluorescence trace and of the electrical signal been proposed.

In an effort to take full advantage of the possibilities inherent in such studies of membrane conformation, we began experiments on the isolated electroplax of *Electrophorus electricus*. The electroplax is ideal for these studies for several reasons. Morphologically the cell is composed of two faces, one of which is excitable and the other inexcitable, offering on the same cell an experimental preparation and a membrane suitable for control experiments. The excitable membrane is very large (15 to 20 mm^2), devoid of Schwann cells, and capable of responding synchronously over its entire surface. Furthermore, the electroplax has been extensively studied electrophysiologically [15, 17], and the biochemical properties of the excitable membrane are currently being investigated [11–13]. Experimentally, the cell is amenable to such techniques as current-clamp, voltage-clamp, and measurement of both impedance changes and ion flux, thus making accessible a large number of variables.

As probes of membrane structure, we have chosen to use fluorescent dyes which might provide reliable information on the environment, the distribution, and the motion of various membrane components. In this paper we report experiments carried out with 1-anilinonaphtalene-8-sulfonic acid (ANS). This dye is a probe for the polarity of its environement [20] and is already known to bind to excitable membrane fragments *in vitro* at hydrophobic sites which are in the near vicinity of membrane proteins [13]. We show that the isolated electroplax is easily stained by ANS without loss of its excitability, and that changes of fluorescence intensity accompany its electrical excitation. Evidence is presented that this change strictly depends on the change of membrane potential.

Materials and Methods

Eels were obtained from Paramount Aquarium (Ardsley, N.Y.) and stored in the Tropical Aquarium of the Musée des Arts Africains et Océaniens in Paris until needed. Single eels were then transported to the Institut Pasteur and used as a source of cells for 4 to 5 days. Single cells were isolated from the organ of Sachs, by the method of Schoffeniels [19] and stored at room temperature in Ringer's solution plus 1.5% glucose until needed.

All solutions were made in Ringer's physiological saline solution [14]. ANS was purchased from Eastman Organic Chemicals and purified before use by the method of Weber [25]. Where indicated, Ba^{++} was added as barium acetate to a final concentration of 2.5 mM with no correction for the change in osmotic strength. Tetrodotoxin (Calbiochem) was added to a final concentration of 0.5 μg/ml.

The potential across the innervated membrane of the electroplax was measured with two microelectrodes, one positioned at the surface of the innervated membrane and one placed inside the cell by penetrating the non-innervated membrane. More frequently, the potential was measured across the whole cell, in which case the microelectrodes were placed on the surface of the innervated and non-innervated membranes. The cell potential was amplified with a Grass model S44 stimulator, isolated from ground with a Grass stimulus isolation unit, and passed to two Ag:AgCl electrodes which straddled the cell. In experiments using the current-clamp techniques, a Grass constant-current unit was also used.

The measurements of fluorescence were performed in the apparatus seen in Fig. 1 (*see* also Ref. [24]). The electroplax was mounted between two pieces of plastic such that a portion of the innervated membrane was visible through a window in one of the pieces of plastic. The plastic sheets containing the cell were then mounted between two metal blocks to form two chambers, one bathing the innervated face and one the non-innervated face. The two metal blocks were electrically isolated, and the temperature regulated with circulating water. Light from a xenon lamp, Osram XBO 250 W, was collected with a spherical mirror and focused on the innervated membrane exposed in the window. The fluorescence light, collected at an angle of 45° to the incident light, was focused on the photocathode of the photomultiplier, RTC XP 1118. A filter between the lamp and the cell was used to select the portion of the spectrum required for excitation of the fluorescent probe; a second filter between the cell and the photomultiplier was used to eliminate light not arising from fluorescence. In all experiments reported in this paper, Kodak 18A and Seavom interference band pass ($\lambda > 400$ nm) filters were used for excitation and analysis, respectively.

The output of the photomultiplier was taken to a Tektronix type 3A9 differential amplifier where a d.c. offset voltage was applied to reduce the resting-state fluorescence signal to about 30 mV. The adjusted signal was then passed to a second oscilloscope to monitor continuously the d.c. level, and to an Intertechnique Didac 800 signal averaging device. This technique produced no detectable distortion of a square wave generated by the stimulator and recorded on the Didac 800 at a sample interval of 50 μsec. A Bioelectric calibrator type CA 5 was then used to calibrate the Didac 800 for a variety of sample sizes. All fluorescence signals are given in units of mV, the potential across the 1 MΩ anode resistance of the photomultiplier.

In a typical experiment, a cell was placed in the chamber, bathed with Ringer's solution, and the recording and stimulating electrodes placed in position. The apparatus was then closed to light, and the voltage applied to the photomultiplier was increased until a suitable signal was obtained, usually 100 to 200 mV. The photomultiplier was then turned off and the appropriate solutions added to the chamber enclosing the

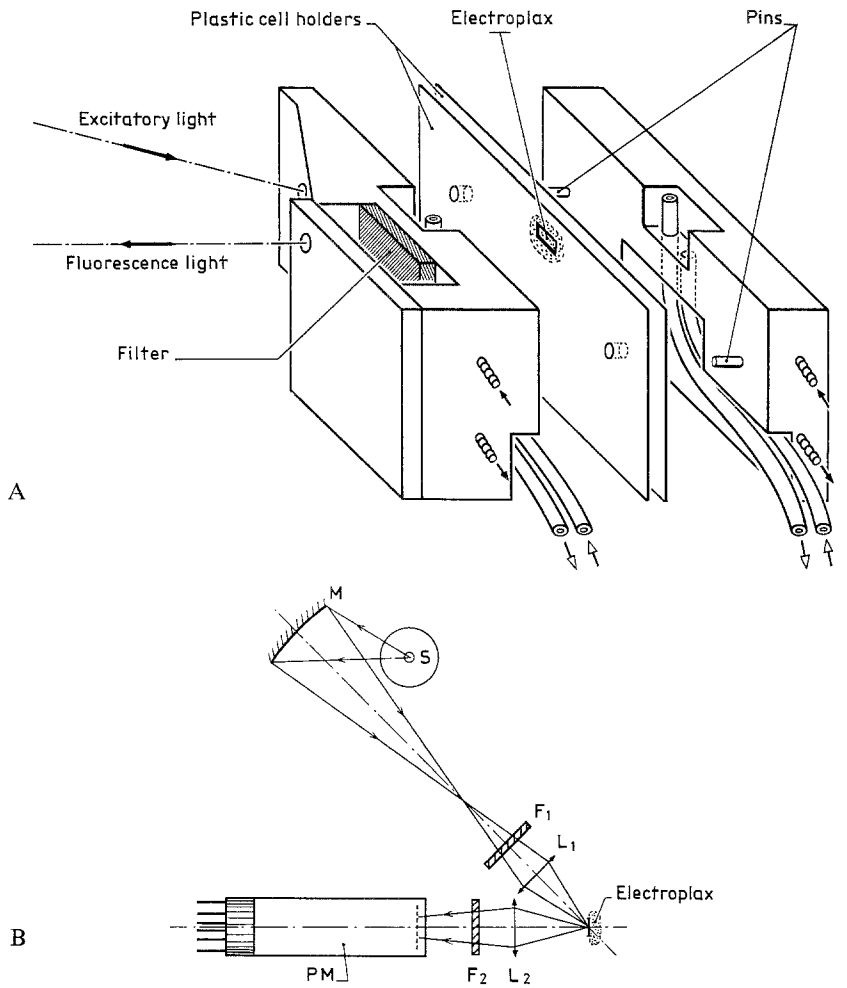

Fig. 1 A and B. Apparatus used for measurements of fluorescence intensity. *A*: Arrangement of the electroplax in the chamber. The tubes entering and leaving the two portions of the chamber allow circulation of the bathing solutions. *B*: Arrangement of the optical system. *S* xenon lamp; L_1 and L_2 lenses; F_1 and F_2, excitation and analyzing filters, respectively; *M* spherical mirror; *PM* photomultiplier

innervated face. The apparatus was then reclosed, the same voltage was applied to the photomultiplier, and the experiment was begun. The cell was stimulated at a frequency of seven to nine stimuli per second, the number of repetitions depending on the cell and the nature of the experiment. The fluorescence signal stored on the Didac 800 was recorded on Polaroid film. When the shape of the cell potential was required, it was recorded on the Didac 800 at the end of the experiment; in all other instances, the magnitude of the potential was simply read off the recording oscilloscope.

Results

Binding of ANS to the Electroplax Membrane at Rest

Fig. 2 shows the result of a typical experiment carried out with the fluorescence apparatus described in Methods. The physiological solution bathing the innervated face of a single electroplax is exchanged with a solution of 10^{-5} M ANS in the same medium. The current of the photomultiplier increases and, after approximately 7 min, reaches a plateau which, in this particular experiment, is stable for the next 7 min. Since the solution of ANS in Ringer's medium is fluorescent, we first tried to estimate what part of the current increase is caused by the fluorescence of free ANS. This quantity is determined in a parallel experiment, under exactly the same conditions except that the electroplax is replaced by a piece of black non-fluorescent plastic. In the presence of 10^{-5} M ANS, the fluorescence of free ANS contributes, on the average, 20% of the total current increase. The major fraction of the current increase is thus due to an increase of fluorescence intensity caused by the interaction of ANS with the electroplax membrane. Fig. 2 further shows that this effect is reversed by the subsequent exchange of ANS solution by Ringer's medium. ANS thus binds reversibly to the electroplax membrane at rest.

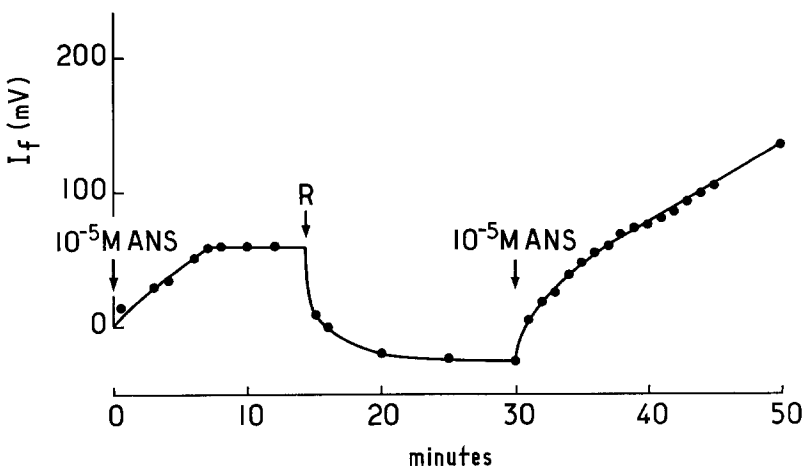

Fig. 2. Interaction of ANS with the excitable membrane at rest. The voltage supplied to the photomultiplier was adjusted to give an output voltage of 200 mV in the absence of ANS. At time zero, a solution of 10^{-5} M ANS was added to the chamber bathing the innervated face. and the increase in photomultiplier output was recorded as a function of time. At time 15 min, the ANS was replaced with Ringer's solution (*R*) and the reversibility of the interaction was followed. At 30 min, the Ringer's solution was replaced with 10^{-5} M ANS, and the interaction of ANS with the membrane was again followed as a function of time

We then attempted to determine the fluorescence parameters of ANS bound to the cell membrane. With our apparatus, in its present state, the emission spectrum of ANS bound to the surface of the electroplax cannot be obtained, although it is clear from the use of filters that this emission occurs at a wavelength larger then 400 nm. However, extensive studies on the fluorescence parameters of membrane-bound ANS have already been performed *in vitro* with excitable membrane fragments purified from the electric organ [12], and the extension of these results to the binding of ANS to the whole cell seems justifiable. These studies have shown that the emission spectrum and quantum yield of ANS bound to the membrane are very similar to those of ANS bound to hydrophobic sites of bovine serum albumin. In other words, ANS binds *in vitro* to highly hydrophobic sites present in the membrane fragments. It is reasonable to expect that, in our *in vivo* experiments, ANS binds to the same type of sites.

In order to evaluate the total number of ANS-binding sites on the membrane at rest, we followed ANS binding as a function of increasing concentrations of ANS. The result of one experiment of this sort is represented in Fig. 3. It should be mentioned that these results happened to be difficult to reproduce quantitatively with a given cell and from one cell to another. The irreproducibility is largely a consequence of two technical difficulties. Below 10^{-6} M ANS, the small increases in fluorescence are obscured by

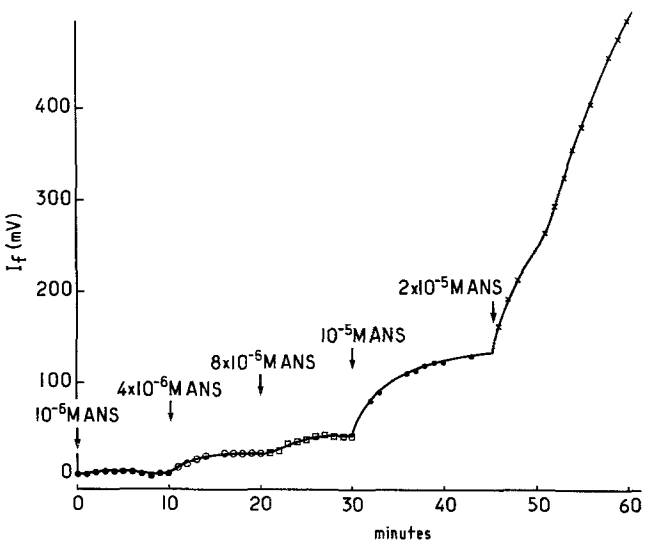

Fig. 3. Effect of increased ANS concentration on the intensity of extrinsic fluorescence. The basal cell fluorescence in the absence of ANS was set at 100 mV, and increasing concentrations of ANS were added as indicated in the figure. The increase (over 100 mV) of fluorescence intensity is plotted as a function of time

Table. *Resting-state fluorescence*

Exp.	Additions[a]	Voltage output of photomultiplier (mV)			
		Cell	Cell 10^{-5} M ANS	ANS[b] solution	ANS[b] bound
1	Ba^{++}, TTX	100	170	9	61
2	Ba^{++}, TTX	100	140	10	30
3	Ba^{++}, TTX	100	140	10	30
4	—	100	190	14	76
5	—	100	135	7	28
6	—	100	140	9	31
7	—	100	250	23	127
8[c]	—	100	300	33	167

[a] Concentrations of Ba^{++} and tetrodotoxin (TTX) were 2.5 mM and 0.5 μg/ml, respectively.

[b] ANS in solution was calculated as described in the text. ANS bound is the difference between the voltage owing to ANS in solution and the increase in photomultiplier output after the addition of ANS.

[c] In this experiment, the cell was placed such that the *non-innervated* membrane faced the photomultiplier.

the various sources of noise in the system. Above 10^{-5} M ANS, and with some cells even at 10^{-5} M ANS, the plateau obtained after 10-min exposure is not stable and the fluorescence increases continuously with time (Figs. 3 & 7). This last effect is interpreted as representing either the slow diffusion of ANS into the digitations of the cell surface or, more likely, the penetration of ANS into the cytoplasm of the cell followed by its binding to cytoplasmic proteins. In any case, this lack of stability with time prevented any accurate measurement of the affinity of ANS for the electroplax membrane at rest and therefore of the total number of ANS-binding sites.

In all these experiments, only the innervated membrane of the cell was exposed to the solution of ANS. In one experiment (Table), the solution of ANS was applied to the non-innervated membrane. In agreement with the *in vitro* experiments of Kasai *et al.* [12], almost the same results were obtained with the non-innervated membrane and with the innervated one.

Fluorescence Changes During the Action Potential

Fig. 4 illustrates that changes of fluorescence intensity accompany the action potential of the isolated electroplax in the presence of 10^{-5} M ANS. The action potentials were elicited in two different ways. Brief depolarizing

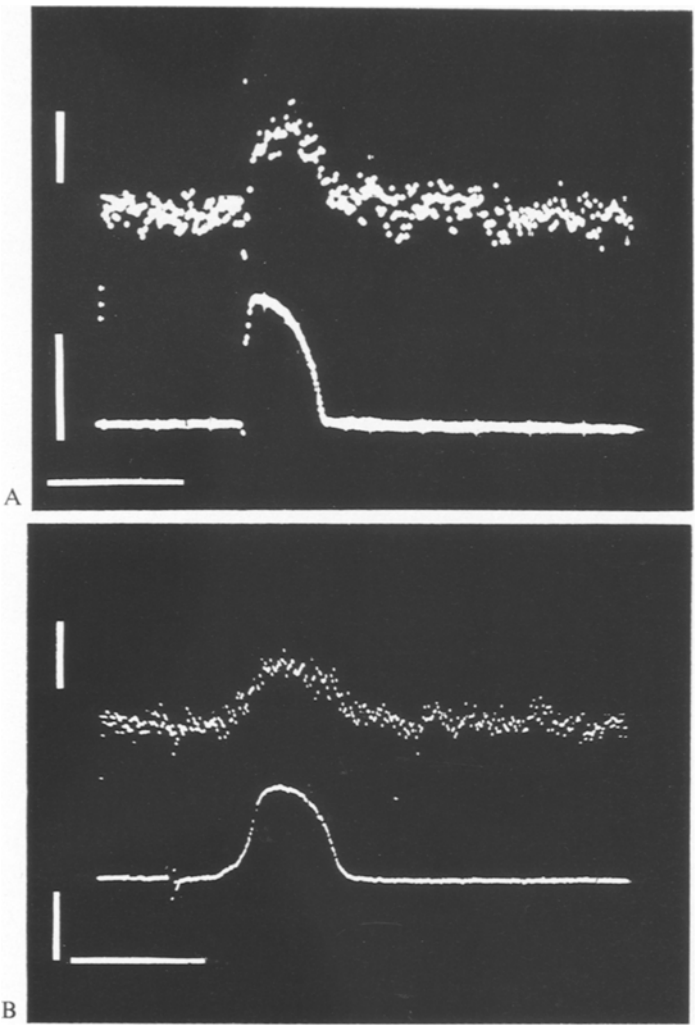

Fig. 4A and B. Changes in fluorescence intensity during excitation of the electroplax in the presence of 10^{-5} M ANS. *A*: The lower trace represents the change in membrane potential following application of a brief depolarizing current. The upper trace is the associated increase in fluorescence intensity averaged over 5,000 repetitions; the vertical bar represents 0.2 and 100 mV for the fluorescence and potential, respectively. The horizontal bar represents 5 msec. *B*: The lower trace indicates the change in membrane potential after stimulation of the nerves innervating the electroplax. The upper trace represents the increase in fluorescence intensity averaged over 2,000 stimuli. The vertical bars represent 0.25 mV and 100 mV for the fluorescence and potential traces, respectively. The horizontal bar represents 5 msec

pulses of 10 μsec stimulate *directly* the electrically excitable membrane of the electroplax. On the other hand, hyperpolarizing current pulses of the same duration but greater magnitudes elicit action potentials at the level

of the nerve terminals which are present on the surface of the cell. The subsequent liberation of acetylcholine at the end plates then generates, *indirectly*, an action potential in the electroplax. In order to extract the fluorescence signal from the noise (*see* Methods), the stimulation was repeated 2,000 or 5,000 times, at a frequency of eight stimuli per second. The upward deflection of the fluorescence traces seen in both cases indicates an *increase* of fluorescence intensity. This increase corresponds to an enhancement of about 8×10^{-3} times the extrinsic fluorescence intensity of the cell at rest in the presence of 10^{-5} M ANS.

The observed increase of fluorescence intensity corresponds to a change of ANS fluorescence since:

(1) No signal was obtained in the absence of ANS, which indicates that the recorded signal is not due to a change of the intrinsic fluorescence of the cell.

(2) Switching the positions of the excitation and analysis filters abolished the signal, which means that it is not caused by a change of the intensity of the light scattered by the cell. It should be mentioned, however, that this control does not deal with a second category of light scattering, i.e., the scattering by the membrane of light emitted by excited ANS molecules. This interpretation is rendered unlikely both by the magnitude of the recorded signal and by its shape (*see* Conclusion).

(3) The position of the electroplax was then inverted, such that its non-innervated side faced the photomultiplier, and was exposed to ANS. Under these conditions, the increase of fluorescence intensity which is associated with the binding to the membrane at rest occurs but no change is recorded during excitation. The increase in ANS fluorescence intensity seen during excitation of the cell in the normal position is thus unique to events occurring at the excitable membrane.

Fluorescence Changes During Current Clamp

In an effort to correlate more clearly the fluorescence signal with events occurring at the cell membrane, the fluorescence intensity was recorded during pulses of constant current. The difference in potential seen in the early and late phases of a current-clamp experiment was maximized by pharmacological inactivation of K conductance with Ba^{++} as described by Ruiz-Manresa [18]. This treatment increases the resistance of the excitable membrane to the same extent as the voltage-induced K inactivation, and, since less current is required to produce a threshold depolarization, a cor-

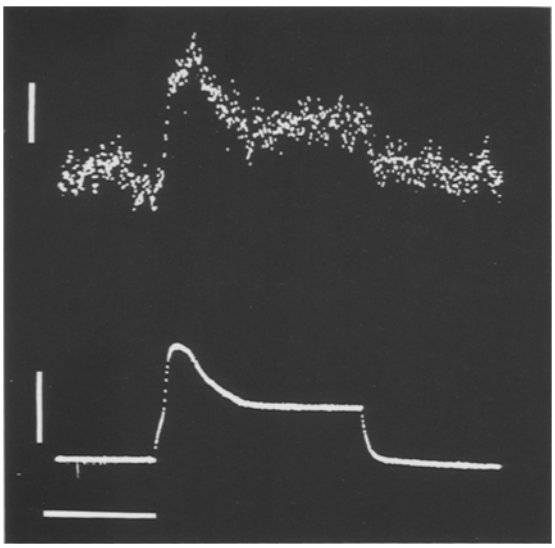

Fig. 5. The change in fluorescence intensity recorded under current clamp. An electroplax, in the presence of 10^{-5} M ANS and 2.5 mM Ba^{++}, was stimulated 5,000 times with a constant depolarizing current, and the associated change in fluorescence intensity was recorded. The vertical bars represent 0.1 mV and 100 mV for the fluorescence signal and membrane potential, respectively. The horizontal bar represents 10 msec

respondingly smaller membrane potential is found in the plateau region of the voltage trace. This in turn facilitates comparison of the variation of the cellular potential with the changes in ANS fluorescence intensity.

The results in Fig. 5 show that the change in fluorescence intensity is similar in form to the variation of cell potential rather than to that of the membrane current, and that it is, furthermore, not unique to any particular phase of the potential. Inhibition of the Na current with tetrodotoxin (*see* Fig. 6) altered the fluorescence signal and the potential in much the same manner, indicating that the plateau region of the fluorescence signal is not a consequence of an excitation event that is simply stabilized by the continued application of current.

Before proceeding with an analysis of the factors governing the change in fluorescence intensity, we first tested the reproducibility of the signal. As mentioned above, the fluorescence of the labelled membrane at rest is unstable and increases with time. We therefore recorded the signal at two different times on the same cell, first 20 min after addition of 10^{-5} M ANS, at a resting-state fluorescence of 115 mV. Then we recorded a second signal, 10 min later, after the resting-state fluorescence had increased by 60%.

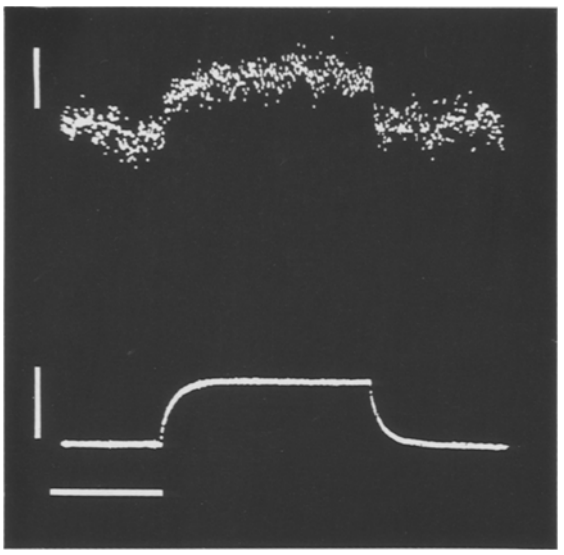

Fig. 6. The effect of tetrodotoxin on the cell potential and on the change in fluorescence intensity during current clamp. A cell in the presence of 10^{-5} M ANS, 2.5 mM Ba^{++}, and 0.5 µg/ml tetrodotoxin was stimulated 5,000 times with a constant depolarizing current, and the associated change in fluorescence intensity was recorded. The vertical bars represent 0.1 mV and 100 mV for the fluorescence and potential traces, respectively. The horizontal bar represents 10 msec

Fig. 7 shows that the two signals do not differ significantly. The fluorescence increase during excitation thus appears to be more reproducible than the fluorescence measurements made in the cell at rest. This is presumably because the signal recorded during excitation comes exclusively from the membrane whereas the fluorescence at rest might include, as previously mentioned, a component derived from ANS in the interior of the cell.

The potential dependent changes in fluorescence intensity recorded under current clamp might be considered a consequence of: (1) a change in the quantum yield of ANS bound to the membrane; (2) an increase in the number of ANS-binding sites; (3) an increase in the affinity of pre-existing ANS-binding sites; or (4) an increased binding of ANS to pre-existing binding sites owing to an increase in the local concentration of ANS, resulting from electrophoresis of ANS in solution.

Cases 3 and 4 may be tested by examining the dependence of the fluorescence signal on the concentration of ANS in solution. In both cases, an increase in ANS concentration should result in binding to pre-existing sites and thus decrease the change in fluorescence intensity seen on depolari-

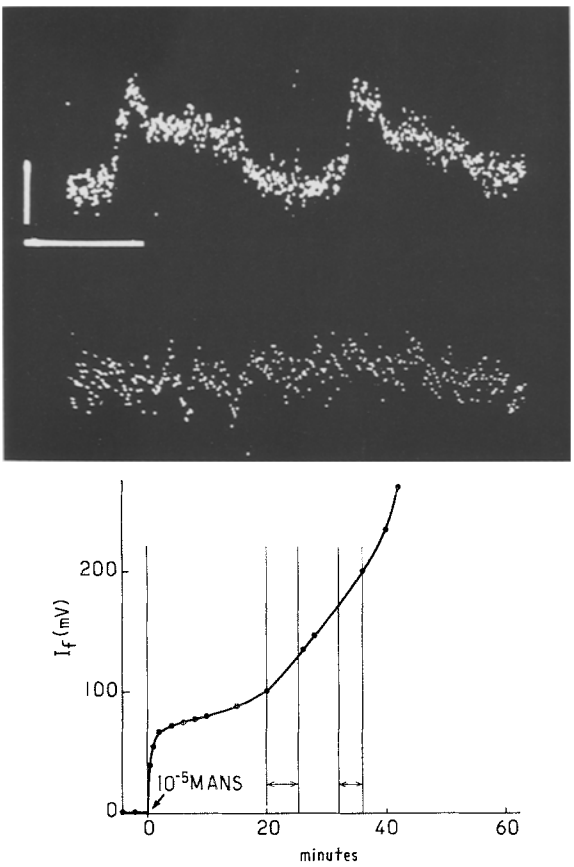

Fig. 7. Reproducibility of the fluorescence signal with respect to the resting-state fluorescence. A cell was placed in the chamber and the voltage supplied to the photomultiplier was adjusted to give a photomultiplier output of 100 mV. A solution of 10^{-5} M ANS and 2.5 mM Ba^{++} was then added, and the increase (over 100 mV) was followed as a function of time. The cell was then subjected to a depolarizing current clamp, and the fluorescence intensity, averaged over 2,000 stimuli, was recorded during the two periods indicated by arrows. During this time, the current was adjusted in order to keep constant the initial peak in membrane potential. The photograph above shows the two fluorescence signals recorded, with the difference between the two signals shown in the lower trace on a time scale expanded twofold. The upper right signal corresponds to the change in fluorescence intensity recorded between 20 and 25 min, while the upper left trace corresponds to that obtained between 32 and 36 min. The vertical bar represents 0.5 mV; the horizontal bar represents 10 msec

zation. The results shown in Fig. 8 are taken from experiments done the same day on two different cells. Although the concentration curve is not a simple absorption isotherm, it does clearly demonstrate saturation and shows no indication of a decrease in the change in fluorescence intensity at high

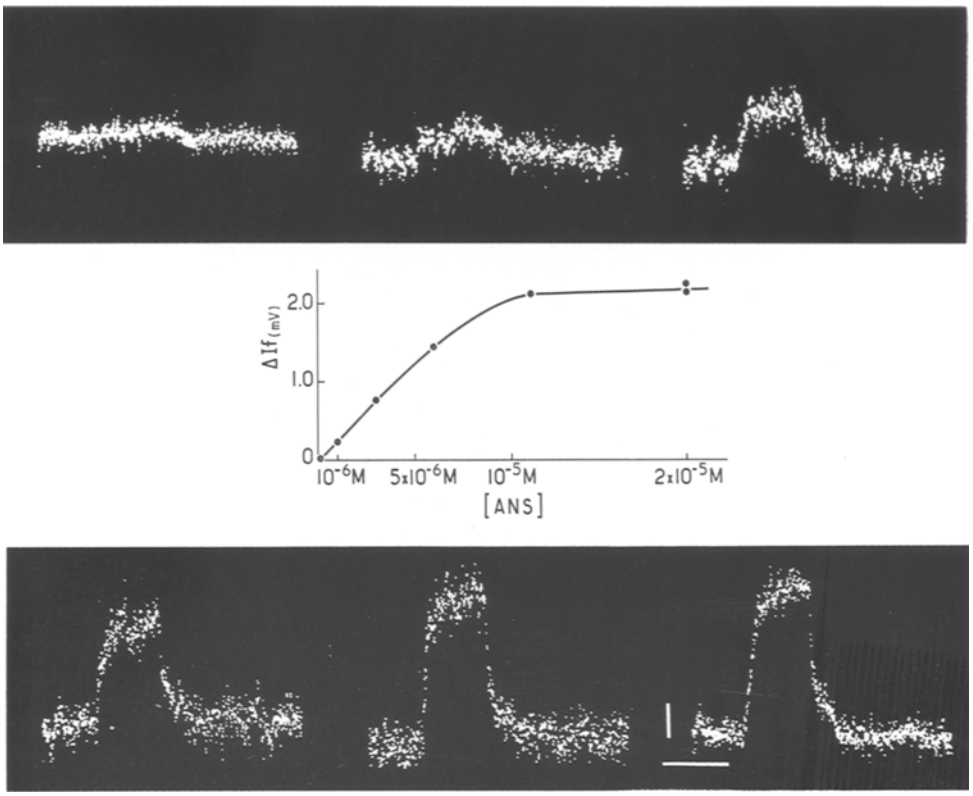

Fig. 8. Magnitude of the fluorescence signal as a function of ANS concentration. A cell was placed in the chamber, a solution of 2.5 mM Ba^{++} and 0.5 μg/ml tetroxotoxin added, and the photomultiplier regulated to give an output voltage of 200 mV. Increasing concentrations of ANS were added and the cell depolarized by 100 mV under current clamp. The depolarization was repeated 2,000 times at each ANS concentration, and the magnitude of the change in fluorescence intensity was plotted as a function of ANS concentration. The magnitude of the change in fluorescence intensity was determined by extrapolating the slow phase of the signal to zero time in order to eliminate variations in the slow phase (*see* text). The photographs show the fluorescence signals at each ANS concentrations. From left to right, upper, 10^{-7} M, 10^{-6} M, 3×10^{-6} M; lower, 6×10^{-6} M, 10^{-5} M, 2×10^{-5} M. The vertical bar in the lower right photograph represents 0.5 mV; the horizontal bar represents 10 msec

ANS concentrations. Although these results effectively eliminate cases 3 and 4, they do not distinguish between cases 1 and 2. The saturation might represent saturation of sites created under current clamp or saturation, in the resting state, of those sites that are sensitive to membrane potential.

Application of a hyperpolarizing current resulted in a decrease in fluorescence intensity (*see* Fig. 9). In the case of hyperpolarization, the fluorescence signal is very similar in form but opposite in sign to that found on depolariza-

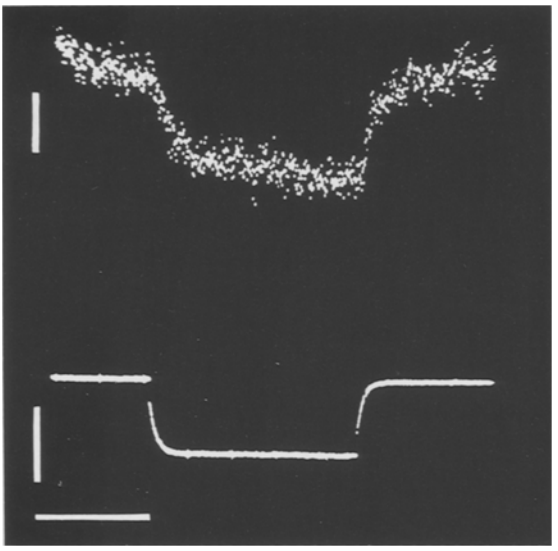

Fig. 9. The change in fluorescence intensity accompanying a hyperpolarizing pulse of constant current. A cell in the presence of 10^{-5} M ANS and 2.5 mM Ba^{++} was hyperpolarized with a constant current. The hyperpolarization was repeated 5,000 times and the change in fluorescence intensity recorded. The vertical bar beside the fluorescence trace represents 0.1 mV; the vertical bar beside the potential trace represents 100 mV. The horizontal bar indicates 10 msec

tion in the presence of tetrodotoxin, further suggesting a dependence of the fluorescence signal on cell potential. The quantitative relationship between fluorescence intensity and the potential imposed across the cell membrane was determined for a cell in the presence of 10^{-5} M ANS. As seen in Fig. 10, the relationship is linear and symmetric about both the resting potential and the zero potential. The change in fluorescence intensity is thus a linear function of the change in membrane potential. We then tried to analyze in some detail the compared time course of the change of fluorescence and of the change in potential under current clamp. In each of these experiments, it is clear that the change in fluorescence intensity lags behind the change in cell potential, both in the initial increase and in the return to the resting-state intensity. A quantitative treatment of the rates of change of fluorescence intensity was hindered, however, by irreproducibility of the fluorescence signal. In an extreme case, the signal seen in Fig. 11 was occasionally encountered, and a variety of rates intermediate between those shown in Fig. 5 and Fig. 11 were normally found.

It is also clear, in these figures, that the increase in fluorescence does not follow a simple exponential and may be separated into a fast and a slow

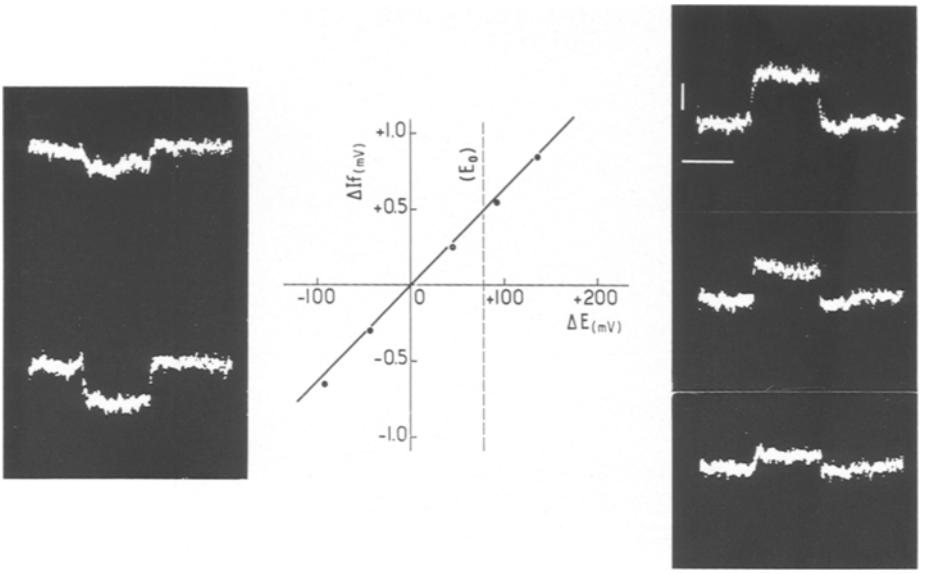

Fig. 10. The magnitude of the fluorescence signal, at constant ANS concentration, as a function of the change in membrane potential. The output of the photomultiplier was adjusted to 200 mV and the Ringer's solution bathing the innervated face was replaced with a solution of 10^{-5} M ANS, 2.5 mM Ba^{++}, and 0.5 μg/ml tetrodotoxin. The potential across the membrane was then changed under conditions of current clamp. The stimulus was repeated 2,000 times at each potential, and the change in fluorescence intensity was recorded. The magnitude of the fluorescence signal was determined and plotted as a function of the change in membrane potential. The dotted line (E_0) indicates the point at which the membrane potential becomes zero. The photographs show the fluorescence signal at each voltage step. On the left side are the signals found with hyperpolarizations of 46 mV (upper photo) and 91 mV (lower photo). The photographs on the right side correspond to depolarizations of 46 mV (lower), 91 mV (middle), and 136 mV (upper). The vertical bar in the upper right photograph represents 0.5 mV; the horizontal bar represents 10 msec

phase. Again, the rate of the fast phase varied from cell to cell, and in many instances the slow phase was completely missing.

It remains, however, that the time course of the fluorescence change does not parallel the time course of the potential response and, in general, is significantly slower.

Conclusion

ANS, a fluorescent probe currently used in protein and membrane studies, binds to the excitable membrane of the isolated electroplax. This result is in agreement with the observations of various authors using preparations of excitable [6, 12, 22, 23] or non-excitable membranes. The fluorescence parameters of ANS bound to the electroplax *in vivo* cannot be determined although, by analogy with the results obtained *in vitro*, it is probable that

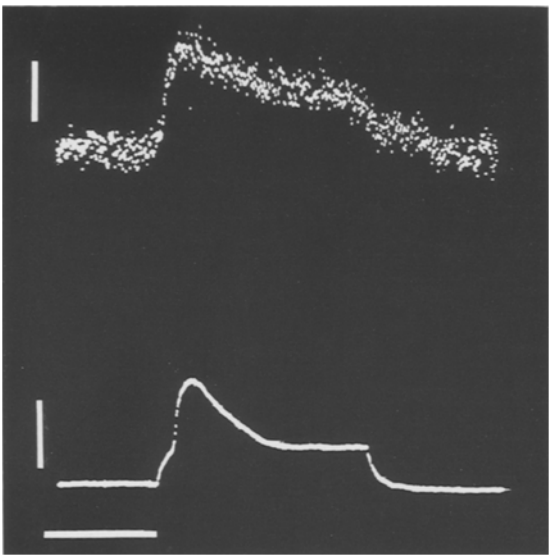

Fig. 11. Change in fluorescence intensity during depolarizing current clamp demonstrating variations in time course of fluorescence signal. The photomultiplier output was regulated to 100 mV in the absence of ANS. A solution of 10^{-5} M ANS and 2.5 mM Ba^{++} was used to replace the Ringer's solution bathing the innervated membrane and the experiment begun. The cell was depolarized 5,000 times under current clamp, and the fluorescence recorded. The vertical bars represent 0.1 mV and 100 mV for the fluorescence and potential traces, respectively. The horizontal bar represents 10 msec

ANS binds to highly hydrophobic sites of the electroplax membrane. Extensive studies of the binding of ANS to the cell at rest are hindered by the lack of stability of the fluorescence increase as a function of time, which is presumably caused by the penetration of ANS within the cytoplasm.

Although knowledge of the number of ANS molecules bound to the membrane at rest would be of considerable interest, we cannot yet provide this information with any precision. We attempted, however, to make an estimate that is valid within the limits of a certain number of assumptions. From the geometry of our apparatus we first calculate the volume of solution excited by the incident light beam. We then assume that: (1) all the fluorescent light seen by the photomultiplier is derived from that portion of the calculated volume which is included in a perpendicular extension of the edges of the window; (2) the fraction of molecules excited by the incident light and the fraction of the emitted light recieved by the photomultiplier is the same for the ANS molecules in solution and the ANS molecules bound to the cell membrane; and (3) the quantum yield of free ANS in Ringer's solution is 0.004 and that of ANS bound to the membrane is 0.75. On the basis of these still unverified assumptions, we can calculate the number of

ANS molecules bound to the membrane. In the presence of 10^{-5} M ANS in one experiment, we find that one ANS molecule is bound per 400 Å^2 of window area. Since the area of the membrane is much larger than the area of the window, because of its very convoluted surface (*see* electron micrographs in Ref. [3]), the actual value may be considerably different. Assuming that the area of the cell membrane is 100-fold larger than the window area, we find one ANS molecule bound per 40,000 Å^2 of excitable membrane. As mentioned above, this might vary widely from one experiment to another.

The changes in fluorescence intensity recorded during the action potential or during membrane excitation under current clamp are quite reproducible. The observed changes in fluorescence are not caused by the scattering by the membrane of the exciting light. In addition, the results of Cohen and associates [5], obtained with the electroplax, make it very unlikely that our results arise from scattering, by the membrane, of light emitted by excited ANS molecules. These authors reported changes in light scattering amounting to 5×10^{-5} the resting-state scattering whereas our results indicate changes about 100 times larger. Furthermore, the change in light-scattering intensity comprises three components: an initial rapid decrease, then an increase, and finally a slow late decrease. In contrast, the changes in fluorescence intensity show only a fast increase and a slower decrease. Thus the change of fluorescence intensity observed is caused by a change in the intrinsic fluorescence of ANS.

We have further demonstrated that the change in ANS fluorescence intensity is not caused by binding, during excitation, to sites of increased affinity. By the same experiment, we demonstrated that the change is not caused by a potential dependent increase in the concentration of ANS in the vicinity of the membrane. It appears highly improbable, on theoretical grounds, that the change occurs because of a direct effect of the electric field on the quantum yield of ANS [16]. The most plausible interpretation of our results is a potential dependent change of membrane structure manifested either by a change of the number of ANS-binding sites or by a change of environment of already bound ANS molecules. An important finding is the fact that under current clamp the change of fluorescence intensity varies linearly with the change of membrane potential, both in amplitude and sign from -100 to $+100$ mV. This symmetry of the fluorescence signal around both resting potential and zero potential cannot be accounted for without postulating both an asymmetry of the cell membrane and an asymmetry in the distribution of ANS in the membrane. It is worth mentioning, in this respect, that a similar requirement for membrane asymmetry was

postulated by Cohen and associates [4] with their potential dependent changes of nerve birefringence during voltage clamp. No relationship between the observed potential dependent changes in fluorescence and the mechanism of membrane excitation has been observed. Inhibition of Na activation and K inactivation by tetrodotoxin and Ba^{++}, respectively, does not eliminate the fluorescence signal. It might be argued however that Ba^{++} simply decreases K conductance without interfering with the molecular events responsible for the potential dependent K inactivation. In this case, the fluorescence might still be a consequence of an inactivation process no longer detectable by electrophysiological techniques. However, Grundfest and associates [15, 17] have shown that a threshold depolarization is required for K inactivation in the electroplax, whereas our fluorescence results do not show any threshold. The observed linear relationship between fluorescence and potential makes it very unlikely that the signal is a consequence of a masked inactivation process. The same reasoning may be applied to Na activation in the presence of tetrodotoxin.

The fact that the change of potential always precedes the fluorescence change is consistent with our conclusion that the fluorescence change is caused by the change of potential and does not report structural changes which cause the membrane potential to change. The observed lag might then be caused by either a slow structural response of the membrane to the changed potential or by a rate-limiting step, such as the binding of ANS to newly created binding sites. Specific probes are needed to bypass this secondary change in fluorescence intensity.

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