MODIFICATION BY PROCAINE OF MEMBRANE ANS FLUORESCENCE CHANGES INDUCED BY ELECTRICAL STIMULATION OF NERVE AND MUSCLE FIBRES.

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SUMMARY

Crab nerve and frog sartorius muscle stained with 1-anilinonaphthalene-8-sulphonate (ANS), show transient increases in fluorescence during action potentials, and transient decreases during imposed hyperpolarisations. Both changes were of the order of 10^{-4} of the background fluorescence. The time-course of both optical and electrical responses were similar.

Procaine ($\sim 10^{-3}$M) reduced or abolished the optical signal, but not the electrical response. The resting state fluorescence of dyed muscle was unaffected by procaine.

The apparent association constant for binding of ANS to muscle was 14μM (±S.D.6μM). The total binding was calculated to be 0.06μmole/g tissue weight. The origin of these effects is discussed.

Axons stained with the fluorescent probe 1-anilinonaphthalene-8-sulphonate (ANS) show small changes in fluorescence during imposed membrane potential steps. This effect appears to be specifically related to the altered voltage profile across the membrane, rather than to conductance changes or charge movement (1). Nevertheless, there is little doubt that the signals originate from ANS molecules either in, or closely associated with, the plasma membrane, and therefore this signal should depend on the state of the membrane.

Local anaesthetics have been shown to produce profound changes in a number of membrane parameters (2). It seemed of interest, therefore, to examine the effects of local anaesthetics on the potential-dependent fluorescence transients of nerve and muscle. Procaine was chosen as a typical member of this group of drugs.

MATERIALS AND METHODS

The walking-leg nerves of shore crabs (Carcinus maenas), and the sartorius muscles of frogs (Rana temporaria and Rana pipiens) were employed.

The tissue baths were constructed from black lucite. The nerve bath had three compartments, and the nerve was mounted through vaselined slits. The first compartment contained a pair of stimulating electrodes, and the responses were recorded between electrodes situated in the second and third compartment. For hyperpolarisation, pulses were applied between the first and second compartment, using an isolated stimulator (Devices, type 2533). Typically, stimuli of 0.5-10volts were employed.

Essentially the same technique was used for muscle hyperpolarisation. A glass window in the second compartment allowed incident ultra violet light, parallel to the plane of the bath, to irradiate a few millimetres of tissue.

Quasi-monochromatic light was obtained from a 24volt, dc-operated, 150 watt, quartz-iodine lamp, in conjunction with a band-pass filter (Kodak Wratten 18A), having a transmission maximum at 365nm. An additional filter of 10% copper sulphate solution was used to absorb red light transmitted by the band-pass filter.

The fluorescent light emitted by the tissue was passed through the glass bottom of the tissue bath, and collected at right-angles to the exciting beam, by a microscope condenser lens. The light was thence passed through a cut-off filter (Kodak Wratten 2A), which transmitted above 420nm, and finally to an 11-stage photomultiplier (EMI 6097F), which was operated with alternate dynodes shorted. With a photomultiplier operating voltage of about 1kV, and a 1megohm load resistor, the fluorescence output was approximately 10V. A brief signal from a light-emitting diode, situated above the irradiated chamber, was adjusted to produce a photomultiplier output 10^{-4} of the background fluorescence. This served as a calibration on the records, and as a test of frequency response.

The photomultiplier load resistance was coupled to an oscilloscope through a 1μF capacitor. The output from the oscilloscope Y-amplifier was, in turn, coupled to a computer of average transients (Biomac 1000), in order to increase the signal-to-noise ratio. Finally, the time-averaged records were fed into an XY-recorder (HR-96, Advance Electronics).

For hyperpolarisation, the exciting beam of light was directed at the nerve or muscle just after it emerged from the first compartment. Because of the cable properties of the fibres, the hyperpolarisation is greatest at that point. In the case of muscle, it was ascertained that good hyperpolarisations (20-60mV) were occurring during the stimulus, by impaling fibres close to the slit with a microelectrode. Conventional intracellular techniques were used. The success of the method depended upon obtaining good inter-compartmental seals.

1-Anilinonaphthalene-8-sulphonate (ANS), as the ammonium salt, and procaine hydrochloride were obtained from Sigma Chemical Co. The physiological saline used for crab nerve had the following ionic composition (in mM): Na^+513, K^+12.9, Ca^{++}11.8, Mg^{++}23.6, Cl^-596, HCO_3^-10, while the Ringer solution used for frog muscle had the following composition: Na^+113, K^+1.9, Ca^{++}1.2, Cl^-115, HCO_3^-2.4, $H_2PO_4^-$0.064. Sucrose Ringer contained an additional 400mM sucrose, and tetrodotoxin was used at 10^{-7}g/ml. The concentration range of ANS was 0.01-0.25mM. Both nerve bundles and frog sartorius muscles were immersed in the dye solution, and experiments were carried out in this solution after equilibration.

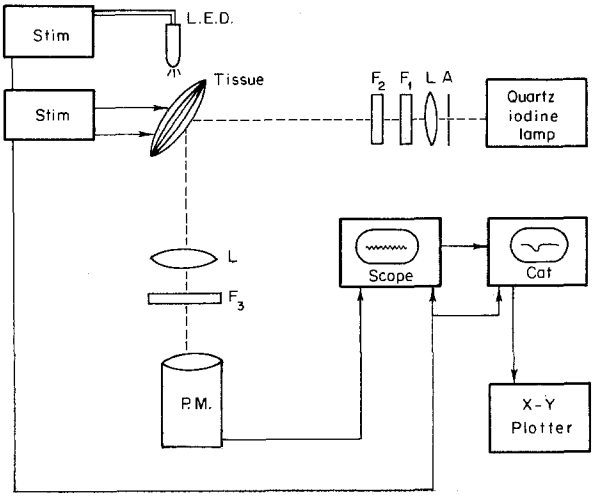
RESULTS

Nerve Action Potentials

In these experiments, ANS was used at 0.25mM, a concentration which produced a slight reduction in the action potential. ANS is known to have some physiological action on axons (3). During an experiment, a further slow, irreversible deterioration in the action potential occurred. Prolonged treatment with ANS alone, or with uv light, did not produce this effect which we therefore ascribe to a photodynamic action (4). During the action potential, there was an increase in the recorded fluorescence of about 10^{-4} of the background fluorescence. This phenomenon has been well documented by Tasaki et al. (5,6,7,8,9). However, 1mM procaine produced a marked reduction in the size of the fluorescence signal, which showed some recovery after wash-out. Although procaine reduced the compound action potential, this reduction was always far less than that of the fluorescence response. Crustacean nerve is known to be relatively resistant to procaine (10).

Nerve Hyperpolarisation

The application of a brief hyperpolarising current pulse to the nerve bundle produced a corresponding transient decrease in fluorescence intensity, the absolute magnitude of which, for a particular stimulus, varied



Optical Arrangement for Fluorescence Experiments

A, aperture; L, lens; F, filter; L.E.D., light-emitting diode; STIM, stimulator; P.M., photomultiplier; C.A.T., computer of average transients.

from preparation to preparation. However, in any one experiment, the fluorescence response was linearly related to the applied voltage.

Experiments with procaine were performed either by draining the bath contents and replacing them with a 10mM solution of procaine in ANS-containing Ringer, or by adding a drop of concentrated procaine solution to the bath. In both cases, the addition of procaine was preceded by an addition or replacement of ANS-containing crab Ringer as a control to check mechanical stability. In every case, procaine inhibited the transient decrease in fluorescence caused by hyperpolarisation.

In some experiments, two brief pulses of differing magnitude were employed in a single sweep, after the manner of Conti and Wanke (11). This reduced the overall exposure of the nerve bundle to uv light, and therefore reduced photodynamic degradation of the axons. Fluorescence signals were recorded before and after the addition of a drop of concentrated procaine. The final bath concentration of anaesthetic was 5mM in each case (Figs.1a and b). In twelve such experiments, the average size of the fluorescence signal after the addition of control solution was 92% ($\pm$S.D.12) of the initial value, while in the presence of 5mM procaine, the average fluorescence response was 41% ($\pm$S.D.16) of the initial value. A few experiments with 3mM cocaine produced qualitatively similar results.

It was not found practical to record intracellularly the hyperpolarisation of the fibres close to the stimulating partition, to check that this was not affected also by procaine. Therefore, a second series of experiments was performed using frog sartorius muscle, where this difficulty could be overcome.

Muscle Hyperpolarisation

Tetrodotoxin (10^{-7}g/ml) was used to suppress action potentials. Where low-sodium Ringer (12) was used to achieve this, the fluorescence responses were inconsistent. Twitch artifacts were eliminated by the addition of 400 mM sucrose to the Ringer (13), and preparations were equilibrated in this solution for at least 15 minutes prior to each experiment.

As in the case of crab nerve, hyperpolarising current pulses produced a voltage-dependent, transient decrease in fluorescence intensity. Again, the fluorescence signals were reduced after the addition of procaine solution (Figs.2a and b). Experiments using a perfusion system were also carried out on sartorius muscle, and a typical result is shown in Fig.3, where it can be seen that the anaesthetic reduced the fluorescence signal to 47% of its initial value. A subsequent wash-out afforded a complete recovery. In some cases, 1mM amethocaine was employed instead of procaine, and qualitatively similar results were obtained.

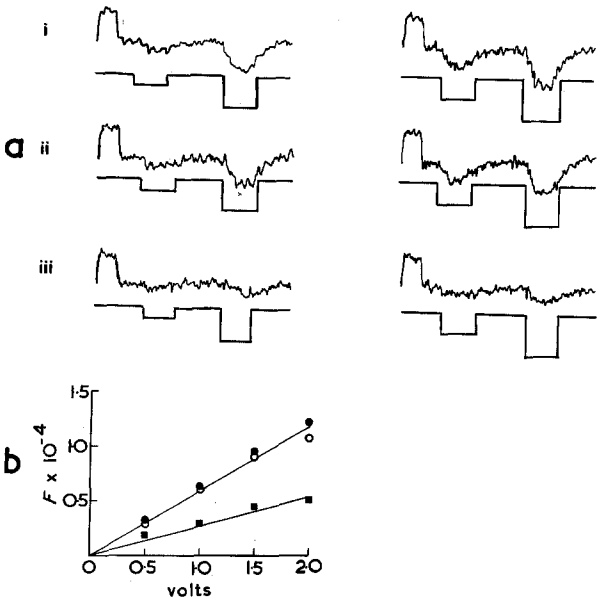


FIG. 1. Effect of procaine on ANS fluorescence changes associated with nerve hyperpolarisation.
(a) Double-pulse records, with 20msec hyperpolarising pulses, and stimuli, from left to right, of 0.5, 1.5, 1.0, 2.0 volts; (i) control fluoresence responses; (ii) after addition of ANS-containing Ringer; (iii) after addition of procaine to give a bath concentration of 5mM; calibration pulse on left of records 10^{-4} of background fluorescence; ANS 0.1mM; 128 sweeps averaged; sweep cycle 160msec.
(b) Plot of hyperpolarising stimulus versus fluorescence intensity change, shown as fraction of background intensity; ● , control; ○ , Ringer added; ■ , procaine added.

Using conventional intracellular recording techniques, the imposed hyperpolarisation of single muscle fibres was measured in the region of uv irradiation. It was found that procaine, in the concentration used for the optical experiments, had no effect on the magnitude of the electrical signal.

ANS Binding to Muscle

Using a perfusion system, the fluorescence output was measured as a function of ANS concentration, in the range 0.001mM-0.08mM. A double-reciprocal plot from four such experiments indicated a deviation from linearity at high concentrations. However, after a correction factor was applied, taking into account the solution path-length and the extinction coefficient of ANS, a linear relationship was obtained, with an apparent association constant of 14μM (±S.D.6μM). This absorption effect was also observed by

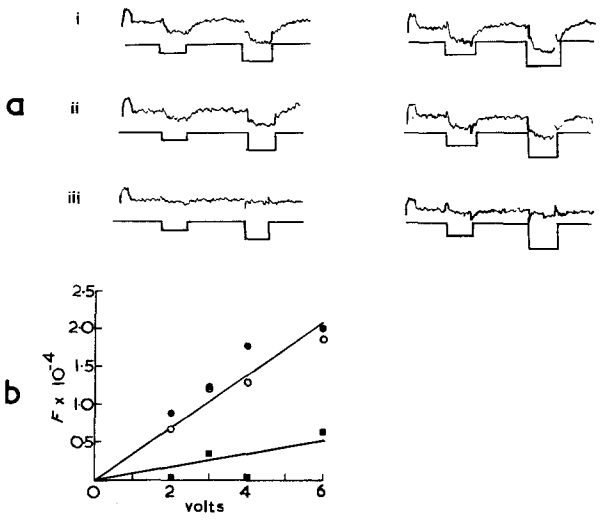


FIG. 2. Effect of stimulus strength and procaine on ANS fluorescence changes associated with muscle hyperpolarisation.
(a) As in FIG. 1., with hyperpolarising stimuli, from left to right, of 2, 4, 3, 6 volts; 256 sweeps averaged.
(b) Plot of hyperpolarising stimulus versus fluorescence intensity change, shown as fraction of background intensity; ● , control; ○ , frog Ringer added; ■ , procaine added.

Gomperts and Stock (14) in their measurements of ANS binding to rat brain microsomes. To estimate the total amount of dye bound, whole sartorius muscles were incubated for one hour in Ringer containing 0.01mM ANS. The muscles were removed, blotted, weighed, and shaken in 5ml of ethanol, aliquots of which were measured on a fluorimeter (λ_{ex}366nm, λ_{em}440nm). The ANS concentrations were found by comparison with standard solutions. From the dose-response data, the amount of ANS bound at the saturating concentration was found to be, on average, 0.06μmole/g tissue weight.

The effect of procaine on ANS binding was determined by measuring the fluorescence output of sartorius muscle in Ringer containing 0.008mM ANS. In the presence of 10mM procaine, no detectable change in muscle fluorescence was observed after 15 minutes incubation.

DISCUSSION

In skeletal muscle, the membrane ANS fluorescence changes produced by changes in membrane potential are reduced or abolished in the presence

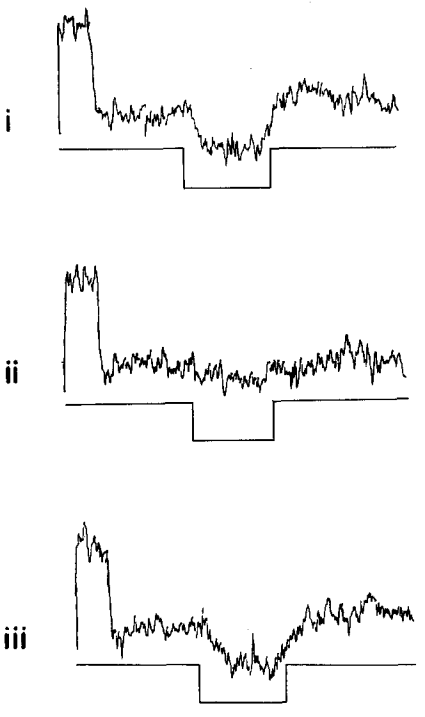


FIG. 3. Perfusion experiment on sartorius muscle, showing ANS fluorescence change during a 30msec hyperpolarising stimulus of 3 volts. (i) in control Ringer; (ii) in 10mM procaine; (iii) after wash-out; calibration signal on left of records; ANS 0.01mM; 128 sweeps averaged.

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of procaine, in concentrations which affect many other aspects of membrane function. Moreover, it has been shown that the effect is not explained by changes in the imposed voltage steps due to procaine. Since most reports suggest that procaine does not greatly alter the resistance of axonal membrane (15), the effects on nerve would seem to be similar. Conti and Wanke (11) found that extremely high procaine concentrations (90-180mM) only reduced by 30% the voltage-induced fluorescence changes in squid giant axon. However, in their experiments, ANS and procaine were applied on opposite sides of the membrane, and at the pH of the medium, procaine would be almost completely ionized, and unable to penetrate the membrane. Therefore, these two observations taken together provide rather strong evidence that the ANS molecules responsible for the fluorescence changes, bind at or near the membrane surface rather than to the bulk lipid phase. Furthermore, the polarity of the ANS signal depends on which side it is applied. ANS

bound to the inside or outside will have opposite polarities relative to the electric field.

Although the effects of procaine can only be properly interpreted when the molecular basis for the ANS change has been established, three possible mechanisms appear plausible. Namely, procaine tends to decrease (a) the binding of 'active' ANS, (b) the field sensed by ANS, (c) the responsiveness of ANS to the field. ANS binding to membrane surfaces is thought to be non-specific (it is seen in bilayers), and Eisenmann (16) has shown it to be quantitatively determined by the surface charge. Protonated procaine will tend to reduce the surface negative charge, and should enhance ANS binding by reducing coulombic repulsion. This would not explain the present observations, although such a phenomenon has been variously reported (17,18,19,20). The ANS participating in the fluorescence change probably represents a negligible fraction of the total bound ANS, and an alteration in the binding of this 'active' ANS might not be detectable. Although there is no direct evidence that the overall binding is to axon membrane, in muscle this is likely, because of the absence of other cellular elements. It is also possible that, as Landowne has suggested for snake skeletal muscle (21), a portion of the fluorescence signal originates from ANS bound to the T-system.

The membrane field could affect the binding, orientation, or quantum yield of fixated ANS. Most of the membrane potential is dropped across the bulk lipid. If only a small fraction is sensed by 'active' ANS, this might explain the low magnitude of the changes. It is conceivable that procaine alters the fraction of the potential dropped across the bulk lipid by, perhaps, altering its dielectric constant.

The most plausible mechanism is that procaine alters the microenvironment of ANS such that the electronic effects induced by the field, are rendered unfavourable. The field could decrease the membrane penetration of ANS so that the polarity of the environment is increased. This would result in a greater transfer from the singlet to the triplet state (22), and thus reduce the quantum yield. A change in local viscosity or lateral pressure, by procaine, could affect the probability of this transfer.

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