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TRANSIENT FLUORESCENCE SIGNALS FROM PYRENE LABELED PIKE NERVES DURING ACTION POTENTIAL POSSIBLE IMPLICATIONS FOR MEMBRANE FLUIDITY CHANGES

Dinu Georgescauld and Hervé Duclohier

Centre de Recherche Paul Pascal (C.N.R.S.)
Domaine Universitaire, 33405 Talence, France

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SUMMARY : *Pike olfactory nerves labeled with pyrene and illuminated at 340 nm showed a highly resolved monomer fluorescence emission and a broad excimer emission band at longer wavelength. The excimer formation being controlled by lateral diffusion in the membrane lipids, the ratio of both maxima emission amplitudes is a fluidity parameter and was found to depend on temperature. When these nerves were stimulated, this ratio (F) underwent a small transient decrease ($\frac{\Delta F}{F}$ range = 10^{-3} to 10^{-4}), synchronous with the propagated impulse. These findings may be interpreted as a transient decreased fluidity of the membrane lipids during excitation*

INTRODUCTION

Optical properties of nerves have been recently studied in an attempt to understand the structural basis of nerve membrane excitation. Among these various experiments, birefringence (as well as thermal) changes suggest a transient increased order of the membrane during action potential (1,2). This hypothetical entropy change may be tested, at the microscopic level, by molecular probes specific for a membrane component and for fluidity changes.

In order to measure the fluidity of the hydrocarbon core of the nerve membrane, we selected a strictly hydrophobic fluorescent probe, pyrene, which has been shown to dissolve in the lipid interior of the membrane (3,4). Detailed photophysical studies in homogeneous solution (5) and in artifical and natural membranes (6,7) point out that excimer formation for pyrene is diffusion controlled and consequently exceedingly sensitive to the microviscosity of its environment.

The quantity of interest is the ratio $F = \frac{I_E}{I_M}$, where I_E and I_M are the intensities of light emitted respectively by pyrene excimer and pyrene monomer. Changes in this ratio reflect changes in the rate of encounter which in turn are sensitive to the local viscosity around each pyrene molecule. In this communication, we report a small transient decrease of the **F** ratio for pyrene labeled nerves during the action potential.

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MATERIALS AND METHODS

The biological material used was the olfactory nerve of the pike "Esox lucius" which has been shown to give large birefringence and thermal spikes (1,2). This unmyelinated nerve presents a high density of axonal membranes and a rather homogeneous size distribution of fibers diameter. Nerve dissection and composition of the pike physiological solution are given in (1).

Labeling procedure was as follows : isolated nerves were incubated two hours in pike solution containing bovine serum albumin (BSA) which has trapped pyrene molecules. Pyrene-BSA complexes were prepared according to (8), allowing a good transfer rate of the highly hydrophobic pyrene molecules from charged BSA to nerve membranes. Then, nerves were washed out several times in order to eliminate remaining complexes and discharged albumins. Below a critical probe concentration ($\frac{\text{pyrene mol.}}{\text{lipids mol.}} : 2.10^{-2}$) the labelled nerves excitability was apparently unaltered as tested with the externally recorded compound action potential.

Fluorescence spectra of labeled nerves in the resting state were obtained from a differential spectrofluorimeter (FICA 55 MK II), which produced spectra corrected in both excitation and emission modes. The nerves were mounted vertically in a calibrated quartz capillary at the centre of a fluorescence cuvette filled with physiological solution. The temperature dependent variations of fluorescence spectra were carried out by means of a water jacketed cell holder which controlled temperature within 0.1°C. For each temperature, if was allowed 15 minutes for equilibrium to be reached. Experiments were usually run in order of increasing temperature. Full details on labeling method and spectroscopic analysis are given elsewhere (9).

Recording of the fast transient fluorescence signals during excitation . The labeled nerve was placed into a lucite chamber provided with isolated partitions filled with pike solution and platinum electrodes for external stimulation and recording of compound action potentials. A quartz window sealed on the side of the central pool allowed nerve illumination. Incident light from a 150 W Xenon arc lamp DC stabilized was made quasi-monochromatic at 340 nm by an ORIEL interference filter and was focused on the nerve by a quartz cylindrical lens. Emitted fluorescence passed (at 90° relative to the incident beam) through a 5 mm long quartz window on the lid of the chamber and the emission filter whose centre was at 400 nm for investigating monomer signals and 480 nm for excimer signals. Small changes in the light detector output (RTC XP 2000 photomultiplier tube) were sent, through an operational amplifier (10 ms rising time), to an averaging computer (Alpha LSI 3/05).

RESULTS AND DISCUSSION

When illuminated at the maximum absorption wavelength of pyrene (340nm), the labeled nerve fibres showed a characteristic fluorescence spectrum (fig. 1), with a highly resolved monomer emission and a broad emission band attributed to the excimer. The fluorescence excitation spectra of both monomer (I-a) and excimer (I-b) were identical with the absorption spectrum of pyrene, indicating that excimer emission did not derive from pyrene aggregates (7).

As shown in fig.2, the $\frac{I_E}{I_M}$ ratio of pyrene labeled nerves increased monotonously with temperature in the physiological range and presented a slope change at about 19°C. This temperature dependence which has also been in -

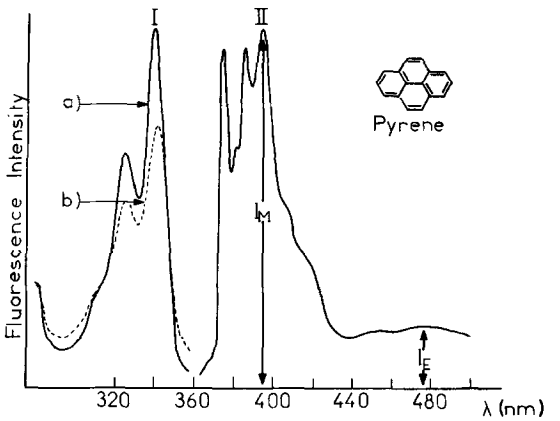

Figure 1 : *Excitation and fluorescence emission spectra of a pike olfactory nerve labeled with pyrene. Concentration of labeling solution : 4.10^{-6}M BSA-pyrene complexes with 10 molecules of pyrene trapped in each albumin. Incubation time : 2 hours, two rinsings, 30 mn each.*

Spectrum I : excitation spectra of monomer (a) and excimer (b) ; respective emission wavelength : 395 and 480 nm ; (b) amplified four times in respect to (a)

Spectrum II : emission spectrum (excitation wavelength : 340 nm)

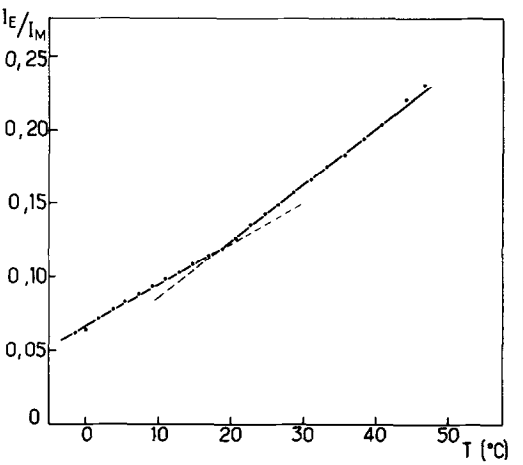

Figure 2 : *Temperature dependence of the $\frac{I_E}{I_M}$ ratio for a pike olfactory nerve labeled with pyrene in the same conditions as for fig. 1*

vestigated on other labeled nerve preparations (10) was very similar to the behaviour of pyrene labeled artificial lipid membranes (11). This result is interpreted as reflecting an increase of the fluidity of the hydrocarbon core of the mer brane with temperature. All experimental evidence points out the normal photophysical properties of pyrene molecules dissolved in the nerve membranes.

Fig. 3 presents the two transient fluorescence signals (at 400nm for monomer and 480nm for excimer) recorded one after another when the labeled nerves were stimulated. Usually, a few tens of sweeps were necessary to improve sufficiently signal to noise ratio and visualization of both optical signals which appeared synchronous with the propagated impulse. Monomer and excimer signals respectively showed a transient increase of monomer emission and a transient decrease of excimer emission ($\frac{\Delta I_M}{I_M} > 0$ and $\frac{\Delta I_E}{I_E} < 0$).

These signals were missing on unlabeled nerves, so there was no interference from light scattering during the nerve impulse. Furthermore, the fluorescence signals were associated in time with action potential, and they were not electro-optical coupling with stimulus artifacts as no signals could be recorded from dead labeled nerves which were stimulated.

These results require an explanation in terms of factors which affect the encounter dynamics. Other explanations, such as quenching of pyrene molecules by oxygen or other species would cause both I_E and I_M to decrease (12). Solvent effects, known to affect other fluorescent probes are remarkably absent in pyrene excimer formation (5). So there must be a transient increase in the local viscosity or alternatively an increase in the volume of the lipid environment which would dilute the probe molecules. Since the thermal and birefringence experiments (1,2) do not support this latter hypothesis, we consider our results confirm on a molecular level, a decreased fluidity of the bulk lipid region of axonal membranes during the action potential (average $\frac{\Delta F}{F} = -5.10^{-4}$ per impulse). Indeed, the monomer-excimer equilibrium at a given temperature : pyrene* + pyrene $\underset{}{\overset{K_{ME}}{\rightleftharpoons}}$ (pyrene - pyrene)* (excited state : *) must be modified during the passage of the nerve impulse, the rate constant K_{ME} being proportional to $\frac{1}{\eta}$, where η is the medium viscosity coefficient.

With regards to the mechanism underlying this decreased fluidity, at this stage, only hypothesis can be put forward. The more common explanation is the effect of the electric field on the bulk of membrane lipids (1,2). An alternative could be a genuine effect of the functioning of ionic channels on the fluidity of boundary lipids which has been recently postulated as an important parameter in the mechanism of nerve excitation (13,14). Our results could be considered as a positive experimental test of this idea although the main criticism is the sparsity of ionic channels compared with the probable homogeneous distribution of pyrene in the whole lipid phase. Nevertheless, the available but partial structural data on nerve membrane and ionic channels allow us to estimate the fraction of lipids that might be affected by the conformational change of the channel. This estimation is based on the following figures : a) the density of sodium channels for the pike olfactory nerve is unknown but we think it to be clo

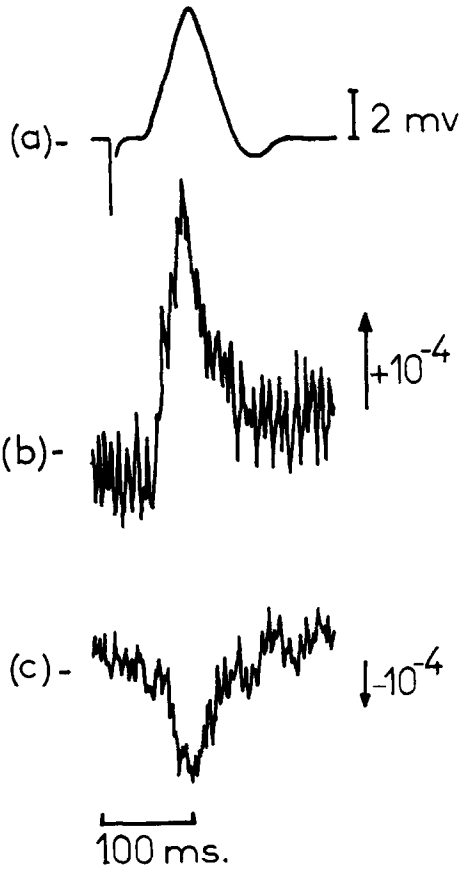

Figure 3 : *Transient fluorescence signals during excitation (same nerves and labeling conditions as in figure 1).*

(a) : oscilloscope photograph of the propagated compound action potential. Delay time between stimulus and the foot of electrical signal has been corrected according to propagation velocity and distance between nerve illuminated portion and recording electrodes. Repetition rate : 1.4 s

(b) : fluorescence signal from monomer (emission filter : 400nm ± 5nm)

(c) : fluorescence signal from excimer (emission filter : 480nm ± 5nm)

60 sweeps averaged for both signals. Vertical arrows on the right : sign and magnitude for a relative intensity change $\frac{\Delta I}{I} = 10^{-4}$ *per sweep. Signal to noise ratio : 0.4 per sweep. T =15°C*

se to the one of garfish olfactory nerve : 40/μm^2 (15) ; b) the number of lipids μm^2 is about 3,6.10^6 for the bilayer (14). The correction for membrane proteins is not taken into account (mean lipid area = 60 Å^2). These first two points give on a average 9.10^4 lipids for a sodium channel. c) if one accept that the sodium

channel is a membrane protein with a molecular weight of about 250 000 and a diameter of 80 Å (16), the number of boundary lipids which form the annulus may be estimated at about 80 for the bilayer. Considering a short range cooperative effect, we can speculate that as much as 3 concentric lipid layers surrounding the channel might be laterally compressed by a volume increase or a shape change of the pore.

Thus, 1°/₀₀ to 3°/₀₀ of the total membrane lipids may "feel" the conformational change of the channel, values consistent with the small transient fluorescence changes reported. It is obvious that these approximations are very speculative and only further experiments on pyrene labeled giant axons under voltage clamp-conditions, which are under way in our laboratory, will confirm or not this tentative hypothesis.

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