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Spectra of voltage-sensitive fluorescence of styryl-dye in neuron membrane

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The voltage sensitivity of fluorescence of an aminobenzstyryl-pyridinium dye (di4-ANEPPS) is characterized in Retzius cells dissociated from the leech. The modulation of the complete spectra of excitation and emission is determined. The spectral changes induced by depolarization are described by a blue shift of the absorption spectrum, by a weaker blue shift and an enhanced width of the fluorescence spectrum and by a decrease of the yield of fluorescence. These changes are attributed tentatively to a superposition of electrochromism and of field-induced resolvation.

Introduction

Fluorescent dyes are used as indicators of fast changes of the membrane potential in neurons [1,2]. Among the best probes being used are homologs of aminostyrylpyridinium ('styryl dyes') [3–6]. The sensitivity of these dyes is low, their photochemical stability is limited and their phototoxicity is often detrimental. Distinctly improved dyes are required to study signal processing within single neurons at high resolution. A molecular design of probes is impossible as long as the mechanism of voltage sensitivity is unknown.

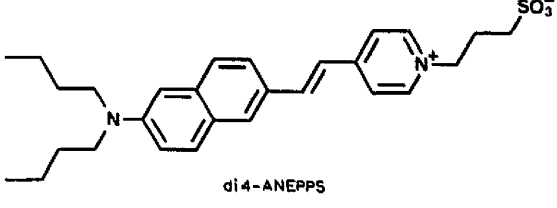
We follow three routes to obtain information about the processes underlying voltage-sensitive fluorescence: (i) Analysis of the photophysics in homogeneous solution [7,8], (ii) analysis of the voltage sensitivity in artificial membranes and (iii) analysis of voltage sensitivity in neurons. In the present paper we describe results concerning the third aspect.

We choose Retzius cells of the leech [9] stained with dibutylaminonapthylethylenepyridiniumpropylsulfonate (di4-ANEPPS) [6]. We determine the voltage sensitivity of the complete spectra of excitation and emission. We describe the data in terms of elementary changes of the spectra as of amplitude, position and width. We discuss the results in terms of possible mechanisms of voltage sensitivity.

Materials and Methods

Neurons and dye

Ganglia of the leech *Hirudo medicinalis* (Biopharm/Swansea) were dissected and pinned on a Sylgard coated dish in Leibowitz-15 medium (Gibco/Eggenstein) with 50 μg/ml gentamycinsulfate (Sigma/Heidelberg) and 6 mg/ml glucose. After opening the tissue capsules the ganglia were incubated in dispase/collagenase (Boehringer/Mannheim; 2 mg/ml L-15 medium) for 1 h at room temperature. The Retzius cells (soma with 50–100 μm of neurite) were dissociated by aspiration into a fire-polished micropipette and washed by transferring them through several drops of L-15 medium [9]. Cover slips were attached to silicone chambers (Flexiperm-mikro 12, Heraeus/Hanau) and coated by an extract of the extracellular matrix of the leech [10,11]. The cells were plated in L-15 medium with 2% foetal calf serum (Gibco).



di4-ANEPPS

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Dibutylaminonaphthylethylenepyridiniumpropylsulfonate (di4-ANEPPS) (Molecular Probes/Junction City) was dispersed in leech Ringer (115 mM NaCl, 1.8 mM $CaCl_2$, 4 mM KCl, 10 mM Tris-maleate (pH 7.4)) at a concentration of 1 mM with 20 mM egg lecithin (Sigma) by sonication (4 × 5 min with intervals of 3 min at 0 °C, Branson sonifier, level 1.5). After centrifugation the suspension of vesicles (diameter 80 nm) was added to the chambers up to a dye concentration of 4–10 μM. The external side of the plasma membrane was stained [11]. Submicroscopic undulations of the plasma membrane lead to a rather random orientation of the dye with respect to a macroscopic normal, whatever the molecular alignment may be.

Optoelectronics

A chamber with a stained neuron was mounted on an inverted microscope (Axiovert, Zeiss/Oberkochen) (Fig. 1). The central part of the arc of a xenon high pressure lamp (150 W, Hamamatsu) was imaged onto the neuron through a continuous interference filter (Veril BL 200, Schott/Mainz), a shutter, a dichroitic beam splitter (FT 510 or FT 580, Zeiss) and an objective of high aperture (Planapo 100x/1,3 Oel pH3, Zeiss). The lower part of the neuron was projected onto the first image plane through the same objective of high aperture, the beam splitter and a cut-off filter (RG 520 or RG 590, Schott). An area of 30–40 μm diameter of the plasma membrane was selected by a diaphragm. The light was focussed onto the cathode (S-20) of a photomultiplier (type C31034, RCA) through a second continuous filter.

The optical set-up excludes an observation of optical effects which are related directly to a reorientation of the dye: (i) Illumination and detection are unselective with respect to the polarization of light. (ii) The aperture angle of illumination and detection is very large, about 120°. (iii) Within a field of 30–40 μm diameter the orientation of the undulated membrane is randomized.

The position of the continuous filters was calibrated by narrow band interference filters (Schott). Wavenumbers $\bar{\nu}_I$ or $\bar{\nu}_D$ of illumination and detection were assigned according to the data sheets. Gaussian transmission curves of halfwidths 1270 cm^{-1} (illumination) and 1040 cm^{-1} (detection) were assigned on the basis of the data sheets. The relative spectrum of yield of the detection system $D(\bar{\nu}_{EM})$ (fraction of emitted quanta which are detected) for a wavenumber of emission $\bar{\nu}_{EM}$ was evaluated as a product of the multiplier response, the characteristics of beam splitter and cut-off filter and the transmission of the continuous filter. The relative spectrum of illumination $I(\bar{\nu}_{EX})$ (quanta per area, per time, per wavenumber interval) at a wavenumber $\bar{\nu}_{EX}$ of excitation was obtained by illuminating a MgO-coated slide [12] in the object plane and measuring the scattered light at every position $\bar{\nu}_I$ without continuous filter in the detection tract. $I(\bar{\nu}_I)$ was determined by correcting for the sensitivity of detection. Due to the flat spectrum of the lamp it was identified with $I(\bar{\nu}_{EX})$ without deconvolution.

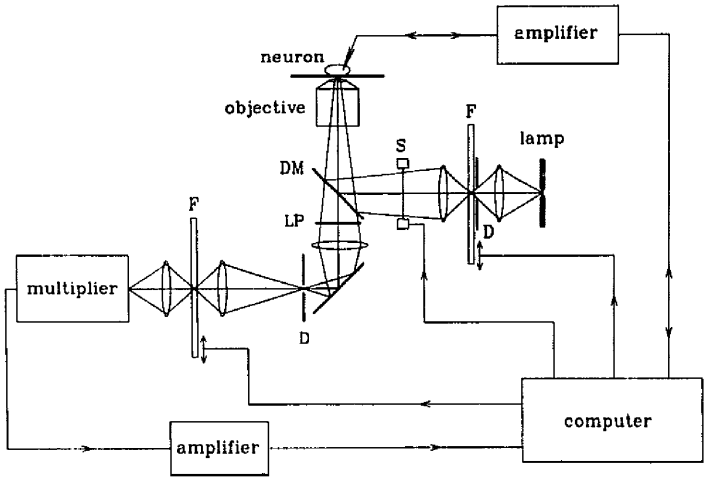

Fig. 1. Opto-electronical set-up. A stained neuron is mounted in the object plane of an inverted microscope. It is illuminated by a high-pressure Xe-lamp through a diaphragm (D), a continuous interference filter (F), a shutter (S), a dichroitic mirror (DM). The fluorescence is detected by a photomultiplier through the dichroitic mirror, a low-pass filter (LP), a diaphragm (D) and a continuous interference filter (F). The cell is stimulated by current injection through a microelectrode. Fluorescence intensity and voltage are recorded at various wavenumbers of excitation and emission.

Emission spectra were measured by scanning $\bar{\nu}_D$ at a selected wavenumber of illumination $\bar{\nu}_I^*$ using beam splitter FT 510 and filter RG 520. Excitation spectra were measured by scanning $\bar{\nu}_I$ at a selected wavenumber of detection $\bar{\nu}_D^*$ using beam splitter FT 580 and filter RG 590. The excitation spectrum reflects the absorption spectrum as the shape of both spectra of di4-ANEPPS are in complete agreement as checked in a series of solvents.

To determine voltage sensitivity we impaled the neuron by a microelectrode (tip diameter < 1 μm filled with 3 M KCl) attached to a micromanipulator (Leitz/Wetzlar). The electrode was used for stimulating action potentials by current injection and for recording the time-dependent membrane potential $V(t)$. Each neuron was tested 1 h after addition of the staining solution by stimulation. We used only neurons with action potentials of a minimal amplitude of 60 mV. The cells were held at a potential of about -60 mV to suppress spontaneous spiking. The voltage sensitivity of the emission spectrum at a given $\bar{\nu}_I^*$ was measured as follows: (1) Selection of wavenumber $\bar{\nu}_D$. Opening of shutter (time zero). (2) Start of current injection. Record of time-dependent fluorescence signal $S(t)$ and of voltage $V(t)$ (time 5 ms). The maximal signal-to-noise ratio (as referred to the change of fluorescence at the peak of the action potential) was about five. (3) Stop of current injection. End of record (time 30 ms). Closing of shutter. After a pause of about 2 s the sequence 1–3 was repeated at a changed wavenumber $\bar{\nu}_D$. The sensitivity of the excitation spectrum was measured similarly by variation of $\bar{\nu}_I$ at fixed $\bar{\nu}_D^*$. For every measurement the relative change of signal was evaluated as $\Delta S/\bar{S} = (S(t) - \bar{S})/\bar{S}$ with respect to the average signal $\bar{S}$. Using this internal normalization we avoided enhanced photodynamic damage by a second illumination without stimulation. The error was negligible. $(S(t) - \bar{S})/\bar{S}$ was plotted versus $V(t)$. The sensitivity (the relative change of fluorescence per voltage change) was evaluated by linear regression.

Evaluation

If N dye molecules in the membrane are excited by monochromatic light of wavenumber $\bar{\nu}_{EX}$ and if the emitted light is observed at a defined wavenumber $\bar{\nu}_{EM}$ the fluorescence signal $S(\bar{\nu}_{EX}, \bar{\nu}_{EM})$ depends on the intensity of illumination $I(\bar{\nu}_{EX})$ (quanta per area, per time, per wavenumber interval), on the absorption spectrum $a(\bar{\nu}_{EX})$ (effective cross section), on the emission spectrum $e(\bar{\nu}_{EM})$ (fraction of the absorbed quanta which are emitted per wavenumber interval) and on the yield of the detection system $D(\bar{\nu}_{EM})$ (fraction of the emitted quanta which are detected) according to Eqn. 1. The spectra $a(\bar{\nu}_{EX})$ and $e(\bar{\nu}_{EM})$ refer to the direction and polarization of incident and detected light of the given set-up. For the present we assume that all N dye molecules have the same properties.

$$S(\bar{\nu}_{EX}, \bar{\nu}_{EM}) = N \cdot I(\bar{\nu}_{EX}) \cdot a(\bar{\nu}_{EX}) \cdot e(\bar{\nu}_{EM}) \cdot D(\bar{\nu}_{EM}) \tag{1}$$

A change ΔV of the membrane potential causes changes of $a(\bar{\nu}_{EX})$ and $e(\bar{\nu}_{EM})$. In the case of small spectral changes the change ΔS of signal is expressed by Eqn. 2. The relative change $\Delta S/S$ is a superposition of the relative changes of absorption and emission according to Eqn. 3. ΔS and $\Delta S/S$ are proportional to ΔV if the spectral changes are linear in the voltage change.

$$\Delta S(\bar{\nu}_{EX} \cdot \bar{\nu}_{EM}) = N \cdot I(\bar{\nu}_{EX})(e \cdot \Delta a + a \cdot \Delta e) \cdot D(\bar{\nu}_{EM}) \tag{2}$$

$$\frac{\Delta S(\bar{\nu}_{EX}, \bar{\nu}_{EM})}{S(\bar{\nu}_{EX}, \bar{\nu}_{EM})} = \frac{\Delta a}{a} + \frac{\Delta e}{e} \tag{3}$$

We describe a spectral change by a relative change of amplitude, by a shift of the maximum and by a change of halfwidth. We obtain Eqn. 4 where ΔA_{EX} and ΔA_{EM} are relative changes of amplitude, ΔM_{EX} and ΔM_{EM} are shifts of the maxima and ΔW_{EX} and ΔW_{EM} are changes of width. The shifts are weighted by the first derivatives a' and e'. The changes of width are weighted by the second derivatives a'' and e''. The latter expressions are strictly valid for Gaussian spectra.

$$\begin{aligned} \Delta S/S = {} & \Delta A_{EX} + \Delta A_{EM} - (a'/a) \cdot \Delta M_{EX} \\ & - (e'/e) \cdot \Delta M_{EM} \\ & + (a''/(a \cdot 8 \cdot \ln 2)) W_{EX} \cdot \Delta W_{EX} \\ & + (e''/(e \cdot 8 \cdot \ln 2)) W_{EM} \cdot \Delta W_{EM} \end{aligned} \tag{4}$$

We illuminate and detect with broad, bell-shaped spectra of illumination and detection centered around wavenumbers $\bar{\nu}_I$ and $\bar{\nu}_D$. The signal $S(\bar{\nu}_I, \bar{\nu}_D)$ and the change $\Delta S(\bar{\nu}_I, \bar{\nu}_D)$ result from integrations over the spectra of illumination and detection. With $I(\bar{\nu}_{EX}) \approx I(\bar{\nu}_I - \bar{\nu}_{EX})$ and $D(\bar{\nu}_{EM}) \approx D(\bar{\nu}_D - \bar{\nu}_{EM})$ we obtain Eqns. 5 and 6 where the brackets denote convolution as e.g.

$$\langle a \rangle = \int \mathrm{d}\bar{\nu}_{EX} a(\bar{\nu}_{EX}) I(\bar{\nu}_I - \bar{\nu}_{EX})$$

and

$$\langle e\rangle = \int d\bar{\nu}_{EM} e(\bar{\nu}_{EM}) D(\bar{\nu}_D - \bar{\nu}_{EM}).$$

$$S(\bar{\nu}_I, \bar{\nu}_D) = N \cdot \langle a\rangle \cdot \langle e\rangle \tag{5}$$

$$\frac{\Delta S(\bar{\nu}_I, \bar{\nu}_D)}{S(\bar{\nu}_I, \bar{\nu}_D)} = \Delta A_{EX} + \Delta A_{EM} - \frac{\langle a'\rangle}{\langle a\rangle} \cdot \Delta M_{EX} - \frac{\langle e'\rangle}{\langle e\rangle} \cdot \Delta M_{EM} + \frac{\langle a''\rangle W_{EX}}{\langle a\rangle \cdot 8 \cdot \ln 2} \cdot \Delta W_{EX} + \frac{\langle e''\rangle W_{EM}}{\langle e\rangle \cdot 8 \cdot \ln 2} \cdot \Delta W_{EM} \tag{6}$$

We scan the detection $\bar{\nu}_D$ at a given wavenumber of illumination $\bar{\nu}_I^*$ and scan the illumination $\bar{\nu}_I$ at a given wavenumber of detection $\bar{\nu}_D^*$. The emission spectrum $S(\bar{\nu}_I^*, \bar{\nu}_D)$ and the excitation spectrum $S(\bar{\nu}_I, \bar{\nu}_D^*)$ are proportional to $\langle e\rangle$ and $\langle a\rangle$, respectively, according to Eqn. 5. The spectra $a(\bar{\nu}_{EX})$ and $e(\bar{\nu}_{EM})$ are obtained by deconvolution of the two spectra up to constant factors.

We characterize voltage sensitivity by scanning $\bar{\nu}_D$ at given $\bar{\nu}_I^*$ and by scanning $\bar{\nu}_I$ at given $\bar{\nu}_D^*$. The sensitivity of emission $\Delta S(\bar{\nu}_I^*, \bar{\nu}_D)/S(\bar{\nu}_I^*, \bar{\nu}_D)$ is fitted by ΔW_{EM}, ΔM_{EM} and a change ΔT_{EM} of total amplitude which comprises ΔA_{EM} and all changes of the excitation spectrum at $\bar{\nu}_I^*$ according to Eqn. 6. On the other hand, the sensitivity of excitation $\Delta S(\bar{\nu}_I, \bar{\nu}_D^*)/S(\bar{\nu}_I, \bar{\nu}_D^*)$ is fitted by ΔW_{EX}, ΔM_{EX} and a change ΔT_{EX} of total amplitude which comprises ΔA_{EX} and the changes of the emission spectrum at $\bar{\nu}_D^*$. From ΔT_{EM} we may evaluate $\Delta A_{EX} + \Delta A_{EM}$ using ΔM_{EX} and ΔW_{EX} according to Eqn. 6. From ΔT_{EX} we may evaluate $\Delta A_{EX} + \Delta A_{EM}$ as well using ΔM_{EM} and ΔW_{EM}.

Different dye molecules may exhibit different spectra and different sensitivities due to a different environment or a different orientation with respect to incident light, detection system and electrical field. We consider here a minimal two state model: N_A of N probe molecules are sensitive and N_B probe molecules form an insensitive background. The spectra of the two types of molecules are assumed to be identical. The observed sensitivity is given by the sensitivity of the active molecules as weighted by the fraction of active molecules $\alpha = N_A/(N_A + N_B)$ according to Eqn. 7. The observed sensitivity is lower than the sensitivity of active molecules. In a set of samples with variable background N_B the largest signal observed reflects best the features of the active probe.

$$\left(\frac{\Delta S(\bar{\nu}_I, \bar{\nu}_D)}{S(\bar{\nu}_I, \bar{\nu}_D)}\right)_{OBS} = \alpha \cdot \frac{\Delta S(\bar{\nu}_I, \bar{\nu}_D)}{S(\bar{\nu}_I, \bar{\nu}_D)} \tag{7}$$

Results

Spectra

We measured the emission spectrum $S(\bar{\nu}_I^*, \bar{\nu}_D)$ of di4-ANEPPS bound to the soma of Retzius cells at a wavenumber of illumination $\bar{\nu}_I^* = 20750\ cm^{-1}$ and the excitation spectrum $S(\bar{\nu}_I, \bar{\nu}_D^*)$ at a wavenumber of detection $\bar{\nu}_D^* = 16400\ cm^{-1}$. Both spectra – averages from several cells – are shown in Fig. 2. The maximum of the emission spectrum was at $M_{EM} = 16550\ cm^{-1}$, the maximum of the excitation spectrum at $M_{EX} = 20650\ cm^{-1}$. Spectra measured on stained connective tissue adherent to the neurons were found to be indistinguishable.

For comparison we measured the spectra in two bulk solvents. The absorption maxima in acetonitrile and chloroform were $M_{EX} = 20250\ cm^{-1}/18550\ cm^{-1}$, the emission maxima were $M_{EM} = 13750\ cm^{-1}/14800\ cm^{-1}$. The neuron appeared to be more polar then acetonitrile with respect to absorption, but appeared to be more unpolar than chloroform with respect to emission. A similar pair of incompatible quasi-polarities was found for di4-ANEPPS bound to cardiolipin vesicles and to bovine serum albumin with $M_{EX} = 20650\ cm^{-1}/20200\ cm^{-1}$ and $M_{EM} = 15500\ cm^{-1}/15350\ cm^{-1}$. Thus the spectra of di4-ANEPPS in the plasma membrane indicate a binding site which may resemble a lipid bilayer, a membrane protein or an interface of lipid and protein. 'Super-polar' absorption and 'super-unpolar' emission are due to anisotropic solvation in lipid or protein [8,13].

Sensitivity

We measured the change $\Delta S(\bar{\nu}_I^*, \bar{\nu}_D)$ of the emission spectrum as induced by 100 mV of depolarization. The wavenumber of illumination was $\bar{\nu}_I^* = 20750\ cm^{-1}$. An example is plotted in Fig. 2. The change was negative for most wavenumbers with a maximal response close to the emission maximum. The change was positive at the blue end of the spectrum. Similarly we measured the change $\Delta S(\bar{\nu}_I, \bar{\nu}_D^*)$ of the excitation spectrum. The wavenumber of detection was $\bar{\nu}_D^* = 16400\ cm^{-1}$. The result for the same neuron is plotted in Fig. 2. The change was negative for all wavenumber with a maximal response towards the red of the excitation (absorption) maximum. The spectra of voltage sensitivity, i.e., the relative change $\Delta S(\bar{\nu}_I^*, \bar{\nu}_D)/S(\bar{\nu}_I^*, \bar{\nu}_D)$ of the emission spectrum and the relative change

$\Delta S(\bar{\nu}_I, \bar{\nu}_D^*)$ of the excitation spectrum, are plotted in Fig. 2. The sensitivity is negative at low wavenumbers and positive at high wavenumbers in the emission spectrum. The sensitivity is negative in the total range of the excitation spectrum.

Considering Fig. 2 it is apparent that the spectral changes cannot be assigned to mere shifts of absorption and emission spectra. In a first approach we assumed a superposition of spectral shift and changed amplitude. We fitted the data according to Eqn. 6 omitting effects of broadening. As shown in Fig. 3 it was possible to describe the general trend of the sensitivity of emission chosing a blue shift $\Delta M_{EM} = 44\ cm^{-1}$ and a drop of total amplitude $\Delta T_{EM} = -2.6\%$. The rms deviation was 1.3%. The sensitivity of excitation was described perfectly by a blue shift $\Delta M_{EX} = 60\ cm^{-1}$ and a drop of total amplitude $\Delta T_{EX} = -3.7\%$. The rms-deviation was 0.8%.

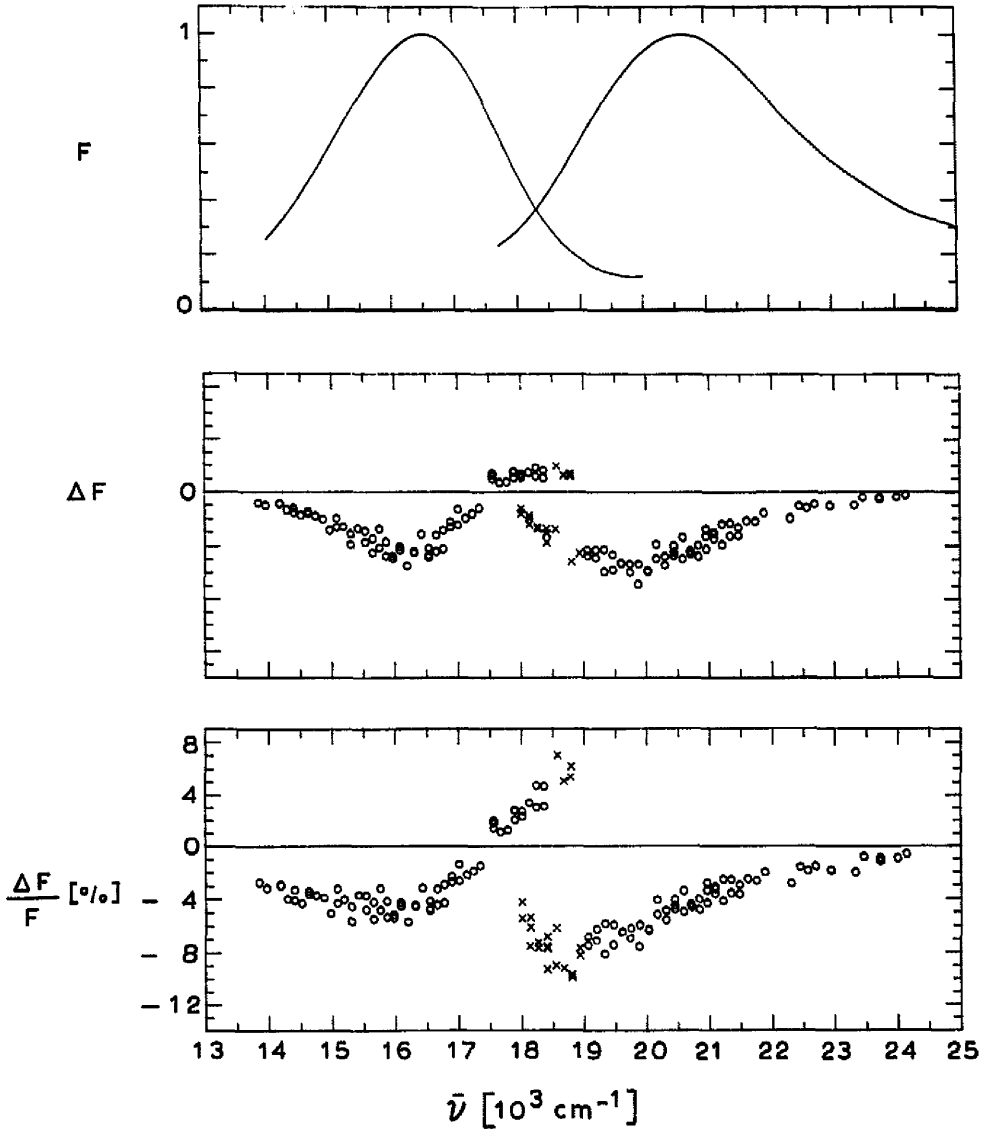

Fig. 2. Fluorescence of di4-ANEPPS in Retzius cell versus wavenumber of illumination and detection. Emission spectra (left) at a wavenumber of illumination $\bar{\nu}_I^* = 20750\ cm^{-1}$ and excitation spectra (right) at a wavenumber of detection $\bar{\nu}_D^* = 16400\ cm^{-1}$. Top: Fluorescence intensities $S(\bar{\nu}_I^*, \bar{\nu}_D)$ and $S(\bar{\nu}_I, \bar{\nu}_D^*)$ (arbitrary units). Center: Change of fluorescence intensity $\Delta S(\bar{\nu}_I^*, \bar{\nu}_D)$ and $\Delta S(\bar{\nu}_I, \bar{\nu}_D^*)$ (arbitrary units) as induced by 100 mV depolarization. Bottom: Relative change of fluorescence intensity $\Delta S(\bar{\nu}_I^*, \bar{\nu}_D)/S(\bar{\nu}_I^*, \bar{\nu}_D)$ and $\Delta S(\bar{\nu}_I, \bar{\nu}_D^*)/S(\bar{\nu}_I, \bar{\nu}_D^*)$. Crosses refer to data points which are affected by stray light.

In a second approach we took into account effects of broadening, i.e., we used the complete Eqn. 6. As shown in Fig. 3 the sensitivity of emission was described perfectly by a blue shift $\Delta M_{EM} = 39\ cm^{-1}$, by a drop of total amplitude $\Delta T_{EM} = -2.2\%$ and by a broadening of width $\Delta W_{EM} = 65\ cm^{-1}$. The rms-deviation was 0.65%. The description of sensitivity of excitation was not improved significantly using a blue shift $\Delta D_{EX} = 58\ cm^{-1}$, a drop of amplitude $\Delta T_{EX} = -3.5\%$ and a broadened width $\Delta W_{EX} = 18\ cm^{-1}$. The rms-deviation was 0.75%. Thus the two-parameter fit was sufficient with respect to the excitation spectrum.

Changes of total amplitude reflect a charge $\Delta A_{EX} + \Delta A_{EM}$ of the intrinsic amplitudes and contributions of shift and broadening according to Eqn. 6. We choose the wavenumber $\bar{\nu}_I^*$ of illumination such that the integral $\langle a' \rangle$ vanished as it affects the emission spectrum and the wavenumber $\bar{\nu}_D^*$ of detection such that the integral $\langle e' \rangle$ vanished as it affects the excitation spectrum. Thus the spectral shifts did not contribute to the total amplitude. As no broadening of excitation was observed we used the relation $\Delta T_{EM} = \Delta A_{EX} + \Delta A_{EM} = -2.2\%$. On the other hand ΔT_{EX} was a superposition of $\Delta A_{EX} + \Delta A_{EM}$ and of a negative contribution of broadening of emission (negative curvature e'' in Eqn. 6). With $\Delta W_{EM} = 65\ cm^{-1}$ we corrected ΔT_{EX} for the broadening effect and obtained $\Delta A_{EX} + \Delta A_{EM} = -2.4\%$. The good agreement of the two values of $\Delta A_{EX} + \Delta A_{EM}$ confirmed the validity the approach. The average was $\Delta A_{EX} + \Delta A_{EM} = -2.3\%$.

In principle a separation of the changes of amplitude of excitation and emission was not possible without direct measurement of light absorption. However, we observed that drastic changes of environment – of the local field in bulk polar solvents – did not affect the oscillator strength of absorption [8], whereas minute changes affected the yield of fluorescence [7,8]. On this basis we assigned the field-induced change of amplitude to a change of emission with $\Delta A_{EM} = -2.3\%$ assuming $\Delta A_{EX} = 0$.

To summarize: The modulation of fluorescence by a depolarization of 100 mV was described by a blue shift of absorption of $\Delta M_{EX} = 60\ cm^{-1}$, a blue shift of fluorescence of $\Delta M_{EM} = 39\ cm^{-1}$, a broadening of fluorescence by $\Delta W_{EM} = 65\ cm^{-1}$ and a drop of fluorescence amplitude by $\Delta A_{EM} = -2.3\%$.

We studied 16 Retzius cells. Qualitatively all measurements were in agreement with respect to a superposition of a drop of quantum yield, of a blue shift of absorption, of a weaker blue shift of emission, of a broadening of emission and a negligible broadening of absorption. We observed, however, some scatter of the data (e.g., with respect to the blue shift of absorption we found values between 40 cm^{-1} and 67 cm^{-1}). We attribute the variability (i) to an imprecise determination of spectral sensitivity as caused by the low signal-

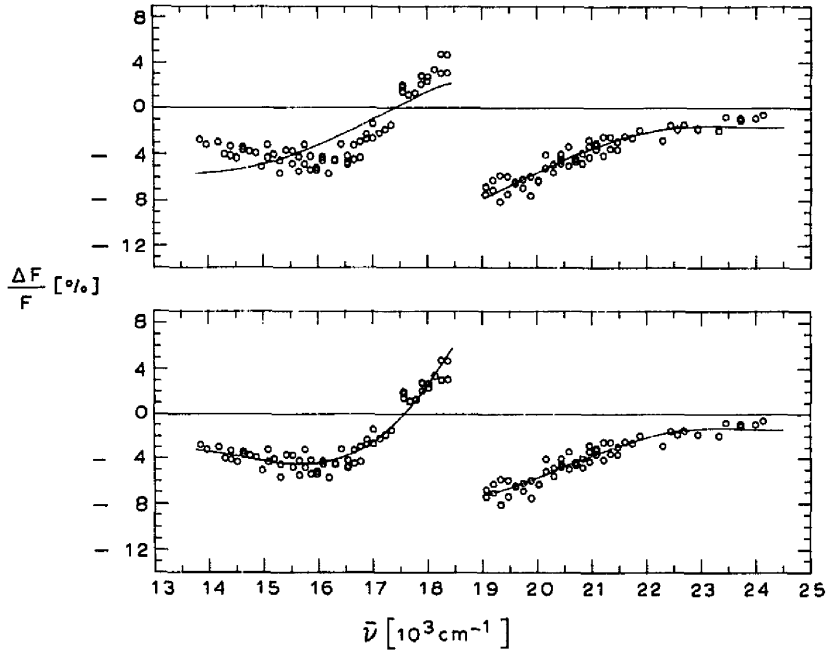

Fig. 3. Relative change of emission spectrum $\Delta S(\bar{\nu}_I^*, \bar{\nu}_D)/S(\bar{\nu}_I^*, \bar{\nu}_D)$ and excitation spectrum $\Delta S(\bar{\nu}_I, \bar{\nu}_D^*)/S(\bar{\nu}_I, \bar{\nu}_D^*)$ for 100 mV depolarization. Top: Fitted by changes of amplitudes and spectral shifts. Bottom: Fitted by changes of amplitudes, spectral shifts and changes of halfwidth. Details in the text.

to-noise ratio and (ii) to variability of the observed sensitivity as due to a variable fraction α of active molecules (Eqns. 7).

Discussion

Sensitivity

By deconvolution of the spectra of excitation $S(\bar{\nu}_I, \bar{\nu}_D^*)$ and emission $S(\bar{\nu}_I, \bar{\nu}_D)$ we obtain the spectra of excitation (absorption) $a(\bar{\nu}_{EX})$ and emission $e(\bar{\nu}_{EM})$ according to Eqns. 1 and 5 up to constant factors. The two-dimensional spectrum $S(\bar{\nu}_{EX}, \bar{\nu}_{EM}) \approx a(\bar{\nu}_{EX}) \cdot e(\bar{\nu}_{EM})$ (Eqn. 1) is plotted in Fig. 4. The maximal intensity is found at $M_{EX} = 20950\ cm^{-1}$ and $M_{EM} = 16300\ cm^{-1}$.

The two-dimensional spectrum of sensitivity $\Delta S(\bar{\nu}_{EX}, \bar{\nu}_{EM})/S(\bar{\nu}_{EX}, \bar{\nu}_{EM})$ is computed from $a(\bar{\nu}_{EX})$ and $e(\bar{\nu}_{EX})$ and from the changes amplitude ΔA_{EM}, the spectral shifts ΔM_{EX}, ΔM_{EM} and the change of width ΔW_{EM} according to Eqn. 4. The result is plotted in Fig. 4. Optimal sensitivity $(\Delta S/S) = -9.2\%$ per 100 mV is found at $\bar{\nu}_{EX} = 18550\ cm^{-1}$ in the red flank of absorption and at $\bar{\nu}_{EM} = 15650\ cm^{-1}$ close to the maximum of emission. The measured spectra of excitation and emission do not cross this point. At optimal sensitivity the blue shift of absorption contributes by -4.6%, the blue shift of emission by -1.1%, the broadening of emission by -1.2% and the drop of amplitude by -2.3%.

The spectra of sensivity $\Delta S/S$ and of intensity S affect both the signal-to-noise ratio. In the case of constant noise – of electronic origin – we have to optimize ΔS, i.e., the product of sensitivity and intensity. In the case of shot-noise of photons – which is proportional to $\sqrt{S}$ – we have to optimize $\Delta S/\sqrt{S}$, i.e., the product of sensitivity and root of intensity.

Mechanism

The usual explanation for spectral shift in membranes is electrochromism: The electrical field interacts with an intramolecular shift of electrical charge in the process of excitation and emission [14–17]. Excitation of di4-ANEPPS shifts positive charge from pyridinium to aminonapthalene [17]. MNDO-CI calculations indicate that an elementary charge is shifted by about 0.3 nm (Ephardt and Fromherz, unpublished). In lipid bilayers the chromophore is aligned approximately normal to the membrane with the naphthalene pointing towards the interior of the membrane [18,19]. If such an alignment may be attributed also to the neuron membrane the charge is shifted against the changing electrical field during a depolarization i.e. the field enhances the energy of excitation and emission. If the voltage of 100 mV drops evenly across a membrane

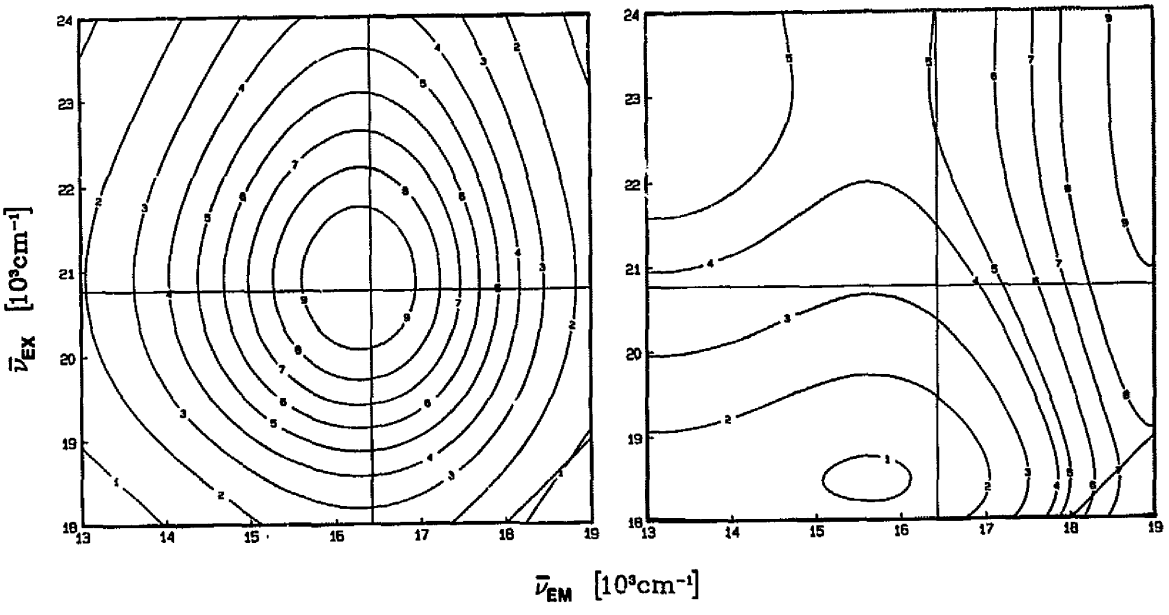

Fig. 4. Fluorescence and voltage-sensitive fluorescence. Left: Fluorescence intensity $S(\bar{\nu}_{EX}, \bar{\nu}_{EM})$ as a function of wavenumbers $\bar{\nu}_{EX}$ and $\bar{\nu}_{EM}$ of excitation and emission. The number code denotes intensities (maximum 10). Right: Relative change of fluorescence $\Delta S(\bar{\nu}_{EX}, \bar{\nu}_{EM})/S(\bar{\nu}_{EX}, \bar{\nu}_{EM})$ as induced by a depolarization of 100 mV. The number code is: 1, −9%, 2, −7%; 3, −5%; 4, −3%; 5, −2%; 6, 0%; 7, 2%; 8, 4% and 9, 8%. The positions of the excitation and emission spectra are indicated by lines. The diagonal of the $\bar{\nu}_{EX}/\bar{\nu}_{EM}$-plane is marked at the lower right of both diagrams.

of thickness of 5 nm we expect a blue shift 50 cm^{-1} which is surprisingly close to the observed blue shift ΔM_{Ex} of absorption.

We postulate a process of field-induced resolvation in the excited state to explain the drop of amplitude ΔA_{EM}, the weakened blue shift ΔM_{EM} and the broadening ΔW_{EM}, of fluorescence. We assume that the electrical field drives the charged napthalene moiety to a changed environment – e.g., towards the surface of the membrane. As a matter of fact an enhanced polarity gives rise to a drop of quantum yield and a red shift of fluorescence of di4-ANEPPS (Ephardt and Fromherz, to be published, cf. Ref. 8). Also the spectral width is extremely sensitive with respect to the environment (e.g., it is enhanced by 1000 cm^{-1} in lecithin as compared to ethanol whereas a systematic change with bulk polarity is not observed) Thus a dislocation of the excited dye could give rise to a drop of amplitude, a partial compensation of the blue shift and to a broadened spectrum.

A changed environment affects the absorption of di4-ANEPPS as well. The spectrum is broadened considerably by polar solvents and by lipids (Ephardt and Fromherz, to be published). The invariant shape of the excitation spectrum indicates that the voltage does not induce significant resolvation in the ground state. A different response of the dye in the ground state as compared to the excited state is not surprising considering the rather different location of the charge in the chromophore.

To summarize: We assign the blue shift of absorption to electrochromism, the weaker blue shift of fluorescence to a superposition of electrochromism and field-induced resolvation, the drop of quantum yield of fluorescence and the broadening of fluorescence to field-induced resolvation. This mechanism must be considered as tentative until a quantitative rationalization of the effects is achieved on the basis of improved measurements.

Conclusion

We measured the modulation of the complete excitation and emission spectra of a styryl-dye in a neuron. We could attribute the modulation to a blue shift of absorption, to a weaker blue shift of fluorescence, to spectral broadening of fluorescence and to a drop of quantum yield of fluorescence. We assigned tentatively the result to a superposition of electrochromism and field-induced resolvation of the excited state. More precise measurements – using voltage clamp of the neuron and defined directions and polarizations of light – may lead to a quantitative rationalization of voltage sensitivity.

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References

1 Cohen, L.B. and Salzberg, B.M. (1978) Rev. Physiol. Biochem. Pharmacol. 83, 35–88.
2 Cohen, L.B. and Lesher, S. (1986) in (pp. 71–99, De Weer, P. and Salzberg, B.M., eds.), Optical methods in cell physiology, Wiley, New York.
3 Grinvald, A., Fine, A., Farber, I.C. and Hildesheim, R. (1983) Biophys. J. 42, 195–198.
4 Grinvald, A., Hildesheim, R., Farber, I.C. and Anglister, L. (1982) Biophys. J. 39, 301–308.
5 Loew, L.M., Cohen, L.B., Salzberg, B.M., Obaid, A.L. and Bezanilla, F. (1985) Biophys. J. 47, 71–77.
6 Fluhler, E., Burnham, V.G. and Loew, L.M. (1985) Biochemistry 24, 5749–5755.
7 Ephardt, H. and Fromherz, P. (1989) J. Phys. Chem. 93, 7717–7725.
8 Ephardt, H. and Fromherz, P. (1991) J. Phys. Chem., in press.
9 Dietzel, I.D., Drapeau, P. and Nicholls, J.G. (1986) J. Physiol. (London) 372, 191–205.
10 Chiquet M. and Nicholls, J.G. (1987) J. Exp. Biol. 132, 191–206.
11 Fromherz, P. and Vetter, T. (1991) Z. Naturforsch., in press.
12 Miller, J.N. (ed.) (1981) Standards in Fluorescence Spectroscopy, Chapman and Hall, London.
13 Loew, L.M., Simpson, L., Hassner, A. and Alexanian, V. (1979) J. Am. Chem. Soc. 101, 5439–5440.
14 Junge, W. and Witt, H. (1968) Z. Naturforsch. 23b, 244–254.
15 Bücher, H. and Kuhn, H. (1969) Chem. Phys. Lett. 3, 508–511.
16 Reich, R. and Schmidt, S. (1972) Ber. Bunsenges. Phys. Chem. 76, 589–598.
17 Loew, L.M., Bonneville, G.W. and Surow, J. (1978) Biochemistry 17, 4065–4071.
18 Loew, L.M. and Simpson, L.L. (1981) Biophys. J. 34, 353–365.
19 Bammel, B.P., Hamilton, D.D., Haugland, R.P., Hopkins, H.P., Schuette, J., Szalecki, W. and Smith, J.C. (1990) Biochim. Biophys. Acta 1014, 61–81.