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Voltage-sensitive fluorescence of amphiphilic hemicyanine dyes in neuron membrane

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Fluorescent amphiphilic hemicyanine dyes were adsorbed to the plasma membrane of isolated Retzius neurons of the leech. Voltage steps were applied to the neuron by the patch-clamp technique in whole-cell configuration. The change of fluorescence was observed as induced by the voltage jump. The relative changes of the excitation spectrum and of the emission spectrum of fluorescence were recorded. The complete set of spectral data for each dye was fitted by five parameters: shifts of the emission and the excitation spectrum, a change of fluorescence quantum yield and changes of the widths of the excitation and of the emission spectrum. The only common feature for all dyes was a blue-shift of the excitation spectrum and a drop of the yield when the neuron was stained from the outside and a positive voltage was applied to the inside. With respect to the shift of the emission spectrum and the changes of width qualitatively different results were obtained for different dyes. It is not attempted to assign a physical mechanism – probably a superposition of several mechanisms – of voltage-sensitivity.

Introduction

Fluorescent dyes are used as indicators for fast changes of the membrane potential in neurons [1,2]. The sensitivity of the dyes is rather low. An optimal matching of the dyes and the optical set-up is crucial to attain a high signal-to-noise ratio, in particular in measurements at a high spatio-temporal resolution as it is required in studies of the arborizations of a single neuron [3].

In the present paper we present the spectra of voltage-sensitivity of fluorescence for eight hemicyanine dyes in isolated Retzius neurons of the leech. Due to the high quality of the measurements with respect to spectral resolution and signal-to-noise ratio we were able to evaluate five parameters which describe the voltage-sensitive fluorescence – the relative change of quantum yield, the shifts of the spectra of excitation and of emission and the changes of spectral width of emission and excitation. The optical set-up was similar to that described in a previous paper [4]. Considerable improvement of the signal-to-noise ratio was attained by applying defined voltage-steps in a whole-cell patch-clamp configuration instead of using stimulated action potentials.

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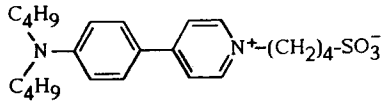
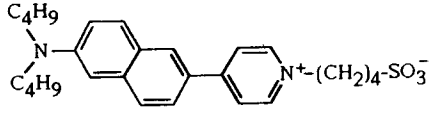
Materials and Methods

Dyes. We used eight amphiphilic hemicyanine dyes, dibutylaminophenylpyridiniumbutylsulfonate (BABP) and its homologs (Fig. 1). Substitution of the aminophenyl and the pyridinium in BABP by aminonaphthyl and by isoquinolinium, respectively, leads to two other biaryl-dyes BNBP and BNBIQ. Insertion of one, two or three conjugated double bonds between the two aromatic moieties in BABP leads to the styryl-dyes RH364, RH160 and RH237. (In RH421 the butyl-groups of RH160 are replaced by pentyl-groups.) Substitution of the aminophenyl by aminonaphthyl and insertion of a double bond leads to the styryl-dye di4ANEPPS. (The butylsulfonate is substituted by propylsulfonate.) The biaryl-dyes were described by Ephardt and Fromherz [5,6]. The styryl-dyes were introduced by Grinvald et al. [7,8] and by Loew et al. [9,10]. These dyes were obtained from Molecular Probes (Junction City, Oregon).

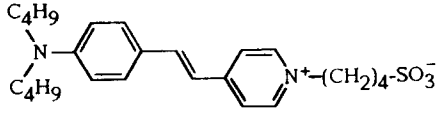
Neurons. Ganglia of the leech *Hirudo medicinalis* (Biopharm/Swansea) were dissected and pinned on a Sylgard coated dish in Leibowitz-15 medium (Gibco) with 50 μg/ml gentamycin sulfate (Sigma) and 6 mg/ml glucose. After opening the tissue capsules the ganglia were incubated in dispase/collagenase (Boehringer/Mannheim; 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



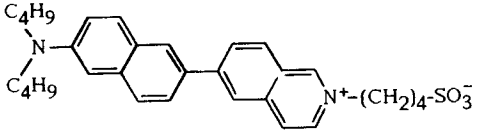
BABP



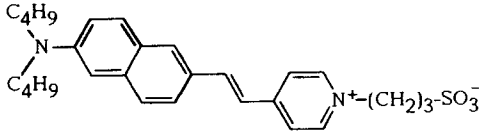
BNBP



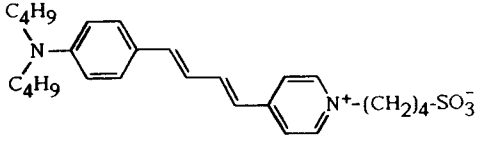
RH 364



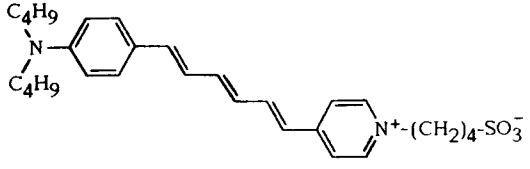
BNBIQ



di 4 ANEPPS



RH 160



RH 237

Fig. 1. Amphiphilic hemicyanine dyes. They are derived from dibutylaminophenyl-pyridiniumbutylsulfonate (BABP) [5] by substitution of aminophenyl and pyridinium by, respectively, aminonaphthyl and isoquinolinium (biaryl-dyes) as in BNBP [6] and BNBIQ [6] or by insertion of double bonds (styryl-dyes) as in RH364 [7], RH160 [7], RH237 [7] and di4ANEPPS [9]. RH421 [8] corresponds to RH160 with pentyl- instead of butyl-groups.

through several drops of L-15 medium [11]. Cover slips were attached to silicone chambers (Flexiperm-mikro 12, Heraeus/Hanau) and coated by an extract of the extracellular matrix of the leech [12,13]. The cells were plated in L-15 medium with 2% foetal calf serum (Gibco).

Electrophysiology. After two days of cultivation the neurons were attached to a patch-pipette (tip diameter 3–4 μm) filled with 140 mM KCl, 1.5 mM $MgCl_2$, 10 mM Hepes and 10 mM EGTA at pH 7.3 [14,15]. The seal resistance in cell-attached configuration was around 0.5 GΩ. The membrane was opened by a voltage pulse. The voltage in the cells was kept constant by a patch-clamp amplifier with respect to an internal reference. The intracellular voltage was controlled by an impaled microelectrode filled with 3 M KCl. The cell was kept at a reference voltage of -60 mV. To record voltage-sensitive fluorescence the cell was depolarized first to $+10$ mV and then hyperpolarized to -130 mV.

Staining. The dyes were 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/Heidelberg) by sonication (three times 5 min with intervals of 5 min at 0°C, Branson sonifier, level 3). After centrifugation the suspension of vesicles (diameter 60 nm) was added to the chambers up to a dye concentration of 4–8 μM. The external side of the plasma membrane of the neurons was stained.

Optics and electronics. A chamber with a stained neuron was mounted on an inverted microscope

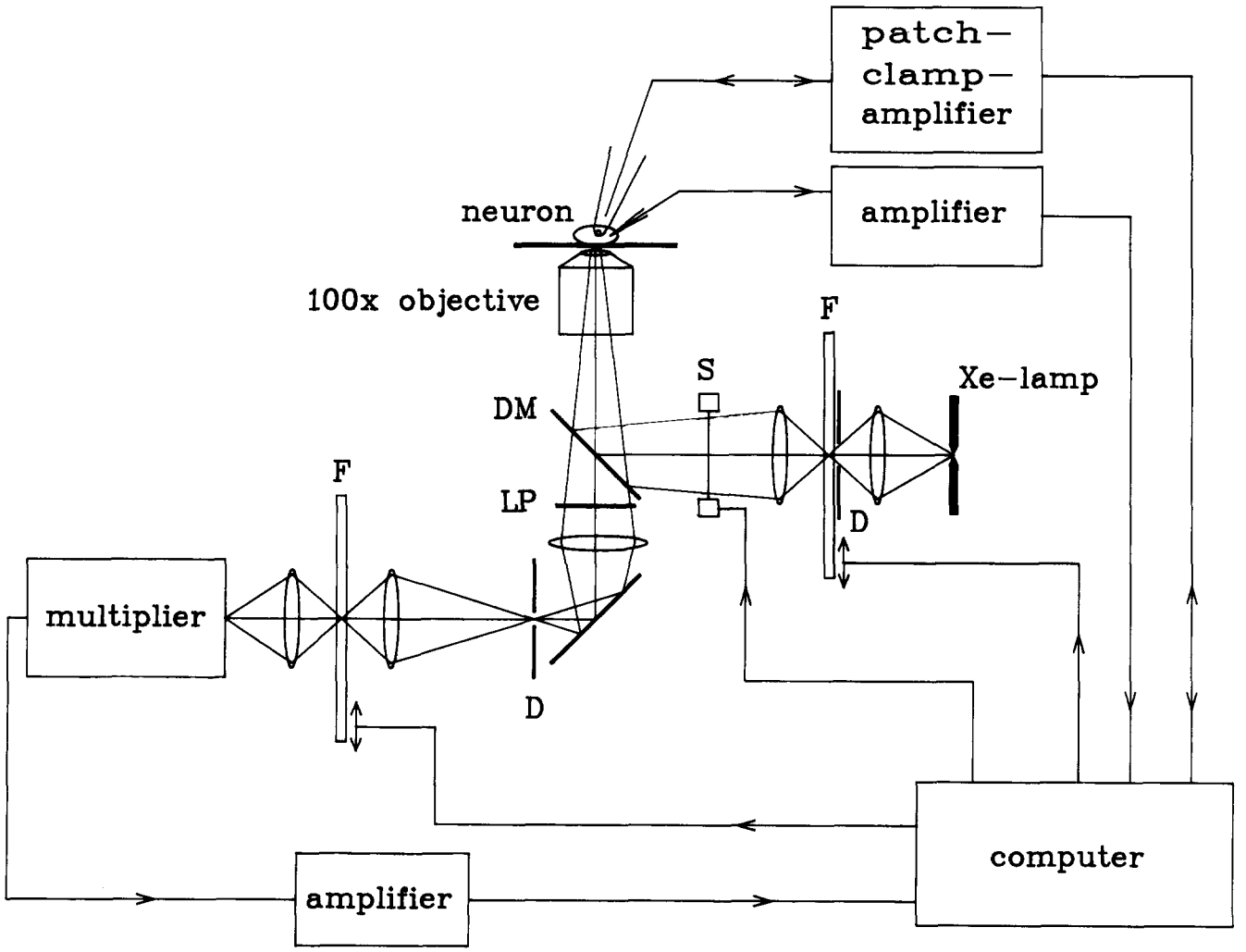


Fig. 2. Optical and electronic set-up. A stained neuron is located in the object plane of an inverted microscope. It is attached to a patch-pipette and impaled by a micropipette. Not drawn is the bath contacted by a third Ag/AgCl electrode. The neuron is illuminated by a Xe-lamp through a diaphragm (D), a continuous interference filter (F), a shutter (S) and 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 fluorescence intensity and the change of fluorescence intensity induced by a voltage step are recorded at various wavenumbers of excitation and emission.

(Axiovert, Zeiss/Oberkochen) (Fig. 2). 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 dichroitic beam splitter (FT 510 or FT 580, Zeiss) and a shutter. The lower part of the neuron was projected onto the first image plane by an objective of high aperture (Neofluar 100 × /1,3 Oel, Zeiss) through the beam splitter, a cut-off filter (RG 520 or RG 590, Schott) and a second continuous filter. An area of 30–40 μm diameter of the plasma membrane was selected by a diaphragm. The light was detected by a photomultiplier with S-20 cathode (C31034, RCA).

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 local orientation of the undulated membrane is randomized.

The Gaussian transmission curves of the continuous interference filters have a halfwidth of about 1270 cm^{-1} in the range of illumination and of about 1040 cm^{-1} in the range of detection. We neglected the convolution of the spectra of the dye with the spectra of illumination and detection as the spectral width of 3500 cm^{-1} of a dye is broadend only by about 150 cm^{-1}. (Note that convolution of two Gaussians leads to the addition of the variances.) The calibration of the spectra of illumination and detection is described in Ref. 4.

Protocol of measurement. After selection of a wavenumber of excitation $\bar{\nu}_{ex}^*$ the voltage-sensitivity of the emission spectrum was measured as follows: (1) Selection of the wavenumber of emission $\bar{\nu}_{em}$. Depolarization to about $V_{dep} = +10$ mV for 20 ms. (2) Opening of the shutter. (3) Record of the time-dependent fluorescence signal $F_{dep}(t)$ and of the voltage $V_{dep}(t)$ for 6 ms. (4) Hyperpolarization to about $V_{hyp} = -130$ mV. (5) After a delay of 6 ms record of the time dependent fluorescence signal $F_{hyp}(t)$ and of the voltage $V_{hyp}(t)$ for 6 ms. (6) Closing of the shutter. (7) Reset of the voltage to -60 mV. After 1–2 s the sequence 1–7 was repeated at a changed wavenumber $\bar{\nu}_{em}$. The sensitivity of the excitation spectrum was measured similarly by variation of $\bar{\nu}_{ex}$ at fixed $\bar{\nu}_{em}^*$.

The excitation spectrum and the emission spectrum themselves were recorded in separate runs after the determination of the spectra of voltage-sensitivity. For the emission spectrum the neuron was illuminated through a band pass filter (450–490 nm) (without continuous interference filter) and the dichroitic mirror FT 510 using a long pass (RG 520) in the emission path. For the excitation spectrum a short pass filter (cut off 650 nm) was added to the illumination path. The dichroitic mirror was replaced by a semitransparent mirror. A long pass (RG 695 nm) was used in the emission beam without continuous interference filter. From these spectra of excitation $a(\bar{\nu}_{ex})$ and of emission $e(\bar{\nu}_{em})$ the first and second derivatives $a'(\bar{\nu}_{ex})$, $e'(\bar{\nu}_{em})$, $a''(\bar{\nu}_{ex})$ and $e''(\bar{\nu}_{em})$ were computed numerically. The spectra $a(\bar{\nu}_{ex})$ and $e(\bar{\nu}_{em})$ were characterized by the wavenumbers M_{ex} and M_{em} of their maximum and by their widths W_{ex} and W_{em} at their semimaximal value. Most excitation spectra had long tails in the blue – due to the contribution of a higher electronic transition or due to inaccurate calibration in that spectral range. To evaluate a characteristic width W_{ex} we determined $W_{ex}/2$ from the maximum of the spectrum to its semimaximal value in the red.

For every sensitivity measurement the time-dependent fluorescences $F_{dep}(t)$ and $F_{hyp}(t)$ were averaged. The averaged values were subtracted from each other, divided by F_{hyp} and divided by $(V_{dep} - V_{hyp})$. The voltage-sensitivity of fluorescence $\Delta F/F\Delta V$ is given by Eqn.1. We express it in %/100 mV, defined with respect to the application of positive voltage to that side of the membrane which is not stained.

$$\frac{\Delta F}{F\Delta V} = \frac{(F_{dep} - F_{hyp})}{F_{hyp}(V_{dep} - V_{hyp})} \tag{1}$$

Two examples of the time course of the fluorescence signal (voltage at the photomultiplier output) and of

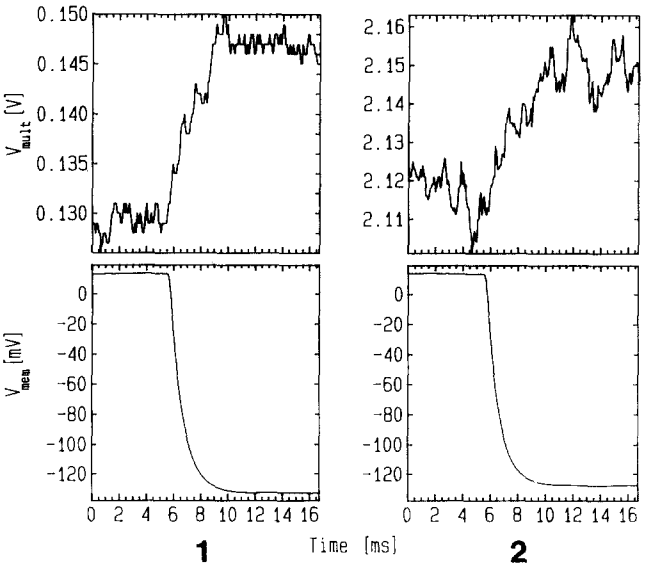


Fig. 3. Fluorescence (voltage output of multiplier) at a wavenumber of excitation 21000 cm^{-1} and intracellular voltage (measured by impaled microelectrode) of BNBIQ versus time. (1) Wavenumber of emission 13830 cm^{-1}. Sensitivity $\Delta F/F\Delta V = -8.2\%/100$ mV. (2) Wavenumber of emission 17790 cm^{-1}. Sensitivity $\Delta F/F\Delta V = -1.1\%/100$ mV. The results of these two measurements are part of the spectral sensitivity of BNBIQ shown in Fig. 5.

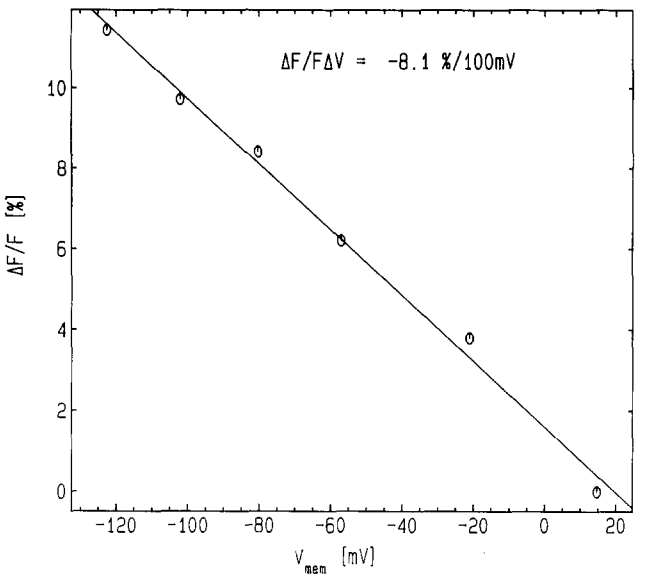


Fig. 4. Linearity of voltage-sensitive fluorescence. The relative change of fluorescence $\Delta F/F$ of BNBIQ induced by hyperpolarization from +15 mV is plotted versus the membrane voltage V_{mem} (Excitation at 19000 cm^{-1}, emission at 16000 cm^{-1}). Linear regression leads to $\Delta F/F\Delta V = -8.1\%/100$ mV.

the membrane voltage are shown in Fig. 3 for the dye BNBIQ. The wavenumber of excitation was 21000 cm^{-1}. The wavenumbers of emission were 13830 cm^{-1} and 17790 cm^{-1}, respectively. In the first case the sensitivity was relatively high with $\Delta F/F\Delta V = -8.2\%/100$ mV. In the second case the sensitivity was lower with $\Delta F/F\Delta V = -1.1\%/100$ mV. The application of defined voltage-steps by patch-clamping improved the quality of averaging as compared to the use of stimulated action potentials [4]. Thus the scatter of the data $\Delta F/F\Delta V$ within a sensitivity spectrum was reduced considerably.

In test runs sequences of voltage steps of various amplitude were applied at a given wavenumber of excitation and emission. In all cases the changes of fluorescence were found to be linear within the range from −120 mV to +15 mV. An example is shown in Fig. 4 for BNBIQ (excitation 19000 cm^{-1}, emission 16000 cm^{-1}).

Evaluation. A change of the membrane voltage affects the spectra of excitation and of emission. The voltage may give rise to relative changes ΔA_{ex} and ΔA_{em} of the amplitudes of the spectra and to shifts ΔM_{ex} and ΔM_{em} of the maxima of the spectra. For given wavenumbers of excitation $\bar{\nu}_{ex}$ and of emission $\bar{\nu}_{em}$ the relative change of the fluorescence intensity is expressed by the first four terms on the right hand side of Eqn. 2 as derived in Ref. 4. The effects of finite spectral width of illumination and detection – as expressed by appropriate convolutions – are omitted in Eqn. 2 (cf. Ref. 4).

$$\frac{\Delta F(\bar{\nu}_{ex},\bar{\nu}_{em})}{F(\bar{\nu}_{ex},\bar{\nu}_{em})} = \Delta A_{ex} + \Delta A_{em} - \frac{a'(\bar{\nu}_{ex})}{a(\bar{\nu}_{ex})}\Delta M_{ex} - \frac{e'(\bar{\nu}_{em})}{e(\bar{\nu}_{em})}\Delta M_{em} + \frac{a''(\bar{\nu}_{ex})W_{ex}}{a(\bar{\nu}_{ex})8\ln 2}\Delta W_{ex} + \frac{e''(\bar{\nu}_{em})W_{em}}{e(\bar{\nu}_{em})8\ln 2}\Delta W_{em} \quad (2)$$

We found that in most cases the data cannot be described by spectral shifts and changes of amplitude alone. The voltage induced a change of spectral shape. As a next approximation we use terms that are proportional to the second derivatives a'' and e'' of the spectra. For spectra of Gaussian shape such terms reflect a change of the half widths W_{ex} and W_{em} of the spectra [4]. In that case the effects of spectral deformation are described by the last two terms on the right hand side of Eqn. 2. As a matter of fact the shape of the emission spectra of the hemicyanine dyes resembles closely to a Gaussian. The excitation spectra resemble to a Gaussian – except for their tail in the blue – around their maximum and in the red. It may be noted that changes of spectral width are quite common for hemicyanine dyes as caused by changes of the environment [6].

As mentioned we determine the voltage-sensitivity experimentally by scanning the wavenumber of excitation $\bar{\nu}_{ex}$ at given wavenumber of emission $\bar{\nu}^*_{em}$ (excitation spectrum) and by scanning the wavenumber of emission $\bar{\nu}_{em}$ at given wavenumber of excitation $\bar{\nu}^*_{ex}$ (emission spectrum). We neglect thus a dependence of the emission spectrum on the wavenumber of excitation. Such a dependence exists in fact for some hemicyanine dyes. A complete two-dimensional spectrum of voltage-sensitivity, however, cannot be determined because of problems with the signal intensity and because of the damage of the membrane during a considerably extended illumination.

We describe the sensitivity $\Delta F/F\Delta V$ of the excitation spectrum according to Eqn. 3 by the specific spectral shift $\Delta m_{ex} = \Delta M_{ex}/\Delta V$ and by the specific spectral broadening $\Delta w_{ex} = \Delta W_{ex}/\Delta V$. A third parameter Δt_{ex} expresses the change of total amplitude. It comprises the intrinsic specific change of amplitude $\Delta a_{ex} = \Delta A_{ex}/\Delta V$ and all changes of the emission spectrum that are effective at the chosen wavenumber $\bar{\nu}^*_{EM}$ according to Eqn. 4.

$$\frac{\Delta F(\bar{\nu}_{ex})}{F(\bar{\nu}_{ex})\,\Delta V} = \Delta t_{ex} - \frac{a'(\bar{\nu}_{ex})}{a(\bar{\nu}_{ex})}\Delta m_{ex} + \frac{a''(\bar{\nu}_{ex})W_{ex}}{a(\bar{\nu}_{ex})8\ln 2}\Delta w_{ex} \quad (3)$$

$$\Delta t_{ex} = \Delta a_{ex} + \Delta a_{em} - \frac{e'(\bar{\nu}^*_{em})}{e(\bar{\nu}^*_{em})}\Delta m_{em} + \frac{e''(\bar{\nu}^*_{em})W_{em}}{e(\bar{\nu}^*_{em})8\ln 2}\Delta w_{em} \quad (4)$$

We describe the sensitivity $\Delta F/F\Delta V$ of the emission spectrum according to Eqn. 5 by the specific spectral shift $\Delta m_{em} = \Delta M_{em}/\Delta V$ and specific spectral broaden-

ing $\Delta w_{em} = \Delta W_{em}/\Delta V$ of emission and a third parameter Δt_{em} which comprises the intrinsic specific change of amplitude $\Delta a_{em} = \Delta A_{em}/\Delta V$ and all changes of the excitation spectrum that are effective at the chosen wavenumber $\bar{\nu}_{ex}^*$ according to Eqn. 6.

$$\frac{\Delta F(\bar{\nu}_{em})}{F(\bar{\nu}_{em})\,\Delta V} = \Delta t_{em} - \frac{e'(\bar{\nu}_{em})}{e(\bar{\nu}_{em})}\Delta m_{em} + \frac{e''(\bar{\nu}_{em})W_{em}}{e(\bar{\nu}_{em})8\ln 2}\Delta W_{em} \qquad (5)$$

$$\Delta t_{em} = \Delta a_{em} + \Delta a_{ex} - \frac{a'(\bar{\nu}_{ex}^*)}{a(\bar{\nu}_{ex}^*)}\Delta m_{ex} + \frac{a''(\bar{\nu}_{ex}^*)W_{ex}}{a(\bar{\nu}_{ex}^*)8\ln 2}\Delta w_{ex} \qquad (6)$$

The amplitude parameters Δt_{ex} and Δt_{em} depend on the sum $\Delta a_{ex} + \Delta a_{em}$ of the intrinsic changes of amplitudes and on the other parameters of spectral modification Δm_{em}, Δw_{em} and Δm_{ex}, Δw_{ex}, respectively. Thus we fit the complete set of data of voltage-sensitivity $\Delta F/F\Delta V$ – for the excitation spectrum and for the emission spectrum together – on the basis of Eqns. 3–6 by the five parameters $\Delta a_{ex} + \Delta a_{em}$, Δm_{em}, Δm_{ex}, Δw_{em} and Δw_{ex} using a least-square procedure. In studies of the spectral properties of the hemicyanine dyes we found that the absorbance was almost unaffected by extreme changes of the environment [6]. On that basis we assume here $\Delta a_{ex} = 0$. We attribute the fitted change $\Delta a_{ex} + \Delta a_{em}$ of amplitude exclusively to a change Δa_{em} of emission, i.e., to a change of the quantum yield of fluorescence.

It cannot be avoided that the experimental sensitivity spectrum of excitation includes a contribution of the voltage-sensitivity of emission and that the experimental sensitivity spectrum of emission includes a contribution of the voltage-sensitivity of excitation. We obtain these interferences from the fit of the data as expressed by $\Delta t_{ex} - \Delta a_{ex}$ (Eqn. 4) and by $\Delta t_{em} - \Delta a_{em}$ (Eqn. 6), respectively. For sake of clarity we use a plot of the data which is corrected for these interferences, i.e., we plot pure sensitivity spectra of excitation and emission. The procedure implies a vertical displacement of the data by $\Delta t_{ex} - \Delta a_{ex}$ and by $\Delta t_{em} - \Delta a_{em}$, respectively. The original data may be reconstructed by reading them with respect to displaced abscissae which are marked in the pertinent figures.

For each dye we measured the sensitivity spectra in 3–10 neurons. The scatter of the fit-parameters was distinctly lower than in the previous study on di4ANEPPS in Retzius cells [4]. The improvement may be due part to patch-clamping, as mentioned above, and part due to an improved preparation of the samples.

A systematic error is introduced into the measurements by dye molecules that are bound to insensitive sites, in particular to connective tissue. That dye contributes to the fluorescence intensity F but not to the fluorescence change $\Delta F/\Delta V$. Thus it lowers the observed voltage-sensitivity $\Delta F/F\Delta V$ as discussed in Ref. 4. In fact the variability of the fit-parameters of different samples is reduced if they are normalized with respect to a selected fit-parameter, e.g., with respect to Δm_{ex}. For presentation we have selected the samples with largest response for each dye to approximate the true voltage-sensitivity.

TABLE I

Wavenumbers M_{em} and M_{ex} of the maxima of the excitation and emission spectra of amphiphilic hemicyanine dyes in the plasma membrane of Retzius cells and widths W_{ex} and W_{em} of the spectra at half of their maximal value

For the excitation spectrum $W_{ex}/2$ is evaluated from the spectral maximum towards the red. This procedure was chosen because of the tailing of the spectra in the blue or because the spectra were not available far in the blue. A similar procedure was chosen also for the emission spectrum of BABP.

Dye	M_{em} (cm^{-1})	M_{ex} (cm^{-1})	W_{em} (cm^{-1})	W_{ex} (cm^{-1})
BABP	18769	23578	4035	4050
BNBP	17461	22383	4196	3952
RH364	17090	20838	3174	3751
BNBIQ	16828	22251	3647	3928
di4ANEPPS	16219	21069	3271	3806
RH160	15856	20079	3119	3941
RH421	15664	20266	3086	3924
RH237	14560	19758	3196	4197

Results

In this section we present the data on the voltage-sensitive fluorescence of the amphiphilic hemicyanine dyes shown in Fig. 1. The data are fitted according to Eqns. 3–6. We start with the three biaryl-dyes of decreasing size BNBIQ, BNBP and BABP. We continue with the two styryl-dyes of increasing size RH364 and RH160. Then we discuss the styryl-dye di4ANEPPS which is a structural neighbour of RH160, RH364, BNBP and BNBIQ (Fig. 1). We end up with RH237, which is derived from RH160 by insertion of a further double bond and with RH421, a homolog of RH160 with pentyl instead of butyl groups. The spectral data and the fit-parameters of voltage-sensitivity are summarized in the Tables I and II, respectively.

BNBIQ

The relative changes of fluorescence intensity $\Delta F/F\Delta V$ for the excitation spectrum (emission at 16000 cm^{-1}) and for the emission spectrum (excitation at 21000 cm^{-1}) are shown in Fig. 5 in comparison to the excitation and emission spectra themselves. The data are fitted according to Eqns. 3–6. We obtain a blue-shift of excitation $\Delta m_{ex} = 75.6$ $cm^{-1}/100$ mV with a broadening $\Delta w_{ex} = 8.9$ $cm^{-1}/100$ mV. (The change of spectral width Δw_{ex} is not significant. An

TABLE II

Fit-parameters of voltage-sensitive fluorescence of amphiphilic hemicyanine dyes: Specific relative change of amplitude of emission Δa_{em}, specific shift of the maximum of emission Δm_{em}, specific shift of the maximum of excitation Δm_{ex}, specific change of spectral width of emission Δw_{em} and specific change of spectral width of excitation Δw_{ex}

The parameters refer to positive voltage applied to the unstained side of the membrane.

Dye	Δa_{em} (%/ 100 mV)	Δm_{em} (cm^{-1}/ 100 mV)	Δm_{ex} (cm^{-1}/ 100 mV)	Δw_{em} (cm^{-1}/ 100 mV)	Δw_{ex} (cm^{-1}/ 100 mV)
BABP	−0.2	−3.4	9.5	3.9	6.9
BNBP	−1.2	−35.6	26.9	−12.2	17.6
RH364	−0.5	−11.1	9.8	−1.3	9.6
BNBIQ	−0.4	72.6	75.6	59.5	8.9
di4ANEPPS	−0.2	25.6	42.0	44.7	6.7
RH160	−1.2	22.3	41.6	30.2	40.5
RH421	−1.9	16.9	48.0	41.4	16.5
RH237	−0.4	34.8	39.5	23.1	48.9

adequate fit may be obtained without it.) We obtain also a blue-shift of the emission spectrum $\Delta m_{em} = 72.6$ $cm^{-1}/100$ mV with a significant change of spectral width $\Delta w_{em} = 59.5$ $cm^{-1}/100$ mV and a change of amplitude $\Delta a_{em} = -0.4\%/100$ mV.

Striking is the large, almost identical blue-shift of the excitation and of the emission spectrum. Striking is also the significant broadening of the emission spectrum. On the other hand the shape of the excitation spectrum is changed only little. The drop of the yield of fluorescence is significant, but modest. The maximal signal that is obtained for an optimal choice of the wavenumbers of excitation (about 19000 cm^{-1}) and of emission (about 15000 cm^{-1}) is above $\Delta F/F\Delta V = -13\%/100$ mV. Also a rather high positive signal $\Delta F/F\Delta V = +9\%/100$ mV is obtained for excitation and emission in the blue ranges of the spectra (excitation at 25000 cm^{-1}, emission at 18500 cm^{-1}).

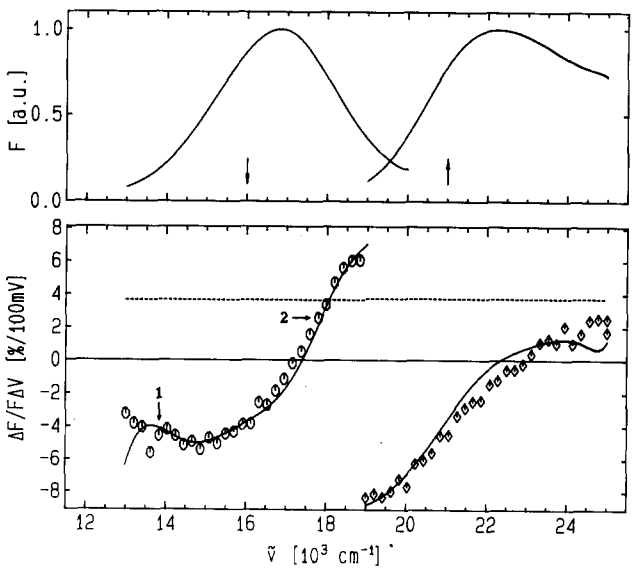


Fig. 5. Voltage-sensitive fluorescence of BNBIQ. (Top) Spectra of emission and excitation. (Bottom) Relative change of the spectra induced by 100 mV (sensitivity spectra). They are fitted according to Eqns. 3–6. The fit parameters are given in Table II. The wavenumbers of excitation $\bar{\nu}_{ex}^* = 21000$ cm^{-1} and of emission $\bar{\nu}_{em}^* = 16000$ cm^{-1} are marked by arrows. The dashed lines are the original abscissae of the experimental data as discussed in the text. The numbers (1) and (2) mark the points that correspond to the data shown in Fig. 3.

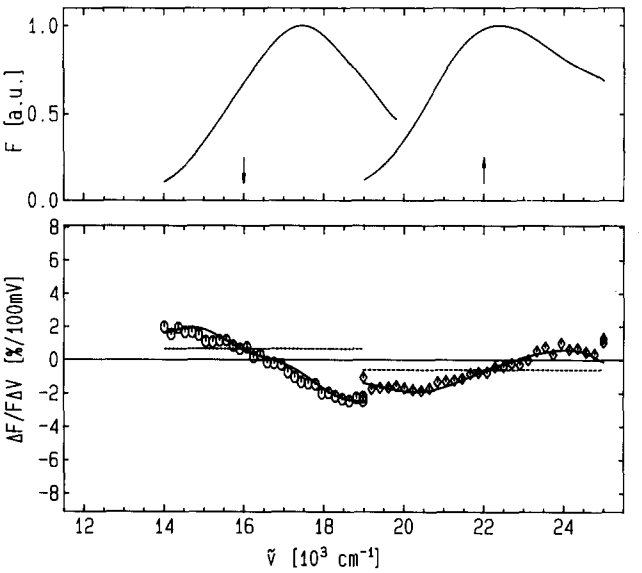


Fig. 6. Voltage-sensitive fluorescence of BNBP with $\bar{\nu}_{ex}^* = 22000$ cm^{-1} and $\bar{\nu}_{em}^* = 16000$ cm^{-1}. For other comments see Fig. 5.

BNBP

The relative changes of fluorescence intensity $\Delta F/F\Delta V$ for the excitation spectrum (emission at 16000 cm^{-1}) and for the emission spectrum (excitation at 22000 cm^{-1}) are shown in Fig. 6. From the fit of the data we obtain a blue-shift of excitation $\Delta m_{ex} = 26.9$ $cm^{-1}/100$ mV with a broadening of $\Delta w_{ex} = 17.6$ $cm^{-1}/100$ mV. We obtain a red-shift of the emission spectrum $\Delta m_{em} = -35.6$ $cm^{-1}/100$ mV with a change of spectral width $\Delta w_{em} = -12.2$ $cm^{-1}/100$ mV and a change of amplitude $\Delta a_{em} = -1.2\%/100$ mV. (The small changes of spectral width are not significant. An adequate fit of the data may be obtained without them.) The maximal negative signal is $\Delta F/F\Delta V = -4.5\%/100$ mV (excitation at 20000 cm^{-1}, emission at 19000 cm^{-1}). The maximal positive signal is $\Delta F/F\Delta V = +2.5\%/100$ mV (excitation at 24000 cm^{-1}, emission at 14500 cm^{-1}).

The dyes BNBIQ and BNBP – which are related closely with respect to their chemical structure – exhibit qualitatively different features of voltage-sensitivity: BNBIQ is dominated by an almost identical blue-shift of excitation and emission whereas in BNBP the

spectral shifts are opposite for excitation and emission.

There are further striking differences between BNBP and BNBIQ: The blue-shift of excitation of BNBP is only about one third of the blue-shift of BNBIQ. On the other hand the drop of amplitude is enhanced by a factor of three. Thus the contribution of the change of amplitude to the total signal is much more pronounced in BNBP than in BNBIQ. The emission spectrum of BNBP is not changed significantly as compared to the considerable broadening in BNBIQ.

We conclude: The replacement of isoquinoline by pyridine has a dramatic effect on the voltage-sensitivity of fluorescence. The chemical modification may affect the intrinsic photophysical processes of the dye and the interaction of the dye with the membrane.

BABP

This is the parent dye of the amphiphilic hemicyanines. The relative changes of fluorescence intensity $\Delta F/F\Delta V$ for the excitation spectrum (emission at 18000 cm^{-1}) and for the emission spectrum (excitation at 22000 cm^{-1}) are shown in Fig. 7. The fit-parameters are given in Table II. The voltage-sensitivity is so low that the fit is not too reliable.

Apparently there is a tendency from BNBIQ to BNBP to BABP: The smaller the dye, the lower is the voltage-sensitivity. As mentioned above, a modification of intrinsic photophysical processes or of the dye–membrane interaction may be involved.

RH364

The size of the chromophore of this styryl-dye corresponds to that of BNBP. The voltage-sensitivity of $\Delta F/F\Delta V$ for the excitation spectrum (emission at

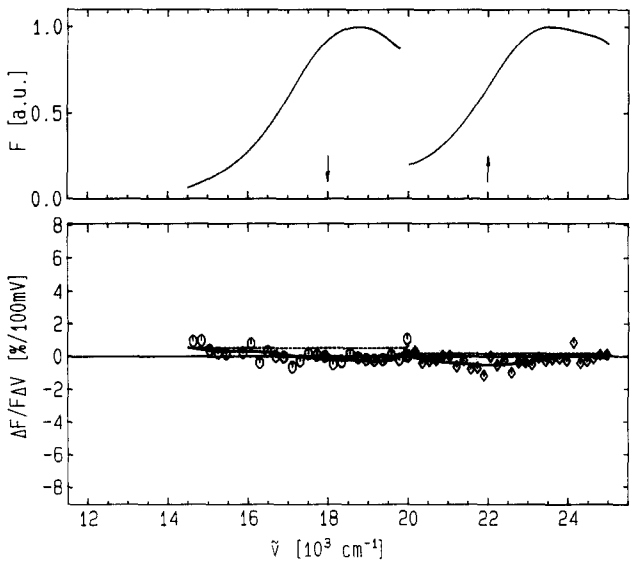


Fig. 7. Voltage-sensitive fluorescence of BABP with $\bar{\nu}_{ex}^{*} = 22000$ cm^{-1} and $\bar{\nu}_{em}^{*} = 18000$ cm^{-1}. For other comments see Fig. 5.

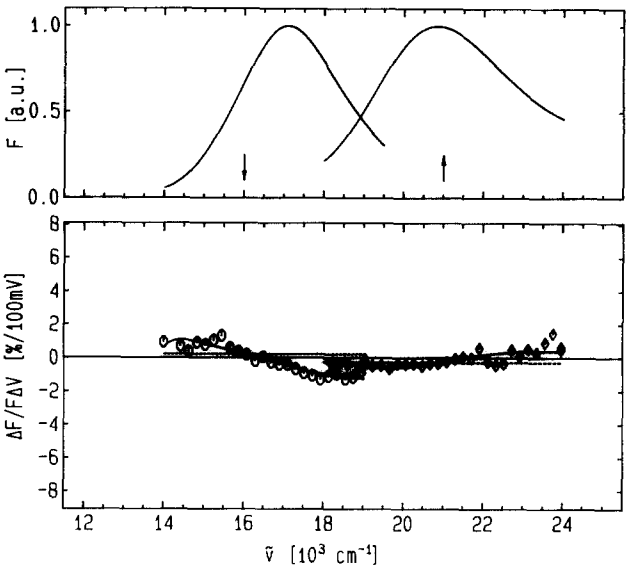


Fig. 8. Voltage-sensitive fluorescence of RH364 with $\bar{\nu}_{ex}^{*} = 21000$ cm^{-1} and $\bar{\nu}_{em}^{*} = 16000$ cm^{-1}. For other comments see Fig. 5.

16000 cm^{-1}) and for the emission spectrum (excitation at 21000 cm^{-1}) is shown in Fig. 8. From the fit of the data we obtain a blue-shift of excitation $\Delta m_{ex} = 9.8$ $cm^{-1}/100$ mV with a broadening $\Delta w_{ex} = 9.6$ $cm^{-1}/100$ mV. We obtain a red-shift of the emission $\Delta m_{em} = -11.1$ $cm^{-1}/100$ mV with the superposition of a change of spectral width $\Delta w_{em} = -1.3$ $cm^{-1}/100$ mV and a change of amplitude $\Delta a_{em} = -0.5\%/100$ mV. (The changes of spectral width are not significant.) The maximal negative signal is $\Delta F/F\Delta V = -2\%/100$ mV (excitation at 19500 cm^{-1}, emission at 18500 cm^{-1}). The maximal positive signal is $\Delta F/F\Delta V = +1.5\%/100$ mV (excitation at 23500 cm^{-1}, emission at 15000 cm^{-1}).

Qualitatively the voltage-sensitivity of RH364 is similar to that of BNBP: Opposite shift of the spectra and relatively large drop of amplitude. However, all effects are distinctly smaller in RH364 such that the overall sensitivity of the biaryl-dye BNBP is by a factor of about three larger than that of the styryl-dye RH364: The replacement of aminonaphthyl by aminostyryl lowers voltage-sensitivity. Again the chemical change may affect the intrinsic photophysical processes as well as the interaction with the membrane.

RH160

This dye may be derived from RH364 by insertion of a second double bond. With respect to its size it corresponds to BNBIQ. The voltage-sensitivity $\Delta F/F\Delta V$ of the excitation spectrum (emission at 16000 cm^{-1}) and of the emission spectrum (excitation at 21000 cm^{-1}) is shown in Fig. 9. From the fit of the data we obtain a blue-shift of the spectrum of excita-

tion $\Delta m_{ex} = 41.6\ cm^{-1}/100\ mV$. A broadening $\Delta w_{ex} = 40.5\ cm^{-1}/100\ mV$ is superposed. The spectrum of emission is shifted to the blue by $\Delta m_{em} = 22.3\ cm^{-1}/100\ mV$. Superposed is a significant broadening of $\Delta w_{em} = 30.2\ cm^{-1}/100\ mV$ and a considerable change of amplitude $\Delta a_{em} = -1.2\%/100\ mV$. The maximal negative signal that may be obtained is $\Delta F/F\Delta V = -5.5\%/100\ mV$ (excitation at 18000 cm^{-1}, emission at 14500 cm^{-1}). The maximal positive signal is $\Delta F/F\Delta V = +7\%/100\ mV$ (excitation at 24000 cm^{-1}, emission at 18000 cm^{-1}).

In a certain sense RH160 is inbetween BNBIQ and RH364 with respect to voltage-sensitivity: The excitation and the emission spectrum both are shifted to the blue as in BNBIQ. The value of the blue-shift of excitation, however, is distinctly smaller than in BNBIQ. The blue-shift of emission is even smaller, only half of the blue-shift of excitation – as if it were compensated partially by a hidden red-shift. The reduction of sensitivity in the transition from the biaryl BNBIQ to the styryl RH160 resembles to the relation of BNBP and RH364. A striking novel issue in RH160 is the extraordinary broadening of the excitation spectrum.

Let us summarize the results up to this point: Qualitatively the features of voltage-sensitivity are similar for the pair of large dyes BNBIQ and RH160 on one hand and for the pair of small dyes BNBP and RH364 on the other hand: Blue-shifts of both spectra for the large dyes, red-shift of emission for the small dyes. The change of chemical nature from biaryl (BNBIQ, BNBP) to styryl (RH160, RH364) affects the overall sensitivity which is higher for the biaryls than for the corresponding styryls.

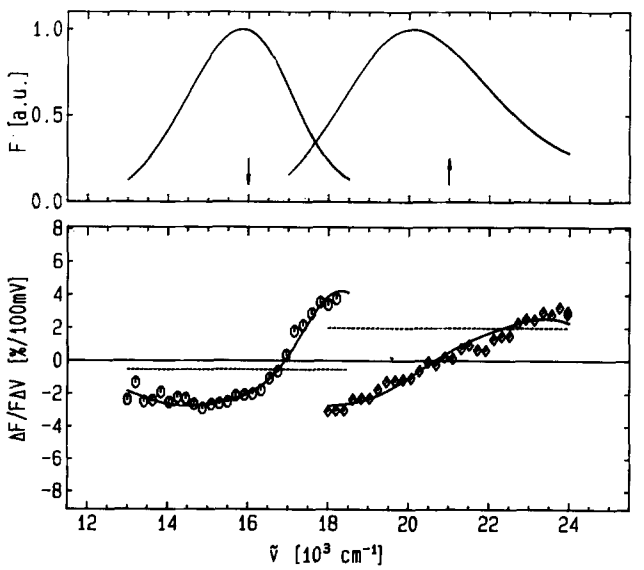


Fig. 9. Voltage-sensitive fluorescence of RH160 for $\bar{\nu}^*_{ex} = 21000\ cm^{-1}$ and $\bar{\nu}^*_{em} = 16000\ cm^{-1}$. For other comments see Fig. 5.

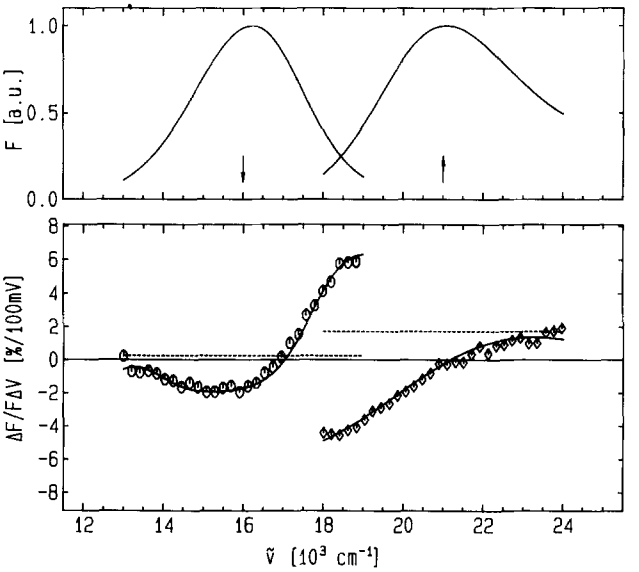


Fig. 10. Voltage-sensitive fluorescence of di4ANEPPS for $\bar{\nu}^*_{ex} = 21000\ cm^{-1}$ and $\bar{\nu}^*_{em} = 16000\ cm^{-1}$. For other comments see Fig. 5.

di4ANEPPS

With respect to its size this dye corresponds to RH160 and BNBIQ. It has a single free double bond like RH364. It possesses an aminonaphthyl- and a pyridinium-group like BNBP. The relative change of fluorescence intensity $\Delta F/F\Delta V$ for the excitation spectrum (emission at 16000 cm^{-1}) and for the emission spectrum (excitation at 21000 cm^{-1}) is shown in Fig. 10. The spectrum of excitation is shifted to the blue by $\Delta m_{ex} = 42.0\ cm^{-1}/100\ mV$. A broadening is superposed of $\Delta w_{ex} = 6.7\ cm^{-1}/100\ mV$. (This change of width is not significant.) The spectrum of emission is shifted to the blue by $\Delta m_{em} = 25.6\ cm^{-1}/100\ mV$. Superposed is a considerable broadening of $\Delta w_{em} = 44.7\ cm^{-1}/100\ mV$ and a small drop of amplitude $\Delta a_{em} = -0.2\%/100\ mV$. The maximal negative signal that may be obtained is $\Delta F/F\Delta V = -6.5\%/100\ mV$ (excitation at 18000 cm^{-1}, emission at 16000 cm^{-1}). The maximal positive signal is $\Delta F/F\Delta V = +8\%/100\ mV$ (excitation at 24000 cm^{-1}, emission at 18000 cm^{-1}).

With respect to its voltage-sensitivity di4ANEPPS resembles to the two large dyes BNBIQ and RH160: Excitation and emission both are shifted to the blue. It resembles on one hand more to the styryl-dye RH160 – considering the unequality of the shift of excitation and emission and the small shift of excitation. It resembles on the other hand more to the biaryl-dye BNBIQ – considering the small drop of amplitude and the unequal broadening of the emission and the excitation spectrum. The sensitivity spectra of di4ANEPPS in the neuron membrane resemble to those in an artificial membrane made of oxidized cholesterol [9].

RH237

This hexatriene-dye is obtained from RH160 by insertion of a further conjugated double-bond. The relative change of fluorescence intensity $\Delta F/F\Delta V$ for the excitation spectrum (emission at 15000 cm^{-1}) and for the emission spectrum (excitation at 21000 cm^{-1}) is shown in Fig. 11. The spectrum of excitation is shifted to the blue by $\Delta m_{ex} = 39.5\ cm^{-1}/100$ mV. A broadening is superposed of $\Delta w_{ex} = 48.9\ cm^{-1}/100$ mV. The spectrum of emission is shifted to the blue by $\Delta m_{em} = 34.8\ cm^{-1}/100$ mV. Superposed is a significant broadening of $\Delta w_{em} = 23.1\ cm^{-1}/100$ mV and a considerable change of amplitude $\Delta a_{em} = -0.4\%/100$ mV. The maximal negative signal that may be obtained is $\Delta F/F\Delta V = -5.5\%/100$ mV (excitation at 18000 cm^{-1}, emission at 13000 cm^{-1}). The maximal positive signal is $\Delta F/F\Delta V = +11\%/100$ mV (excitation at 24000 cm^{-1}, emission at 17000 cm^{-1}).

The features of voltage-sensitivity of RH237 are related on one hand closely to RH160, but they are related to BNBIQ, too. The value of the blue-shift of excitation and the similar broadening of excitation and emission are similar to RH160. The identity of the blue-shift of emission and excitation as well as the small drop of yield are similar to BNBIQ.

RH421

The chromophore of this dye is identical to that of RH160. The length of the hydrophobic chains is enhanced. The relative change of fluorescence intensity $\Delta F/F\Delta V$ for the excitation spectrum (emission at 16000 cm^{-1}) and for the emission spectrum (excitation at 21000 cm^{-1}) is shown in Fig. 12. The spectrum of

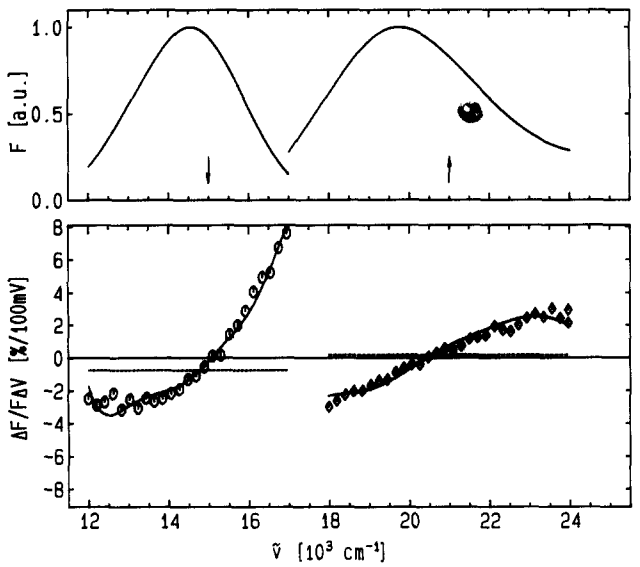


Fig. 11. Voltage-sensitive fluorescence of RH237 for $\tilde{\nu}^*_{ex} = 21000\ cm^{-1}$ and $\tilde{\nu}^*_{em} = 15000\ cm^{-1}$. For other comments see Fig. 5.

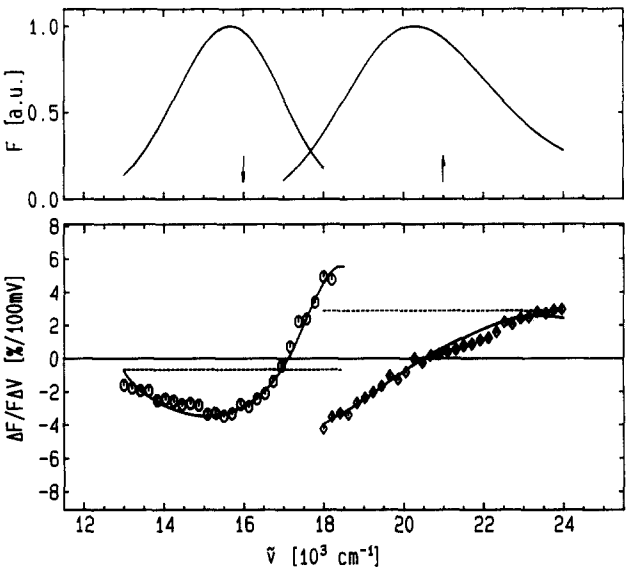


Fig. 12. Voltage-sensitive fluorescence of RH421 for $\tilde{\nu}^*_{ex} = 21000\ cm^{-1}$ and $\tilde{\nu}^*_{em} = 16000\ cm^{-1}$. For other comments see Fig. 5.

excitation is shifted to the blue by $\Delta m_{ex} = 48.0\ cm^{-1}/100$ mV. A broadening is superposed of $\Delta w_{ex} = 16.5\ cm^{-1}/100$ mV. The spectrum of emission is shifted to the blue by $\Delta m_{em} = 16.9\ cm^{-1}/100$ mV. Superposed is a broadening of $\Delta w_{em} = 41.4\ cm^{-1}/100$ mV and a large change of amplitude $\Delta a_{em} = -1.9\%/100$ mV. The maximal negative signal is $\Delta F/F\Delta V = -7.5\%/100$ mV (excitation at 18000 cm^{-1}, emission at 15500 cm^{-1}). The maximal positive signal is $\Delta F/F\Delta V = +8.5\%/100$ mV (excitation at 24000 cm^{-1}, emission at 18000 cm^{-1}).

The voltage-sensitivity of RH421 is similar to that of RH160. The effect of amplitude is somewhat larger and the broadening of excitation is somewhat smaller.

Discussion

The most striking aspect of the measurements of voltage-sensitive fluorescence of the hemicyanine dyes is the individuality of the dyes. We observe only two common features: (a) All excitation spectra are shifted to the blue. (b) All changes of fluorescence yield are negative.

With respect to the shift of the emission spectra we may distinguish three classes: (i) Identical blue-shift of emission and excitation (BNBIQ and RH237). The shifts of BNBIQ are almost twice that of RH237. Both dyes exhibit a relative small change of yield. (ii) Reduced blue-shift of emission as compared to the blue-shift of excitation (di4ANEPPS and RH160). Here the change of quantum yield of RH160 is large compared to that of di4ANEPPS. (iii) Opposite (red) shift of emission – similar in magnitude to the blue-shift of

excitation (BNBP and RH364). Here the sensitivity of BNBP is more than twice the sensitivity of RH364.

There is no visible correlation between the spectral shifts and the change of yield.

The short chromophores (BNBP and RH364) exhibit a red-shift of the emission spectra whereas the larger chromophores (BNBIQ, di4ANEPPS and RH160) exhibit a blue-shift.

The biaryl-dyes (BNBIQ and BNBP) are more sensitive than the styryl-dyes of similar size (RH160/di4ANEPPS and RH364).

There is no trend among the chromophores of similar size (BNBIQ, di4ANEPPS and RH160): Immobilization of one double bond (RH160 to di4ANEPPS) suppresses the change of quantum yield. Immobilization of the second double bond (di4ANEPPS to BNBIQ) enhances the spectral shifts of excitation (factor two) and of emission (factor three). Due to the latter effect BNBIQ is the most sensitive dye in the series.

A significant broadening of emission occurs only when the emission spectrum is shifted to the blue. Occurrence (RH160, RH237) or absence of significant broadening of excitation is unrelated to other effects.

Considering the individual features of voltage-sensitivity of the various dyes we are not able to present a general concept for the physical mechanism, i.e., we are not able to answer the question whether the electrical field (i) interacts with intramolecular shifts of charge or (ii) affects the location and orientation of the charged chromophore with respect to the matrix [2]. We add, however, a few remarks concerning several feasible effects.

Excitation of BNBIQ gives rise to the shift of an elementary charge along the axis of the chromophore by 0.5 nm [6]. If the chromophore is oriented normal to the membrane and if the voltage drop is homogenous across the membrane we expect a blue-shift of 81 $cm^{-1}/100$ mV. This number is close to the experimental shift 76 $cm^{-1}/100$ mV of the excitation spectrum. The coincidence may be a hint that electrochromism plays a role [16]. However, the argument is weak as long as we have no information on the orientation of the chromophore (cf. Ref. 17). In the case of BNBP (charge shift by 0.33 nm [6]) the expected spectral shift is 53 $cm^{-1}/100$ mV as compared to the experimental shift of 26.9 $cm^{-1}/100$ mV. Within the concept of electrochromism the discrepancy is to be assigned to an imperfect orientation of the chromophore.

The reduced blue-shift of emission (di4ANEPPS and RH160) and the red-shift of emission (BNBP and RH364) reveal clearly that – besides a possible electrochromic effect – another process must be involved that affects the position of the spectra: For an interaction of the electrical field with an intramolecular charge shift we expect identical effects for a given electronic transition in a given matrix, i.e., for excitation and emission. A dislocation/reorientation of the dye or a reorganization of the matrix may be responsible.

The significant changes of amplitude reveal clearly that even a third process plays a role in voltage-sensitivity. We found that the fluorescence yield of the hemicyanines is affected strongly by the polarity of the environment – polar solvents suppress fluorescence [5,6,18]. The effect was assigned to the twist around a C-C single-bond [5,6,18,19]. We could attribute the field-induced drop of yield to an enhanced polarity around the chromophore. Such a change might be caused by displacement/reorientation of the dye or by reorganization of the matrix. This idea, however, is rather vague as the description of solvation at the membrane/water interface in terms of solvation by bulk solvents is not straightforward [6].

It should be noted in this context that opposite spectral shifts of emission and excitation – though small (3–10 $cm^{-1}/100$ mV) – and considerable drops of amplitude (around $-1\%/100$ mV) were found for various styryl-dyes in planar lipid bilayers (Dambacher, K.H. and Fromherz, P., unpublished observations). From the complete sensitivity spectra – measured in spherical bilayers (Fromherz, P. and Schenk, O., unpublished observations) fit-parameters were evaluated as described in the present paper, e.g., for di4ANEPPS in glycerolmonooleate $\Delta a_{em} = -2.8\%/100$ mV, Δm_{em} -6.3 $cm^{-1}/100$ mV and $\Delta m_{ex} = 33$ $cm^{-1}/100$ mV [2]. The different sensitivity of the same dye in the neuron membrane and in a lipid bilayer suggests that the interaction of the dye with the membrane – location, solvation, mobility – is important, indeed.

If relaxation processes – displacement/reorientation of the dye or reorganization of the matrix – after excitation play a role with respect to the red-shift of fluorescence and to the drop of fluorescence yield, the lifetime of the emitting molecular state is relevant: processes of relaxation have less chance to affect fluorescence in the case of a short lifetime. We have no time-resolved data of fluorescence in the neuron membrane. However, in lecithin bilayers the lifetime of the dye BNBP – which exhibits a red-shift of fluorescence and a large drop of amplitude – is relatively long (1.3 ns [6]) whereas the dye BNBIQ – which exhibits no red-shift and only little drop of amplitude – is shorter (0.65 ns [6]).

The spectrum of voltage-sensitivity is only one of the features that has to be considered with respect to optical recording. In the selection of a dye and of the wavelengths of excitation and emission one has to take into account, of course, the spectra of excitation and emission themselves, the spectral intensity of the light source and the spectral sensitivity of the detector. For example, BNBIQ – the best hemicyanine – cannot be used with the usual set-up for optical recording with a mercury lamp because of its low absorbance at 535 nm.

Conclusions

The combination of patch-clamp technique and fluorescence microscopy allowed the determination of the voltage-sensitivity of hemicyanine dyes in a neuron membrane. The data could be fitted by a change of fluorescence yield, shifts of the emission and excitation spectra of fluorescence and changes of spectral width. The values of the five parameters provide a certain classification of the dyes. However, at present moment the change of yield, the spectral shift and the changes of width are mere fitting parameters: In no case we know the physical origin for the spectral modifications.

We hope that the spectra of voltage-sensitivity for the hemicyanine dyes in neuron membranes together with similar data in artificial lipid membranes will enable us to elucidate the physical mechanism of voltage-sensitivity and the development of optimized fluorescent probes. Independent of any physical interpretation, however, the spectral data are the basis for an optimization of optical recording with respect to the choice of a dye for a given optical configuration or with respect to an optimal choice of lamps, detectors and filters.

Acknowledgments

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