Fluorescent Probe Based on Intramolecular Proton Transfer for Fast Ratiometric Measurement of Cellular Transmembrane Potential

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Development of fast-response potentiometric probes for measuring the transmembrane potential V_m in cell plasma membranes remains a challenge. To overcome the limitations of the classical charge-shift potentiometric probes, we selected a 3-hydroxychromone fluorophore undergoing an excited-state intramolecular proton transfer (ESIPT) reaction that generates a dual emission highly sensitive to electric fields. To achieve the highest sensitivity to the electric field associated to V_m, we modified the fluorophore by adding two rigid legs containing terminal polar sulfonate groups to allow a deep vertical insertion of the fluorophore into the membrane. Fluorescence spectra of the new dye in lipid vesicles and cell membranes confirm the fluorophore location in the hydrophobic region of the membranes. Variation of V_m in lipid vesicles and cell plasma membranes results in a change of the intensity ratio of the two emission bands of the probe. The ratiometric response of the dye in cells is ≈15% per 100 mV, and is thus quite large in comparison with most single-fluorophore, fast-response probes reported to date. Combined patch-clamp/fluorescence data further show that the ratiometric response of the dye in cells is faster than 1 ms. Analysis of the excitation and emission shifts further suggests that the probe responds to changes in V_m by a mechanism based on electrochromic modulation of its ESIPT reaction. Thus, for the first time, the ESIPT reaction has been successfully applied as a sensing principle for detection of transmembrane potential, allowing to couple classical electrochromic band shifts with changes in the relative intensities of the two well-separated emission bands. The fast two-band ratiometric response as well as the relatively high sensitivity of the new probe are the key features that make it useful for rapid detection of V_m changes in cell suspensions and single cells. Moreover, the new design principles proposed in the present work should allow further improvement of the probe sensitivity.

Introduction

Transmembrane potential (V_m) is the most dynamic electrostatic component of cell plasma membranes and plays essential roles in a variety of cellular functions related to bioenergetics, ion transport, motility, and cell communication. It is highly important to monitor this potential, notably in neuronal cell assemblies, because this can provide clues to understand the mechanisms of brain activity.[1,2] Intracellular microelectrode (or patch-clamp) recording and optical microscopy are the two major methods used to monitor V_m in cells. Microelectrodes allow recording V_m with extremely high precision and temporal resolution. However, since they work on the principle of "point measurement", observation of the spatial distribution of V_m within a single cell or cell assemblies using microelectrodes is complicated and requires multi-electrode techniques.[3] This crucial limitation can be partly overcome using optical methods and fluorescent probes that allow recording of V_m in single cells and multicellular assemblies with both high temporal and spatial resolution.[1,3,4] However, the weak point of fluorescence-based measurements of V_m is directly connected with the intrinsic limitations of available voltage-sensitive fluorescent probes, which provide lower signal-to-noise ratio compared to the microelectrode technique.[5] For instance, the initially developed probes with strong response to V_m were too slow to respond to the fast variations of V_m in nerve cells.[6] New generation dyes introduced by Grinvald et al.[7] showed improved speed of response (microsecond time scale), but exhibited other drawbacks such as a response in emission intensity, which is difficult to calibrate and dependent on probe concentration.

A new methodology of V_m measurement based on fluorescence resonance energy transfer (FRET) was recently developed[8] using the hydrophobic oxonol dye as a FRET partner. This dye undergoes a V_m driven translocation between the two leaflets of the cell membrane, allowing fast (microsecond time scale) and strong (up to 50% per 100 mV) responses to V_m variations.[8] This method has been used in a number of cellular applications,[8b−8d] but requires double labeling of the cell plasma membrane with precise relative concentrations of the FRET components.

In contrast to the slow-responding dyes, the styryl dyes developed by Loew et al.[9] have a fast and ratiometric response to variations of V_m, but suffer from their low sensitivity to changes in V_m (≈7% per 100 mV). This ratiometric response is recorded from the band shift of the excitation spectrum, while in emission, this response is less pronounced.[9] However, for fluorescence ratiometric imaging techniques, probes with a ratiometric response in emission are much more convenient. The styryl dyes were introduced about 15 years ago, but only recently their sensitivity was 2-fold improved by increasing the fluorophore length and rigidity, reaching the physical limits of the electrochromic response.[10]

To overcome the limits associated with voltage-sensitive dye responses in cell membranes, we selected another basic fluo-

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rophore, 3-hydroxychromone, for the development of new probes. Dyes of the 3-hydroxychromone family exhibit an excited-state intramolecular proton transfer (ESIPT) reaction and consequently a dual fluorescence with two emission bands, corresponding to the initially excited normal (N*) and the ESIPT reaction product, tautomer (T*) forms.[11] Due to the very different dipole moments of these two emissive states,[12] the dual emission of 3-hydroxychromones is highly sensitive to the polarity[13] and electric fields[14] of their surroundings. Thus, the electric field of an attached proximal charge was recently shown to induce an intramolecular Stark effect in the 3-hydroxychromone fluorophore that shifts the two emission bands and strongly modifies their relative intensities.[14a] Indeed, this electric field interacts differently with the two dipole moments and thus changes dramatically the relative energies of the two emissive states. As a result, the ESIPT equilibrium between the N* and T* states is shifted and the relative fluorescence intensities of the two bands are modified.[14] Thus, due to the ESIPT reaction, the classical electrochromic shifts in 3-hydroxychromones are coupled with a strong variation in the relative intensities of the two emission bands, which provides a new approach in the design of ratiometric probes.[14a] This approach has been successfully applied to develop new fluorescent ratiometric probes highly sensitive to the external electric field associated with the dipole potential in model[15] and cellular[16] membranes. Therefore, 3-hydroxychromone is a highly promising fluorophore for development of fluorescent probes for measuring transmembrane potential.

To achieve the highest sensitivity to the electric field associated with V_m, the fluorophore must be introduced as deeply as possible into the hydrophobic region of the bilayer, where the dielectric constant of the bilayer is minimal. This requirement cannot be easily met with hemicyanine and styryl pyridinium dyes[9,10] because their fluorophore bears a positively charged group that anchors the fluorophore at the polar interface of the bilayer. In contrast, 3-hydroxychromones are uncharged molecules of relatively low polarity, which can be introduced at any position within the bilayer.[17]

In the present work, we developed a 3-hydroxychromone derivative containing two rigid legs with terminal anionic groups, which allow vertical insertion of the fluorophore deeply into the hydrophobic region of the bilayer. Characterization of the new dye in lipid vesicles and cell suspensions demonstrates that it responds to voltage changes by variation of the intensities of its two emission bands. Moreover, in combined fluorescence microscopy and whole-cell patch clamp recordings from single cells, fast responses of the probe to V_m variations were obtained. This dye is the first transmembrane potential probe operating on the basis of an ESIPT reaction, so that development of other new improved potentiometric probes can be expected.

Experimental Section

Materials. All chemicals and biochemicals were purchased form Aldrich, Fluka, or Sigma. For fluorescence measurements, the solvents were of spectroscopic grade.

Synthesis of the probe di-SFA. 2-(6-diethylaminobenzo-[*b*]furan-2-yl)-3-hydroxy-6-ethoxymethylchromone ($C_{24}H_{25}NO_5$) (1). One mol of 6-diethylaminobenzo[*b*]furan-2-carbaldehyde[13b] and 1 mol of 5′-chloromethyl-2′-hydroxyacetophenone[17] were dissolved in ethanol followed by an addition of 4 mol of 50% aqueous sodium hydroxide. After stirring for 3 h, 15 mol of sodium methoxide and 12 mol of 30% hydrogen peroxide were added. The mixture was refluxed for 3 min, cooled to room temperature and poured into water. The formed precipitate was filtered and washed with water. It was purified by re-crystallization from acetonitrile. Yield 20%. ^{1}H NMR (200 MHz, DMSO-D6) 1.20−1.40 (9H, m), 3.48 (4H, m), 3.60 (2H, q, J 7.1), 4.92 (2H, s), 6.79 (1H, m), 6.90 (1H, s), 7.51 (1H, d, J 8.8), 7.65−7.80 (3H, m), 8.22 (1H, s); ESI (CH_3CN, 1% HCOOH) *m*/*z* (M^++1) 408.2.

6-[2-(3,5-dibromophenyl)-vinyl]-2-(6-diethylaminobenzo-[*b*]furan-2-yl)-3-hydroxychromone ($C_{29}H_{23}Br_2NO_4$) (4). Chromone **1** in excess 48% hydrobromic acid was heated on an oil bath at 100 °C for 3 h. The cooled solution was neutralized with 10% sodium carbonate. The resultant precipitate of chromone **2** (6-bromomethyl-2-(6-diethylaminobenzo[*b*]furan-2-yl)-3-hydroxychromone) was filtered and washed with water. Yield of the crude product was 95%, which was used directly in the next step. A mixture of chromone **2** and excess thriethyl phosphite was heated at 160 °C for 3 h. The excess of triethyl phosphite was removed in vacuo and the product **3** ([2-(6-diethylaminobenzofuran-2-yl)-3-hydroxy-4-chromon-6-ylmethyl] phosphonic acid diethyl ester) was used directly in the next step. One mol of chromone **3** and 1.1 mol of 3,5-dibromobenzaldehyde (synthesized from 1,3,5-tribromobenzene, butyllithium and dimethylformamide[18]) was dissolved in dry THF. Two mol of NaH was added to the resultant mixture stirred in an ice bath. After addition, the mixture was stirred for 2 h at room temperature. After addition of water, the precipitate was filtered and crystallized from butanol. Yield 35%, ^{1}H NMR (200 MHz, $CDCl_3$) 0.89 (6H, t, J 6.9), 3.49 (4H, q. J 6.9), 6.79 (1H, d, J 8.9), 6.89 (1H, s), 7.13 (1H, d, J 12.7), 7.51 (1H, d, J 8.3), 7.58−7.75 (6H, m), 7.84 (1H, d, J 8.3), 8.34 (1H, s). ESI (CH_3-CN, 1% HCOOH) *m*/*z* (M^++1) 612.0, 610.0, 608.0.

Di-SFA ($2Na^+[C_{45}H_{35}NO_{10}S_2]^{2-}$). A mixture of 5 mg of chromone **4**, 10 mg (6 mol excess) of p-styrenesulfonate, 1 mg of tri-o-tolylphosphine, 0.5 mg of palladium (II) acetate, and 0.05 mL of diisopropylethylamine in 0.5 mL of dry dimethylformamide was stirred at 105 °C for 2 h. After cooling, an excess of methanol was added, and the formed precipitate was filtered. The impurities in the precipitate were removed by extraction with toluene under reflux followed by two extractions with acetonitrile under reflux. The obtained product was pure according to thin-layer chromatography. Yield 60%, ^{1}H NMR (200 MHz, DMSO); ^{1}H NMR (200 MHz, $CDCl_3$) 1.16 (6H, t, J 6.9), 3.49 (4H, m), 6.79 (1H, d, J 8.9), 6.92 (1H, s), 7.30−7.75 (20 H, m), 7.85 (1H, d, J 8.3), 8.12 (1H, s), 8.31 (1H, s); ESI (MeOH, 5% H_2O) *m*/*z* (M^{2-}/2) 406.6.

Preparation of Lipid Vesicles. The procedure was adapted from Mantana and Loew.[9] Buffer solutions of 20 mM HEPES, pH 7 (22 °C) containing either 100 mM K_2SO_4 (high K^+ buffer) or 100 mM Na_2SO_4 and 1 mM K_2SO_4 (low K^+ buffer) were prepared. A suspension of unilamellar lipid vesicles was made from 10 mg of egg yolk phosphatidylcholine (Sigma; type XI-E) in 0.5 mL of high K^+ buffer using a Lipex Biomembrane extruder. The size of the filters was first 0.2 μm (7 passages) and thereafter 0.1 μm (10 passages). Typically, this procedure generates monodisperse unilamellar vesicles with a mean diameter of 0.11 μm, as measured with a Zetamaster 300 (Malvern Instruments). Next, 10 μL of the resultant vesicle suspension was diluted into 1 mL of the appropriate buffer containing 1 μM of the probe di-SFA and 2 mg/mL of β-cyclodextrin. Transmembrane potential was generated by addition of valinomycin in ethanol solution (final concentration of ethanol <0.02%) to a final concentration 90 nM.

Cell Preparation. For cell suspensions, human lymphoblastoid CEM cells (kindly provided by Dr Kunzelmann, Faculté de Médecine, Strasbourg), were suspension-cultured in synthetic

medium X-Vivo 15 (Cambrex) at 37 °C in a humidified 5% CO_2 atmosphere. For loading of the dye, cells were incubated for 10 min in Ringer solution containing inter alia 1.5 mM K^+, 2 mg/mL β-cyclodextrin and 4 μM di-SFA. After centrifugation (5 min at 1500 rpm) cells were suspended at 2×10^6 cells/ml in Ringer solution containing 1.5 mM K^+. Valinomycin in ethanol solution was added to the cell suspension to a final concentration 1.5 μM (final concentration of ethanol <0.02%). The concentration of valinomycin in cells was increased in comparison to lipid vesicles in order to obtain comparably fast generation of transmembrane potential.

For patch-clamp experiments, A172 cells (a human astrocytoma cell line) and L929 cells (a mouse fibroblast line) obtained from the American Type Culture Collection were cultured as described previously.[16,19] Cells were plated in Petri dishes with fibronectin-coated glass bottoms. To decrease photodegradation effects, astaxanthin (Sigma) was used as an antioxidant.[8a] Astaxanthin was dissolved at 2 mM in THF and 100 μL of this solution was added to 20 mL of DMEM with vortexing. Normal extracellular medium was replaced by the astaxanthin-containing medium at room temperature, and then the cells were placed in the 37 °C incubator for 24 h before measurements. For loading of the dye (15 min at room temperature), the culture medium was replaced by a Ringer solution containing inter alia 1.5 mM K^+, 2 mg/mL β-cyclodextrin, and 5 μM di-SFA. Cells were rinsed with the 1.5 mM K^+ Ringer solution.

Instrumentation. Absorption and fluorescence spectra were recorded on a Cary 400 (Varian) and FluoroMax 3.0 (Jobin Yvon) spectrophotometer, respectively. Time based measurements of the fluorescence ratio from lipid vesicles or cell suspensions were acquired on an SLM-Aminco 48000 spectrofluorometer equipped with two photomultipliers (PTI) and monochromators. The long-wavelength channel was set to 605 nm, and the short-wavelength channel to 527 nm (for lipid vesicle measurements) or 510 nm (for cellular measurements). To obtain high signal-to-noise ratio, the monochromator slits were set to 32 nm. Excitation wavelength was 450 nm.

The combined patch-clamp[20] and fluorescence experiments on single adherent cells were run at room temperature, on an inverted microscope (Nikon Diaphot). Excitation light from a 100 W Xe arc lamp (Osram) was limited using a neutral density filter to minimize bleaching, and band-pass (10 nm) filtered at 440 nm. A dichroic mirror (455 nm) allowed separation of excitation and emission intensities. Additional removal of excitation light from the fluorescence signal was provided by a long-pass filter with a 460 nm cutoff. Emitted epifluorescence was collected using a 40x UV-fluor oil immersion objective (n.a. 1.3, Nikon) and limited to a single cell using an adjustable rectangular diaphragm. Emitted fluorescence was separated with a dichroic mirror (565 nm). Additional removal of the short-wavelength light at the long-wavelength channel was provided with the long-pass filter with a 520 nm cutoff. Signals were recorded simultaneously using two photomultipliers (Hamamatsu R374) with matched photometers (P1, Nikon). The ratio of emitted fluorescence was obtained with an analogue divider and acquired at 1 kHz. Conventional whole-cell patch-clamp recordings were made in voltage-clamp mode.[20] Pipets were pulled from thin-wall borosilicate glass and filled with a standard internal Cs^+ solution (pipet resistance 7−10 MΩ). The bath contained 1.5 mM K^+ Ringer solution. Variations in V_m were imposed on single cells using the patch-clamp amplifier (List EPC-7). The resultant changes in di-SFA emitted fluorescence were acquired as described above simultaneously with the voltage command signal and stored on a computer using a CED 1401 A/D interface and CED software.

Results and Discussion

Probe Design and Synthesis. The following principles were used to design a new efficient fluorescence probe for measuring cellular transmembrane potential. First, the fluorophore should be located vertically in the bilayer, so that its dipole moment is aligned with the electric field in the bilayer. Second, the fluorophore should be located deeply in the hydrophobic part of the bilayer where the dielectric constant is the lowest and thus the dielectric screening of the electric field induced by V_m is minimal. As a consequence, this location should optimize the sensitivity of the fluorophore to changes in V_m. Third, the probe molecules should be anchored at the outer leaflet of the cell membrane, so that the fluorophores always exhibit the same direction with respect to the electric field. Finally, the fluorophore itself must show a large change in dipole moment upon electronic excitation. This last point can be achieved by selection of charge transfer fluorophores with the largest conjugation. It was commonly accepted that the best charge-transfer fluorophores for measuring electric field are styrylpyridinium dyes.[9] However, these fluorophores cannot be inserted deeply into the hydrophobic part of the bilayer due to their highly polar pyridinium ionic group (Figure 1).

4′-(Dialkylamino)-3-hydroxyflavone dyes are more suitable for getting a deep vertical insertion into the membranes, since their fluorophore is neutral and rather hydrophobic (calculated logarithm of *n*-octanol/water partition coefficient[21] LogP ≥ 2.19). Moreover, due to the high sensitivity of its ESIPT to electric fields, this fluorophore exhibits a strong electrochromism[14] with large changes of the relative intensities of its two emission bands. However, according to our data in organic solvents,[13] insertion of 4′-(dialkylamino)-3-hydroxyflavone dyes deeply into the bilayer, where the dielectric constant is low ($\epsilon = 2-4$), should result in an extremely low intensity of the short-wavelength band, so that the desired dual emission would not be observed. Therefore, to design a new potentiometric probe, we started from a recently developed analogue of 4′-(dialkylamino)-3-hydroxyflavone, 2-(6-diethylaminobenzo[*b*]furan-2-yl)-3-hydroxychromone[22] (dye FA) that shows well resolved dual emission in highly apolar environments.[23] Moreover, due to extended conjugation, this dye exhibits a relatively high excited-state dipole moment,[12,24] which makes it more attractive as an electrochromic dye. In addition, the absorption and emission of this dye are significantly shifted to the longer wavelengths as compared to its 3-hydroxyflavone analogue,[22] making it more suitable for biological applications. Thus, following the above design principles, we synthesized a new dye, di-SFA, for measuring V_m (Figure 1). The vertical orientation and deep location of the 3-hydroxychromone fluorophore in the bilayer is provided by two rigid legs oriented at 120° with respect to each other (and, consequently, 120° with respect to the fluorophore dipole) and containing terminal polar sulfonate groups. The latter groups anchor the molecule at the polar interface of the bilayer, imposing the apolar part of the molecule to protrude deeply into the bilayer (Figure 1). Furthermore, due to the two sulfonate groups, the probe has a double negative charge in aqueous media, a feature that should diminish significantly its penetration through the cell membrane, since the cell resting potential is negative. Noticeably, the 3-hydroxychromone fluorophore was substituted at 6-position in order to position the substituent parallel to the fluorophore dipole. This substitution should not modify significantly the

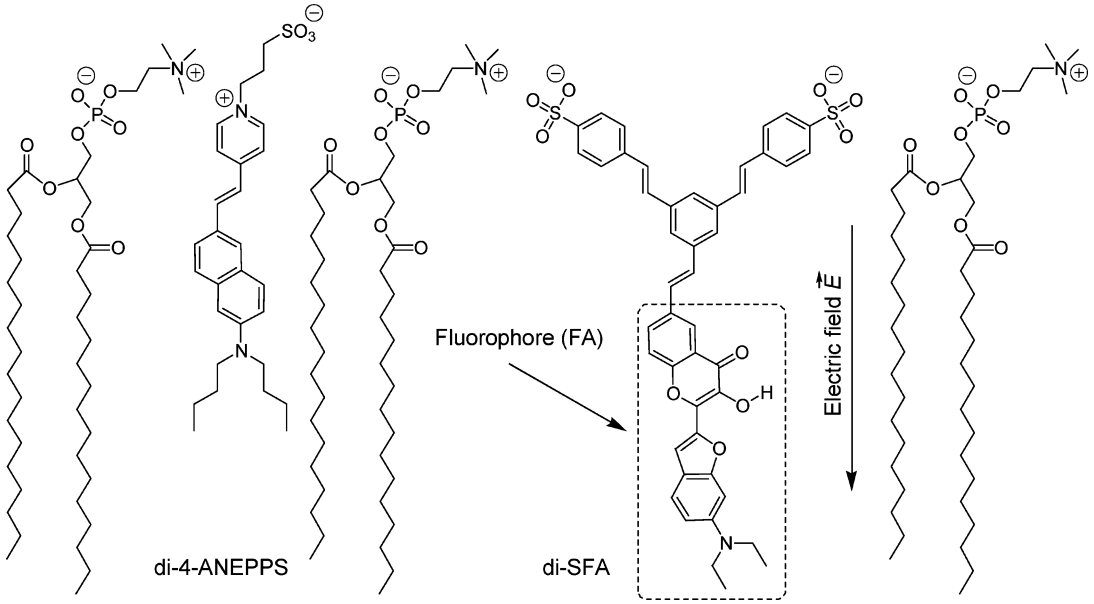

Figure 1. Assumed location of di-4-ANEPPS (a styrylpyridinium dye) and di-SFA (a 3-hydroxychromone derivative) in lipid bilayers. The fluorophore part of the probe di-SFA is boxed. Only half of the bilayer is shown.

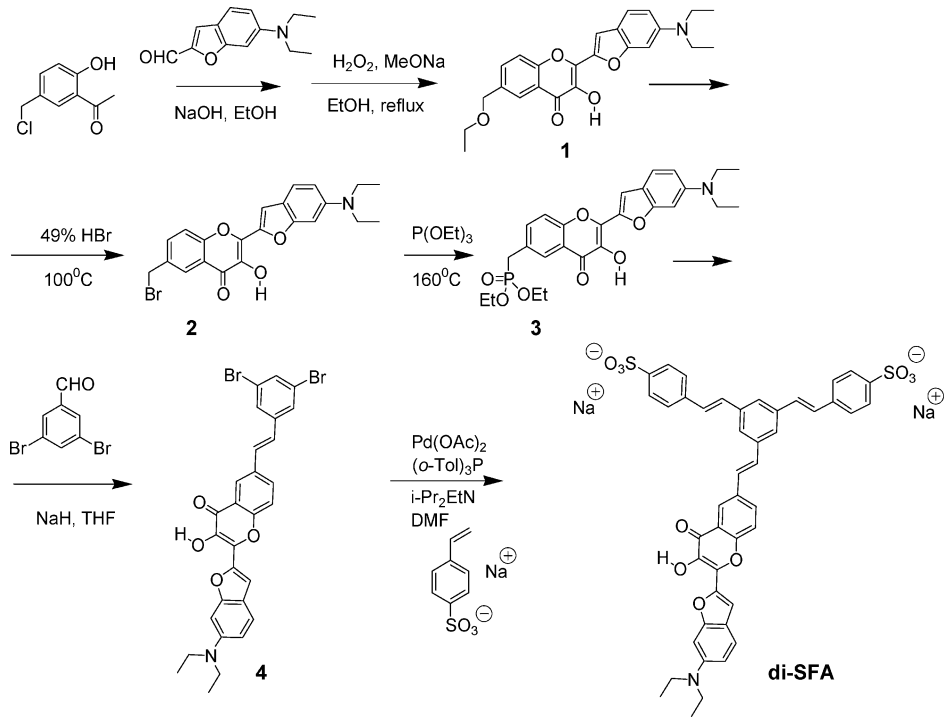

Figure 2. Synthesis of the probe di-SFA.

fluorescent properties of the fluorophore, since it is in *meta*-position with respect to the 4-carbonyl group of the fluorophore (Figure 1).

The synthesis of the dye di-SFA was performed according to the scheme described in Figure 2. 4′-chloromethyl-2′-hydroxyacetophenone was reacted with the corresponding aldehyde in basic conditions and then further transformed into 6-ethoxymethyl-3-hydroxychromone derivative using the modified Algar−Flynn−Oyamada methodology.[25] This last derivative was converted quantitatively into the corresponding 6-bromomethyl derivative, which was then transformed into the corresponding phosphonate. A Wadsworth−Emmons reaction of the phosphonate with 3,5-dibromobenzaldehyde afforded the 3,5-dibromostyrene derivative of 3-hydroxychromone. The latter was converted into the target dye di-SFA by Heck coupling with p-styrenesulfonate.

Spectroscopic Properties of Probe di-SFA in Organic Solvents and Lipid Vesicles. In a first step, we tested how the substituent at 6-position of the fluorophore FA modified its fluorescence properties. For this purpose, we compared the fluorescence properties of di-SFA and its FA fluorophore in solvents of different polarities (Table 1). We observe that the absorption spectra of these two dyes are very similar though the absorption band of di-SFA is slightly red shifted by 5−7 nm (260−360 cm^{-1}) in all the studied solvents (Table 1). The similarity of the absorption spectra of these two dyes is an indication that the introduction of the large aromatic substituent at 6-position does not considerably modify the fluorophore of

TABLE 1: Comparison of the Absorption and Fluorescence Band Maxima (λ_{Max}) of Probe Di-SFA with Respect to Its FA Fluorophore[a]

	absorption band			N* band			T* band		
	λ_{max}, nm			λ_{max}, nm			λ_{max},nm		
solvent	FA	Di-SFA	$\Delta\nu$ cm^{-1}	FA	Di-SFA	$\Delta\nu$ cm^{-1}	FA	Di-SFA	$\Delta\nu$ cm^{-1}
dioxane	439	445	310	509	516	270	611	619	210
THF	439	444	260	546	554	260			
acetonitrile	438	445	360	580	589	260			

[a] $\Delta\nu$ designates the difference in the band position between the two dyes in the energy scale. In THF and acetonitrile, the T* band of the dyes is not resolved due to its very low relative intensity.

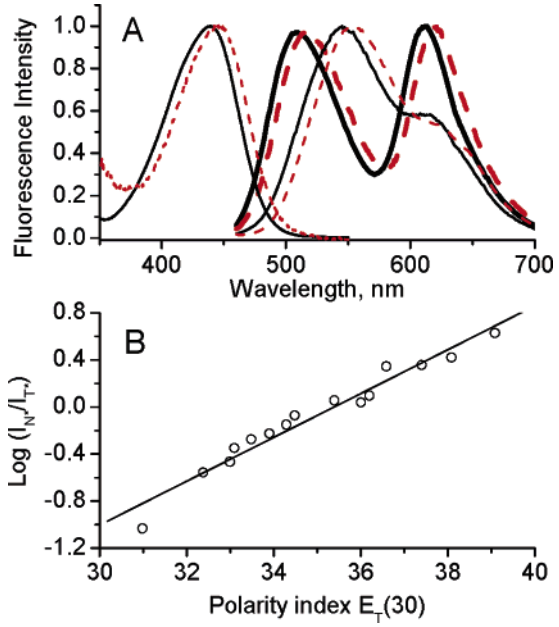

Figure 3. Spectroscopic properties of probe di-SFA and its fluorophore FA in different solvents. A. Absorption (left) and fluorescence (right) spectra of probe di-SFA (red dashed curves) and its fluorophore FA (black solid curves) in tetrahydrofurane (thin curves) and dioxane (thick curves). B. Logarithm of I_{N^*}/I_{T^*} vs. polarity index $E_T(30)$ of different organic solvents. Correlation equation: $\log(I_{N^*}/I_{T^*}) = -6.59 + 0.186E_T(30)$, correlation coefficient $r = 0.979$, standard deviation, SD = 0.092. Data are from ref 23.

the di-SFA probe. Moreover, the fluorescence spectra of these two dyes are also very close, with a similar red shift of the di-SFA bands by 6−8 nm (260−270 cm^{-1} for the N* band and 210 cm^{-1} for the T* band) (Tables 1, Figure 3). Especially important is the nearly identical solvatochromism of the N* band of the di-SFA and FA dyes, Indeed, the N* band of di-SFA shifts to the red to the same extent as that of FA with increasing solvent polarities (dioxane < tetrahydrofuran (THF) < acetonitrile), as can be seen from the constant difference in the band positions for these two dyes in the studied solvents (Table 1). This indicates that the dipole moment of the 3-hydroxychromone fluorophore in di-SFA dye is identical to that of the FA fluorophore. In the low polar dioxane, both dyes exhibit dual emission of nearly identical relative intensities (Figure 3A), indicating that the ESIPT behavior in these two dyes is also nearly identical. These results allow us to use the well-characterized dye FA[23] as a reference to estimate the polarity of environment of di-SFA fluorophore in lipid membranes. This polarity can be estimated from the logarithm of the intensity ratio of N* and T* bands of dye FA which varies linearly with the solvent polarity index $E_T(30)$ (Figure 3B).[23,24]

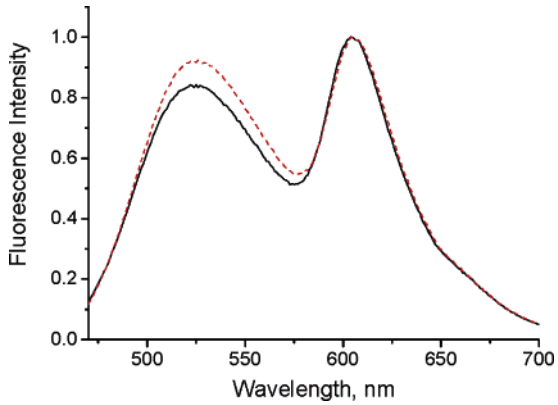

Figure 4. Fluorescence emission spectra of di-SFA in lipid vesicles at different transmembrane potentials: 0 mV (solid black line) and −118 mV (dashed red line). V_m was created by a K^+ gradient (200 mM inside vs 2 mM outside the liposomes) in the presence of 90 nM valinomycin and estimated from the Nernst equation. Probe concentration was 1 μM. Ratiometric probe response is 8% per 100 mV. Excitation wavelength was 450 nm. The spectra are normalized at the peak maxima.

The fluorescence spectrum of di-SFA bound to lipid vesicles exhibits two well-resolved bands of similar intensity (Figure 4). According to numerous studies of 3-hydroxychromone dyes,[13,15−17,23] the short-wavelength band can be unambiguously assigned to the emission of the N* band, while the long-wavelength band corresponds to the T* emission. The excitation spectra recorded at the two emission bands are identical, confirming that both emissive states originate from the same ground-state species. From the ratio of the two emission bands in this spectrum, we can estimate the polarity of the probe surrounding from the linear correlation (Figure 3B) of the FA fluorescence intensity ratio vs the empirical polarity index E_T-(30).[23] The obtained value, $E_T(30) = 35.0$, corresponds to a dielectric constant $\epsilon \approx 4$, which is close to the dielectric constant values commonly accepted for the hydrophobic region deeply inside the lipid bilayer. This result suggests a deep imbedding of the probe fluorophore into the lipid bilayer close to the fatty acid residues, in line with the expectations from the probe molecular design (Figure 1). In contrast to the ANEPPS dye, where the estimation of the polarity of its surrounding is biased by the highly anisotropic nature of the lipid bilayer,[26] the di-SFA probe likely enables a faithful estimation of its fluorophore surrounding in membranes. Indeed, unlike the ANEPPS dye, the fluorophore of di-SFA is neutral and should thus not undergo differential solvation in the bilayer.[26] In addition, the apolar environment of the deep location of the di-SFA fluorophore in the lipid bilayer is much less anisotropic than the polar water−bilayer interface where the ANEPPS fluorophore is located.

Fluorescence Response of the Probe Di-SFA to Transmembrane Potential in Lipid Vesicles. A transmembrane potential, V_m, was generated in lipid vesicles using the well-established technique based on valinomycin induced K^+ diffusion potential.[9b] Hyperpolarization of the vesicles ($V_m = -118$ mV) significantly changes the fluorescence spectrum of the probe, with an increase of the relative intensity of the N* band and a slight red shift of both bands (Figure 4). The ratio of the two bands increases by ≈9.5%, indicating a probe sensitivity of 8% per 100 mV. In the meantime, the absolute fluorescence intensity of the probe does not considerably change on addition of valinomycin. A red shift is also observed in the excitation spectra (not shown). The magnitude and the direction of the observed spectral shifts correspond to those observed for the

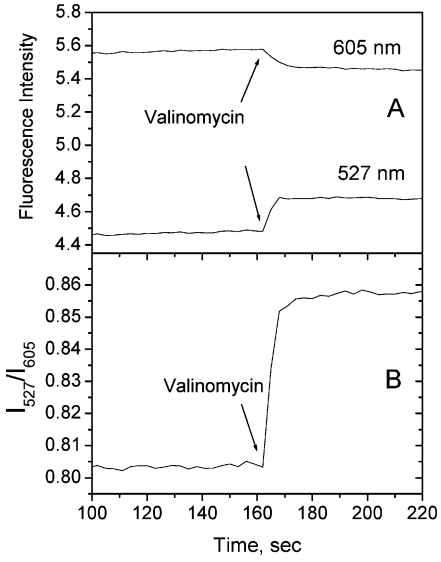

Figure 5. Time-dependence of di-SFA response to hyperpolarization of lipid vesicles. The fluorescence intensities at 527 and 605 nm (A) and the fluorescence intensity ratio I_{527}/I_{605} (B) of di-SFA were recorded from lipid vesicles before and after addition of 90 nM valinomycin (which generated a V_M value of −118 mV, due to a K^+ gradient). Excitation wavelength was 450 nm.

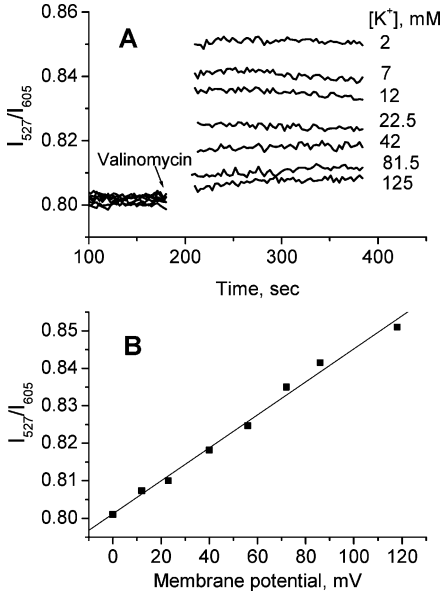

Figure 6. A. Quantitative analysis of di-SFA response to changes in the external K^+ concentrations. The fluorescence ratio of the probe di-SFA was recorded at different external concentrations of K^+ in a suspension of lipid vesicles (internal [K^+] was 200 mM). The ratio of the two bands was recorded before and after addition of valinomycin. B. Plot of the I_{527}/I_{605} ratio as a function of the V_m value calculated from the Nernst equation. Probe concentration was 1 μM. Excitation wavelength was 450 nm.

styrylpyridinium dye di-4-ANEPPS in similar lipid vesicles,[9b] indicating an electrochromic mechanism for our dye (see below).

The time-dependence of the probe response to hyperpolarization of the lipid vesicles was monitored by recording the emitted fluorescence intensity as a function of time simultaneously at 527 and 605 nm for the N* and T* bands, respectively (Figure 5). Addition of valinomycin results in a fast increase in the 527 nm signal and a simultaneous decrease in the 605 nm signal, in accordance with the spectroscopy data (Figure 3). We observed that the intensity ratio, I_{527}/I_{605} (i.e., I_{N*}/I_{T*}), increases upon addition of valinomycin with a rise-time of ≈10 s (Figure 5). However, this response time was likely limited by the diffusion of valinomycin, in line with the literature,[9b] and thus does not represent the true probe response time.

Quantitative analysis of the probe response was investigated by varying the external K^+ concentration (Figure 6a). The ratiometric probe response upon valinomycin addition decreases with increasing external K^+ concentration and is nearly zero when the external and internal K^+ concentrations are equal. This last observation excludes any direct effect of valinomycin on the probe fluorescence. Expected V_m values for different external K^+ concentrations were calculated using the Nernst equation, enabling the construction of a calibration curve for the probe response. Our results show that the I_{N*}/I_{T*} ratio of the dye is a linear function of V_m in lipid vesicles (Figure 6B), confirming the applicability of di-SFA for quantitative measurements. The slope of the curve corresponds to a ≈6% ratio response of the probe per 100 mV. This somewhat smaller response as compared to that calculated from the spectra (see above) is probably connected with the signal recording. In the latter case, to obtain time resolution of seconds with low noise, we increased the monochromator slits to 32 nm, including thus regions of the spectrum where the sensitivity to V_m changes is less than at the band maximum.

Cell Suspensions. The response of the di-SFA probe to V_m in cell membranes was then tested in a suspension of CEM cells. The transmembrane potential was controlled by addition of valinomycin and varying the external K^+ concentration.[27] In cell suspensions, the fluorescence spectrum of di-SFA also exhibits two bands (Figure 7A). However, compared to lipid vesicles, the spectrum is blue-shifted by ≈12 nm and the relative intensity of the N* emission band is significantly lower (I_{N*}/I_{T*} is 0.55 in cells, while it is 0.85 in lipid vesicles). This suggests that the dye senses a more hydrophobic environment in cells. According to the solvent polarity correlation curve for the parent FA fluorophore (Figure 3B), the estimated polarity in cells is $E_T(30) = 34.0$ (corresponding to a dielectric constant $\epsilon \approx 3$), which is significantly lower than that in lipid vesicles. The lower polarity of the environment of di-SFA fluorophore in the cell plasma membrane as compared to the vesicle bilayer is probably associated with the lipid composition of the outer leaflet of the plasma membrane. Indeed, in addition to phosphatidylcholine, the outer leaflet contains sphingomyelin and cholesterol,[28] which could significantly increase the membrane hydrophobicity.[29]

Addition of valinomycin to a cell suspension loaded with di-SFA increases the relative intensity of the N* emission band (Figure 7A). This effect is similar in direction but considerably smaller in amplitude to that observed in lipid vesicles (Figure 4), likely due to the cell resting potential. In line with this hypothesis depolarization of cells by addition of external K^+ (with an isotonic high K^+ buffer) results in a pronounced decrease in the relative intensity of the N* band and a slight shift to the blue (by ≈2 nm). The T* band (Figure 7B) as well as the excitation band (Figure 7C) also exhibit shifts in the same direction as that of the N* band, in accordance with the expected electrochromic effect (see below).[14] As predicted by the Nernst equation (assuming that internal K^+ was 140 mM), addition of valinomycin hyperpolarizes cells to −116 mV, while addition of external K^+ depolarizes them to −46 mV. Thus, the ratiometric response of the probe to V_m in cell plasma membranes is ≈15% per 100 mV, which is considerably higher than the response observed in lipid vesicles. The higher sensitivity of the probe to V_m in cells is probably due to the

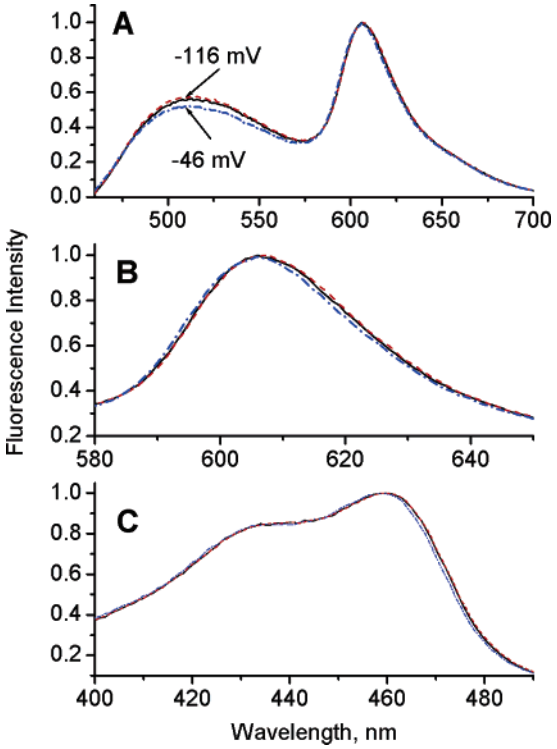

Figure 7. Response of di-SFA to transmembrane potential in CEM cell suspensions. Fluorescence emission (A and B) and excitation (C) spectra of di-SFA from a suspension of CEM cells at the resting potential (black), following hyperpolarization due to the addition of 1.5 μM valinomycin (red dashed), and after depolarization by addition of an isotonic solution containing 150 mM external K^+ buffer (blue dash-dotted). Membrane potentials were estimated by the Nernst equation. Fluorescence emission spectra were recorded at 450 nm excitation. Excitation spectra were recorded at 610 nm emission.

less polar environment of the fluorophore ($\epsilon \approx 3$ vs 4) in the cell membrane which reduces its dielectric screening from the electric field associated with V_m. Importantly, the ratiometric response of 15% per 100 mV is among the largest reported to date, for a single-fluorophore probe with a fast response to cellular V_m. Only recent studies on anellated hemicyanine dyes having an extremely long and rigid fluorophore demonstrates higher sensitivity to V_m, ranging from 17% per 100 mV, when excited close to the band maximum, up to 35% per 100 mV, when excited at the very red edge, where the absorption cross section is as much as 100 times smaller than at its peak.[10]

Time-dependent ratiometric measurements of V_m in cell suspensions were also performed (Figure 8). The fluorescence ratio of the probe in cells was stable with time. After addition of valinomycin, a fast increase in the I_{510}/I_{605} (i.e., I_{N*}/I_{T*}) ratio was observed (Figure 8A), corresponding to a hyperpolarization, in accordance with the spectroscopy data (Figure 7A). Then, subsequent addition of external K^+ resulted in a stepwise decrease in the I_{510}/I_{605} ratio (Figure 8A), consistent with the cell depolarization. In this experiment the observed sensitivity of the dye corresponded to 12% per 100 mV, which due to instrumental reasons (see above) is somewhat lower than that obtained from the spectra. From the observed correlation of the I_{510}/I_{605} ratio vs V_m, we estimated that the resting V_m of CEM cells was ca. −75 mV. This is slightly larger than the value reported in the literature (−60 mV) probably due to the use of 1.5 mM K^+ external buffer, while the reported values were obtained with 3 mM K^+ external buffer.[30] In the control experiment (Figure 8B), addition of the correspondent quantities of external Na^+ does not change the I_{510}/I_{605} ratio, while

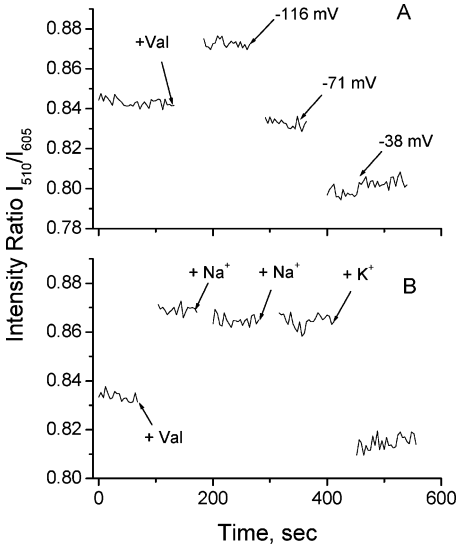

Figure 8. Fluorescence ratiometric recordings of the dye di-SFA from a suspension of CEM cells. (A) The fluorescence ratio increases upon valinomycin addition to resting cells (hyperpolarization), and then consequently decreases upon increasing external $[K^+]$ to 8.5 and 31.2 mM (depolarization by addition of aliquots of 150 mM K^+ buffer to the 1 mL cuvette). Membrane potentials were estimated by the Nernst equation assuming an internal $[K^+]$ of 140 mM. (B) The probe showed no sensitivity to the same experimental protocol using aliquots of 150 mM Na^+ buffer.

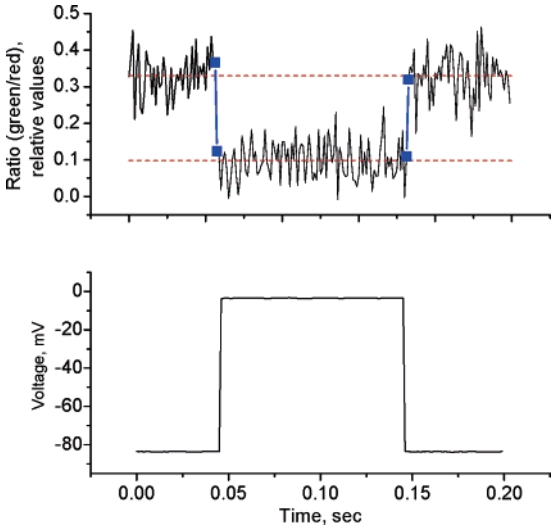

Figure 9. Fluorescence ratiometric response of the dye di-SFA (A) to a repeated step depolarization (B) from a single patch-clamped astrocytoma cell. (A) Average of 300 responses (black curve). The dashed red lines correspond to the fluorescence signal before and after depolarization. The blue parts of the curve correspond to the fluorescence response within 1 ms after the potential step. (B) Corresponding imposed voltage command (100 ms, 80 mV), from a holding potential of −80 mV. Ratio was acquired as a relative value.

subsequent addition of external K^+ results in the expected decrease of this ratio.

Whole-Cell Patch-Clamp Experiments. To perform fast recording of the fluorescence response of di-SFA probe to known rapid V_m variations in cells, we made simultaneous whole-cell patch-clamp[19] and fluorescence recordings (Figure 9). Our patch-clamp setup combined with a fluorescence microscope allows fast fluorescence recording of two emission channels simultaneously with controlled membrane potential changes. Experiments were done on single, adherent A172 astrocytoma cells[19] and L929 fibroblasts. After probe loading,

both types of cells exhibited orange fluorescence exclusively at the plasma membrane (not shown), and internalization of the dye inside the cell was detectable only 2 h after the start of experiments. Single A172 cells were voltage-clamped at −80 mV and the fluorescence ratio from signals in the short- (green) and long-wavelength (red) emission channels was recorded. Cells were subjected to 100 ms long depolarizing steps of 80 mV amplitude, and in response to these step depolarizations, the probe shows fast systematic changes in the fluorescence ratio (Figure 9). We applied up to 500 pulse repetitions per cell (200 ms per cycle), and the response of the probe was continuously observed. The duration of experiments (about 10−20 min) was limited by seal breakdown and probe photodegradation. The response of the probe was clearly detectable in all cells that were successfully voltage-clamped. Importantly, the amplitude of the fluorescence response detected within 1 ms of the collection time is 0.226 ± 0.016 when voltage step is applied (Figure 9, see regions marked blue), which corresponds well to the average overall response (0.232 ± 0.054) of the probe. This demonstrates that the probe exhibits 100% of its response within 1 ms, so that its response time is below 1 ms.

Mechanism of the Probe Response and New Possibilities for Improved Probe Design. The present work is the first successful attempt to use the ESIPT reaction for probing transmembrane potential. For this purpose, we used the unique 3-hydroxychromone fluorophore which exhibits two emission bands due to two ESIPT-coupled emissive species. By grafting two rigid legs to this fluorophore, we succeeded to position it deeply inside the bilayer as indicated by the fluorescence spectroscopy data. This deep location of the fluorophore imposed by two identical anchoring legs implies its vertical insertion into the bilayer parallel to the electric field of V_m (Figure 1). Generation of a transmembrane potential in the lipid bilayer results in a direct interaction of the electric field ($\vec{E}$) with the excited state dipoles of the dye (Figure 10). Importantly, the dipole moment of the N* state is significantly larger than that of the T* state,[12,13,24] and it is probably oriented more parallel with respect to the electric field (Figure 10). Thus, according to semiempirical AM1 calculations, the dipole moment of the N* state is 11.0 D, while that of the T* state is only 2.2 D.[12] Therefore, an increase in V_m ("minus" charge inside the cell) likely decreases the energy of the N* state with respect to the T* state, shifting the ESIPT equilibrium[13,14] (N*↔T*) toward the former and increasing its relative intensity. Moreover, since the excited N* state is characterized by a much larger dipole moment than the ground N state (by AM1 method: 11.0 D in the excited state vs 3.7 D in the ground state),[12−14,24] an increase in V_m should also shift the absorption (or excitation) and the N* emission band to the red. The T* band should also demonstrate some red shift since its ground (T) state is polarized against the electric field $\vec{E}$ ("minus" charge on 3-oxygen and "plus" on protonated 4-oxygen, see Figure 10).[14a] In line with these expectations, our present data in lipid vesicles and cells show that an increase in V_m shifts of the excitation and emission bands to the red and increases the relative intensity of the N* band.

Since the fluorophore of di-SFA is highly solvatochromic,[23,24] an alternative mechanism of the probe response could be proposed. Indeed, since the probe bearing two sulfonate groups is negatively charged in aqueous media, an increase in V_m could result in an electrostatic repulsion of the dye to a shallower region of the bilayer, so that the surrounding polarity of the fluorophore could increase. This polarity increase could, in turn,

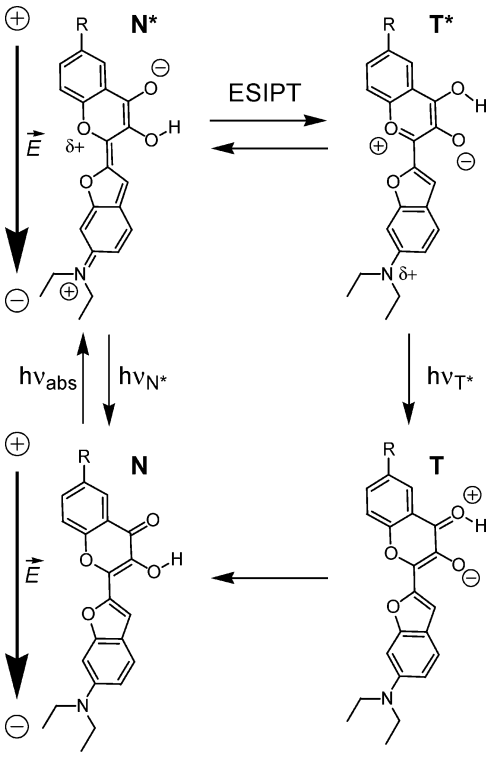

Figure 10. ESIPT in di-SFA fluorophore as the mechanism of the probe response to the electric field ($\vec{E}$). The four-level diagram of the ground- and excited-state transformations of the dye is presented based on the previous studies.[13,14]

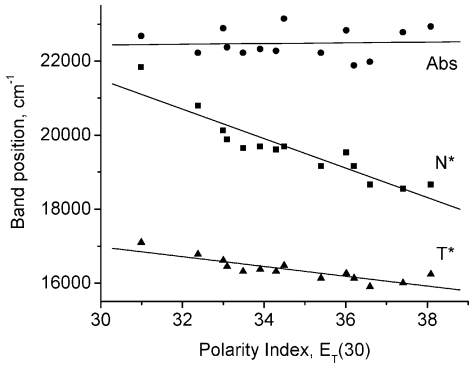

Figure 11. Position of the absorption (●), N* (■), and T* (▲) emission bands as a function of the polarity index $E_T(30)$ of different organic solvents. Experimental data are from ref 23.

explain the increase in the relative intensity of the N* band as well as its red shift. However, a careful examination of the shifts of the excitation and the T* emission bands allows us to discard this mechanism. Indeed, according to our extensive solvatochromic data on the parent FA fluorophore,[23,24] an increase in solvent polarity shifts the T* band nearly 3-fold less than the N* band, and does not shift the absorption band (Figure 11). In contrast to these data, changes in V_m shifts both the absorption (excitation) and the N* emission bands to a similar extent, while the T* band is shifted about half as much as the N* band. For example, in cell suspensions, an increase in V_m by 70 mV shifts the excitation, N* and T* bands to the red by 41, 47, and 24 cm^{-1}, respectively. The shift in the excitation band and the relatively strong shift of the T* band cannot be due to polarity changes, so that an electrochromic mechanism is more likely. This conclusion is further substantiated by our previous studies on the intramolecular Stark effect in 3-hydroxychromone derivatives and probes for dipole potential in lipid membranes

showing that the electric field shifts the absorption (or excitation) band to nearly the same extent as the N* band, and shifts the T* band half as much as the N* band,[14a,15] in full agreement with our observations on probe di-SFA in biomembranes. In addition, the absolute value of these shifts is close to that observed in the potentiometric styrylpyridinium dye di-4-ANEPPS, which also operates by an electrochromic mechanism.[9] Finally, the observed fast two-band ratiometric response of the probe (<1 ms) in our combined patch clamp-fluorescence measurements provides additional support for the ESIPT modulation by an electrochromic mechanism which operates in a nanosecond time-scale. This time scale is determined by the fast ESIPT kinetics[14] and the probe fluorescence lifetime.

Though di-SFA is somewhat less sensitive than the last representatives of the hemicyanine (ANNINE) family,[10] the new design principles involving deep imbedding of the ESIPT fluorophore into the bilayer provide considerable room for further improvement of probe sensitivity. Indeed, the location of the fluorophore of di-SFA is still far from the middle of the bilayer, where the dielectric constant is minimal, and thus the sensitivity to the electric field of V_m is the highest. Therefore, a likely improvement will be to design a probe with a longer rigid spacer that would constrain the fluorophore to locate deeper in the bilayer. In parallel, the fluorophore itself can be improved to further increase the dipole moment of its N* state with respect to the T* state, which may significantly increase the sensitivity of the fluorophore to the electric fields.

Conclusions

We have developed and characterized a new two-color fluorescent probe for measuring the transmembrane potential across cell membranes. The di-SFA probe exhibits a change in the intensity ratio of its two emission bands in response to variations of V_m. The fluorescence ratiometric response of the new probe to V_m in cells is rapid (<1 ms) and the probe sensitivity is up to 15% per 100 mV, which is relatively large in comparison to single-fluorophore, fast response probes reported to date. Consequently, this new probe should be useful for the rapid detection of V_m changes in cell suspensions as well as in single cells, in particular nerve cells having fast electrical activity. Moreover, unlike the charge shift probes (ANEPPS and ANNINE probes), the di-SFA probe responds to membrane potential not only by band shifts, but also by variation of the relative intensities of its two well separated emission bands. This feature allows a more convenient and accurate two-band ratiometric detection in spectroscopy and microscopy of living cells as compared to the recording of the ratiometric effects on the edges of a single band for the charge shift dyes. Finally, the new design methodology presented here provides possibilities for further improvement of the probe response by structural modifications.

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References and Notes

(1) (a) Malonek, D.; Grinvald, A. *Science* **1996**, *272*, 551−554. (b) Tsodyks, M.; Kenet, T.; Grinvald, A.; Arieli, A. *Science* **1999**, *286*, 1943−1946.

(2) (a) Briggman, K. L.; Abarbanel, H. D.; Kristan, W. B., Jr. *Science* **2005**, *307*, 896−901. (b) Cacciatore, T. W.; Brodfuehrer, P. D.; Gonzalez, J. E.; Jiang, T.; Adams, S. R.; Tsien, R. Y.; Kristan, W. B., Jr.; Kleinfeld, D. *Neuron* **1999**, *23*, 449−459.

(3) Grinvald, A.; Hildesheim, R. *Nat. Rev. Neurosci.* **2004**, *5*, 874−885.

(4) Momose-Sato, Y.; Mochida, H.; Sasaki, S.; Sato, K. *Neuroscience* **2003**, *116*, 407−423.

(5) *Handbook of Fluorescent Probes and Research Products*, 8th ed; Haugland, R. P, Ed..; Molecular Probes, Inc.: Eugene, Oregon, 2001.

(6) Cohen, L. B.; Salzberg, B. M.; Davila, H. V.; Ross, W. N.; Landowne, D.; Waggoner, A. S.; Wang, C. H. *J. Membr. Biol.* **1974**, *19*, 1−36.

(7) Shoham, D.; Glaser, D. E.; Arieli, A.; Kenet, T.; Wijnbergen, C.; Toledo, Y.; Hildesheim, R.; Grinvald, A. *Neuron* **1999**, *24*, 791−802.

(8) (a) Gonzalez, J. E.; Tsien, R. Y. *Chem. Biol.* **1997**, *4*, 269−277. (b) Cacciatore, T. W.; Brodfuehrer, P. D.; Gonzalez, J. E.; Jiang, T.; Adams, S. R.; Tsien, R. Y.; Kristan, W. B., Jr.; Kleinfeld, D. *Neuron* **1999**, *23*, 449−459. (c) Briggman, K. L.; Abarbanel, H. D.; Kristan, W. B. Jr. *Science* **2005**, *307*, 896−901. (d) Kuznetsov, A.; Bindokas, V. P.; Marks, J. D.; Philipson, L. H. *Am. J. Physiol. Cell Physiol.* **2005**, *289*, C224−229.

(9) (a) Gross, D.; Loew, L. M. *Methods Cell Biol.* **1989**, *30*, 193-218. (b) Montana, V.; Farkas, D. L.; Loew, L. M. *Biochemistry* **1989**, *28*, 4536−4539.

(10) (a) Kuhn, B.; Fromherz., P. *J. Phys. Chem. B* **2003**, *107*, 7903−7913. (b) Kuhn, B.; Fromherz, P.; Denk, W. *Biophys J.* **2004**, *87*, 631−639.

(11) Sengupta, P. K.;. Kasha, M. *Chem. Phys. Lett.* **1979**, *68*, 382−385.

(12) Yesylevskyy, S. O.; Klymchenko, A. S.; Demchenko, A. P. *J. Mol. Struct.: Theochem* **2005**, *755*, 229−239.

(13) (a) Klymchenko, A. S.; Demchenko, A. P. *Phys. Chem. Chem. Phys.* **2003**, *5*, 461−468. (b) Klymchenko, A. S.; Pivovarenko, V. G.; Ozturk, T.; Demchenko, A. P. *New. J. Chem.* **2003**, *27*, 1336−1343.

(14) (a) Klymchenko, A. S.; Demchenko, A. P. *J. Am. Chem. Soc.* **2002**, *124*, 12372−12379. (b) Shynkar, V. V.; Mély, Y.; Duportail, G.; Piémont, E.; Klymchenko, A. S.; Demchenko, A. P. *J. Phys. Chem. A* **2003**, *107*, 9522−9529.

(15) Klymchenko, A. S.; Duportail, G.; Mely Y.; Demchenko, A. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11219−11224.

(16) Shynkar, V. V.; Klymchenko A. S.; Duportail G.; Demchenko, A. P.; Mély Y. *Biochim. Biophys. Acta* **2005**, *1712*, 128−136.

(17) Klymchenko, A. S.; Duportail, G.; Ozturk, T.; Pivovarenko, V. G.; Mély, Y.; Demchenko, A. P. *Chem. Biol.* **2002**, *9*, 1199−1208.

(18) Xi, H.; Gibb, C. L. D.; Gibb, B. C. *J. Org. Chem.* **1999**, *64*, 9286−9288.

(19) (a) Rondé, P.; Giannone, G.; Gerasymova, I.; Stoeckel, H.; Takeda, K.; Haiech, J. *Biochim. Biophys. Acta* **2000**, *1498*, 273−280. (b) Giannone, G.; Rondé, P.; Gaire, M.; Haiech, J.; Takeda, K. *J. Biol. Chem.* **2002**, *277*, 26364−26371.

(20) (a) Hamill, O. P.; Marty, A.; Neher, E.; Sakmann, B.; Sigworth, F. J. *Pflügers Arch.* **1981**, *391*, 85−100. (b) Takeda, K.; Schini, V.; Stoeckel, H. *Pflügers Arch.* **1987**, *410*, 385−393. (c) Barbara, J. G.; Takeda, K. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9905−9909.

(21) Rekker, R. F.; Mannhold, R. *Calculation of Drug Lipophilicity;* VCH: Weinheim, 1992.

(22) Klymchenko, A. S.; Ozturk, T.; Pivovarenko, V. G.; Demchenko, A. P. *Tetrahedron Lett.* **2001**, *42*, 7967−7970.

(23) Ercelen, S.; Klymchenko A. S.; Demchenko A. P. *Anal. Chim. Acta* **2002**, *464*, 273−287.

(24) Ercelen, S.; Roshal A. D.; Demchenko, A. P. Klymchenko, A. S. *Polish J. Chem.* **2002**, *76*, 1287−1299.

(25) Klymchenko, A. S.; Ozturk, T.; Demchenko, A. P. *Tetrahedron Lett.* **2002**, *43*, 7079−7082.

(26) Loew, L. M.; Simpson, L.; Hassner, A.; Alexanian V. *J. Am. Chem. Soc.* **1979**, *101*, 5439−5440.

(27) Laris, P. C.; Pershadsingh, H. A.; Johnstone, R. M. *Biochim Biophys Acta*. **1976**, *436*, 475−488.

(28) Calderon, R. O.; DeVries, G. H. J. Neurosci. Res. **1997**, *49*, 372−380.

(29) Bagatolli, L. A.; Gratton, E.; Fidelio, G. D. *Biophys. J.* **1998**, *75*, 331−341.

(30) Zegarra-Moran, O.; Rasola, A.; Rugolo M.; Porcelli, A. M.; Rossi, B.; Galietta, L. J. *J. Immunol.* **1999**, *162*, 5359−5366.