pH-Insensitive FRET Voltage Dyes

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Many high-throughput ion channel assays require the use of voltage-sensitive dyes to detect channel activity in the presence of test compounds. Dye systems employing Förster resonance energy transfer (FRET) between 2 membrane-bound dyes are advantageous in combining high sensitivity, relatively fast response, and ratiometric output. The most widely used FRET voltage dye system employs a coumarin fluorescence donor whose excitation spectrum is pH dependent. The authors have validated a new class of voltage-sensitive FRET donors based on a pyrene moiety. These dyes are significantly brighter than CC2-DMPE and are not pH sensitive in the physiological range. With the new dye system, the authors demonstrate a new high-throughput assay for the acid-sensing ion channel (ASIC) family. They also introduce a novel method for absolute calibration of voltage-sensitive dyes, simultaneously determining the resting membrane potential of a cell. (Journal of Biomolecular Screening XXXX:xx-xx)

Key words: ion channels, high-throughput screening, cell-based assays, Förster resonance energy transfer, membrane potential

INTRODUCTION

MODERN APPROACHES FOR ION CHANNEL DRUG SCREENING include electrophysiology, radioactive flux or tracer assays, binding assays, ligand displacement assays, and optical assays using fluorescent or absorbent ion and membrane potential indicators. Although electrophysiological methods are definitive for characterizing channels and modulators, the extremely low throughput precludes their use in high-throughput screening (HTS), in which compound libraries consisting of thousands to millions of discrete chemical entities are tested for binding to or modification of function of the target. Optical methods are well suited to HTS assays, due to their speed, low cost, and ability to perform multiple experiments in parallel.

The membrane potential of a cell can be detectably altered by the activity of as few as 100 ion channels per cell, so it is a particularly sensitive measure of ion channel function. Several classes of membrane-potential-sensitive indicators have been described. Electrochromic dyes employ intramolecular charge movement through the membrane, causing voltage sensitivity of the emission and/or excitation spectra. Although they have very fast responses (< 1 µs), their sensitivities are too low for HTS (0.01%-0.1%/mV). Redistribution dyes are charged, generally lipophilic dyes that exhibit Nernstian intracellular concentration with high sensitivity (~10%/mV) but slow response times (~100 s). By restricting the detection of a redistribution dye to the plasma membrane using Förster resonance energy transfer (FRET), moderate voltage sensitivity (1%-2%/mV) can be combined with response times as fast as 1 ms.

Since the original description of FRET voltage-sensitive dyes, little improvement has been made in the method (although see Wolff et al. and Farinas et al.). In the most widely used incarnation, an oxonol (DiSBAC$_x$ (3), where $x = 2, 4,$ or 6; see Fig. 1B) is the mobile acceptor. A coumarin-linked phospholipid donor (CC2-DMPE; see Fig. 1A) is bound to the outer face of the cell membrane. Hydroxycoumarins are relatively poor choices for FRET donors due to their strong pH dependence in the physiological range. The peaks of the excitation spectra of the protonated forms (depicted for CC2-DMPE in Fig. 2) are shifted 40 to 50 nm lower relative to the charged forms. Although adding the chloride to the hydroxycoumarin shifts the pK$_a$ from ~7.5 to 6.2, small changes in local pH during the assay can lead to artifacts. Hydroxycoumarins are also sensitive to local surface potential, which can be altered by lipid composition and polarity. Because a drop in overall fluorescence of coumarin is proportionally reflected by a drop in oxonol emission, these artifacts are largely canceled out by the standard ratiometric analysis. However, the pH dependence precludes the use of assays for which a lower pH is desired—for example, for acid-gated or modulated channels such as the acid-sensing ion channel (ASIC) family.
In principle, virtually any fluorophore pair can be used for FRET as long as the emission spectrum of the donor has some overlap with the excitation spectrum of the acceptor. Other fluorophores that have been used for FRET include various oxonols, fluoresceins, rhodamines, Texas red, variants of green fluorescent protein (GFP), and so on. Thus, there is a rich body of possibilities for investigating new dyes with superior properties.

We have identified pyrenes as interesting candidates for donor fluorophores for voltage-sensitive FRET. The simple pyrene moiety is amenable to chemical modification, often without affecting the fluorescence properties. We explored molecules exemplified by 8-octadecyloxypyrene-1,3,6-trisulfonic acid (PTS$_{18}$; see Fig. 1C). Other dyes used in this study differed only in the length of the hydrocarbon tail (PTS$_x$, where $x$ is the number of carbons in the tail). These dyes have several features that allow their use as donors for voltage-sensitive FRET. The acidic sulfoxide groups ($pK_a < 2$) keep the fluorophore embedded at the phospholipid-water interface of the membrane, prevent migration through the membrane, and eliminate pH sensitivity in the physiological range. The heavily charged groups also convey good water solubility and tend to prevent excimer formation. The long hydrocarbon chains firmly anchor the dye to the membrane. The excitation/emission spectra of oxypyrenes are similar to those of CC2-DMPE, allowing their direct substitution in assays developed using the coumarin.

![FIG. 1. Dyes used in voltage-sensitive Förster resonance energy transfer: (A) CC2-DMPE, (B) DiSBAC$_3$, and (C) PTS$_{18}$.](image)

![FIG. 2. Excitation and emission spectra for cells stained with CC2-DMPE and PTS$_{18}$. Dot-dash lines (---) are the spectra before adding 3 µM oxonol; solid lines (—) are the spectra after adding oxonol. Fluorescence emission was monitored at 450 nm for the excitation scan; the excitation wavelength was 400 nm for the emission scan. The spectra were normalized to the fluorescence intensities before adding oxonol at the peak wavelengths. Dotted lines (····) are the excitation and emission spectra for oxonol in octanol. Fluorescence emission was monitored at 590 nm for the excitation scan; the excitation wavelength was 500 nm for the emission scan. (A) Spectra for cells stained with 6 µM CC2-DMPE. (excitation peak 415 nm, emission peak 455 nm). (B) Spectra for cells stained with 2.5 µM PTS$_{18}$ (excitation peak 405 nm, emission peak 435 nm). Excitation and emission spectra for cells stained with PTS$_{13}$, PTS$_{14}$, and PTS$_{16}$ were indistinguishable from those of PTS$_{18}$, although each dye had a slightly different dependence of Förster resonance energy transfer efficiency on dye concentration.](image)
Here, we introduce the use of oxypyrene/oxonol dye pairs for voltage-sensitive FRET. Using a novel potassium titration method, we characterized their voltage sensitivities. We show the pH insensitivity of the voltage dependence and demonstrate its utility in an assay for an acid-gated ion channel.

MATERIALS AND METHODS

Reagents

DiSBAC$_2$(3), CC2-DMPE, and voltage assay background suppression compound–1 (VABSC-1) were obtained from Invitrogen (Carlsbad, CA). Cell culture reagents were from Gibco (Carlsbad, CA). Pyrene dyes, Pluronic F-127, assay buffer ingredients, and gramicidin were from Sigma (St. Louis, MO).

The assay buffer consisted of the following (quantities in mM): 130 NaCl, 4 KCl, 2 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, and 5 dextrose. The assay buffer consisted of the following: 134 NaCl, 2 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, and 5 dextrose. The assay and high-potassium buffers were adjusted to 300 mOs and pH 7.4 and were mixed to obtain various potassium concentrations. Buffer solutions for altering pH were made by adding 20 mM N-tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid (TAPS), 2-(N-morpholino)ethanesulfonic acid (MES), or acetic acid ($pK_a$ = 8.5, 6.2, and 4.7, respectively); adjusting the pH (to 9, 6, or 4, respectively); and then adjusting the osmolarity to 300 mOs with water. Various mixtures of these altered pH buffers with normal assay buffer were used to achieve variable final pH values.

Dye stock solutions were 10 mM dye in dry DMSO. Donors were solubilized at 2× final concentration by adding the appropriate volume of stock solution plus an equal volume of 10% Pluronic F127 in DMSO into a plastic 50-mL centrifuge tube. Assay buffer was added to the dye while vortexing. DiSBAC$_2$(3) was solubilized at 2× final concentration by adding the appropriate volume of stock solution into a plastic 50-mL centrifuge tube. Assay buffer was added to the dye while vortexing. The blocking dye VABSC-1 was added to the oxonol solution at 1 mM. Test compounds were also added at 2× to the oxonol solution where applicable.

Cell culture

HEK-293 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum in tissue culture flasks at 37 °C in a 5% CO$_2$–95% air-humidified atmosphere. Cells were subcultured every 2 to 3 days. Freshly dissociated HEK-293 cells were plated at 50,000 cells/well in 100 µL/well of growth medium in 96-well polystyrene-coated plates (BioCoat, Becton-Dickinson, Franklin Lakes, NJ). For some experiments, 384-well plates were used (25,000 cells in 50 µL/well). No differences were seen in the results obtained from the 2 different types of plate.

Optical assays

Growth medium was replaced with assay buffer using a BioTek (Winooski, VT) ELx405 plate washer. After adding donor dye, the cells were incubated for 30 min at room temperature in the dark. The oxonol/test compound solution was added following a 2nd rinse with the plate washer. The cells were then incubated again for 30 min at room temperature in the dark. The oxonol and VABSC remained in the wells during the assays.

Static fluorescence intensities and excitation/emission spectra were obtained using a Molecular Probes (Carlsbad, CA) Gemini plate reader. Fluorescence peaks are described as the peak wavelength ± the half-width at half-maximal intensity. Extinction coefficients were measured using a Beckman (Fullerton, CA) DU 530 spectrophotometer. Dynamic changes in fluorescence intensities were measured using a Voltage-Ion Probe Reader (VIPR™, Aurora Biosciences Corp., San Diego, CA). The optical filters were 400 ± 8 nm for excitation and 460 ± 15 nm (donor) and 580 ± 30 nm (acceptor) for emission. Unless otherwise specified, donor and acceptor emission intensities were recorded at 1 Hz. Background signals were obtained using multiwell plates containing the assay buffer only. For potassium addition assays, fluorescence emissions were recorded for 10 s to establish a baseline fluorescence ratio. Then, a volume of stimulus buffer equal to the volume already in the well (100 µL for 96-well plates, 50 µL for 384-well plates) was added.

Data analysis

Primary analysis was performed using VIPRData 2.0 from Aurora Discovery (San Diego, CA). The normalized fluorescence ratio $\Phi(t)$ is defined as the background-subtracted donor/acceptor ratios normalized to unity at the start of an assay:

$$\Phi(t) = \frac{D(t) - D_0}{A(t) - A_0} \frac{A(0) - A_0}{D(0) - D_0},$$

where $D(t)$ and $A(t)$ are the donor and acceptor emission intensities at time $t$, and $D_0$ and $A_0$ are the background intensities. Changes in $\Phi(t)$ are linearly related to changes in the cell membrane potential $V_m$. A positive change in membrane potential pulls oxonol away from the outer leaflet of the membrane, leading to an increase in donor signal and a decrease in acceptor signal. Therefore, positive $\Phi(t)$ indicates a depolarization of the membrane potential.

Linear and nonlinear fitting was performed using Origin 7 (Originlab, Northampton, MA).

mRNA expression of human ASIC in HEK293

Total RNA was extracted from HEK293 cells using RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized
from 5 µg RNA using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer’s protocol. Real-time PCR was performed on Smart Cycler (Cepheid, Sunnyvale, CA) using LightCycler DNA Master SYBR Green I (Roche, Basel, Switzerland). All pairs of gene-specific primers are intron spanned, and their sequences were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hASIC1</td>
<td>5'-GCCATCTGCTGACGACTCTCC-3'</td>
<td>5'-GAATCACTCGAGGCATGTAC-3'</td>
</tr>
<tr>
<td>hASIC2</td>
<td>5'-GCCACCTTCATAGGGTCCGAC-3'</td>
<td>5'-GCTTGTTCAGTAAGTACCTC-3'</td>
</tr>
<tr>
<td>hASIC3</td>
<td>5'-GTCGCCACCTTTGACATTCG-3'</td>
<td>5'-GGCCATTGCGCCATGTCC-3'</td>
</tr>
<tr>
<td>hASIC4</td>
<td>5'-CTACTAGTGTCGCTGCTCGA-3'</td>
<td>5'-GGCTAGCCCTCTGTGGGGA-3'</td>
</tr>
</tbody>
</table>

After initial denaturation for 2 min at 95 °C, thermal cycles consisted of 5 s at 95 °C, 7 s at 60 °C, and 12 s at 72 °C for 40 cycles. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and confirmed by sizing and specific restriction patterns.

**RESULTS**

**Dye characterization**

**Cell staining.** HEK-293 cells stained with CC2-DMPE and PTS4 appear as bright rings, indicating that only the cell membrane is stained. Although PTS10 also initially showed ring staining, this dye desorbed from the membrane with a timescale of ~10 min. This effect was not seen with CC2-DMPE or the other pyrene dyes over the duration of experiments.

**Excitation/emission spectra.** FRET between 2 fluorophores was demonstrated by comparing fluorescence scans of the individual dyes with those of the combined dyes. Figure 2 illustrates the excitation and emission spectra for HEK cells stained with a FRET donor (either 6 mM CC2-DMPE or 2.5 mM PTS10) with and without 3 mM DiSBAC2(3). The spectra were normalized to the peak intensities in the absence of oxonol. Before adding oxonol, CC2-DMPE (Fig. 2A) showed a single broad excitation peak at 418 nm ± 33 nm (half-width at half maximum) and a single emission peak at 453 ± 27 nm. Upon adding oxonol, the emission at 453 nm dropped in amplitude by 69%, whereas a 2nd emission peak grew at 562 ± 17 nm. This 2nd emission peak corresponds to the fluorescence spectrum of DiSBAC2(3) (thin lines). The shift in emission from the donor (pyrene) to the acceptor (oxonol) emission indicates that FRET occurred.

PTS10 had a more complex excitation spectrum (Fig. 2B), with 2 peaks at 405 ± 12 and 378 ± 30 nm. The emission spectrum of PTS10 is dependent on the solvent polarity surrounding the pyrene moiety, shifting from 435 nm in water to 420 nm in a lipid environment. As shown in Figure 2B, the emission had a single peak at 435 ± 25 nm, indicating that the fluorophore in stained cells was in a polar environment. Both excitation peaks showed similar emission spectra. Upon adding oxonol, the emission at the 435-nm peak dropped in amplitude by 65%, whereas a 2nd emission peak grew at 562 ± 17 nm. Both excitation peaks were reduced in amplitude uniformly when oxonol was added. The other pyrenes showed similar behavior.

**Dye loading is an important factor in designing a successful assay.** Acceptor concentration must be high enough to obtain efficient FRET, and both dye fluorescence intensities must be high enough to avoid contamination with background fluorescence. To study this issue, HEK-293 cells in 384-well microplates were stained with cross gradients of oxonol and a donor dye. HEK-293 cells endogenously express potassium channels that confer a negative membrane potential. The cells were challenged with an addition of high-potassium saline, so that the potassium concentration changed from 4 to 69 mM (potassium reversal potential changed from –90 to –17 mV). Figure 3 shows the time course of the normalized fluorescence ratio Φ. After the addition, the cells depolarized, pulling the negatively charged oxonol from the outer leaflet to the inner leaflet. The reduced oxonol concentration near the donor decreased the efficiency of FRET, so the donor emission increased at the expense of the acceptor emission. In the presence of gramicidin, the cells were already depolarized at the start of the experiment, so there was no change in membrane potential or FRET resulting from the addition of elevated potassium.

**Figure 4** shows the results of the dye matrix experiments for CC2-DMPE and PTS10. This experiment was performed using PTS12, PTS14, and PTS16 as donors with similar results. PTS10 was not included due to the destaining phenomenon. Donor and acceptor signals were normalized to the plate background, which in our experience provides a convenient and reproducible fluorescence standard.

The patterns in donor (Fig. 4b) and acceptor (Fig. 4c) emission intensities were very similar for all the donors, PTS4 and CC2-DMPE alike. As the donor dye concentration increased, both donor and acceptor emission intensities increased. At any given donor concentration, increasing oxonol caused a decrease in donor and an increase in acceptor intensities. These observations are consistent with FRET. For dilute donor, when the acceptor concentration is constant, the fraction of energy transfer remains constant. Increasing the donor concentration thereby gives proportional increases to both dye emissions. However, when the acceptor dye concentration is increased, FRET efficiency increases and causes a rise in acceptor fluorescence at the expense of donor fluorescence.

The normalized ratio change (Fig. 4a) shows that the voltage sensitivity increases with donor concentration, even when the staining was well above background. There also appears to be an optimal oxonol concentration in the range 1 to 5 µM. As expected, this roughly corresponds to 50% FRET efficiency based on the drop in donor fluorescence as a function of oxonol concentration (Fig. 4b).
**pH dependence.** Dyes in aqueous solution were prepared at 1 µM in assay buffer at various pH values. The assay buffer was supplemented with 10 mM MES (pKₐ = 6.1) and 10 mM TAPS (pKₐ = 8.4) to stabilize the pH above and below the effective range for HEPES (pKₐ = 7.5). Figure 5 summarizes the fluorescence intensity at 400-nm excitation and 460-nm emission as functions of pH for CC2-DMPE and PTS18. Although the fluorescence of the pyrene was unaffected by changes in pH, CC2-DMPE has pKₐ = 6.2 and is virtually nonfluorescent at 400 nm below pH 5.

We examined the pH dependence of the dyes loaded into cells in a set of assays in which the pH was changed from pH 7.4 to a test value, and we monitored the fluorescence emissions at 460 nm and 580 nm using VIPR™. To avoid any pH-dependent changes in membrane potential, 1 µM gramicidin was included in the assay buffer. The gramicidin essentially locked the membrane potential at zero, so that any changes in fluorescence intensities were solely due to the dyes. Figure 6 shows the normalized fluorescence change Φ, as well as the donor and acceptor intensity changes caused by the pH change during the assay. Whereas the pyrene dyes show no significant pH dependence, the loss of fluorescence of cells stained with CC2-DMPE at low pH mirrors the effect in saline (Fig. 5).

**Demonstration of voltage sensitivity**

A well-established assay for determining the activity of potassium channels involves engineering a cell line in which the resting potential is set by the target channel. While monitoring the voltage-sensitive fluorescence, a high-potassium buffer is added. If the potassium channel is active, a large membrane potential change is seen; if it has been blocked by a test compound, the membrane potential change is reduced or absent. HEK-293 cells endogenously express potassium channels that generate a negative membrane potential.19,20

The PTS₇ donors were compared to CC2-DMPE using this high-potassium assay configuration. In the dye matrix experiment described above, an addition of elevated potassium was performed in VIPR™ while monitoring the FRET ratio Φ(t). The time course of the response was similar to the traces in Figure 3A. Figure 4Aa and Figure 4Ba show Φ measured 38 s after the high-potassium addition for CC2-DMPE and PTS₁₈ as the FRET donors, respectively. Both donors show weak maxima in response at around 2 to 5 µM oxonol. This roughly corresponds to the 60% to 70% reduction in donor fluorescence at 3 µM oxonol (Fig. 2). In theory, maximal voltage sensitivity should occur near 50% FRET efficiency, where the slope of FRET versus acceptor concentration is greatest.

To further compare the voltage sensitivity of the various dyes, we determined the response of HEK-293 cells to high potassium in the presence of gramicidin, a nonselective monovalent cation ionophore. The gramicidin conductance competes with the endogenous potassium currents, so that at high gramicidin levels, the membrane potential approaches zero. Thus, the membrane potential (and therefore the FRET ratio) does not change upon the addition of elevated potassium. Figure 3A shows the time course of cells loaded with PTS₁₈ and oxonol with and without gramicidin. A large and reproducible increase in the FRET ratio Φ was seen in response to elevated potassium. Except for a small addition artifact, gramicidin completely blocks this response. Responses using the different donors were indistinguishable. Figure 3B shows the response...
as a function of the gramicidin concentration. For all donor dyes, the gramicidin gave IC$_{50}$ values ranging from 200 to 250 nM. Thus, the PTS dyes appear to be interchangeable with CC2-DMPE for use in VIPR™.

Assays are normally constructed to raise the final potassium concentration as high as possible to achieve the largest possible membrane potential change and therefore the largest signal. However, by changing the extracellular potassium to intermediate levels, the final membrane potential can also be set to intermediate values. With an appropriate model of the behavior of the resting potential, an accurate relationship between fluorescence and membrane potential can be established, allowing measurements of voltage dye sensitivity and resting membrane potential. Because the conductances are
so voltage sensitive, an alternative approach to the Goldman-Hodgkin-Katz equation was used. We used this method to characterize and compare the voltage sensitivity of the FRET dyes. Similar methods have been described previously for calibrating dyes using the ionophore gramicidin or valinomycin rather than the endogenous channels to clamp membrane potential.

HEK-293 cells endogenously express several potassium currents, which set the resting potential to –40 to –60 mV. Multiple transient outward (Iₒ) and noninactivating delayed rectifier (Iₜₐ₉) subtypes have been detected. In our hands, whole-cell electrophysiology revealed that the average potassium currents could be well described by conductances of \( g_K = 1.7 \text{nS}(1 + \exp((-V_m -1)/16)) \) and \( g_{A} = 1.5 \text{nS}(1 + \exp((-V_m + 8)/8)) \) (\( n = 8 \), average membrane capacitance 12 pF). These values are comparable to those previously reported.

### FIG. 5

(A) Fluorescence in saline for CC2-DMPE and PTS, with 400 nm excitation and 460 nm emission ( ▽ CC2-DMPE; O PTS₁₀; V PTS₁₆; Δ PTS₁₂; ◇ PTS₁₀). The solid line is a fit to the CC2-DMPE data and indicates that \( pK_a = 5.6 \). (B) Fluorescence excitation spectra (emission wavelength 490 nm) for CC2-DMPE in saline at pH 3.8 (⋯), 6.2 (-----), and 7.4 (−−−).

### FIG. 6

The pH behavior of Förster resonance energy transfer (FRET) donors loaded into cells, in the presence of 1 µM gramicidin. (▽ CC2-DMPE; O PTS₁₀; V PTS₁₆; Δ PTS₁₂; ◇ PTS₁₀). Data are the normalized changes in fluorescence (excitation at 400 nm) when the buffer is changed from pH 7.4. (A) Donor fluorescence (460 nm) as a function of final pH. (B) Acceptor fluorescence (580 nm). (C) Fluorescence ratio \( \Phi \).
We constructed a numerical model of the currents in a cell near the resting membrane potential. Although the Boltzman forms of the potassium conductances above are fairly accurate representations in the voltage range around 0 mV and above, the resting potential is actually near –50 mV. In this range, all currents are very small, and the channels are nearly completely deactivated. Extrapolating the behavior of the channels to such small currents is inherently inaccurate. So, although the following model cannot be numerically verified, the validity and results of the model rely only on the existence of a substantial potassium conductance when the cell is depolarized with elevated potassium. Only a single, “averaged” potassium current is included for simplicity. The total current as a function of membrane potential \( V_m \) is given by

\[
I = g_L V_m + g_k (V_m - V_k) \left(1 + \exp \left( \frac{-V_m + V_{1/2}}{V_d} \right) \right)^{-1}.
\]  

(2)

The resting potential is the point where \( I = 0 \); this equation must be solved numerically. With normal assay buffer at 4 mM K\(^+\), and assuming an intracellular concentration of potassium at 130 mM, the reversal potential of potassium is given by the Nernst equation to be \( V_k = -89 \) mV at room temperature. Using representative values for the leak conductance \( g_L = 50 \) pS, \( g_k = 2 \) nS, \( V_{1/2} = 0 \), and \( V_d = 15 \) mV, the net current is zero at a resting potential of –51 mV. The potassium and leak currents at this potential are only \( \pm 3 \) pA. The cell input resistance at this membrane potential is approximately \((100 \text{ pS})^{-1} = 10 \text{ G}\Omega\). The solution to equation (2) is fairly sensitive to changes in the channel parameters, so it cannot be used to predict the resting potential in low extracellular potassium.

In a typical high-potassium addition assay, the voltage-dependent fluorescence is monitored while the extracellular potassium concentration is raised from normal saline (4 mM K\(^+\)) to an elevated level (usually an equal volume mixture of normal saline and 130 mM K\(^+\)). At an extracellular potassium concentration of 72 mM, the potassium reversal potential as predicted by the Nernst equation is –15 mV. In this case, the total current in equation (2) also goes to zero at \( V_m = -15 \) mV. At elevated membrane potentials, above the threshold for potassium channel activation, the channel conductance is substantially greater than the leak conductance. Then, the 1st term in equation (2) can be neglected, and the solution to equation (2) is \( V_m = V_k \).

Consider a high-potassium addition assay in which the final potassium concentration is intermediate between 4 and 72 mM. If the potassium reversal potential does not exceed the low-potassium resting potential, very little change in membrane potential will be seen. At higher potassium levels, the final membrane potential approaches asymptotically the potassium reversal potential. Thus, assuming a linear relationship between the fluorescence change and the membrane potential change, \( \theta \) the slope of the response versus final potassium reversal potential is a direct measure of the dye sensitivity. Furthermore, the intercept of the response at zero fluorescence change gives the resting membrane potential in the starting saline.

**Figure 7** shows the results of potassium titration experiments for HEK-293 cells stained with 3 \( \mu \text{M oxonol} \) and either 6 \( \mu \text{M CC2-DMPE} \) or 2.5 \( \mu \text{M PTS}_{18} \). The data at the 3 highest reversal potentials were used to generate linear least squares fits (---). The dashed line (----) indicates the control response when no change in bath solution is made. The intercept of the fits with the control responses indicates the resting membrane potential.

**Assay for acid-sensing ion channels.** The ASICs are novel and exciting therapeutic targets with unusual biophysical properties. They show extremely sharp pH dependence below the physiological range and pass cations with little selectivity. ASICs apparently gate by rapid exchange of protons for tightly bound calcium in the pore. When activated, they exhibit a rapid transient opening followed by a slow sustained component. The 4 main proteins (ASIC1-4) and their splice variants could be...
involved in pain, mechanosensation, and/or neurotransmission. We have developed an optical assay for rapid, accurate determination of the effects of test compounds as modulators of ASICs.

The biophysical properties of ASICs represent unusual challenges for building an HTS-compatible assay. ASICs are activated by protons, with pKa ranging from pH 6.5 to 4.5 for the various subtypes. Each subtype has rapidly desensitizing (less than 1 s) and slowly or nondesensitizing components in response to activation. So, the recording method must be fast enough to detect subsecond kinetics with minimal pH-induced artifacts.

In addition to several potassium channel subtypes, HEK-293 cells were reported to express endogenously the acid-sensing ion channel ASIC1a. This amiloride-sensitive, sodium-selective channel was reported to have a pEC$_{50}$ = 6.45 with rapid (~1 s) activation and inactivation kinetics. Activation of this channel should lead to a depolarizing response. In our hands, reverse transcription PCR (RT-PCR) using probes for the 4 human ASIC genes revealed the presence of mRNA for ASIC1, 3, and 4; ASIC2 was absent (see Fig. 8). Because ASICs readily form heteromers that mix the pH sensitivities and kinetics of the individual subunits, the responses are mixtures of a wide distribution. Using whole-cell patch clamp recording, we detected acid-evoked currents with similar kinetics and pH dependence as previously reported.

FRET donors were evaluated for their usefulness in an assay for this channel. Wild-type HEK-293 cells stained with 3 µM DiSBAC$_2$(3) and various FRET donors were challenged in VIPR™ with low pH. Figure 9A shows the time course of the fluorescence ratio of HEK-293 cells stained with PTS 18/oxonol when the buffer was reduced from pH 7.4. Below pH 7.0, a large transient response appeared, approaching the sodium

| Table 1. Voltage Sensitivities and Resting Membrane Potentials Calculated for HEK-293 Cells Stained with 3 µM DiSBAC$_2$(3) and Various FRET Donors |
|---------------------------------|---------------------------------|-----------------|-----------------|-----------------|
| Dye | Staining Concentration (µM) | Excitation/Emission Maxima (nm) | ε (M$^{-1}$cm$^{-1}$) | Dye | Sensitivity (%/mV) | Resting Potential (mV) |
| CC2-DMPE | 6 | 415/435 | 81,000 | 1.71 ± 0.02 | –46.0 ± 1.5 |
| PTS$_{10}$ | 1.25 | 404/432 | 182,000 | 1.81 ± 0.02 | –51.3 ± 3.1 |
| PTS$_{12}$ | 1.25 | 402/434 | 128,000 | 1.92 ± 0.07 | –46.5 ± 7.8 |
| PTS$_{14}$ | 1.25 | 404/434 | 125,000 | 1.80 ± 0.07 | –45.8 ± 4.9 |
| PTS$_{16}$ | 1.25 | 404/434 | 114,000 | 1.87 ± 0.09 | –47.8 ± 9.6 |
| PTS$_{18}$ | 2.5 | 405/435 | 114,000 | 1.81 ± 0.02 | –46.1 ± 2.8 |

Excitation and emission maxima were determined for HEK-293 cells stained only with the donor. Extinction coefficients were measured at 400 nm with 1 µM dye in assay buffer. Dye sensitivities and resting potential were calculated using linear regressions to the response as a function of the final potassium titration data, as in Figure 7. The uncertainty values are the standard errors of the regressions. FRET, Förster resonance energy transfer.
reversal potential. This transient response was present at all final pH values below 7.0. The 10% to 90% rise time of the transient was 3 s, substantially slower than the 0.5-s rise time seen in patch clamp experiments.33 This effect is due to the ~1-s response time of the voltage dyes.9

Below pH 6.6, a sustained response appeared. Figure 9B shows the fluorescence ratio change of this sustained response (35 s after the stimulus) as a function of the final pH. PTS18, PTS16, PTS14, and PTS12 gave very similar results, a relatively pH-independent depolarization with a threshold at pH 7.0. A Boltzmann fit to the PTS18 data gave a midpoint at pH 6.7 and a width of 0.2 pH units.

FIG. 9. Activation of the endogenous acid-sensing ion channel (ASIC) in HEK-293 cells. (A) Time course of response for HEK-293 cells loaded with PTS18 and oxonol, recorded at 10 Hz. The cells started at pH 7.4, and the pH bathing the cells was changed at t = 12 s after the start of the assay. One representative trace for each of pH 7.4, 6.6, and 6.0 is shown. The scale on the right indicates the membrane potential, estimated from the potassium titration experiments. (B) Sustained response t = 45 s after the addition of low pH buffer as a function of final pH using different donor fluorophores (□ CC2-DMPE; O PTS16; V PTS14; ▽ PTS12; ◇ PTS10). The curve is a Boltzmann fit to the PTS18 data; it has a midpoint at pH 6.7 and a width of 0.2 pH units.

FIG. 10. Amiloride dose response. Wild-type HEK-293 cells stained with PTS18 and oxonol were challenged with pH 6.0 in the presence of varying concentrations of amiloride. Symbols are mean ± SEM for n = 4 data points. Curves are Boltzmann fits for the transient (•; IC50 = 21 µM, slope = 1.3) and sustained (○; IC50 = 21 µM, slope = 1.3) components of the response.

DISCUSSION

FRET can occur when a donor fluorophore is in close proximity to a 2nd acceptor fluorophore with a longer excitation wavelength. The energy transfer rate is inversely proportional to the 6th power of the separation between the fluorophores.7,34 Dye pairs in common use typically have R0 in the 10- to 100-Å range, making FRET one of the few techniques capable of probing distances in the 10- to 100-Å range. Using assumptions similar to previous work8 and the spectra in Figure 2, we used the Förster theory7 to estimate R0 = 41 Å for PTS18/DiSBAC2(3), compared to R0 = 48 Å for the CC2-DMPE/DiSBAC2(3) pair.
In contrast with most applications using FRET, voltage sensing does not involve molecular interaction or binding between fluorophores. Rather, proximity is achieved by attaining a high concentration of acceptor molecules. Voltage sensitivity is achieved by altering the average distance between dye molecules via Nernstian-driven changes in concentration in the outer leaflet of the cell membrane. Dye concentrations necessary to achieve this level of FRET correspond to a molecular density of ~1 mol percent in the membrane. A detailed analysis of the FRET efficiency as functions of donor and acceptor concentrations at such high densities must consider factors including restricted dimensionality, existence of multiple acceptors and donors within the range of interaction, and diffusion; this is beyond the scope of this work. In any case, we would expect voltage sensitivity to drop as the FRET efficiency exceeds 50%. The dye titrations shown in Figure 4 suggest that this occurs above a bath concentration of 2 to 5 µM oxonol.

Multiple factors must be considered in choosing dye pairs. Although many combinations of mobile and fixed acceptors and donors exist, we consider the case of a mobile, charged acceptor and a donor fixed to the outer leaflet of the membrane. The Förster distance must be on the order of or less than the thickness of the membrane to avoid significant FRET from both sides of the membrane. In general, this requires a significant separation between the donor emission and acceptor excitation wavelengths. Neither dye should be susceptible to aggregation or formation of excimers. The donor dye must be sufficiently water soluble for loading yet lipophilic enough to remain in the membrane for the 1- to 2-h duration of typical experiments. The donor must not cross the membrane, whereas the acceptor must cross quickly. Finally, neither dye should be sensitive to environmental conditions encountered in the assays.

Uncharged pyrenes at a high concentration trend to form excimers through stacking of multiple molecules into aggregates. Pyrene excimer formation is highly concentration dependent and typically results in a 60- to 70-nm red shift and loss of structure of the emission spectrum. Spectra of the trisulfonated pyrenes showed no evidence of concentration-dependent spectral changes in the absence of oxonol, either in aqueous solution or when loaded into cells. The spectral changes in Figure 2B somewhat resemble excimer formation, in that the emission at 436 nm shifted to the red when oxonol was added. Although we were unable to exclude absolutely the possibility of excimer formation induced by the addition of oxonol, the behavior of the combined PTS/oxonol system was entirely consistent with voltage sensitive FRET. 1) Adding the acceptor to PTS-stained membranes caused a simultaneous drop in emission at 436 nm and an increase in emission at 562 nm. 2) The 525- to 600-nm emission spectrum was identical to the spectrum for free oxonol. 3) The transfer of emission at 436 nm to emission at 562 nm was dependent on oxonol concentration and 4) on cell membrane potential, in a manner consistent with the negatively charged mobile acceptor.

In summary, we have introduced here a new class of donor dyes for FRET-based detection of membrane potential. In some respects, the performance of these pyrenetrisulfonate dyes is nearly identical to CC2-DMPE. PTS, and CC2-DMPE readily stain the outer leaflet of cells in the 1- to 10-µM range with comparable brightness, provide ~1.8%/mV voltage sensitivities in combination with oxonol, and possess similar fluorescence spectra. The environmental insensitivity of pyrenes relative to coumarins should reduce addition artifacts in many assays. In particular, for assays requiring changes in pH to detect channel activity, the low pKa of the pyrene trisulfonate fluorophore is a significant improvement over coumarin-based donors. As an example, we designed an assay for the endogenous ASIC channels in HEK-293 cells that is capable of separately detecting the transient and proton-evoked sustained activity. Using VIPR™, we have used this assay in a 384-well format and could easily screen plates in HTS mode, with a read time of approximately 10 min per plate, and consistently obtain screening windows z’ > 0.5.

We also introduced a simple method for simultaneously determining the resting potential of cells and the voltage sensitivity of the dye(s). This method exploits the fact that the membrane potential of a cell with substantial potassium channel conduc-
tance will follow the potassium reversal potential. By measuring the response at different potassium concentrations, we obtained an absolute calibration of the voltage response of the dyes.

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