A Novel Membrane Potential-Sensitive Fluorescent Dye Improves Cell-Based Assays for Ion Channels

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ABSTRACT

The study of ion channel–mediated changes in membrane potential using the conventional bisoxonol fluorescent dye DiBAC₄(3) has several limitations, including a slow onset of response and multistep preparation, that limit both the fidelity of the results and the throughput of membrane potential assays. Here, we report the characterization of the FLIPR Membrane Potential Assay Kit (FMP) in cells expressing voltage- and ligand-gated ion channels. The steady-state and kinetics fluorescence properties of FMP were compared with those of DiBAC₄(3), using both FLIPR and whole-cell patch-clamp recording. Our experiments with the voltage-gated K⁺ channel, hElk-1, revealed that FMP was 14-fold faster than DiBAC₄(3) in response to depolarization. On addition of 60 mM KCl, the kinetics of fluorescence changes of FMP using FLIPR were identical to those observed in the electrophysiological studies using whole-cell current clamp. In addition, KCl concentration-dependent increases in FMP fluorescence correlated with the changes of membrane potential recorded in whole-cell patch clamp. In studies examining vanilloid receptor-1, a ligand-gated nonselective cation channel, FMP was superior to DiBAC₄(3) with respect to both kinetics and amplitude of capsaicin-induced fluorescence changes. FMP has also been used to measure the activation of K_ATP1 and hERG.2 Thus this novel membrane potential dye represents a powerful tool for developing high-throughput screening assays for ion channels.

INTRODUCTION

ION CHANNELS ARE CRITICAL in setting the resting membrane potential and controlling excitability of myocytes and neurons. They also play important physiological roles in cell proliferation, hormone secretion, and homeostasis of water and electrolytes. Defects in ion channel function result in diseases referred to as channelopathies.3 Most notably, mutations of the HERG gene and other K⁺ channels expressed in cardiac tissues cause long Q–T syndrome, which leads to increased risk of ventricular tachycardia and sudden death.4,5 Channelopathies are also manifested in such diseases as familial periodic paralyses, myasthenia gravis, epilepsy, ataxia, and diabetes. Thus ion channels represent key molecular targets for drug discovery.

The search for molecules that modulate ion channel function has been hindered by the lack of direct electrical measurements in a high-throughput screening (HTS) format. Membrane excitability in cell-based assays is a dynamic phenomenon that requires precise and accurate measurements to gather high-content information. The standard for monitoring ion channel activity has been patch-clamp recording.6–8 Although this technique allows detailed biophysical characterization of ion channel activation, inactivation, gating, ion selectivity, and drug interactions, the throughput and ease of use of patch-clamp instrumentation is quite low. The demand of ion channel HTS requires robust instrumentation and high signal:background assays combined with ease of use. Historically, ion channel HTS is equated with decreased information content, emphasizing the need for new methods to measure membrane potential changes in various cell types.

Current technologies for ion channel screening include radioligand binding, radioactive flux assays, cell viability assays in yeast and mammalian cell lines, automated patch-clamp techniques, and optical recording techniques such as bioluminescence and fluorescence detection. Among these methods, optical readouts of ion channel function are favorable for HTS

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because they are potentially sensitive, versatile, and amenable to miniaturization and automation. Fluorescence readouts are used widely both to monitor intracellular ion concentrations and to measure membrane potentials. For example, large transient increases in intracellular $\text{Ca}^{2+}$ concentration through activation of ion channels can be monitored using fluorescent probes such as Fluo-3 and Calcium Green. In addition to ion-selective fluorescent indicators, there are several fluorescent dyes that are sensitive to changes in membrane potential, including styryl, bisoxonol, and fluorescence resonance energy transfer–based voltage-sensitive dyes.

The bisoxonol fluorescent dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol, or DiBAC$_4$(3), has been the reagent of choice for measuring membrane potential. Redistribution of the dye as a result of depolarizing or hyperpolarizing stimuli in cells causes changes in fluorescence. However, utilization of DiBAC$_4$(3) has several limitations, including slow kinetics as well as fluctuations in response to temperature and dilution of the dye. In addition, screening experiments using bisoxonol dyes require multistep preparations and take at least 30–60 min for dye loading, compromising the fidelity and throughput of DiBAC$_4$(3)-based screening assays.

Here, we report the characterization of a novel membrane potential dye (FLIPR Membrane Potential dye [FMP]) and compare it with DiBAC$_4$(3) using FLIPRTM (Molecular Devices, Sunnyvale, CA) in cells expressing either voltage- or ligand-gated ion channels. We find that this dye is superior to DiBAC$_4$(3) in kinetics, and its fluorescence correlates well with the membrane potential determined directly by patch-clamp recording. These results have been previously reported in abstract form.

**MATERIALS AND METHODS**

**Cell culture and transfection**

CHO-K1 cells and HEK293 cells were cultured in Ham’s F-12 medium (Life Technologies, Grand Island, NY) and in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD), respectively, both supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT) and 2 mM L-glutamine (BioWhittaker). Cells were plated onto 10-cm dishes 24 h before transfection using Lipofectamine Plus™ (Life Technologies). CHO-K1 cells were transfected with 6 μg of cDNA encoding human ether-a-go-go–like K$^+$ channel (hElk-1; GenBank accession number AY053503) in the pMET7 vector. HEK293 cells were transfected with 6 μg of human vanilloid receptor-1 cDNA (hVR-1; GenBank accession number AJ277028) in the pCDNA3.1 vector. Cells were transfected with 6 μg of appropriate vector cDNA alone to serve as controls. In all cases, cells were replated 24 h after transfection and plates were used for experiments 24 h later. For FLIPR experiments, CHO-K1 cells (32,000 cells/well) were added to 96-well plates (Becton Dickinson, Bedford, MA) while HEK293 cells (64,000 cells/well) were plated in poly-d-lysine–coated 96-well plates (Becton Dickinson).

**Electrophysiology**

Standard whole-cell patch-clamp recordings were performed in CHO-K1 cells plated onto 0.5-cm glass coverslips (BELLCO, Vineland, NJ) coated with 1 mg/ml protamine (Sigma Chemical Co., St. Louis, MO). As with the FLIPR experiments, all recordings were performed 48 h after transfection. Electrodes

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**FIG. 1.** Voltage-dependent activation of human Elk-1 transiently expressed in CHO-K1 cells. Whole-cell patch-clamp experiments were performed 48 h after transfections. Cells were depolarized from −60 mV to +50 mV at 10-mV increments for 50 ms, followed by hyperpolarization to −120 mV for 20 ms. The holding potential was −80 mV. No outward current was recorded from CHO-K1 cells transfected with vector cDNA using the same voltage step protocol. Scale bars indicate duration (ms) and current amplitude (nA).
were pulled using a Sutter P-97 (Sutter Instruments, Novato, CA) and had initial resistances of 3–5 MΩ. The extracellular recording solution was Hank’s balanced salt solution (HBSS) (Life Technologies) containing 5.5 mM D-glucose and 10 mM HEPES (pH 7.4). The intracellular recording solution contained 140 mM KCl, 10 mM HEPES, 10 mM ethyleneglycoltetraacetic acid, and 0.5 mM MgCl₂ (pH 7.3). Whole-cell patch-clamp recordings were performed using an EPC9 amplifier (Heka Elektronik, Lambrecht/Pfalz, Germany). The volume of the recording chamber (Warner Instrument, Hamden, CT) was 100–150 µl. For current clamp recordings, the resting membrane potential was first determined in whole-cell configuration. KCl (7.5–120 mM) was then perfused into the chamber by gravity, and cell membrane depolarization was recorded.

Dye loading

**FLIPR membrane potential assay kit.** Cells were washed 3 times with assay buffer (HBSS containing 20 mM HEPES, pH 7.4), ensuring that no residual solution was left in the wells after each wash. Then, 50 µl of assay buffer was added to each well, allowing cells to equilibrate for 10 min. Finally, 50 µl of FMP (Molecular Devices, order number 8034), reconstituted as described in the product insert (final concentration = 20 µM) was added at 37°C for 30 min before experiments.

**DiBAC₄(3).** To avoid dilution of DiBAC₄(3) during the experiments, cells were washed as described above with assay buffer containing 5 µM DiBAC₄(3). All additions to the wells contained 5 µM DiBAC₄(3) to prevent further dilution of the dye. Moreover, all plastics except the cell plates were prerinse with assay buffer containing 10 µM DiBAC₄(3), because DiBAC₄(3) adheres to surfaces. Cells were loaded by incubation with 100 µl of 5 µM DiBAC₄(3) at 37°C for 1 h.

**Measurement of membrane potential changes using FLIPR**

The instrument used was the FLIPR-I, and filters for the various dyes were selected according to the manufacturer’s instructions. For hElk-1 experiments, background fluorescence was measured every 20 s for 2 min before addition of KCl or assay buffer. Immediately after adding KCl (60 mM unless otherwise noted) or assay buffer, fluorescence was measured every second for FMP or every 20 s for DiBAC₄(3). For hVR-1 experiments, background fluorescence was measured every 20 s for 2 min, and, on addition of capsaicin, fluorescence was measured every 20 s for 7–10 min for both FMP and DiBAC₄(3).

**Measurement of intracellular calcium changes using FLIPR**

HEK293 cells transiently expressing hVR-1 were washed once with Ca²⁺ flux assay buffer (HBSS, 20 mM HEPES, 1.3 mM Ca²⁺, 2.5 mM probenecid [pH 7.4]) without leaving any residual solution after the wash. Cells were then loaded with 4 µM Fluo-3, a fluorescent Ca²⁺ indicator dye, in the presence of 0.08% pluronic acid (both from Molecular Probes) in Ca²⁺ flux assay buffer and incubated for 1 h at 37°C. After incubation, cells were washed 3 times with 200 µl of assay buffer, leaving 100 µl in each well after the last wash. Background fluorescence was measured every second for 2 min, followed by addition of 100 µl of 400 nM capsaicin (final concentration = 200 nM) or assay buffer. Immediately after addition, flu-

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**FIG. 2.** Comparison of ΔmV and Δfluorescence in hElk-1–transfected CHO-K1 cells in response to KCl depolarization. (A) Electrophysiology: Resting membrane potentials were measured using current clamp for cells transfected with hElk-1 (Elk) or vector alone (Vector). The resting membrane potentials for hElk-1 and vector-transfected cells were ~63 mV and ~32 mV, respectively. Then 60 mM KCl was perfused onto the cells for 1 min, followed by washing. (B) FLIPR: After measuring baseline fluorescence for 120 s, 60 mM KCl was added. KCl induced a much larger increase in fluorescence in cells transfected with hElk-1 (Elk) compared with vector-transfected cells (Vector).
the rat ether-a-go-go–like K⁺ voltage-gated channels, we cloned and studied the human ortholog of the rat.

Membrane potentials returned to resting levels after polarization of cells transfected with hElk-1 (from contrast, 60 mM KCl resulted in a persistent 50–55-mV decrease as a result of endogenous channels in the CHO-K1 cells. In a slight depolarization in vector-transfected cells (10–15 mV) showed a more negative resting membrane potential (200–650), encompassing the transmembrane regions, pore loop, and cyclic nucleotide binding domain. We used standard whole-cell patch clamp to examine the voltage dependence and kinetics of channel activation of hElk-1. Cells transfected with hElk-1 showed a slowly activating, noninactivating, and slowly deactivating outward K⁺ current at depolarizing voltages from −30 mV to +50 mV (Fig. 1), as described previously for rElk-1. Cells transfected with vector alone showed no currents at any voltage tested. We also used whole-cell current clamp to measure membrane potential changes in cells transfected with hElk-1 or vector alone (Fig. 2A). Cells transfected with hElk-1 showed a more negative resting membrane potential (−65 ± 12 mV, n = 3 cells) than vector-transfected cells (−33 ± 8 mV, n = 5 cells). Perfusion with 60 mM KCl for 60 s induced a slight depolarization in vector-transfected cells (10–15 mV) as a result of endogenous channels in the CHO-K1 cells. In contrast, 60 mM KCl resulted in a persistent 50- to 55-mV depolarization of cells transfected with hElk-1 (from −63 mV to −10 mV), in accordance with the noninactivating nature of this channel. Membrane potentials returned to resting levels after removal of 60 mM KCl.

Next, we tested the ability of FMP to detect the changes in membrane potential induced by hElk-1 activation in FLIPR experiments. Fluorescence signals were measured from cells pre-loaded with 20 μM FMP. The baseline fluorescence recorded from cells expressing hElk-1 was lower than that seen in vector-transfected cells (data not shown), reflecting the more negative resting membrane potential in the hElk-1–transfected cells (Fig. 2A). Addition of 60 mM KCl resulted in a slight increase in fluorescence in vector-transfected cells (500–1000 FU), probably resulting from the 10- to 15-mV depolarization induced by KCl. However, a sustained fluorescence increase (2000–3000 FU) was measured in hElk-1–transfected cells stimulated with 60 mM KCl (Fig. 2B) that could be blocked completely by 3–10 mM BaCl₂ (data not shown), indicating that this resulted from the activation of K⁺ channels. The increases in FMP fluorescence were reversed on removal of 60 mM KCl. By comparison of the FLIPR experiments with the electrophysiological data, it was estimated that a 10%–12% increase in FMP fluorescence corresponded to each 10-mV depolarization of the membrane. Furthermore, in cells expressing hElk-1, direct measurements of the membrane potential (recorded using whole-cell current clamp; Fig. 2A) were compared to FMP fluorescence changes measured with FLIPR (Fig. 2B). The onset of the response to 60 mM KCl using these two different methods is essentially synchronous, suggesting that “real-time” changes in FMP fluorescence occur in response to changes in membrane potential (Fig. 3). Similarly, the FMP fluorescence observed with FLIPR in response to increasing concentrations of KCl showed a strong linear correlation (r = 0.993) with direct measurements of membrane potential induced by corresponding concentrations of KCl (Fig. 4).

Finally, we compared FMP and DiBAC₄(3) with respect to the kinetics of fluorescence changes in the FLIPR assay under the same experimental conditions. Historically, DiBAC₄(3) has been characterized by considerable latency and nondynamic responses to changes in membrane potential. Figure 5 shows that the response to 60 mM KCl in hElk-1–transfected cells was 14-fold faster with FMP (t½ = 17 s) than with DiBAC₄(3)
We also noted several additional advantages of FMP over DiBAC₄(3):

1. The dye loading time for FMP was as short as 15 min, while the minimal incubation time for DiBAC₄(3) was 1 h.
2. There was no difference in fluorescence between cells loaded with FMP at 37°C versus room temperature (data not shown).
3. FMP fluorescence was not significantly affected by dilution with assay buffer or other reagents, while DiBAC₄(3) was highly sensitive to these manipulations (Baxter and Xie, unpublished data).

**FMP experiments with a ligand-gated non-selective cation channel**

The above results show that FMP is superior to DiBAC₄(3) in measuring the activity of a noninactivating voltage-gated K⁺ channel, hElk-1. In order to determine the versatility of FMP with other classes of ion channels, we examined FMP fluorescence changes in response to activation of the vanilloid receptor (VR-1). Rat VR-1 was initially cloned on the basis of activation by capsaicin, the pungent ingredient in hot peppers, and the human gene was identified by homology. VR-1 is expressed in sensory neurons, and VR-1 from both rats and humans is responsive to heat and acidic pH as well as capsaicin. Therefore, VR-1 acts as a molecular integrator of several painful stimuli. VR-1 shows sequence and structural homology to the transient receptor potential channel family. Electrophysiological studies reveal that VR-1 is a rapidly desensitizing, nonselective cation channel with high Ca²⁺ permeability. Therefore, Ca²⁺-sensitive fluorescent dyes such as Fura-2, Fluo-3, and Fluo-4 can be used to monitor capsaicin-evoked responses via VR-1. We wanted to determine whether membrane potential–sensitive dyes would also be suitable for detecting capsaicin-induced responses from hVR-1–transfected HEK293 cells in FLIPR experiments.

In cells loaded with Fluo-3, addition of 200 nM capsaicin evoked a rapid and sustained increase in fluorescence resulting in a transient depolarization on addition of capsaicin. FMP fluorescence decayed rapidly on cell repolarization as a result of desensitization of the VR-1 channel. The data are representative of 4–8 experiments.
from Ca\(^{2+}\) influx on channel opening. Prolonged measurements of Fluo-3 fluorescence (10 min) showed no rapid decrease with time, suggesting that Ca\(^{2+}\) levels remain high after channel closing. In hVR-1–transfected cells loaded with FMP, 200 nM capsaicin induced a fast increase in fluorescence; however, the onset and amplitude of the response with FMP was similar to that seen in Fluo-3–loaded cells (Fig. 6). The synchronous responses measured with Fluo-3 and FMP show that “real-time” changes in FMP fluorescence occurred in response to Ca\(^{2+}\) influx, confirming data obtained with the hElk-1–transfected cells (see Fig. 3). In contrast to the Fluo-3 experiments, the FMP fluorescence decayed almost immediately on reaching peak signal and returned to baseline within 200–300 s as a result of repolarization of the membrane on desensitization of the channel. Because FMP measures the primary event of channel opening, this offers increased sensitivity over secondary measurements of intracellular calcium levels (i.e., Fluo-3). Finally, we compared DiBAC\(_4\)(3) with FMP and Fluo-3 in response to capsaicin. In contrast to FMP or Fluo-3, the response using DiBAC\(_4\)(3) was much slower and of lower amplitude, and the signal decayed more slowly on receptor desensitization. Addition of 200 nM capsaicin did not induce any responses in vector-transfected HEK293 cells for all three dyes (data not shown).

CONCLUSIONS

FMP is broadly applicable to measuring membrane potential changes induced by a variety of different ion channels. The kinetics and the amplitude of FMP fluorescence correlate well with the changes of membrane potential recorded in whole-cell patch clamp in cells transfected with the hElk-1 K\(^{+}\) channel. FMP is superior to DiBAC\(_4\)(3) in measuring the amplitude and kinetics of membrane potential changes following Ca\(^{2+}\) influx via hVR-1. Most notably, the kinetics of FMP fluorescence changes on depolarization shows an almost exact temporal correlation with direct measurement of membrane potential and Ca\(^{2+}\) flux, respectively. It will be of great interest to test the ability of this dye to report rapidly inactivating currents, such as those produced by A-type K\(^{+}\) channels. Two recent papers published in this journal, while this paper was under review, have further demonstrated the usefulness of FMP in monitoring ion channel activity and the applicability of this dye for HTS.1,2 The results reported here provide further confirmation of the utility of this technique in HTS. In addition, our preliminary studies show that FMP may also be applied to the screening of certain electrogenic transporters that generate net charge movement during the transport cycle (Baxter et al., manuscript in preparation).

In addition to the advantages conferred by the kinetics parameters, we found that FMP is more favorable than DiBAC\(_4\)(3) with respect to ease of use for HTS. Cells can be loaded with FMP for as little as 15 min, and this assay can be performed at room temperature, whereas DiBAC\(_4\)(3) requires loading for at least 1 h at 37°C. FMP is also subject to much less artifact following solution addition than DiBAC\(_4\)(3). Although the studies of FMP reported here were done using the FLIPR-1 (96 wells), similar results were obtained with the FLIPR 384 instrument, and our studies reveal that FMP is also suitable for use with a conventional fluorescence plate reader (Baxter and Xie, unpublished data). Thus this novel membrane potential–sensitive dye will be a useful tool for developing HTS assays for different types of ion channels and electrogenic transporters in a variety of different formats.

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