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Cell-Based Fluorescence Screen for K⁺ Channels and Transporters Using an Extracellular Triazacryptand-Based K⁺ Sensor

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We report the synthesis of a water-soluble, dextran-conjugated fluorescent K⁺ sensor, TAC-Lime_{dex}, whose green fluorescence strongly increases with [K⁺], and demonstrate its utility for assay of cellular K⁺ transport. K⁺ channels and K⁺-coupled ion transporters represent an important group of targets for drug discovery.^{1–3} K⁺ channels are involved in cardiac and neuronal excitability, epithelial fluid transport, extracellular and intracellular ionic homeostasis, and cell proliferation.² K⁺-coupled ion transporters are involved in transepithelial fluid secretion and absorption, and in cell volume regulation and ionic homeostasis. Patch-clamp is the gold standard for assay of K⁺ channel function, though technically tedious for high-throughput measurements. Radioactive Rb⁺ uptake is generally used to assay electrically silent K⁺-coupled transporters such as the K⁺/Cl[−] symporter. Membrane voltage-sensing probes have also been used to assess K⁺ channels. There is need for a robust fluorescence assay of K⁺ transport for screening applications as an alternative to patch-clamp and radioactive Rb⁺.

We previously introduced the long-wavelength, K⁺-sensitive fluorescent indicator, TAC-Red, consisting of a K⁺-binding triazacryptand ionophore (TAC) coupled to a red fluorescing xanthylium chromophore.⁴ The K⁺ sensing mechanism of TAC-Red, and that of a newer K⁺ indicator TAC-Crimson,⁵ involves charge-transfer quenching in which K⁺-triazacryptand binding prevents electron-transfer-type chromophore quenching. These dyes have bright fluorescence, excellent K⁺-selectivity, and millisecond response kinetics to changes in [K⁺].^{4,5} However, they partition significantly into many cell types, limiting their utility as an extracellular K⁺ sensor.

After testing many chromophores and conjugation strategies, we devised a synthetic route to generate the K⁺ sensor, TAC-Lime_{dex}. TAC-Lime_{dex} consists of a triazacryptand K⁺ ionophore in direct conjugation with a green fluorescent chromophore, connected through an amide linkage to amino dextran via succinimidyl ester chemistry (Figure 1a). The synthesis involved conversion of TACCHO **1** to the TAC-Lime (Bodipy dye) methyl ester **2** by reaction of aldehyde **1** with methyl 3-(2,4-dimethyl-1*H*-pyrrol-3-yl) propanoate. Oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) followed by treatment with boron trifluoride (BF₃) yielded the triazacryptand bodipy dye, TAC-Lime ester **2**. Hydrolysis of the methyl ester and conversion of the free acids to the disuccinimidyl ester gave TAC-Lime-DiSE **3**, which was reacted with amino dextran to give TAC-Lime_{dex}.

TAC-Lime_{dex} green fluorescence was strongly K⁺-sensitive, increasing by 50% with increasing [K⁺] from 0 to 2 mM (Figure 1b). As found for TAC-Red, TAC-Lime_{dex} fluorescence was not sensitive to pH in the biologically relevant range of 5 to 9 or to anions or other cations, with the exception of the K⁺ analogues Cs⁺ and Rb⁺. TAC-Lime_{dex} was stable in saline solution at room temperature for 14 days.

The principle of the method to assay cellular K⁺ transport is diagrammed in Figure 2a. TAC-Lime_{dex} is used as an extracellular K⁺ sensor to detect cellular K⁺ efflux. TAC-Lime_{dex} is membrane impermeant (<3% cell-associated fluorescence after 1 h). With appropriate solution ionic composition and electrochemical gradients, increasing TAC-Lime_{dex} fluorescence provides a quantitative measure of K⁺ channel/transporter function. Figure 2b shows a representative single measurement in which HT-29 cells, after K⁺ channel activation by ATP, were exposed to a K⁺-free solution containing TAC-Lime_{dex}. K⁺ efflux produced a time course of increasing TAC-Lime_{dex} fluorescence, from which [K⁺] and K⁺ efflux rates, d[K⁺]/dt, are deduced.

Control validation studies are shown in Figure 2c, with K⁺ efflux data summarized in Figure 2d. K⁺ efflux in HT-29 cells was relatively slow under control conditions and greatly increased by incubation with the K⁺/H⁺ ionophore nigericin, which provides a rapid pathway for electroneutral K⁺ efflux. Preincubation with a K⁺-selective ionophore, valinomycin, also increased K⁺ efflux, indicating that K⁺ conductance is rate-limiting. The valinomycin preincubation was done in a high K⁺, cytoplasmic-like solution to prevent cellular K⁺ depletion.

The electroneutral K⁺/Cl⁻ cotransporter (KCC) is involved in ionic and osmotic homeostasis in many cell types and in cell growth and tumor invasion. KCC function has been measured previously by radioactive Rb⁺ uptake.^{6,7} Figure 3a shows TAC-Lime_{dex} assay of KCC function in SiHa cells, a human cervical cancer cell line with hypotonicity-stimulated KCC activity.⁷ K⁺ efflux was increased 3-fold following hypotonic challenge (200 mosm/L), with the increase in K⁺ efflux inhibited by the KCC inhibitor (R)-(+)-[(dihydroindenyl)oxy]alkanoic acid (DIOA). The results demonstrate utility of the TAC-Lime_{dex} assay in measuring electroneutral K⁺ transport.

Figure 3b demonstrates the utility of the TAC-Lime_{dex} assay in measuring K⁺ channel activity in HT-29 cells, which express a Ca²⁺-activated K⁺ channel responsive to ATP, carbachol, and Ca²⁺ ionophores.⁸ ATP treatment greatly increased K⁺ efflux, which was inhibited by the K⁺ channel blocker tetraethylammonium (TEA) or by pretreatment with the cytoplasmic Ca²⁺ chelator, 1,2-bis(*o*aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM). With 3 mM K⁺ in the outside solution, the signal was 54% of that at 0 K⁺.

Last, the measurement in Figure 3b was repeated using a commercial fluorescence platereader, in which the K⁺-free, TAC-Lime_{dex}-containing solution was delivered by syringe pump to freshly washed cells (with K⁺-free buffer) in a 96-well plate. Inclusion of ATP increased the fluorescence response, which was blocked by TEA (Figure 3c). Initial curve slopes from multiwell measurements were (fluorescence units/s ± SD): 0.17 ± 0.01 (control), 0.33 ± 0.03 (ATP), and 0.22 ± 0.02 (+TEA).

Our results establish a simple cell-based fluorescence assay for plasma membrane K⁺ transport. The assay takes advantage of the strong fluorescence enhancement of TAC-Lime_{dex} to small increases in [K⁺]. Using TAC-Lime_{dex} as an extracellular K⁺ sensor, the kinetics of increasing TAC-Lime_{dex} fluorescence provides a quantitative readout of K⁺ accumulation into an initially K⁺-free, extracellular solution. The TAC-Lime_{dex} signal is sufficiently bright and robust for measurements using commercial fluorescence platereaders. As such, the assay should be amenable to high-throughput screening applications for discovery of modulators of plasma

membrane K⁺ transporters. Because the readout is K⁺ efflux rather than membrane potential or electrical current, both electro-genic and electrically silent K⁺-coupled transporters can be assayed.

For assay of K⁺ channels, certain limitations apply because K⁺ efflux into a K⁺-free extracellular solution is measured. Cell membrane potential is generally hyperpolarized under this condition. The K⁺ conductance to be assayed should be sufficiently high and sustained with an interior-negative membrane potential. Also, counterion conductance should be sufficiently high such that plasma membrane K⁺ conductance is rate-limiting under assay conditions.

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Figure 1.

TAC-Lime_{dex} synthesis and K⁺ sensitivity. (a) Synthesis procedures: (i) Methyl-3-(2,4-dimethyl-1*H*-pyrrol-3-yl) propanoate, TFA, DCM, rt, 18 h followed by addition of DDQ, 4 h; (ii) BF₃ Et₂O, DIEA, DCM, 0 °C, 2 h; (iii) 0.2 M aq NaOH, 100 °C, 2 h; (iv) *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate, DIEA, DMF; (v) amino-dextran, TEA, H₂O/DMSO. (b) Fluorescence spectra of TAC-Lime_{dex}·K⁺ titrations done at 500 nm excitation wavelength (for emission spectra) and 510 nm emission wavelength (for excitation spectrum). Solutions contained 5 μM TAC-Lime_{dex} (5 mM HEPES, pH 7) in balanced KCl/NaCl to maintain constant ionic strength at 200 mM.

Figure 2.

Principle of cell-based assay of K⁺ transport. (a) K⁺ efflux measurement method showing accumulation of K⁺ in an initially K⁺-free extracellular solution resulting from K⁺ efflux from cells. (b) Single measurement of ATP-induced K⁺ efflux in HT-29 cells (cell density 6.3×10^5 cells/cm², cell/bath volume ratio 0.27). (c) K⁺ efflux in HT-29 cells under control conditions, and after incubations with a K⁺ ionophore (valinomycin) or K⁺/H⁺ antiporter (nigericin). Cells were preincubated in high K⁺ buffer where indicated. Each data point is mean $\pm$ SE ($n = 3$). (d) Deduced K⁺ efflux rates, $d[K^+]/dt$, from data in (c). * $P < 0.05$.

Figure 3.

Application of TAC-Lime_{dex} for assay of K⁺-ion-coupled transporters and K⁺ channels. (a) KCC (K⁺/Cl⁻) cotransport in SiHa cells was activated by hypotonicity (200 mosm/L) in the absence or presence of 100 μ M DIOA (SE, $n = 4$). Fluorescence data (top) and summary of K⁺ efflux rates (bottom). * $P < 0.05$. (b) Calcium-activated K⁺ channels in HT-29 cells were activated by 100 μ M ATP and inhibited by 10 mM TEA or 50 μ M BAPTA (SE, $n = 3$). (c) Fluorescence plate reader assay of ATP-stimulated K⁺ efflux for the cell system studied in (b).