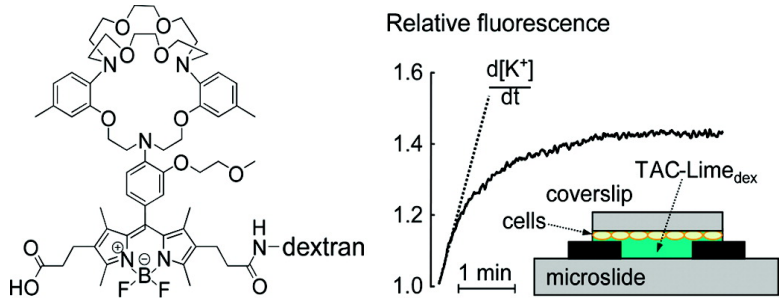
Communication

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

Wan Namkung, Prashant Padmawar, Aaron D. Mills, and A. S. Verkman

J. Am. Chem. Soc., **2008**, 130 (25), 7794-7795 • DOI: 10.1021/ja8014499 • Publication Date (Web): 31 May 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 2 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 05/31/2008

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

Wan Namkung, Prashant Padmawar, Aaron D. Mills, and A. S. Verkman*

Departments of Medicine and Physiology, University of California, San Francisco, California 94143-0521

Received March 3, 2008; E-mail: alan.verkman@ucsf.edu

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 TAC-CHO **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 (BF_3) 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

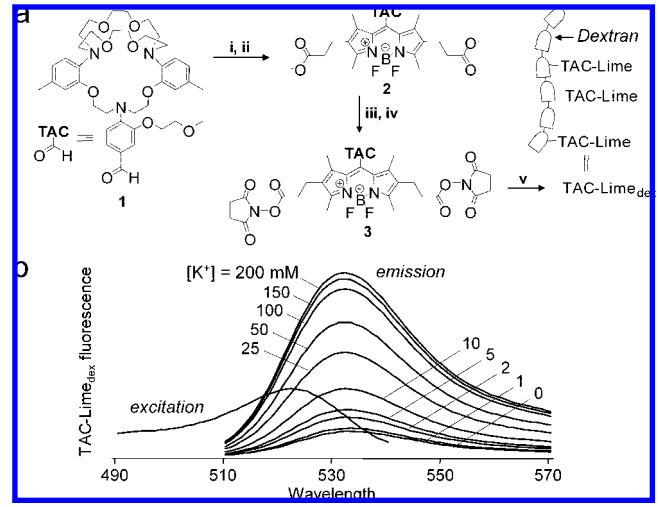


Figure 1. TAC-Lime$_{dex}$ synthesis and K^+ sensitivity. (a) Synthesis procedures: (i) Methyl-3-(2,4-dimethyl-1*H*-pyrrol-3-yl) propanoate, TFA, DCM, rt, 18 h followed by addition of DDQ, 4 h; (ii) BF_3 Et_2O, DIEA, DCM, 0 °C, 2 h; (iii) 0.2 M aq NaOH, 100 °C, 2 h; (iv) *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, DIEA, DMF; (v) amino-dextan, TEA, H_2O/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.

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

10.1021/ja8014499 CCC: $40.75

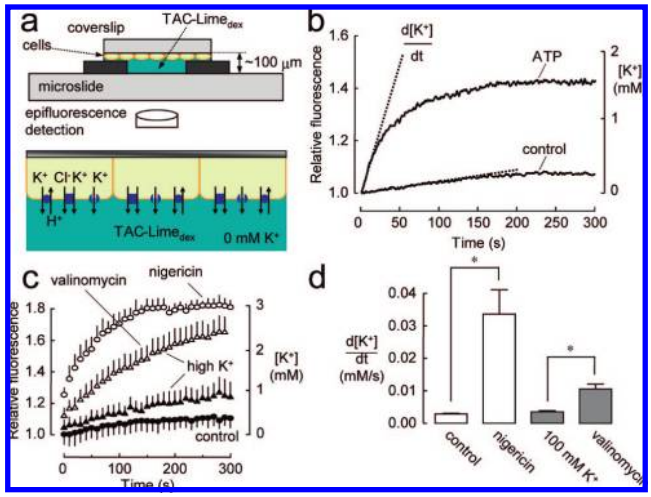


Figure 2. Principle of cell-based assay of K^+ transport. (a) K^+ efflux measurement method showing accumulation of K^+ in an initially K^+-free extracellular solution resulting from K^+ efflux from cells. (b) Single measurement of ATP-induced K^+ efflux in HT-29 cells (cell density 6.3×10^5 cells/cm^2, 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 ±SE ($n = 3$). (d) Deduced K^+ efflux rates, $d[K^+]/dt$, from data in (c). $*P < 0.05$.

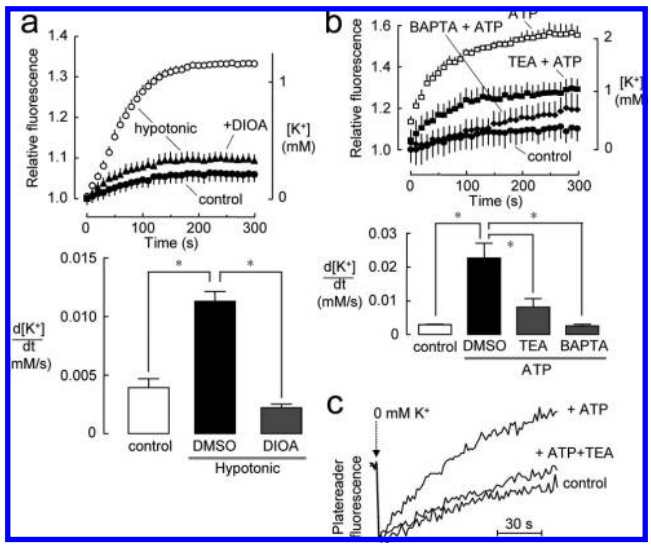


Figure 3. Application of TAC-Lime$_{dex}$ for assay of K^+-ion-coupled transporters and K^+ channels. (a) KCC (K^+/Cl^-) cotransport in SiHa cells was activated by hypotonicity (200 mosm/L) in the absence or presence of 100 μM DIOA (SE, $n = 4$). Fluorescence data (top) and summary of K^+ efflux rates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

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 Ca^{2+}-activated K^+ channel responsive to ATP, carbachol, and Ca^{2+} ionophores.[8] ATP treatment greatly increased K^+ efflux, which was inhibited by the K^+ channel blocker tetraethylammonium (TEA) or by pretreatment with the cytoplasmic Ca^{2+} 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 electrogenic 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.

Acknowledgment. Supported by NIH Grants EB00415, HL73856, DK72517, HL59198, DK35124, and EY13574, and grants from the Cystic Fibrosis Foundation.

Supporting Information Available: Experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Shieh, C. C.; Coghlan, M.; Sullivan, J. P.; Gopalakrishnan, M. *Pharmacol. Rev.* **2000**, *52*, 557–594.
(2) Wickenden, A. D. *Pharmacol. Ther.* **2002**, *94*, 157–182.
(3) Villalonga, N.; Ferreres, J. C.; Argilés, J. M.; Condom, E.; Felipe, A. *Recent Patents Anticancer Drug Discovery* **2007**, *2*, 212–223.
(4) Padmawar, P.; Yao, X.; Bloch, O.; Manley, G. T.; Verkman, A. S. *Nat. Methods.* **2005**, *2*, 825–827.
(5) Magzoub, M.; Padmawar, P.; Dix, J. A.; Verkman, A. S. *J. Phys. Chem. B.* **2006**, *110*, 21216–21221.
(6) Gillen, C. M.; Brill, S.; Payne, J. A.; Forbush, B., III *J. Biol. Chem.* **1996**, *271*, 16237–16244.
(7) Shen, M. R.; Chou, C. Y.; Hsu, K. F.; Hsu, Y. M.; Chiu, W. T.; Tang, M. J.; Alper, S. L.; Ellory, J. C. *J. Biol. Chem.* **2003**, *278*, 39941–39950.
(8) Wu, H.; Franklin, C. C.; Kim, H. D.; Turner, J. T. *Am. J. Physiol.* **1991**, *260*, C35–C42.

JA8014499