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A Genetically Encoded Bioluminescent Indicator for the Sodium Channel Activity in Living Cells

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Voltage-gated Na^+ channels (Na_vs) are activated by membrane depolarization and are responsible for the initiation and propagation of action potentials in excitable cells. Na_vs are widely expressed in neuronal, neuroendocrine, muscle, and cardiac muscle cells, and their dysfunction causes a variety of diseases. The patch-clamp technique, which has been used for more than 30 years, provides high sensitivity and high temporal resolution; however, it cannot allow spatial analysis and is not suitable for high-throughput chemical screening. A few synthetic fluorescent indicators for Na^+, such as benzofuran isophthalate (SBFI), have been developed to reveal intracellular Na^+ behavior involved in biological functions.[1,2] However, since they are diffusive and hardly targeted to specific intracellular locations, they are not applicable to monitoring of Na^+ channel activity. The generation of genetically encoded luminescent indicators could potentially overcome this limitation, an example of which is illuminated in Ca^{2+} channel indicator.[3] The purpose of this study was to develop a novel genetically encoded bioluminescent indicator for Na_vs in which Na^+-sensitive *Gaussia* luciferase is fused with the Na_v (Figure 1). *Gaussia* luciferase obtained from

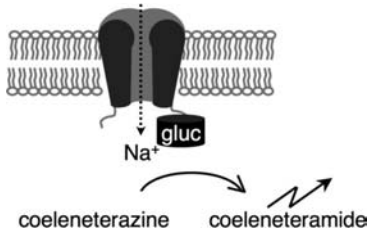

Figure 1. Schematic principle of a genetically encoded indicator for Na^+ channels.

Gaussia princeps is a recently identified secretory photoprotein. It oxidizes its substrate, coelenterazine, in a Na^+-dependent manner and yields a higher luminescence quantum than *Renilla* luciferase.[4−6] Because of high sensitivity and low background in luminescent assays, the absence of toxicity, and a wide linear dynamic range, this luciferase can be used to generate a novel genetically encoded Na^+ channel indicator.

To apply *Gaussia* luciferase to a Na^+ channel indicator, it should be held in living cells. To disguise the *N*-terminal secretion signal sequence, a myc-His tag was fused to the *N*-terminus of the signal sequence (designated as m-gluc) (Figure S1A in the Supporting Information). Luminescence intensities of the mutant measured both in cell lysate and in cell media indicated that m-gluc is successfully retained in cells and holds sufficient enzymatic activity (Figure S1B). We next examined the Na^+ dependency of m-gluc. The luminescence intensity of m-gluc increased with increasing Na^+ concentration at various concentrations of Cl^- ions (Figure S1C). K^+ is another cation that is abundantly present and dynamically changes its concentration in cells; K^+ did not affect the m-gluc luminescence intensity (Figure S1D). We further confirmed the Na^+ dependence of m-gluc in a physiological cytosolic ionic composition containing Ca^{2+} and Mg^{2+} (Figure S1E) and the thermostability of gluc (Figure S2). Collectively, these data led us to expect that fusion of the Na_v to the *N*-terminus of *Gaussia* luciferase would make it unsecretable while maintaining sufficient enzymatic activity and Na^+ dependence.

To construct a genetically encoded bioluminescent indicator for the Na^+ channel, we used the rat skeletal muscle Na^+ channel (μ1), a well-characterized Na_v.[7−9] We connected the cytosolic *C*-terminus of μ1 with the *N*-terminus of *Gaussia* luciferase through a flexible 15 amino acid linker; the resulting species is designated as μ1-gluc (Figure 2A).

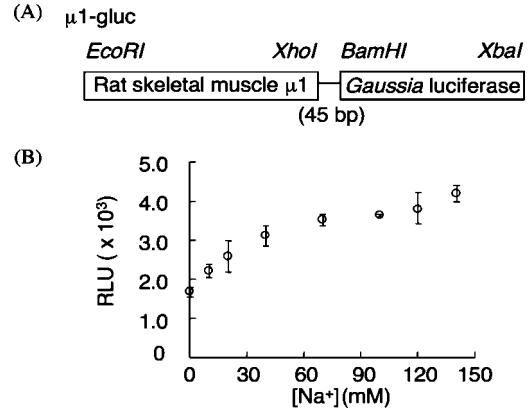

Figure 2. Genetically encoded Na^+ channel indicator μ1-gluc. (A) Structure of μ1-gluc. (B) Na^+-dependent luminescence activity of μ1-gluc in a physiological cytosolic ionic composition ($[K^+]$ = 140 mM, $[Cl^-]$ = 1.6 mM, $[Ca^{2+}]$ = 0.002 mM, $[Mg^{2+}]$ = 0.8 mM).

The rationale for this indicator is that Na^+ flowing through the pores of μ1 would activate *Gaussia* luciferase in μ1-gluc, resulting in emission of bioluminescence (Figure 1).

We first confirmed retainability of μ1-gluc in cells (Figure S3). The cells expressing μ1-gluc emit a weak luminescence compared with the cells expressing gluc or m-gluc alone. We speculate that this is because μ1 is a large membrane protein with molecular weight (MW) of >200 kD, so it could be expressed less efficiently than gluc or m-gluc. We next confirmed that μ1-gluc produces a light-emission response over a 2-fold range that correlates with Na^+ concentration in the physiological range 0−150 mM in the presence of K^+, Ca^{2+}, Mg^{2+}, and Cl^- (Figure 2B).

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We then used whole-cell patch-clamp experiments to examine whether μ1-gluc retained Na^+ transport activity. μ1-gluc expressed rapidly activating and inactivating Na^+ currents, which are indistinguishable from those of wild-type μ1 (Figure S4). Although the current density was decreased (μ1, 453.5 $\pm$ 88.5 pA/pF; μ1-gluc, 160 $\pm$ 30.9 pA/pF), the current kinetics of μ1 and μ1-gluc were not different (Figure S4).

Knowing that μ1-gluc retains both Na^+-dependent luciferase activity and Na_v function, we examined if μ1-gluc could work as an indicator for the Na_v. We analyzed changes in the luminescence intensity of μ1-gluc in response to membrane depolarization induced by high extracellular K^+ in HEK293T cells. The luminescence intensity increased by 1.47 $\pm$ 0.04 in response to high-K^+-induced depolarization (Figure 3). A Na^+ channel blocker, tetrodotoxin,

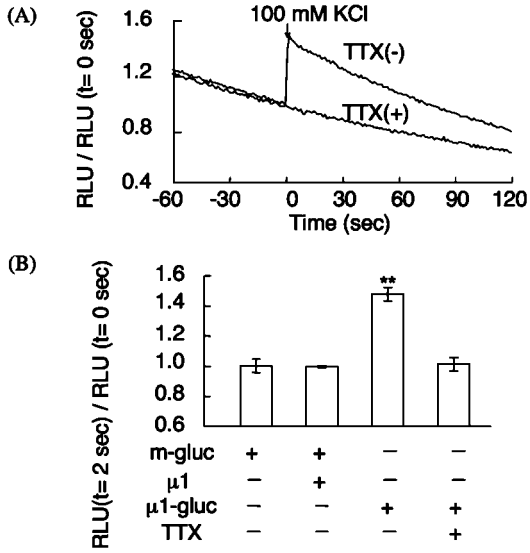

Figure 3. Response of μ1-gluc to Na^+ flow upon depolarization in living cells. (A) Representative time courses of the luminescent intensities of μ1-gluc upon high-K^+-induced depolarization with (+) or without (−) tetrodotoxin (TTX). The HEK293T cells expressing μ1-gluc were seeded in 96-well microplates with 150 μL of medium. Coelenterazine (23.6 μM in phosphate buffered saline, 40 μL) was added to each well at $t = -300$ s (i.e., 300 s prior to the addition of 10 μL of 2 M KCl at $t = 0$). Luminescence intensities were measured every 1 s. The luminescence intensities were normalized to those at $t = 0$. (B) Magnitude of luminescence response induced by K^+-depolarization. The luminescence intensities at 2 s, which exhibited the maximum intensities in the majority of experiments, were normalized to the intensities at $t = 0$. m-gluc was used as a control because the fusion protein blocked the gluc secretion signal. ** indicates $P < 0.05$.

which occludes channel pores and disturbs Na^+ flow,[7,8] abolished the increase in luminescence intensity in response to high-K^+-induced depolarization (Figure 3).

This study illustrates the first development of a genetically encoded bioluminescent indicator for the Na^+ channel. We believe that the indicator is capable of detecting Na^+ flow through the pores of Na^+ channels but not the cytosolic Na^+ rise in response to Na^+ channel activation, because a Na^+ channel pore blocker, tetrodotoxin, eliminated luminescence changes and co-transfection of m-gluc with μ1 failed to detect depolarization-induced luminescence changes. We also believe that μ1-gluc sensed Na^+ flow through μ1 pores rather than that the gluc part of μ1-gluc traveled across the membrane in response to depolarization and detected extracellular high [Na^+]; this belief is based on the following reasons: (1) luminescence intensity was not detected in the cell medium of HEK293T cells transfected with μ1-gluc (Figure S3); (2) since voltage-dependent Na^+ channels could permeate hydrophilic molecules with sizes of <15 Å[10] but could not permeate those with MW >600 Da,[11] it is physically impossible for gluc (MW = 20 kDa) to permeate through the channel pore; and (3) if one assumes that gluc travels across the membrane via a pathway other than the channel pore upon K^+-induced depolarization, then even in the presence of the channel blocker tetrodotoxin, K^+-induced depolarization should have increased the luminescence intensity, which was not the case.

The present investigation promises potentially important applications. It may provide a high-throughput screening system for drug discovery against Na^+ channels, which should be useful in controlling lethal cardiac arrhythmias, epileptic seizures, and intolerable pain associated with terminal stages of cancer. It may also offer a system for monitoring the Na^+ channel activity in living cells, which may be useful in illuminating neuronal activity in vivo. However, the light emission of μ1-gluc is currently not high enough for real-time in vivo imaging; for this purpose, further optimization of the indicator and detection system will be needed.

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Supporting Information Available: Experimental procedures, luminescence data, and electrophysiological data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Harootunian, A. T.; Kao, J. P.; Eckert, B. K.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 19458.
(2) Minta, A.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 19449.
(3) Mori, M. X.; Erickson, M. G.; Yue, D. T. *Science* **2004**, *304*, 432.
(4) Lee, R. F.; Nevenzel, J. C.; Paffenhöfer, G. A. *Science* **1970**, *167*, 1510.
(5) Tannous, B. A.; Kim, D. E.; Fernandez, J. L.; Weissleder, R.; Breakefield, X. O. *Mol. Ther.* **2005**, *11*, 435.
(6) Verhaegent, M.; Christopoulos, T. K. *Anal. Chem.* **2002**, *74*, 4378.
(7) Trimmer, J. S.; Cooperman, S. S.; Tomiko, S. A.; Zhou, J.; Crean, S. M.; Boyle, M. B.; Kalen, R. G.; Sheng, Z.; Barchi, R. L.; Sigworth, F. J.; Goodman, R. H.; Agnew, W. S.; Mandel, G. *Neuron* **1989**, *3*, 33.
(8) Sunami, A.; Dudley, S. C., Jr.; Fozzard, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14126.
(9) Kazen-Gillespie, K. A.; Ragsdale, D. S.; D'Andrea, M. R.; Mattei, L. N.; Rogers, K. E.; Isom, L. L. *J. Biol. Chem.* **2000**, *275*, 1079.
(10) Lee, P. J.; Sunami, A.; Fozzard, H. A. *Circ. Res.* **2001**, *89*, 1014.
(11) Weber, F.; Brinkmeier, H.; Aulkemeyer, P.; Wollinsky, K. H.; Rudel, R. *J. Neurol.* **1999**, *246*, 955.

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