J|A|C|S
COMMUNICATIONS

Published on Web 04/11/2007

Photochemical Gating of Intracellular Ca^{2+} Release Channels

Jiahong Ni,[†] Darryl A. Auston,[†,‡] David A. Freilich,[†,§] Sukumaran Muralidharan,[†] Eric A. Sobie,[||] and Joseph P. Y. Kao*[,†,‡,§]

Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, Maryland 21201, Department of Physiology and Program in Neuroscience, University of Maryland School of Medicine, Baltimore, Maryland 21201, and Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, New York 10029

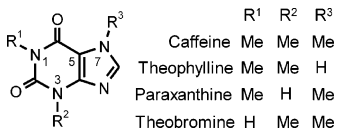
Received December 29, 2006; E-mail: jkao@umaryland.edu

Electrically excitable cells abundantly express a channel that mediates release of Ca^{2+} from intracellular calcium stores. These release channels are blocked by the alkaloid, ryanodine, and are therefore called ryanodine receptors (RyRs).[1] In nerve and muscle cells, electrical excitation of the cell membrane opens voltage-dependent Ca^{2+} channels, which conduct Ca^{2+} ions from the extracellular solution into the cytosol. In turn, these Ca^{2+} ions can bind to, and thus trigger the opening of, RyRs to permit massive Ca^{2+} flux from intracellular stores into the cytosol to raise the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). This process is known as "Ca^{2+}-induced Ca^{2+} release" (CICR). The increased $[Ca^{2+}]_i$ consequent to CICR is crucial to cardiac muscle contraction.[2] CICR can also regulate electrical excitability in neurons by controlling the opening of Ca^{2+}-activated K^+ channels.[3] In the course of our research on neuronal excitability, we wished to open RyRs rapidly without activating the voltage-dependent Ca^{2+} influx that normally serves as a trigger. To this end, we designed and synthesized a "caged" agonist that, when photolyzed intracellularly, activates RyRs rapidly and robustly.

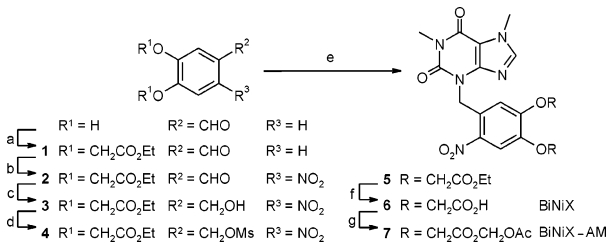
A "caged" molecule is an inert but photolabile molecule that is transformed by photolysis into a biologically active molecule; this process is often called "uncaging" or "photorelease".[4] Because the spatial resolution of photorelease is limited only by the ability to focus light, diffraction-limited (sub-micrometer) resolution is potentially achievable. Moreover, fast photochemical kinetics enable fast photorelease. Therefore, focal photolysis of caged molecules is an excellent tool for controlling biological processes with high spatiotemporal precision. The most common approach to caged molecules is to attach a photolabile protective group to an essential functional group in a biomolecule and thus abolish its biological activity. Photolytic cleavage of the protective group unmasks the functional group to restore bioactivity.

Methylxanthines, such as theophylline and caffeine, are RyR agonists (Chart 1).[5] They bind to RyRs and induce channel opening even at resting levels of $[Ca^{2+}]_i$ ($\leq 0.1\ \mu M$). As a starting point, we sought a methylxanthine that has (1) good potency and efficacy for activating RyRs, (2) high water solubility, (3) high membrane permeancy to permit easy passage through cell membranes, and (4) accessibility to chemical modification. Paraxanthine (PX; 1,7-dimethylxanthine) uniquely satisfied these criteria.[5] As a photolabile protective group, we used 4,5-bis(carboxymethoxy)-2-nitrobenzyl (BcmNb), which, when attached to the 3 position of PX, would yield a highly water-soluble caged molecule bearing two carboxylate groups (3-BcmNb-PX, or BiNiX for brevity). Finally, we wished to mask the carboxylates on the caged molecule as acetoxymethyl

Chart 1. Methyxanthines That Activate Ryanodine Receptors



Scheme 1. Synthesis of BiNiX and Its Acetoxymethyl Ester[a]



[a] Reagents and conditions: (a) $BrCH_2CO_2Et$, K_2CO_3, DMF, 0 °C to room temp; (b) KNO_3, TFA, 0 °C to room temp; (c) $NaBH_4$, CH_2Cl_2/EtOH/HOAc (35:5:1), 0−5 °C; (d) MsCl, Et_3N, CH_2Cl_2, 0 °C to room temp; (e) 1,7-dimethylxanthine, K_2CO_3, *n*-Bu_4NHSO_4, DMF, room temp; (f) (i) 1 N NaOH, CH_2Cl_2/MeOH (1:1), room temp; (ii) 1 N HCl; (g) $BrCH_2OAc$, Et_3N, DMF, room temp.

(AM) esters, which are labile to cleavage by intracellular esterases.[6] The AM ester, being lipophilic and thus membrane-permeant, can enter a cell by diffusion across the cell membrane. Thereafter, intracellular esterases rapidly cleave the AM esters to expose the carboxylates on BiNiX, which, being a dianion at physiologic pH, cannot readily cross the cell membrane, and is therefore trapped inside the cell. Thus, BiNiX can be accumulated intracellularly.

The synthesis of BiNiX and the corresponding AM ester is outlined in Scheme 1. The reagent for introducing the BcmNb protective group was prepared from 3,4-dihydroxybenzaldehyde in four steps: *O*-alkylation with ethyl bromoacetate, nitration, reduction of the aldehyde to the benzylic alcohol, which was then converted to the mesylate. Alkylation of paraxanthine with the mesylate, followed by hydrolysis, yielded water-soluble BiNiX. Reaction of BiNiX with bromomethyl acetate afforded the lipophilic AM ester.

In aqueous solution, BiNiX has a near-UV absorption peak at $\lambda_{max} = 346$ nm ($\epsilon = 5.72 \times 10^3\ M^{-1}\ cm^{-1}$; spectrum in Supporting Information). Photolysis of BiNiX in sodium phosphate buffer (0.1 M, pH 7.4) at 355 nm proceeded with a quantum efficiency of $\phi = 0.0058 \pm 0.0002$. To study the kinetics of photolysis, we used transient absorption spectroscopy to monitor the photolytically generated, short-lived *aci*-nitro intermediate,[7] whose decay is concomitant with cleavage of the protective group and release of product.[8] The transient absorption decay followed a single-exponential time course, with a time constant of $\tau = 0.26\ \mu s$ (or $t_{1/2} = 0.18\ \mu s$; see Supporting Information). Thus, flash photolysis

[†] University of Maryland Biotechnology Institute.
[‡] Department of Physiology, University of Maryland School of Medicine.
[§] Program in Neuroscience, University of Maryland School of Medicine.
[||] Mount Sinai School of Medicine.

10.1021/ja069361q CCC: $37.00

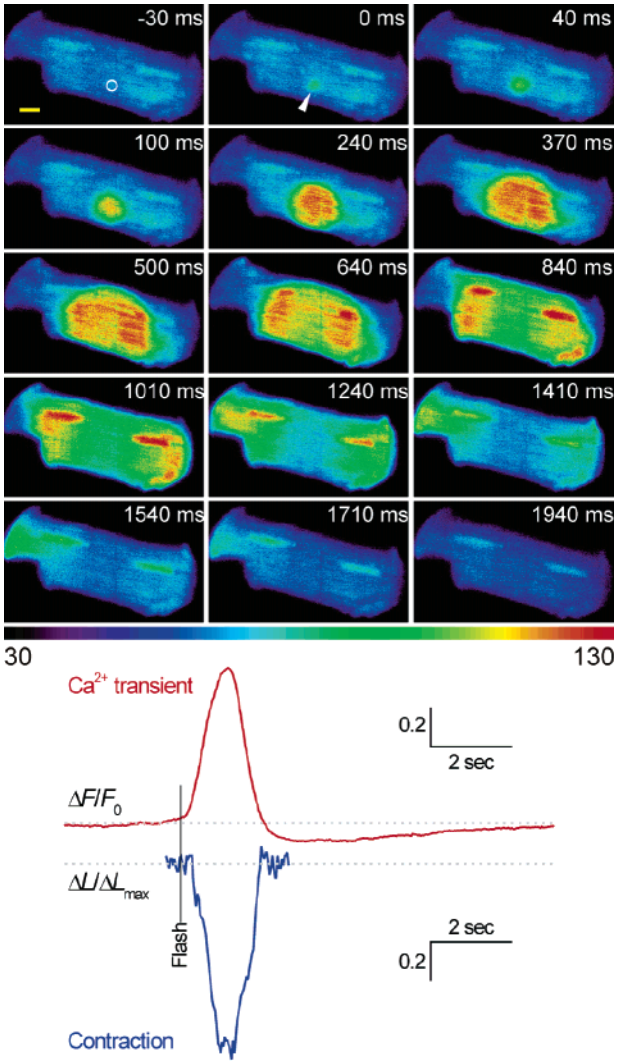


Figure 1. In a cardiac myocyte, subcellular photorelease of paraxanthine in a 5-μm spot (white circle) triggers a Ca^{2+} wave and consequent contraction of the myocyte (yellow scale bar = 10 μm). An 8-ms flash of 355-nm light from a quasi-continuous Nd:YAG laser was delivered immediately before acquiring the image at 0 ms. Intracellular [Ca^{2+}] was monitored by imaging Fluo-3 fluorescence. Fluorescence intensity is encoded in pseudocolor according to the color-scale bar. Changes of fluorescence and cell length relative to baseline are shown as red and blue line traces, respectively.

of BiNiX liberates PX with modest quantum efficiency but at a very high rate—under pulse photolysis conditions, photorelease of PX is essentially complete in <1 μs.

To test the biological efficacy of the caged RyR agonist, we loaded BiNiX into adult rat cardiac myocytes by incubation with the AM ester. Cardiac myocytes have an abundance of RyRs on their intracellular Ca^{2+} storage compartment, the sarcoplasmic reticulum (SR).[9] When activated, the RyRs release Ca^{2+} from the SR to cause a marked increase in $[Ca^{2+}]_i$ which, in turn, triggers contraction of the myocyte.[2] Indeed, as the montage in Figure 1 shows, in a cardiac myocyte loaded with BiNiX and the fluorescent Ca^{2+} indicator, Fluo-3, an 8-msec pulse of 355-nm light delivered focally to a 5-μm spot (white circle) near the middle of the cell triggered local Ca^{2+} release (marked by white arrowhead), which was amplified regeneratively by CICR into a wave of elevated $[Ca^{2+}]_i$ that swept through the cell. The Ca^{2+} wave triggered contraction of the myocyte, as illustrated by the length-versus-time trace in Figure 1 (a time-lapse video of the experiment is available as Supporting Information). The light-evoked Ca^{2+} release was completely abolished in the presence of 10 μM ryanodine, a specific inhibitor of RyRs. In cells loaded with Fluo-3 but not BiNiX, identical light flashes evoked no response (figure in Supporting Information). These findings indicate that the observed response was not artifactual, but rather resulted from specific RyR activation.

Two examples illustrate the potential utility of BiNiX in cellular physiology. First, gating of RyRs depends on both cytosolic and SR luminal [Ca^{2+}]. Activating RyRs with photoreleased PX, instead of a cytosolic [Ca^{2+}] rise, allows the regulation by SR luminal [Ca^{2+}] to be probed independently. This is important for studying spontaneous Ca^{2+} waves that can trigger cardiac arrhythmias. Second, Ca^{2+} released through RyRs can profoundly affect ion channels and electrical excitability in neurons; however, little is known about the spatial distribution of RyRs in these anatomically complex cells. In conjunction with fluorescent Ca^{2+} indicators such as Fluo-3, focal photorelease of PX affords a means for mapping the distribution of RyRs in neurons.

In summary, we have designed, synthesized, and demonstrated the biological utility of BiNiX, a photoactivatable RyR agonist. Through incubation with the AM ester form, BiNiX can be easily loaded into cells. Thereafter, focal photolysis permits activation of RyRs with high spatiotemporal precision. Therefore, BiNiX is a unique tool for selectively probing RyR function in living cells.

Acknowledgment. We thank Mr. Marcus Palmer for preparing the cardiac myocytes. This research was supported by the NIH (Grants GM056481 and GM64706 to J.P.Y.K.).

Supporting Information Available: Details of chemical syntheses, physical chemical measurements, biological experiments, and characterization data for all intermediates; video (avi). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Fill, M.; Copello, J. A. *Physiol. Rev.* **2002**, *82*, 893−922.
(2) Bers, D. M. *Nature* **2002**, *415*, 198−205.
(3) (a) Cordoba-Rodriguez, R.; Moore, K. A.; Kao, J. P. Y.; Weinreich, D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 7650−7657. (b) Moore, K. A.; Cohen, A. S.; Kao, J. P. Y.; Weinreich, D. *J. Neurophysiol.* **1998**, *79*, 688−694.
(4) For reviews of caged molecules and biological applications: (a) Kao, J. P. Y. *Curr. Protoc. Neurosci.* **2006**, *37*, 6.20.1−6.20.21. (b) Lawrence, D. S. *Curr. Opin. Chem. Biol.* **2005**, *9*, 570−575. (c) Shigeri, Y.; Tatsu, Y.; Yumoto, N. *Pharmacol. Ther.* **2001**, *91*, 85−92. (d) Marriott, G.; Walker, J. W. *Trends Plant Sci.* **1999**, *4*, 330−334. (e) Nerbonne, J. M. *Curr. Opin. Neurobiol.* **1996**, *6*, 379−386. (f) Kao, J. P. Y.; Adams, S. R. In *Optical Microscopy: Emerging Methods and Applications*; Herman, B., Lemasters, J. J., Eds.; Academic Press: San Diego, CA, 1993; pp 27−85. (g) Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755−784.
(5) Rousseau, E.; Ladine, J.; Liu, Q. Y.; Meissner, G. *Arch. Biochem. Biophys.* **1988**, *267*, 75−86.
(6) Tsien, R. Y. *Nature* **1981**, *290*, 527−528.
(7) (a) Yip, R. W.; Wen, Y. X.; Gravel, D.; Giasson, R.; Sharma, D. K. *J. Phys. Chem.* **1991**, *95*, 6078−6081. (b) Zhu, Q. Q.; Schnabel, W.; Schupp, H. *J. Photochem.* **1987**, *39*, 317−332. (c) Schupp, H.; Wong, W. K.; Schnabel, W. *J. Photochem.* **1987**, *36*, 85−97. (d) Yip, R. D.; Sharma, D. K.; Giasson, R.; Gravel, D. *J. Phys. Chem.* **1985**, *89*, 5328−5330.
(8) (a) McCray, J. A.; Trentham, D. R. *Annu. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 239−270. (b) Walker, J. W.; Reid, G. P.; McCray, J. A.; Trentham, D. R. *J. Am. Chem. Soc.* **1988**, *110*, 7170−7177.
(9) Franzini-Armstrong, C.; Protasi, F.; Ramesh, V. *Biophys. J.* **1999**, *77*, 1528−1539.

JA069361Q