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Optogenetic Control of Voltage-Gated Calcium Channels

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Abstract: *Voltage-gated Ca^{2+} (Ca_V) channels mediate Ca^{2+} entry into excitable cells to regulate a myriad of cellular events following membrane depolarization. We report the engineering of RGK GTPases, a class of genetically encoded Ca_V channel modulators, to enable photo-tunable modulation of Ca_V channel activity in excitable mammalian cells. This optogenetic tool (designated optoRGK) tailored for Ca_V channels could find broad applications in interrogating a wide range of Ca_V-mediated physiological processes.*

Voltage-gated Ca^{2+} (Ca_V) channels constitute the major route of Ca^{2+} entry into cells of the nervous and cardiovascular systems, as well as other electrically excitable cells.[1] Ca_V channels respond to membrane depolarization to permit Ca^{2+} influx, thereby playing instrumental roles in Ca^{2+}-dependent physiological processes, including neurotransmitter/hormone release, gene expression, and muscle contraction.[1b,c] Deregulated Ca_V channels can give rise to pathophysiological conditions ranging from cardiovascular disorders to psychiatric conditions.[2] Consequently, Ca_V channels are important targets for therapeutic intervention and physiological regulation.[3] Currently widely used Ca_V channel

blockers (e.g., dihydropyridines) have prominent drawbacks, including off-target toxicity, lack of spatial control, and non-reversibility.[3b,4] New interventional approaches to control Ca_V channels are thus needed.

Optogenetics, which involves the incorporation of synthetic photosensitive modules into cells of living tissues to control cellular activities with high spatiotemporal precision, provides an ideal solution to overcome the drawbacks associated with conventional Ca_V channel blockers.[5] The Ras-like GTPases Rad/Rem/Gem/Kir (RGK), which function as negative regulators of Ca_V channels, are considered to be the prime candidates for generating optogenetic tools to modulate Ca_V channels.[6] Given that membrane anchoring is necessary for RGKs to exert their suppressive effects on Ca_V channels,[6b,7] we reasoned that Ca_V channels could be remotely modulated by harnessing light to control the translocation of RGK to the plasma membrane (PM). We therefore engineered a set of optogenetic constructs by using a light-sensitive heterodimerization system to control the subcellular localization of engineered Rem (Scheme 1 and Figure S1 in the Supporting Information). We chose the optical dimerizer pair iLID (LOV2-ssrA) and sspB because of their small size, fast photoresponsive kinetics, low background interaction, and minimized perturbation to endogenous signaling pathways.[8]

To enable light-inducible cytosol-to-PM translocation of engineered Rem, we set out to install sspB into different positions of Rem_{1-266} tagged with the red fluorescent protein mCherry (mCherry-Rem_{1-266}) via flexible linkers with varying lengths (Figures S1–3). In parallel, we tethered iLID tagged with the yellow fluorescent protein Venus (Venus-iLID) to

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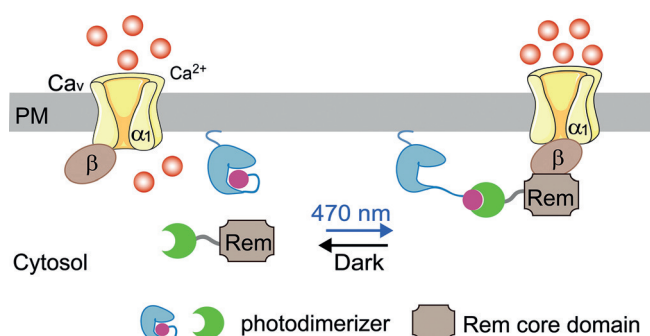
Scheme 1. Design of optoRGK to photo-tune CaV channel activity. Spatiotemporal control of the Rem1 core domain is achieved by utilizing the LOV2-ssrA/sspB optical dimerizer pair. The light-inducible cytosol-to-PM translocation of Rem enables inducible suppression of Ca_V channel activity. Green crescent = 1st photodimerizer component (sspB), blue structure = membrane tethered LOV2, pink sphere = 2nd photodimerizer component (ssrA), LOV2 + ssrA = improved light-induced dimer (iLID) protein.
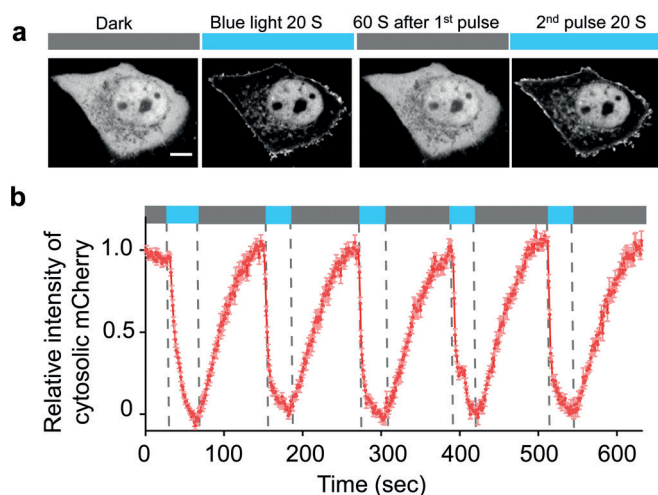
**Figure 1.** Visualization of the reversible recruitment of the Rem core domain (Rem$_{1-266}$, without the C-terminus) to the PM in response to blue-light illumination. a) Representative confocal images showing light-inducible cytosol-to-PM translocation of mCherry-Rem1$_{1-266}$–sspB in HeLa cells co-expressing Venus-iLID-CAAX. The images represent the same cell in the dark (black bar) or exposed to blue light at 470 nm (40 µW mm^{-2}; blue bar). Scale bar: 5 µm. b) Quantification of cytosolic mCherry signals of optoRGK over five repeated light–dark cycles. $n = 34$ cells from three independent experiments. Error bars denote the SEM.

the PM with a C-terminal PM-targeting sequence (CAAX) derived from KRas4B[8] or the PM-tethering motif (Lyn11) from the tyrosine-protein kinase Lyn.[9] We then co-transfected these two constructs into HeLa cells and examined the reversible recruitment of cytosolic Rem$_{1-266}$ to the PM (Figure 1a, Figures S1–4). In the dark, mCherry-Rem$_{1-266}$–sspB was evenly distributed in the cytosol (Figure 1a). Upon blue light illumination, photoexcitation produced a covalent adduct between residue C450 of LOV2 and the cofactor FMN, thereby exposing the caged ssrA component of iLID to restore its interaction with sspB. Consequently, mCherry-Rem$_{1-266}$–sspB was recruited toward the PM within seconds ($t_{1/2, on} = 3.2 \pm 1.0$ sec). Upon withdrawal of light, mCherry-Rem$_{1-266}$–sspB dissociated from the PM-resident ssrA and diffused back to the cytosol ($t_{1/2, off} = 23.0 \pm 2.4$ sec; Figure 1, Figure S3, and Movie S1). After screening over 20 constructs with different combinations of key elements (various Rem fragments, linkers, PM-targeting motifs, and insertion sites), we identified mCherry-Rem1$_{1-266}$–sspB/iLID-CAAX as an ideal candidate because it exhibited an optimal performance with high sensitivity and relatively fast kinetics (Figures S1–S3). To enable a near 1:1 co-expression of the two components using a single construct within the same cells, we used a multicistronic expression system by utilizing the self-cleaving 2A peptide (P2A) to generate fluorescent protein (FP)-tagged (mCherry or CFP) Rem1$_{1-266}$–sspB-P2A-iLID-CAAX.[10] We named the system optoRGK and used the FP-tagged constructs for further characterization and applications in excitable cells.

To determine whether optoRGK could photo-modulate Ca$_V$ channels, we expressed optoRGK in HEK293 cells stably expressing the human Ca$_V$1.2 channel components (HEK-

Ca$_V$1.2)[11] and evaluated membrane-depolarization-induced Ca^{2+} entry with a red genetically encoded Ca^{2+} sensor jRGCaMP1b[12] (Figure 2a,b) or the green Ca^{2+} dye Fluo-4 (Figure S3). 50 mM potassium chloride (KCl) was employed to induce membrane depolarization. In the dark, addition of
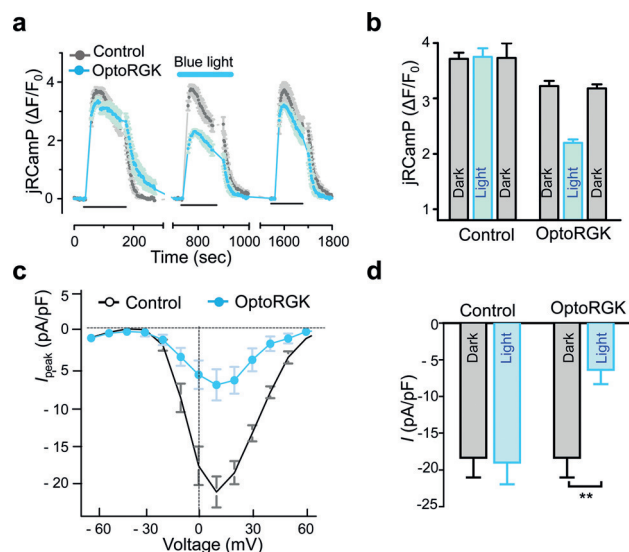
Figure 2. OptoRGK-mediated photoswitchable inhibition of Ca^{2+} entry through Ca$_V$1.2 channels. a) Ca^{2+} influx in HEK-Ca$_V$1.2 cells transiently expressing optoRGK and the red Ca^{2+} sensor jRCaMP1b with and without blue-light stimulation. Cells transfected with the empty vector are used as control. Membrane-depolarization-induced Ca^{2+} entry was elicited by adding 50 mM KCl (black line below the curves; three repeated cycles) to transfected cells. Blue line represents light stimulation under 470 nm with a power density of 40 µW mm^{-2}. b) Bar graphs showing the statistical results of mean Ca^{2+} entry for each cycle. c) The current–voltage relationships of Ca$_V$ channels in HEK-Cav1.2 cells transfected with optoRGK. Cells were either shielded from light or exposed to blue light prior to electrophysiological recording. d) Bar graphs showing the statistical results of peak whole-cell currents induced by pulses of +10 mV depolarization (pA/pF) in HEK-Ca$_V$1.2 cells before and after photo-stimulation. All data were presented as mean ± SEM. **$P < 0.01$ (paired Student's t-test).

KCl elicited a pulse of Ca$_V$-mediated Ca^{2+} influx in both control and optoRGK-expressing cells (Figure 2, 1st cycle). Upon blue-light illumination, cells overexpressing optoRGK showed a significant reduction in KCl-induced Ca^{2+} entry compared to control cells (Figure 2a, 2nd cycle). Furthermore, Ca^{2+} influx could be restored in the absence of blue light (Figure 2a, 3rd cycle), thereby attesting to the reversibility of optoRGK in the regulation of Ca$_V$ channels. In parallel, we performed electrophysiological studies to independently confirm optoRGK-mediated, light-switchable modulation of Ca$_V$ channels in HEK293-Ca$_V$1.2 cells. In the dark, cells expressing optoRGK showed robust whole-cell currents with a typical Ca$_V$ I-V relationship and amplitudes similar to those of control cells (maximal peak current density ≈ 21.2 pA/pF, $n = 12$; Figure 2c,d). However, upon receiving blue-light stimulation, the amplitudes of depolarization-induced whole-cell currents were significantly diminished (maximal peak current density ≈ 6.3 pA/pF, $n = 15$; Figure 2c,d). These

results clearly established optoRGK as a genetically encoded light-switchable channel modulator that allows optical inhibition of Ca_V channels in excitable cells.

We next moved on to test optoRGK in C2C12 cells, a mouse myoblast cell line[13] with functional Ca_V channels.[6b] Again, we observed light-dependent inhibition of Ca^{2+} influx in this excitable cell line (Figure S5). To further test optoRGK in more physiologically relevant systems, we introduced it into HL-1 cardiomyocytes, a well-characterized atrial myocyte culture line derived from the adult mouse that retains many of the differentiated properties of cardiac cells,[14] including rhythmic oscillations of cytosolic Ca^{2+} (Movie S2). By using Fluo-4 as the Ca^{2+} indicator, we first evaluated rhythmic Ca^{2+} oscillations in HL-1 cells with and without overexpression of full length Rem or its truncated version Rem_{1-266} (Figure S6, Movies S3 and S4). Both control cells and HL-1 cells transfected with $mCherry-Rem_{1-266}$–sspB (Figure S6b) exhibited rhythmic Ca^{2+} oscillations, while cardiomyocytes expressing the full-length Rem failed to evoke Ca^{2+} transients (Figure S6a). These findings are consistent with the results obtained from C2C12 myoblast cells (Figure S5) and other types of excitable cells.[15]

Having validated the use of HL-1 cardiomyocytes to test our tool, we next examined the rhythmic oscillations of cytosolic Ca^{2+} (Fluo-4 signals as readout) in HL-1 cells expressing optoRGK. Upon blue-light illumination, $mCherry-Rem1_{1-266}$–sspB translocated from the cytosol to close to the PM within several seconds (Figure S7), accompanied by the attenuation of oscillatory Ca^{2+} signals (Figure 3a, bottom, and Movie S5). By contrast, the control cells displayed regular Ca^{2+} oscillations under blue light. To further validate whether such action was reversible in HL-1 cells, we used the red Ca^{2+} indicator Cal-590 (excitation at 562 nm without pre-activating optoRGK) rather than Fluo-4 to monitor Ca^{2+} oscillations (Figure 3b). HL-1 cells expressing optoRGK showed regular Ca^{2+} spikes in the dark. However,

upon blue-light illumination, the rhythmic oscillations were substantially attenuated. Notably, regular Ca^{2+} oscillations were restored in the same HL-1 cardiomyocyte after removal of the light source (Figure 3b).

Taken together, compared with traditional small-molecule Ca_V channel blockers that often lack reversibility, selectivity, and tissue-specificity, engineered RGK proteins could serve as promising candidates to enable spatiotemporal control of Ca_V channels with a simple pulse of light. This study complements the recent development of engineered stromal interaction molecule 1 (STIM1) to photo-regulate endogenous Ca^{2+} channels in mammalian cells (e.g., optoSTIM1[16] and Opto-CRAC[17]). We anticipate that the optoRGK tool developed in the current study will find broad applications in interrogating a wide range of Ca^{2+}-dependent physiological processes in mammals.

Proof-of-concept experiments have already demonstrated the potential of using RGK to treat heart disease.[18] To test potential in vivo applications, we plan to express optoRGK in the atrioventricular node of rodent models with atrial fibrillation disease,[19] and examine whether photostimulation could suppress aberrant atrioventricular nodal conduction to intervene atrial fibrillation.

Given that RGK proteins primarily exert suppressive effects on high-voltage-activated Ca^{2+} (Ca_V1/Ca_V2) channels[20] and that LOV2-based photoswitches have relatively slow kinetics, the efficacy of optoRGK will likely depend on the distribution and endogenous levels of Ca_V channel subtypes in different cell types and tissues. In its current configuration, optoRGK is well suited to modulate cardiomyocytes because of the abundant expression of L-type $Ca_V1.1/Ca_V1.2$ channels and the relatively long duration of cardiac action potentials. Its compatibility with neurons and other types of electrically excitable cells (e.g., pancreatic beta cells) remains to be tested.

Conflict of interest
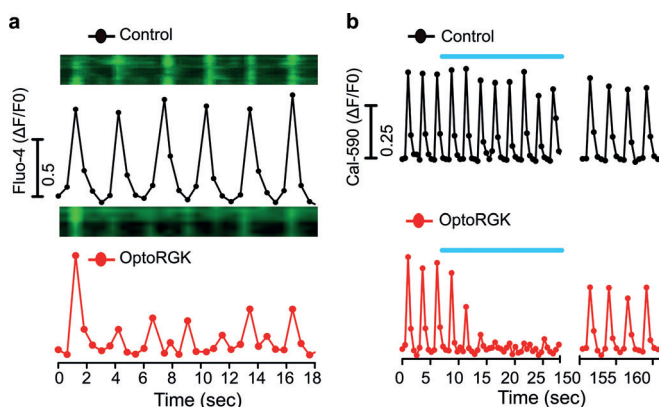
The authors declare no conflict of interest.

Figure 3. OptoRGK-mediated light-inducible inhibition of the rhythmic oscillations of cytosolic Ca^{2+} in cardiac cells. a) Ca^{2+} oscillations reported by Fluo-4 in HL-1 cells with (bottom) and without (top) expression of optoRGK. Kymographs of Fluo-4 signals in a representative HL-1 cell are shown above the traces. Excitation was set at 488 nm to record Fluo-4 signals while simultaneously photoactivating optoRGK. b) Ca^{2+} oscillations in HL-1 cells monitored by Cal-590. Blue bar = blue light illumination at 470 nm (40 μW mm^{-2}).

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