Ca\textsuperscript{2+}-Mediated Synthetic Biosystems Offer Protein Design Versatility, Signal Specificity, and Pathway Rewiring

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SUMMARY

Synthetic biosystems have been engineered that enable control of metazoan cell morphology, migration, and death. These systems possess signal specificity, but lack flexibility of input signal. To exploit the potential of Ca\textsuperscript{2+} signaling, we designed RhoA chimeras for reversible, Ca\textsuperscript{2+}-dependent control over RhoA morphology and migration. First, we inserted a calmodulin-binding peptide into a RhoA loop that activates or deactivates RhoA in response to Ca\textsuperscript{2+} signals depending on the chosen peptide. Second, we localized the Ca\textsuperscript{2+}-activated RhoA chimera to the plasma membrane, where it responded specifically to local Ca\textsuperscript{2+} signals. Third, input control of RhoA morphology was rewired by coexpressing the Ca\textsuperscript{2+}-activated RhoA chimera with Ca\textsuperscript{2+}-transport proteins using acetylcholine, store-operated Ca\textsuperscript{2+} entry, and blue light. Engineering synthetic biological systems with input versatility and tunable spatiotemporal responses motivates further application of Ca\textsuperscript{2+} signaling in this field.

INTRODUCTION

Synthetic biosystems engineered at the molecular level have been reported that enable control of metazoan cell morphology (Levskaya et al., 2009; Yeh et al., 2007), migration (Wang et al., 2010; Wu et al., 2009), and apoptosis (Gray et al., 2010) using exogenous chemicals or light. Synthetic proteins and protein networks allow the reprogramming of cells to perform diverse tasks that they are not otherwise capable of performing. Already, reprogrammed cells are powerful tools for research in embryonic development (Wang et al., 2010), drug discovery (Gray et al., 2010), and therapeutic interventions in diseases such as leukemia (Kalos et al., 2011). These systems were designed using specific allosteric interactions such as the caging of Rac1 by LOV2 in PARac (Wu et al., 2009) or with protein domains and peptides that are responsive to specific exogenous chemicals such as rapamycin in the FRB/FKBP12 heterodimerization of SNIPer (Gray et al., 2010). These approaches provide signal specificity but necessarily limit the flexibility in providing alternate input signals to these systems, such as different wavelengths of light or exogenous chemicals, without a complete redesign. Although these biosystems are hard wired to a particular input stimulus, endogenous biosystems based on Ca\textsuperscript{2+} signaling are inherently modular—that is, proteins responding to and transporting Ca\textsuperscript{2+} are interchanged and interconnected in diverse ways across cell types and subcellular locations to create signal complexity and specificity (Berridge et al., 2000).

Ca\textsuperscript{2+} is a ubiquitous second messenger that is involved in physiological processes in different cells depending on the presence of Ca\textsuperscript{2+}-dependent proteins to function.

In this study, we report a way to reprogram certain mammalian cells such that several exogenous stimuli (i.e., blue light, thapsigargin, and acetylcholine) trigger blebbing morphology and cell migration. The reprogramming is done via CaM and its binding peptides (Crvici and Ikura, 1995), which translate the varied stimuli into the same physiological effect: RhoA activation leading to bleb formation and actinomyosin-based migration (Fackler and Grosse, 2008). Local Ca\textsuperscript{2+} signals were created using Ca\textsuperscript{2+}-generating proteins that are specific to the plasma membrane (PM) such as nAChR channels (Zhou et al., 2010a, 2010b) that enable specific activation...
of the CaM/RhoA chimera. The toolbox of Ca\textsuperscript{2+}-generating proteins used also includes channelrhodopsin-2 (ChR2), which can translate 440 nm light into cytoplasmic Ca\textsuperscript{2+} (Petreanu et al., 2007) and thus morphology changes and migration.

**RESULTS**

**An Embedded Peptide Chimera Enables Ca\textsuperscript{2+}-CaM Control over RhoA**

Ca\textsuperscript{2+}-dependent control over RhoA (a protein not normally directly sensitive to Ca\textsuperscript{2+}) was achieved by genetically embedding CaM-binding peptides within the secondary structure of RhoA(Q63L) (Figure 1A; see Figure S1 available online). Genetically embedding peptides within a surface-exposed loop in the secondary structure of a protein allowed the chimera to bind a new peptide-specific partner (in this case CaM) while having minimal impact on the protein’s existing binding partners (specifically ROCK) in the absence of the peptide-specific partner. Introducing a synthetic CaM-binding site in RhoA enabled control over the strength and Ca\textsuperscript{2+} dependence of the CaM-chimera interaction, in contrast to our previous work, where the interaction was restricted to a naturally occurring CaM-binding site in RhoA (Mills et al., 2010). The surface-exposed loop in RhoA between amino acids 49 and 50 was chosen for chimera construction (Figure S1) for several reasons: first, it is sandwiched between regions of well-defined secondary structure; second, it is distal from the switch I and II regions (Hakoshima et al., 2003); and third, it is near the N terminus in 3D space, and thus an N-terminally fused CaM can bind to any CaM-binding peptides inserted there. An IQ-motif peptide (IQp) was chosen as the embedded peptide so that CaM would bind to the RhoA/IQp chimera at resting Ca\textsuperscript{2+} (IQp binds CaM Ca\textsuperscript{2+} independently with a micromolar dissociation constant; Trybus et al., 2007). This should prevent RhoA binding to effector proteins at basal Ca\textsuperscript{2+}. To force CaM dissociation from the chimera at high Ca\textsuperscript{2+}, a second peptide should be available that binds CaM in a Ca\textsuperscript{2+}-independent manner with a stronger affinity, such as from myosin light chain kinase (MLCKp, nanomolar dissociation constant; Lukas et al., 1986). Finally, CaM was retained in the fusion to minimize the disturbance to the cell’s balance of CaM and binding partners. Thus, the fusion would be CaM-MLCK-RhoA(Q63L)/IQp (Figure 1A; Figure S1). YFP was added as
CaR-Q Can Mediate Ca\textsuperscript{2+}-Sensitive Cell Morphology Changes

CaR-Q caused Ca\textsuperscript{2+}-induced morphology changes consistent with switchable RhoA activity in several mammalian cell lines including HEK293, HeLa, and CHO cells (Figure 1; Figures S1–S3; Movies S1–S3). Ca\textsuperscript{2+}-dependent activation of CaR-Q could not be measured in vitro using a rhotekin Rho-binding domain (RBD)-GST pull-down assay, likely because CaR-Q was poorly folded in Escherichia coli. Instead, CaR-Q was fused with a membrane localization tag and coexpressed with RBD-GST sandwiched around monomeric red fluorescent protein (mRFP); in live cells, RBD-mRFP-GST appeared to colocalize with membrane-localized CaR-Q after stimulation with ionomycin and Ca\textsuperscript{2+} (Figure S2). Pairs of YFP and RFP images were analyzed using the Pearson correlation (PC) coefficient before and after ionomycin stimulation. Soluble cyan fluorescent protein (CFP) was used as a spectrally distinct volume indicator (Correia et al., 2008; Robinson et al., 2002). PC correlation between RBD and CaR-Q increased by 26%, whereas the correlation change was less than 1% for the dominant-negative CaR-M design to not affect RhoA activity at basal Ca\textsuperscript{2+} when expressing membrane-localized CaR-Q after stimulation with ionomycin (Figure 2). This shows that the embedded peptide approach to synthetic protein control can create a C-terminal fusion for visualization (Rekas et al., 2002); for brevity, we will refer to the construct as CaR-Q (Ca\textsuperscript{2+}-responsive RhoA protein with IQ-motif peptide).

To assay the physiological activity of CaR-Q, we utilized the observation that overexpression of cytosplasmic RhoA(Q63L)-YFP causes dynamic nonapoptotic blebbing in certain epithelial-like cell lines such as HEK293, HeLa, and CHO cells (Figure 1; Figures S1 and S3). Cells expressing CaR-Q were no more likely to bleb than YFP-expressing control cells at basal Ca\textsuperscript{2+}, and much less likely to bleb than cells expressing RhoA(Q63L)-YFP, consistent with the design of CaR-Q to suppress RhoA activity at basal Ca\textsuperscript{2+} (Figure 1B). When cells expressing CaR-Q were stimulated with ionomycin and Ca\textsuperscript{2+}, these cells began to show dynamic blebs consistent with RhoA activation that were not present in control cells expressing YFP or CaM-YFP, a dominant-negative mutant of CaR-Q (RhoA-T19N), or when CaR-Q was incubated with Y-27632, an inhibitor of the RhoA effector Rho kinase, or the CaM inhibitor CDZ (Figures 1C–1F; Figures S1–S3; Movies S1 and S2). The blebbing was fully reversible, as cells returned to their normal morphology when the ionomycin transient expired (usually after 10–15 min) or upon adding EDTA to chelate free Ca\textsuperscript{2+} (Figures 1D–1G).

Mutations to disrupt binding between CaR-Q and GTPase-accelerating proteins (GAPs) (E93H, N94H) had little effect on either blebbing morphology at the basal level or the likelihood of blebbing induction after Ca\textsuperscript{2+} (Figures 1B and 1C), for both cytoplasmic and membrane-localized CaR-Q, in contrast to the reported results with PARac (Wu et al., 2009). However, the caging mechanism reported for PARac is substantially different from the CaM/peptide approach taken here; these results suggest that CaR-Q does not act as a “GAP sink.”

There was a dose-dependent relationship between free Ca\textsuperscript{2+} and the blebbing morphology. The cell medium was buffered at specific free Ca\textsuperscript{2+} concentrations between 0.1 and 1,000 μM, and the likelihood of blebbing morphology was observed after addition of ionomycin to cells expressing membrane-localized CaR-Q. The free concentration of Ca\textsuperscript{2+} resulting in 50% change (EC\textsubscript{50}) was 10\textsuperscript{−4.56}, or 27 μM (Figure 2). The quality of the fit is good: $r^2 = 0.977$. An EC\textsubscript{50} greater than 1 μM is reasonable, because previous studies have shown that the modulation of the CaM-binding peptide interface in protein Ca\textsuperscript{2+} biosensors can tune the Ca\textsuperscript{2+} sensitivity between 0.6 and 160 μM (Palmer et al., 2006). In our analysis, we forced the slope of the sigmoid to be 1 (i.e., it did not allow for cooperativity between Ca\textsuperscript{2+} binding and morphology change). With this restraint lifted, the calculated EC\textsubscript{50} is only slightly different at 25 μM.

The embedded peptide design is robust: replacing the embedded peptide with one that binds CaM in a Ca\textsuperscript{2+}-dependent manner can reverse the direction of the Ca\textsuperscript{2+}-induced RhoA switch (Figures 1D and 1G). To test the flexibility of our embedded peptide design, we replaced the embedded IQ-motif peptide with MLCKp and removed the N-terminally fused MLCKp to create a CaM-RhoA(Q63L)/MLCKp chimera, termed CaR-M. When CaR-M was expressed in HEK293 cells, the percentage of blebbing cells was not significantly different from cells expressing RhoA(Q63L)-YFP (49.4% ± 18.3% and 47.2% ± 15.0%, respectively). This is consistent with the CaR-M design to not affect RhoA activity at basal Ca\textsuperscript{2+} when there is little interaction between CaM and MLCKp. When cells expressing CaR-M were stimulated with ionomycin, blebbing slowed and retracted in some cells, consistent with a loss of RhoA activity (Figure 1G; Movie S3). This shows that the embedded peptide approach to synthetic protein control can create a switch in any direction (i.e., on-off or off-on), depending on the characteristic interaction between CaM and the embedded peptide.
Spatially Localized Ca$^{2+}$ Signals Can Regulate Cell Morphology via CaR-Q

CaR-Q is sensitive to local Ca$^{2+}$ signals and can be differentially activated by different spatial patterns of Ca$^{2+}$ signaling (Figures 3 and 4; Figure S3; Movies S4–S6). To determine whether CaR-Q could respond to short Ca$^{2+}$ transients, in addition to long periods of high Ca$^{2+}$ as with ionomycin, we stimulated HEK293 cells expressing CaR-Q with the exogenous stimulus acetylcholine (ACh). ACh signaling was mediated using the $\alpha_4$ subunit of nAChR to create a plasma membrane Ca$^{2+}$ channel (Nashmi et al., 2003). This experiment was also designed to test whether CaR-Q could operate as a robust Ca$^{2+}$-signaling module, that is, to determine whether different upstream Ca$^{2+}$-generating elements could activate CaR-Q. Cells cotransfected with nAChR-$\alpha_4$ and CaR-Q did not bleb in response to ACh stimulation (Figure 3A; Movie S4). We suspected that CaR-Q may be more likely to respond to ACh on the plasma membrane given that the Ca$^{2+}$ influx originated there, and that RhoA is localized to the plasma membrane during activation in vivo (Pertz et al., 2006). CaR-Q was fused to a membrane tag derived from Lyn kinase (see Supplemental Experimental Procedures for peptide sequence) to create pLyn-CaR-Q (Figure 3B), and this construct reliably induced blebbing in response to ACh (Figures 3C and 3D; Movie S5). As with ionomycin, blebbing did not occur with a dominant-negative mutant of pLyn-CaR-Q (RhoA T19N), or in the presence of Y-27632 or CDZ (Figure 3C; Movie S6). Further, pLyn-CaR-Q induced blebbing when HEK293 cells were stimulated with ATP (Ca$^{2+}$ generated by the IP$_3$ pathway) but CaR-Q did not (Figure 3E). To determine the temporal relationship between the Ca$^{2+}$ signal and appearance of blebbing, cells were cotransfected with pLyn-CaR-Q, nAChR-$\alpha_4$, and a FRET-based Ca$^{2+}$ sensor, TNXL (Mank et al., 2006). These experiments showed that ACh induced a Ca$^{2+}$ transient with a 10–20 s peak, followed by a 2–3 min delay before the onset of morphology change and then 5–15 min of blebbing morphology (Figure 3F). This shows that a short Ca$^{2+}$ event can cause a relatively prolonged physiological change. Taken together, this set of results demonstrates that two essential features of Ca$^{2+}$ signaling are functioning in the case of CaR-Q: first, that CaR-Q and its localized derivatives can respond to Ca$^{2+}$ generated from several upstream modules, and second, that CaR-Q is sensitive to changes in local or global Ca$^{2+}$, depending on its subcellular localization.

CaR-Q, when localized to the ER, can induce morphology change in response to SOCE (Figure 4; Movies S7 and S8).
SOCE is a homeostatic mechanism employed by cells when the intracellular Ca\(^{2+}\) stores (e.g., in the ER lumen) are depleted (Zhou et al., 2010a, 2010b). Two proteins involved in SOCE are Stim1 and Orai1, which act as a Ca\(^{2+}\) sensor (Zhou et al., 2010a) and Ca\(^{2+}\) channel, respectively (Zhou et al., 2010b). Given that SOCE is an important physiological pathway of Ca\(^{2+}\) entry, we tested whether CaR-Q could respond to SOCE induced by thapsigargin (Tg). Cells coexpressing Orai1 and CaR-Q in Ca\(^{2+}\)-free medium were stimulated with Tg and Ca\(^{2+}\), but blebbing was not observed (Figure 4A). Given our insights into the spatially sensitive nature of CaR-Q response to Ca\(^{2+}\), CaR-Q was localized to the cytoplasmic-facing membrane of the ER using Stim1 or Stim1-CaR-Q (Figure 4B). Treatment with Tg alone induced punctum formation, consistent with Stim1 and Orai1 oligomerization (Zhou et al., 2010a), but did not induce blebbing until addition of Ca\(^{2+}\) to the extracellular medium (Figures 4C and 4D; Movie S7). Blebbing did not occur in the case of a dominant-negative Stim1-CaR-Q (RhoA T19N) or when we used an alternate plasma membrane Ca\(^{2+}\) channel, ChR2 (Petreanu et al., 2007), as the source of Ca\(^{2+}\) influx for Stim1-CaR-Q (Figure 4A; Movie S8). As with the nAChR-\(\alpha4\) experiments, the induced SOCE experiments were repeated with a cotransfection of Stim1-CaR-Q, Orai1, and TNXL to correlate Ca\(^{2+}\) signal with morphological change. In this case, blebbing only occurred after the SOCE Ca\(^{2+}\) transient, with a typical 2–3 min duration, which was comparable to the duration of the SOCE Ca\(^{2+}\) transient (Figure 4E). This result was in contrast to the above results where a shorter transient resulted in a longer morphological change and is likely a property of the kinase cascade involved in regulating blebbing downstream of RhoA (Fackler and Grosse, 2008). Taken together, these results further demonstrate that CaR-Q can be activated by a variety of Ca\(^{2+}\)-generating modules, and can be differentially regulated based on the spatial pattern of Ca\(^{2+}\) signaling. The experiments with ChR2 suggest that not only is Stim1-CaR-Q sensitive to plasma membrane Ca\(^{2+}\) but even more specifically is sensitive to a particular protein complex that is generating the Ca\(^{2+}\) influx.

### Buffers Proteins Alter the Temporal Relationship between Ca\(^{2+}\) Signal and Morphology Change

The duration of blebbing morphology induced by the nAChR-\(\alpha4\) Ca\(^{2+}\) transient can be decreased using the cytoplasmic...
Figure 5. Effect of PAV on Blebbing Duration

(A) Representative ACh-induced Ca²⁺ transient in HEK293 cells expressing nAChR-α4 and the TNXL biosensor. Typically, the peak duration was 10–20 s and the width of the shoulder (from end of peak to half maximum) was 30–40 s.

(B) Representative transient in HEK293 cells expressing nAChR-α4, PAV-mRFP, and the TNXL biosensor. Typically, the peak duration was 20–30 s and the width of the shoulder was 1–2 min.

(C) Average duration of blebbing (time elapsed from onset of first bleb to retraction of all blebs) after ACh stimulation in cells expressing pLyn-CaR-Q, nAChR-α4, and PAV, where indicated. The data are presented as the mean ± standard deviation (n = 12 cells; *p = 0.033 by Student’s t test).

Ca²⁺-buffering protein PAV (Schwaller et al., 2002) (Figure 5). We have observed that Ca²⁺ transients caused by SOCE or ionomycin that last several minutes result in shorter blebbing duration than Ca²⁺ transients that last several seconds, as those caused by ACh or ATP. We sought to determine whether the characteristic output (i.e., blebbing morphology) duration of a given input could be changed by tuning the duration of the intermediate Ca²⁺ signal, either by shortening the SOCE transient or lengthening the nAChR-α4 transient. When PAV (fused to mRFP or PAV-mRFP) was coexpressed with nAChR-α4, the characteristic Ca²⁺ peak after ACh stimulation tended to be elongated by several seconds with an increased shoulder peak duration (Figures 5A and 5B), which is consistent with the role of PAV in buffering Ca²⁺ release and modulating the duration of Ca²⁺ transients (Schwaller et al., 2002). When PAV, nAChR-α4, and pLyn-CaR-Q were coexpressed, the average duration of blebbing after ACh stimulation was significantly decreased from 12.2 ± 5.0 min without PAV to 8.3 ± 3.4 min (n = 12 cells, p = 0.033; Figure 5C). These results indicate that the Ca²⁺-responsive system presented here is sensitive to the duration of Ca²⁺ signal and that by tuning the duration of that signal, the average duration of the morphology output can be tuned. However, it should be noted that there may be some effect of PAV on spatial buffering of the nAChR-α4 Ca²⁺ signal that was not detected in our measurements.

CaR-Q-Regulated Morphology Changes Enhance Cell Migration

Blebbing morphology and cell migration can be controlled by light when a light-sensing module is present to generate a Ca²⁺ signal to regulate CaR-Q (Figure 6). Photoactivatable molecules are valuable research tools because they can be regulated with spatiotemporal precision, and photoactivatable proteins have been developed recently to control cell morphology and migration (Levskaya et al., 2009; Wu et al., 2009; Yeh et al., 2007) and DNA binding (Strickland et al., 2008). Several proteins exist that generate Ca²⁺ signals based on particular wavelengths of light (e.g., ChR2 with blue light [Petrerau et al., 2007] or VChR1 with green light [Zhang et al., 2008]) and may be suitable Ca²⁺-generating modules to activate CaR-Q. Indeed, cells coexpressing ChR2 and pLyn-CaR-Q developed a blebbing morphology when exposed to flashing blue light (438/24 nm from a xenon lamp, 10 s period, 3% duty cycle) (Figure 6A). Given the role of actinomycin contractility and blebbing in ameboid-like cell migration (Fackler and Grosse, 2008; Yang et al., 2006), we hypothesized that long-term activation of pLyn-CaR-Q would enable light-activated cell migration. Cells coexpressing pLyn-CaR-Q and ChR2 showed significant migration over 24 hr in a wound closure assay (Figures 6B–6K). The migration was significantly less when the ChR2 module was not present, when cells were incubated with Y-27632, or when the T19N mutant of pLyn-CaR-Q was used (p < 0.001, n = 9 wounds). Cells migrating into the wound tended to move as clusters, often with only one or two transfected cells per cluster of five to ten cells. This suggests that ameboid-like migration can direct cell migration in groups, which is supported by recent studies showing that photoactivation of Rac1 in a single cell can affect the direction of a cluster of adhered cells (Wang et al., 2010). This experiment demonstrates that light-activated cell migration can be achieved with an engineered Ca²⁺-signaling network by combining a light-sensitive module, ChR2, with a module that regulates a morphology known to be involved in migration, CaR-Q.

DISCUSSION

In this study, we have demonstrated the versatility and potential utility of engineered biomolecular systems based on Ca²⁺ signaling. In nature, Ca²⁺ signaling can simultaneously regulate many diverse cellular systems; cells are able to interpret the complexity of Ca²⁺ signaling because such signals are well defined in time (e.g., frequency and waveform; Berridge, 1998; De Koninck and Schulman, 1998) and space (through microdomains and buffering proteins; Schwaller et al., 2002; Wei et al., 2009), and because Ca²⁺-generating and Ca²⁺-responsive protein modules interact in different ways based on cell type or localization (Berridge et al., 2000). We showed that the CaR-Q protein could be activated by a variety of Ca²⁺-generating modules including nAChR-α4, purinergic receptors, and the SOCE gate formed by Orai1 and Stim1. However, in each of these cases, CaR-Q was only functional when localized to the site of Ca²⁺ entry (PM or ER, respectively), showing that CaR-Q responds primarily to local Ca²⁺ signals. This is advantageous...
for an engineered system because it means that Ca\textsuperscript{2+} signaling need only be perturbed in part of the cell to activate CaR-Q. Engineered proteins such as CaR-Q that respond to local Ca\textsuperscript{2+} signals may be useful tools to investigate the effect of local and global Ca\textsuperscript{2+} signaling in systems such as the NSCaTE element in Ca\textsuperscript{V} channels (Dick et al., 2008) or calpains, which are activated at Ca\textsuperscript{2+} concentrations not observed globally in the cytoplasm (Perrin and Huttenlocher, 2002). The ChR2 Ca\textsuperscript{2+}-generating module was combined with pLyn-CaR-Q to enable photoactivation of the blebbing morphology, without making any changes to the CaR-Q module itself. We showed that long-term activation of the blebbing morphology increased cell motility and migration through a wound closure assay. At first pass, this appears to be in contrast to the generally accepted notion that overexpression of dominant-positive Rho proteins inhibits cell migration (Charras and Paluch, 2008). Even though there was no directed activation of CaR-Q, cells tended to migrate into the open space of the wound because they could not migrate into the monolayer. This system may be further improved in the future to enable specific, direction-oriented cell migration either with a point-source light or perhaps by using a biochemical gradient with CaR-Q and a module that can translate a particular biochemical signal into a Ca\textsuperscript{2+} signal (e.g., EGF binding to EGFR; Tinhofer et al., 1996). Finally, the particular strategy employed here to enable allosteric, Ca\textsuperscript{2+}-dependent control over RhoA may be a generalizable approach for engineering protein-level systems. Whether the embedded peptide approach can be expanded beyond the Ras superfamily will depend on the careful selection of binding partners and structural analysis.

Figure 6. Light-Activated Cell Migration Using pLyn-CaR-Q and ChR2
(A) The percent of morphologically normal HEK293 cells that developed a blebbing morphology during flashing blue light illumination, under the indicated transfection and inhibitor conditions (n = 3 experiments each, >10 observed cells). Cells were cotransfected with ChR2-mCherry. Data are the mean and error bars are the standard deviation.
(B) Wound width immediately after wound (dark bars) and after 24 hr (light bars). (1) RhoA(DP)-YFP not illuminated, (2) pLyn-CaR-Q + ChR2 illuminated, (3) pLyn-CaR-Q(T19N) + ChR2 illuminated, (4) pLyn-CaR-Q + ChR2 + Y-27632 illuminated, (5) pLyn-CaR-Q illuminated, (6) pLyn-CaR-Q + ChR2 not illuminated.
(C) Data from (B) displayed as percent wound closure (initial width minus width at 24 hr divided by initial width) under the same conditions as in (B). The differences between condition 2 and conditions 3–6 were significant (p < 0.001). For (B) and (C), n = 9 experiments.
(D–K) Representative images from wound closure assays immediately after scraping (D–G) and 24 hr after scraping (H–K). HEK293 cells were expressing RhoA(DP)-YFP (D and H) or coexpressing ChR2 with pLyn-CaR-Q(T19N) (E and I) or pLyn-CaR-Q (F, G, J, and K). Bright-field and fluorescence images are presented for the same cells in the latter case (F and G at 0 hr, J and K at 24 hr). Scale bars represent 100 \mu m.
SIGNIFICANCE

We have developed a synthetic biosystem to control cell morphology and migration using Ca\(^{2+}\) signaling. Compelling reasons to use Ca\(^{2+}\) signaling in synthetic biosystems engineering are 3-fold. First, engineering a synthetic binding site in RhO and changing the calmodulin-binding peptide (i.e., MLCK or IQ) enable the direction of the blebbing to be reversed from off-on to on-off. This shows that once a suitable site is identified in potentially any protein, we can make it turn on or off by changing the CaM-binding peptide. This shows the protein design versatility possible with Ca\(^{2+}\) signaling. Second, an emerging theme in Ca\(^{2+}\) signaling to explain how a ubiquitous Ca\(^{2+}\) signal can regulate so many diverse cellular events is the spatial localization of the Ca\(^{2+}\) signal. Using this knowledge, we created a system that can distinguish between local and global Ca\(^{2+}\) signals by targeting the RhO chimera to the plasma membrane or endoplasmic reticulum so that it is only activated by plasma membrane Ca\(^{2+}\) fluxes or store-operated Ca\(^{2+}\) entry, respectively. This shows Ca\(^{2+}\)-signaling specificity can be engineered. Third, Ca\(^{2+}\)-mediated systems can exploit the inherent modularity of Ca\(^{2+}\) signaling to create multimodal systems where light and exogenous chemicals generated Ca\(^{2+}\) signals, which in turn activated the RhO chimera. Three distinct modules were used to induce morphology changes, but many other modules are possible candidates for future applications including red-shifted opsins and appropriate cytokine receptors. This shows that pathway rewiring can be achieved by Ca\(^{2+}\) signaling. Engineering synthetic biological systems with input versatility and tunable spatiotemporal responses are key motivations for further applications of Ca\(^{2+}\) signaling in this field.

EXPERIMENTAL PROCEDURES

Plasmid Construction

CaR-Q, its derivative constructs, and other fusions were created by cloning with the pCPTvx3 vector as described previously (Truong et al., 2003). Primers used to amplify the 5′ and 3′ fragments of RhO in CaR-Q are given in Supplemental Experimental Procedures. Plasmids encoding RhO(A63L), RBD, Chr2, Oral1, Stim1, and nAChR-α4 are Addgene plasmids 12968, 12602, 20938, 19756, 19754, and 15245, respectively. The dominant-negative mutant CaR-Q, RhO (T19N), was created using the self-hybridizing overlap PCR method. The amino acid sequences of peptides used in this study are given in Supplemental Experimental Procedures.

Cell Culture and Transfection

COS7, HeLa, and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 25 mM D-glucose, 1 mM sodium pyruvate, and 4 mM L-glutamine (Invitrogen) in a T-25 flask. Cells were passaged at 95% confluency using 0.05% trypsin with EDTA (Sigma-Aldrich) and seeded onto glass-bottom dishes at 1:15 dilution (Mattek). Cells were transiently transfected using Lipofectamine 2000 according to the manufacturer’s directions (Invitrogen).

Reagents

Cells treated with Y-27632 (5 μM) and CDZ (50 μM) were preincubated with inhibitor in PBS with CaCl\(_2\) for 1 hr prior to imaging. ATP (10 μM), ACh (1 mM), ionomycin (1 μM), and Tg (2 μM; BioShop) were added by diluting 1:10 in imaging medium (PBS with CaCl\(_2\), except where noted otherwise) after a 5 min control period. Rhodamine-phalloidin (Invitrogen) was used according to the manufacturer’s protocol. The Ca\(^{2+}\)-buffering kit was used according to the manufacturer’s protocols (Biotium). All reagents, except where noted, were from Sigma.

Illumination, Imaging, and Wound Assay

Imaging was performed using an inverted IX81 microscope with a Lambda DG4 xenon lamp source and QuantEM 512SC CCD camera with a 20×, 40×, and 100× oil immersion or 10× objectives (Olympus). Filter excitation (ex) and emission (em) band-pass specifications were as follows: for CFP, exc: 438/24 nm; em: 482/52 nm; for YFP, exc: 500/24 nm; em: 542/27 nm; for RFP, exc: 580/20 nm; em: 630/60 nm (Semrock). For ChR2, excitation was done using the CFP excitation filter normally with a 300 ms flash every 10 s. FRET emissions were recorded by simultaneously imaging the CFP and YFP channels using a DualView SpecEM system (Olympus). Image acquisition was done with MetaMorph Advanced (Olympus).

Most imaging experiments were conducted in PBS with CaCl\(_2\), except SOCE experiments, where CaCl\(_2\) was added to Ca\(^{2+}\)-free PBS after Tg, and ChR2 experiments, where complete growth medium was used. Wound assays were conducted by growing transfected HEK293 cells to confluence, scraping the dish with a 1 μl pipette tip, washing three times in PBS, and incubating in complete growth medium overnight. Overnight illumination was provided using an iPod programmed to flash blue light for 1 s every 15 s. The power output of the iPod display was measured at 0.80 mW/cm\(^2\) for white light and 0.12 mW/cm\(^2\) for blue light (450 nm) using a Konica-Minolta CS-200 spectroradiometer (DisplayMate) in a dark room with the iPod running on AC power. The power output of the xenon lamp at the microscope stage is 25 mW/cm\(^2\). For TNXL biosensor experiments, 1 μM ionomycin was added at the end of experiments to establish R\(_{\text{max}}\) and then 2 mM EDTA was added to establish R\(_{\text{min}}\).

Data Analysis

A cell was counted as blebbing if at least one circular bleb appeared at any point within 20 min of stimulus onset, unless it was also blebbing before stimulus, in which case the cell was not considered. Data regarding the frequency of blebbing after a stimulus are the mean of at least three independent experiments over at least ten cells. Data regarding the frequency of cell blebbing before stimulus are the mean of at least ten independent experiments over at least 100 cells. Data are presented as the mean ± standard deviation. Significance, where discussed, was determined using an independent Student’s t test and p < 0.05 was considered significant.

PC values for image correlation between pLyn-CaR-Q and RBDR-mRFP-GST were calculated using MetaMorph. Images were cropped to remove nonco-transfected cells, and the background was subtracted using a 32 x 32 pixel square median filter. Cropped, background-subtracted images were used for PC calculation without any further processing.

Curve fitting for EC\(_{50}\) estimation was done using MS Excel by minimizing the sum of squares between the generic sigmoid of the form shown in Figure 2 and the data collected in Figure 2. The squared Pearson correlation coefficient (r\(^2\)) was calculated by comparing the values of the data and the best fit.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and eight movies and can be found with this article online at doi:10.1016/j.chembiol.2011.09.014.

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