Cell Calcium 48 (2010) 195–201

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Structure based design of a Ca^{2+}-sensitive RhoA protein that controls cell morphology

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ARTICLE INFO

Article history:
Received 16 August 2010
Accepted 31 August 2010

Keywords:
Calcium signaling
RhoA signaling
Synthetic biology

ABSTRACT

The Rho proteins are important regulators of cell morphology, and the prototypical protein RhoA is known to regulate contraction, blebbing and bleb retraction. We have identified and experimentally confirmed that RhoA has a binding site for calmodulin, a ubiquitous transducer of the Ca^{2+} second messenger. Using structural modeling, a fusion protein was designed wherein RhoA activity was controlled by Ca^{2+} via calmodulin. Living cells transfected with this synthetic protein underwent Ca^{2+} sensitive and calmodulin-dependent bleb retraction within minutes. Further, the modularity of Ca^{2+} signaling was exploited to induce bleb retraction in response to blue light (using channelrhodopsin-2) or exogenous chemicals (with acetylcholine receptor), showing input signal versatility. The widespread use of Ca^{2+} signaling in nature suggests that fully exploring its signaling potential may allow powerful applications to other synthetic biological systems.

1. Introduction

The Rho GTPases RhoA, Rac1 and Cdc42 are small protein switches that have been intensely studied because of their role in regulating cell morphology and motility [1–6]. RhoA is known to regulate actin–myosin contractility, blebbing and bleb retraction via the Rho kinase (ROCK-1) pathway [7–9], and is also implicated in apoptosis and proliferation [10]. The design of synthetic control pathways for other Rho-family proteins such as Rac1 [3,5] and Cdc42 [2,4] has resulted in systems where those proteins can be directly controlled using a stimulus that can be applied with varying degrees of spatial or temporal control. However a similar control strategy for RhoA has not been achieved. We have identified a previously unreported binding site in the carboxy-terminal region of RhoA (amino acids 168–190) for calmodulin (CaM), a ubiquitous transducer of the Ca^{2+} second messenger [11,12]. The newly discovered site was used to design a RhoA fusion protein that can be deactivated on Ca^{2+} influx into the cytoplasm by calmodulin binding to and destabilizing RhoA, resulting in bleb retraction in a variety of cell lines. Ca^{2+} is widely used by cells for signal transduction [11–14] and we have exploited the existence of Ca^{2+}-modulating proteins such as channelrhodopsin-2 (ChR2) [15] and acetylcholine receptor (nAChR) [16] to create light- and chemical-sensitive morphology changes that use Ca^{2+} as an intermediate messenger. By leveraging on the newly discovered interaction site between calmodulin and RhoA, we have shown that the RhoA morphology of blebbing can be brought under the control of Ca^{2+}.

2. Results

2.1. Identification of a calmodulin binding site in RhoA

Bioinformatic analysis [11] identified a putative CaM binding site in residues 168–190 of RhoA (Supplementary Fig. S1(A–C)), which corresponds to the carboxy-terminal α-helix and polybasic tail but does not include the prenylation motif sequence. Structural examination of RhoA (PDB ID: 1A2B) showed that this region was surface exposed and thus, may support CaM binding. A CaM–sepharose pull-down assay was used with subsequent fluorescent SDS-PAGE to verify that RhoA could bind CaM (Fig. 1(A and B)). RhoA bound to CaM in a Ca^{2+}-dependent fashion and wild type as well as dominant negative (Thr19Asn: 'DN') and dominant positive (Gln63Leu: 'DP') mutants bound to CaM. To verify that the identified sequence was sufficient for CaM binding the carboxy-terminal peptide was synthesized and confirmed to be sufficient to pull down a fluorescent protein, Venus [17] (Fig. 1(B) and Supplementary Fig. S1(D)).

The interaction between RhoA and CaM lends itself to the development of a control system where Ca^{2+} can be used to manipulate RhoA, which unlike the related GTPases Rac1 and Cdc42,

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doi:10.1016/j.ceca.2010.08.009

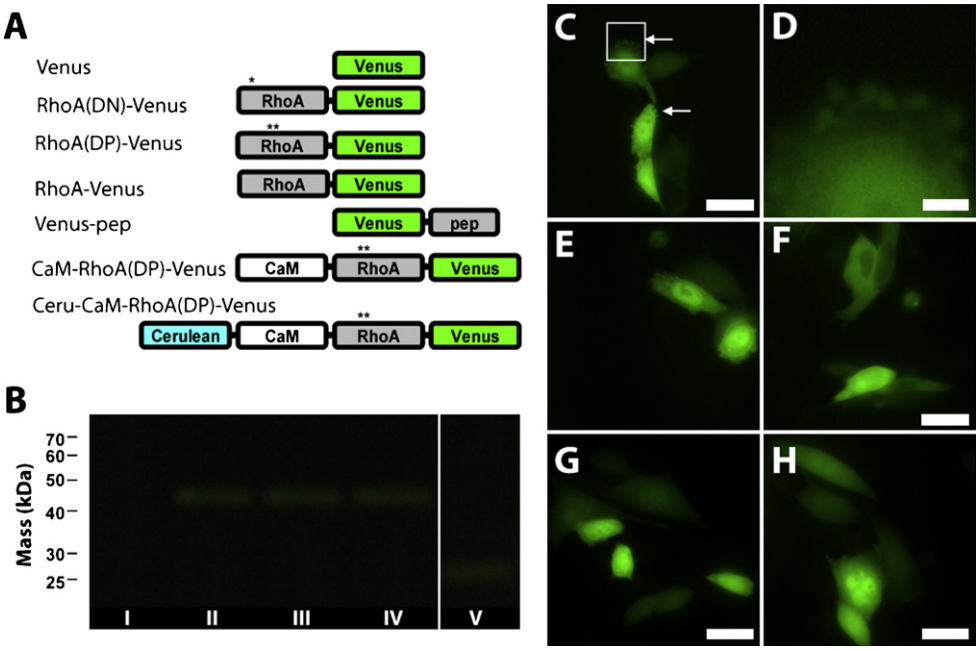

Fig. 1. RhoA has a CaM binding site. (A) Schematic layout of selected protein constructs used in this study. *Denotes Thr19Asn mutation; **denotes Gln63Leu mutation. (B) Fluorescent SDS-PAGE after CaM–sepharose pulldown of *E. coli* lysate containing: I, Venus; II, RhoA(DN)–Venus; III, RhoA(DP)–Venus; IV, RhoA–Venus; V, Venus-pep(167–181). (C–H) Images of CHO cells transfected with RhoA(DP)–Venus (C, white box magnified in D), RhoA–Venus (E), RhoA(DN)–Venus (F), Venus (G), and CaM–Venus (H). The white arrows in (C and D) show plasma membrane blebs associated with RhoA(DP)–Venus. Scale bars are 30 μm in (C, E–H) and 5 μm in (D). RhoA: truncated variant (4–181), pep: carboxy-terminal peptide from truncated RhoA (167–181).

has not been controlled to date. RhoA(DP) overexpression in a set of epithelial-like cell lines (CHO, HeLa and HEK293), caused distinctive non-apoptotic blebbing [18,19] (Fig. 1(C and D) and Supplementary Fig. S2) which is absent when RhoA cannot interact strongly with its downstream effectors (e.g. ROCK-1), as when RhoA(WT) or RhoA(DN) were overexpressed (Fig. 1(E and F) and Supplementary Fig. S2). RhoA overexpression caused blebbing in this context and not stress fibre formation because the lack of lipid modification likely altered its localization and morphological effect. Cells transfected with Venus or CaM–Venus did not bleb, which shows that blebbing is not spontaneous in these cells, nor is it a result of CaM overexpression (Fig. 1(G and H)). A switch of RhoA activity may be created in these cell lines because a morphology change could be easily observed after Ca^{2+} stimulation. Ca^{2+} is a good candidate for this type of study because it can be delivered easily by endogenous pathways or ionophores [20,21] and can be measured accurately with a variety of protein-based biosensors [22–24]. A potential drawback of using Ca^{2+} in this application is the fact that Ca^{2+} is already used by cells to regulate many processes [13]. However, this also means that there is a large biological toolbox available to manipulate intracellular Ca^{2+} by a variety of stimuli including by light with channelrhodopsin-2 [15] and chemically by nicotinic acetylcholine receptor [16]. In this way, a Ca^{2+}-sensitive RhoA protein could be coupled to many different stimuli whereas previous studies [2–5] have been limited to a single input signal.

2.2. Structure-based design of a Ca^{2+}-sensitive RhoA

Structural analysis revealed that a linear, tandem fusion, CaM–RhoA(DP)–Venus, would likely allow CaM sufficient flexibility to bind the carboxy-terminal binding site of RhoA (Fig. 2). The proximity of RhoA and CaM in this fusion protein results in a high local concentration, and allows binding that may be less favourable when both proteins are free in solution. The N- and C-termini of the truncated RhoA are in proximity (approximately 16 Å), and a small linker could provide sufficient flexibility for CaM to bind the site. When Ca^{2+}-loaded, CaM exposes a hydrophobic pocket that can be filled with a target binding peptide, depending on the binding mode [11,12]. Visual inspection of the binding site sequence suggests that the binding motif is most likely 1–10 with Val170 and Leu179, and possibly Ala174, forming the hydrophobic anchors, in a similar manner as Ca^{2+}/CaM-dependent kinase II [11,12,25] (Fig. 2(A and B)). If this were the case, then CaM binding would either require the rearrangement of the three nearby β-strands (residues 5–12 and 39–60) to accommodate the amino- and carboxy-terminal lobes of CaM forming a hydrophobic binding pocket around the site, or the binding site itself may be "pulled" away from its current location to enter CaM's hydrophobic channel, as modeled here (Fig. 2(D)) [26]. In order for the CaM–RhoA interaction to occur as modeled there must be a significant hydrophobic-driven rearrangement of RhoA to account for the repositioning of the helix. Given the large conformational change in RhoA in our model, we believe that the CaM binding destabilizes RhoA and renders it unable to interact with its effector proteins, such as ROCK-1. A fluorescence resonance energy transfer (FRET)-based probe, using the FRET donor Cerulean [27] was designed to determine if Ca^{2+} has any effect on the conformation of the fusion protein. A measurable change in FRET efficiency ($p = 0.002$, calculation described in Section 5) occurred when the Cerulean–CaM–RhoA(DP)–Venus fusion was loaded with Ca^{2+}, which indicates that a conformational change occurred in the protein (Fig. 2(C)).

Taken together the FRET data suggested that the fusion allows CaM to efficiently bind RhoA in the presence of Ca^{2+}, but this is not direct evidence that CaM binding affects RhoA interaction with effector proteins. *In vitro* analyses of RhoA-effector binding were not feasible here because of the difficulty of creating a RhoA pulldown system using protein components purified from *Escherichia coli*. Instead, RhoA binding with effectors can be tested after expression in metazoan cells by observing the effect of Ca^{2+} on specific morphologies associated with RhoA, in this case, blebbing. The effect of Ca^{2+} and CaM on blebbing independent of RhoA can be determined using careful controls, as described below.

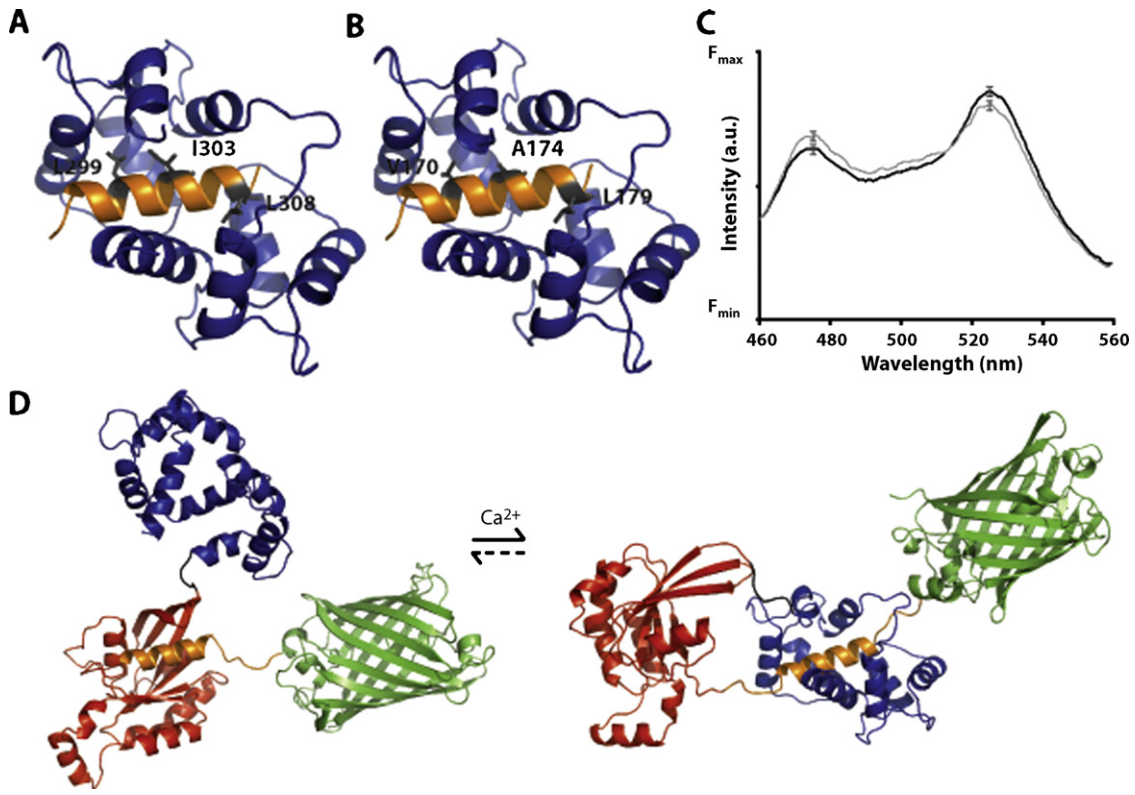

Fig. 2. Structural modeling of CaM–RhoA interaction. (A) CaM is shown binding to the Ca^{2+}/CaM-dependent kinase II peptide (PDB: 1CM1) with the hydrophobic anchors highlighted in grey. (B) The suspected CaM-binding peptide from RhoA is shown with the likely hydrophobic anchors highlighted in grey, based on the peptide in the 1CM1 structure. (C) The fluorescence spectra of Cerulean–CaM–RhoA(DP)–Venus excited at 440 nm in the absence (grey) and presence (black) of Ca^{2+} shows an increase in FRET efficiency upon Ca^{2+} binding. These curves are the means of three independent experiments, error bars for selected points are the standard deviation. (D) Model of the CaM–RhoA(DP)–Venus fusion in the absence of Ca^{2+} (left) and the presence of Ca^{2+}, showing CaM bound to the carboxy-terminal helix of RhoA (right), using FPMOD [26]. CaM: blue, RhoA: red, CaM-binding peptides: orange, Venus: green, flexible CaM–RhoA linker: black.

2.3. Bleb-retraction by CaM–RhoA is Ca^{2+}- and CaM-dependent

When cells transfected with CaM–RhoA(DP)–Venus undergoing non-apoptotic blebbing were stimulated with chemicals that elevate intracellular Ca^{2+}, efficient bleb retraction was observed in all three cell lines studied (CHO, HeLa and HEK293) (Fig. 3). The blebbing that occurred after transfection of CaM–RhoA(DP)–Venus was similar to blebbing induced after transfection with RhoA(DP)–Venus (Supplementary Fig. S2). CHO, HeLa and HEK293 cells were stimulated to induce Ca^{2+}-transients (Supplementary Fig. S3) to observe if this could control the bleb morphology of transfected cells.

Transfected CHO cells stimulated with ATP exhibited dynamic blebbing for approximately 10 min after ATP stimulation, and then began rapid bleb retraction over 3–5 min until the blebs had fully retracted and were no longer noticeable (Fig. 3(A, D–G) and Supplementary Movie 1, Figs. S4 and S7). Bleb retraction was quantified by counting the number of blebs and calculating the percent change in the number of blebs between 0 and 20 min after ATP stimulation (see Section 5 for more detail). Stimulation with ATP or the Ca^{2+}-ionophore ionomycin resulted in significantly more bleb retraction than the case with no stimulation ($p < 0.001$ in each case). Stimulation with ATP also resulted in significantly more retraction than when cells were pretreated with Y-27632 ($p = 0.001$) or the calmodulin inhibitor CDZ ($p < 0.001$). There was no significant difference between stimulating with ATP or ionomycin, which is likely because the ATP transient in CHO cells can be very long (Supplementary Fig. S3). ATP stimulation did not cause bleb retraction in CHO cells transfected with RhoA(DP)–Venus compared to the fusion with CaM ($p < 0.001$). Further, when cells were transfected with Venus, CaM–Venus or CaM–RhoA(DN)–Venus, there was no blebbing at any point before, during or after stimulation with ATP or ionomycin. As a further test, we designed a fusion protein where CaM was given a close, high-affinity binding site that could compete with the binding site in RhoA: CaM–MLCKp–RhoA(DP)–Venus where MLCKp is the CaM-binding peptide from myosin light chain kinase [11]. When blebbing cells transfected with this construct were stimulated with ATP, the bleb retraction was efficiently prevented ($p < 0.001$). This is further evidence that CaM binding to RhoA is responsible for the observed morphology effects.

Results were similar with UTP stimulation in HeLa cells transfected with CaM–RhoA(DP)–Venus (Fig. 3(B, H–K) and Supplementary Movie 2, Figs. S5, S7): UTP and ionomycin stimulation resulted in significantly more bleb retraction than in unstimulated cells ($p < 0.001$ and $p = 0.003$, respectively), Y-27632 and CDZ pretreatment reduced bleb retraction ($p < 0.001$ for both) and there was no bleb retraction after UTP stimulation of RhoA(DP)–Venus transfected cells ($p = 0.001$). The dominant negative constructed did not cause any blebbing. As well, when cells transfected with the MLCKp-containing construct discussed above were stimulated with UTP, there was significantly less bleb retraction ($p < 0.001$). Some transfected HeLa cells under UTP stimulation showed total bleb retraction while others showed partial retraction and then resumed blebbing 15–20 min after stimulation, and a few cells showed no retraction at all. However, the ionomycin-induced bleb retraction occurred more reliably and completely than the UTP-induced bleb retraction and occurred in all HeLa cells observed. We suspected that the different responses to these chemicals was due to the inherent variability in UTP-induced

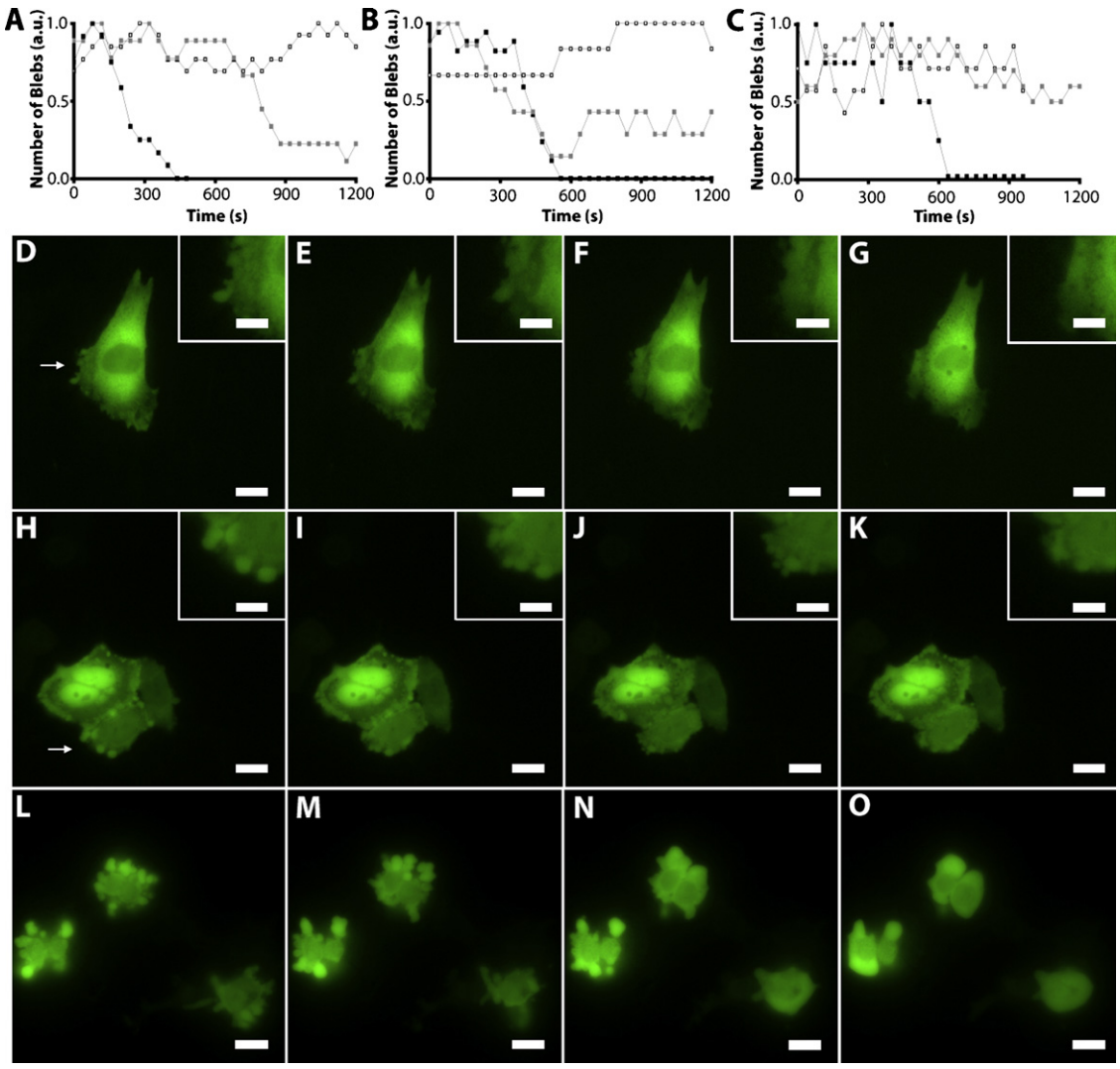

Fig. 3. Ca^{2+}-induced bleb retraction in CHO, HeLa and HEK293 cells. (A–C) The number of blebs is shown as a function of time for CHO, HeLa and HEK293 cells, respectively, transfected with CaM–RhoA(DP)–Venus after no stimulation (open squares), ATP stimulation (UTP for HeLa) (grey squares) or ionomycin stimulation (black squares). While these data are not continuous, a line has been drawn between data points to help the reader follow the trend. (D–G) Time course of bleb retraction in CHO cells showing images immediately before stimulation, at the start of the morphology change, when the morphology change was almost complete, and then 20 min after stimulation, respectively. The white arrow indicates the area enlarged in the inset. (H–K) A similar time course of images for HeLa cells, and (L–O) HEK293 cells. Scale bars are 30 μm (D–O) and 6 μm in the insets (D–K).

Ca^{2+}-transients in HeLa cells, some of which tend to be short or oscillatory on the order of several minutes, and only occur in ~70% of cells [28]. This is also what likely led to greater variability in the extent and timing of bleb retraction in these cells.

HEK293 cells showed highly dynamic, large blebs that appeared and disappeared randomly on the order of seconds. This dynamic morphology change has been observed in the context of ROCK-1 [29], but it has not been reported specifically in the context of RhoA (Supplementary Movie 3). When these cells were stimulated with ATP, the blebbing continued without any apparent change. However, there was rapid near total bleb retraction with ionomycin stimulation (Fig. 3(C, L–O) and Supplementary Movie 3, Figs. S6, S7). We believe that ATP was not sufficient to cause bleb retraction in HEK293 cells because the ATP-induced Ca^{2+} transient in these cells is very short (Supplementary Fig. S3). The ionomycin-induced bleb retraction occurred after a delay of approximately 4–6 min and resulted in rapid, total bleb retraction within approximately 3 min, which is consistent with the bleb retraction observed in HeLa and CHO cells whether due to ionomycin, ATP or UTP.

2.4. Bleb retraction in response to a variety of input signals

Cells cotransfected with a monomeric red fluorescent protein (mRFP) variant of the CaM–RhoA fusion, CaM–RhoA(DP)–mRFP, and either ChR2 [15] or nAChR [16] fused to yellow fluorescent proteins became sensitive to blue light (440 nm) or acetylcholine (ACh), respectively (Fig. 4). An advantage of a protein switch sensitive to Ca^{2+} is that it can be made sensitive to many other external stimuli because Ca^{2+} signaling is naturally modular [14] (Fig. 4(A)). ChR2 is an algal light-gated cation channel that allows an influx of Ca^{2+} from the extracellular medium [15], while ACh stimulation of nAChR causes a Ca^{2+} transient, also by influx from the extracellular medium [16]. Efficient bleb retraction occurred in CHO cells cotransfected with the Ca^{2+}-sensitive RhoA fusion and either ChR2 (Fig. 4(B–E)) after 440 nm exposure (but not 560 nm exposure) or nAChR (Fig. 4(F–I)) after application of 1 mM ACh, over a similar timescale as was observed using nucleotide triphosphates and ionomycin (Fig. 3). The ability to engineer a functional connection between a Ca^{2+}-dependent process and a separate light- or chemically-dependent mechanism shows that Ca^{2+} can be a

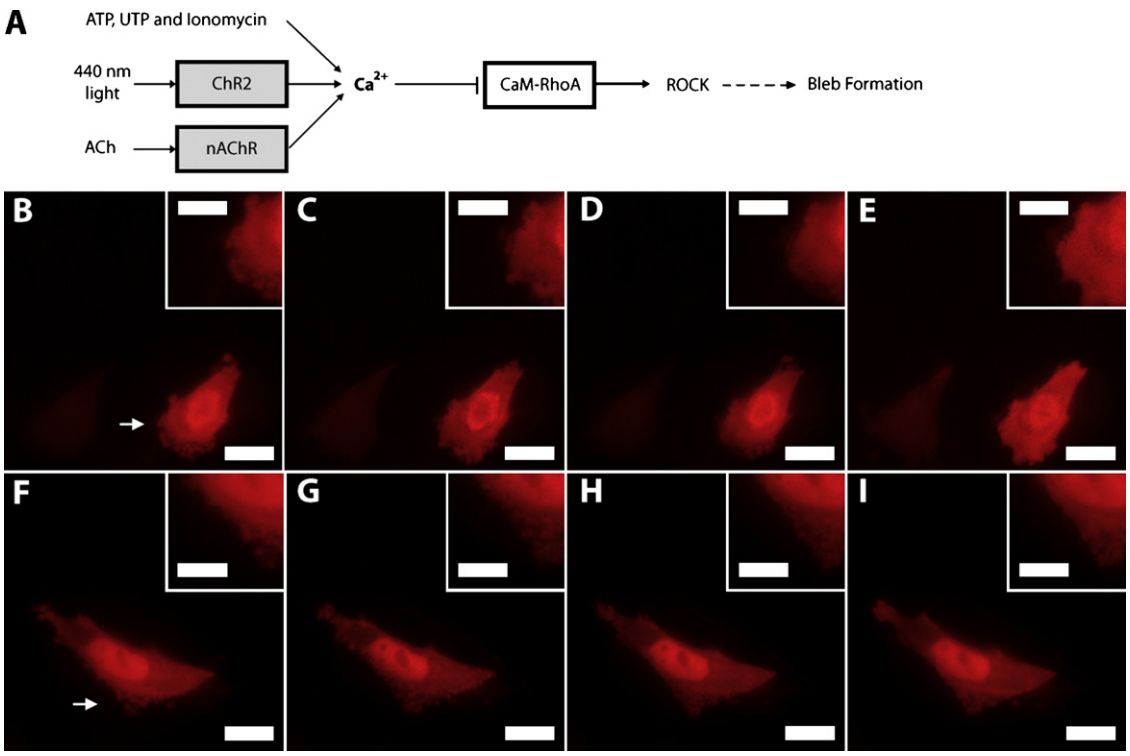

Fig. 4. Light and chemical induced bleb retraction through modular Ca^{2+} design. (A) Schematic diagram of Ca^{2+}-based bleb retraction: the Ca^{2+} dependent process can be made sensitive to a wide range of stimuli due to the prevalence of Ca^{2+} signaling in nature. Here, we have demonstrated this with blue light (via ChR2) and ACh (via nAChR), but many other stimuli may be candidates. (B–E) CHO cells co-transfected with ChR2 plasmid and CaM–RhoA(DP)–mRFP demonstrate bleb retraction after stimulated with blue light (0, 5, 10 and 20 min after irradiation onset, respectively). (F–I) CHO cells co-transfected with nAChR plasmid and CaM–RhoA(DP)–mRFP demonstrate bleb retraction after stimulation with ACh (0, 5, 10 and 20 min after addition of ACh). Scale bars in (B–I) are 30 μm and 6 μm in the insets.

valuable tool for synthetic biological applications where stimulus flexibility is required.

3. Discussion

The Ca^{2+}-based control of RhoA that we have demonstrated here can be compared to other methods of controlling Rho GTPases such as Rac1 [3,5] and Cdc42 [2,4]. In some of these designs, spatiotemporal control of Rac1 or Cdc42 was achieved using a light input. In theory, Ca^{2+}-based designs are capable of a similar level of spatiotemporal control. In this particular study, we have demonstrated temporal control over bleb retraction on the order of a few minutes, but spatial control could also be achieved using photo-activation of caged IP_3 or ChR2 with a narrow beam of light. A drawback of using Ca^{2+} to control engineered proteins lies in the fact that the Ca^{2+} signal affects many pathways simultaneously. However there are inherent strengths in the use of Ca^{2+} as a control stimulus such as versatility of input signal (demonstrated here for blue light and acetylcholine), ease of measurement and delivery, and potential applicability to structurally similar target proteins. For example, this approach may be applicable to similar GTPases such as Rac1 or Cdc42 even though they lack CaM-binding sites: the main hydrophobic anchors (Val170 and Leu179) are identical between the three proteins and CaM's wide specificity for target peptides means only limited mutagenesis may be necessary to develop Ca^{2+}-sensitive Rac1 and Cdc42 fusions.

Ca^{2+} loading of CaM–RhoA prevents new blebs from forming which allows for the cell's natural bleb retraction to operate over the observed 2- to 3-min time scale. Our experiments, and others, have shown that RhoA(DP) has a causal role in blebbing in certain cell lines, and the literature has established that the suppression of RhoA and ROCK-1 abolishes blebbing [9,19]. However, it is also known that RhoA and ROCK-1 are involved in bleb retraction once an actin network has formed in a bleb at the end of the bleb lifecycle, which explains why bleb retraction was less likely to occur in our experiments where cells had been incubated with Y-27632 [18]. In addition to ROCK-1, many other proteins are involved in bleb retraction such as several actin tethering and bundling proteins including ezrin, moesin, fimbrin and coronin [8,9]. In our experiments bleb retraction did not occur when CaM binding to RhoA was prevented (either by CDZ or MLCKp). This suggests that either CaM binding to RhoA directly causes bleb retraction, or that by destabilizing RhoA, CaM binding prevents new blebs from forming which allows native mechanisms to initiate bleb retraction [8,9,31]. Our structural model supports RhoA deactivation through CaM binding. Further, the timeline for bleb retraction that we have observed (both above and in Supplementary Fig. S7) agrees well with reported timelines for natural bleb retraction in several cell lines. Given that our structural model supports RhoA deactivation through binding to CaM, it is most likely that CaM binding to the RhoA fusion stops new blebbing from occurring which then allows the cell's endogenous bleb retraction mechanisms to take over [8,9].

The ability of a simple CaM–RhoA fusion to control the blebbing function of the RhoA suggests that CaM may be involved in regulating RhoA to some extent in nature. The existence of an accessible CaM binding site in RhoA is most likely used by cells where Ca^{2+}, CaM and RhoA are in proximity. For instance, it is known that both CaM and RhoA are involved in ATP induced blebbing via the $P2X_7$ Ca^{2+} channel [32]. In this natural context, it is likely that Ca^{2+}, CaM and RhoA are in proximity to mediate Ca^{2+} dependent blebbing.

An application of this work, beyond a proof-of-concept demonstration, lies in the potential to exploit the connection between blebbing and cell motility [19,33]. Cell migration *in vivo* is often guided by extracellular cues that induce a Ca^{2+} response in the migrating cell [33,34]. The CaM–RhoA fusion protein could enable transfected cells to explore their extracellular environment by

randomly blebbing and then to sense nearby cells secreting Ca^{2+}-inducing chemicals by no longer blebbing, in a process similar to how apoptotic cells release ATP to attract macrophages for clearance [34]. This "stop here" signal may be the first step toward engineering cells to perform more complicated tasks.

4. Conclusion

We have demonstrated the structure-based design of a Ca^{2+}-sensitive RhoA protein. Using a previously unreported CaM binding site near the carboxy-terminus of RhoA, a fusion protein was engineered that abolishes RhoA-dependent morphology effects in response to Ca^{2+}. Secondary proteins that change the intracellular Ca^{2+} concentration (ChR2 and nAChR) were used to achieve the same cellular effect of bleb retraction using either blue light or exogenous chemicals. The modular design applied here using Ca^{2+} enables versatility in the control of proteins in living cells. The large toolbox of proteins involved in Ca^{2+} signaling has no doubt contributed to, and is reinforced by, its widespread use in nature as a signal with high fidelity and spatiotemporal accuracy. By taking advantage of these characteristics of Ca^{2+} signaling we have designed a system where multiple extracellular stimuli can be applied to cause the same cellular effect. Fully exploring the versatility of Ca^{2+} signaling components may allow finer, more robust control of synthetic cellular systems.

5. Materials and methods

5.1. Peptide construction and subcloning

All subcloning was performed using the cassette system described previously [35]. RhoA proteins (WT, DN and DP) were cloned from Addgene plasmids 12965, 12967 and 12968, respectively using primers RhoA_53 and RhoA_35 shown below. CaM was cloned from human cDNA using the primers CaM_53 and CaM_35 also shown below. The MLCKp, and minimal CaM binding peptide from RhoA were made using the self hybridizing primers shown in Supplementary Materials and inserted into the cassette system. ChR2 was from Addgene plasmid 15753 and nAChR α subunit was from Addgene plasmid 15245.

5.2. CaM pulldown and fluorescent SDS-PAGE

CaM–sepharose beads were prepared according to the manufacturer instructions (Strategene, La Jolla, CA, USA). Plasmids encoding for the respective proteins were expressed in *E. coli* DH5α and protein was extracted using mechanical disruption. A NuPage Bis–Tris gel was used in NuPage 1× SDS-MOPS buffer (Invitrogen). The gel was examined using 488 nm excitation and 525 nm emission filters (Illumatool Lighting System, Light Tools Research, Encinitas, CA, USA) and a photograph was taken on a Canon A350 digital camera. The gel was then stained overnight using PageBlue to visualize the PageRuler ladder (Fermentas, Burlington, ON, Canada).

5.3. Cell culture and transfection

Cells were cultured in a 37 °C 5% CO_2 incubator in high glucose DMEM supplemented with 10% FBS (Invitrogen). Cells were passaged at 95% confluency by washing in PBS (Invitrogen) and incubating with 1 mL Trypsin–EDTA for 3–5 min at 37 °C (Sigma–Aldrich, St. Louis, MO, USA). All transfection was done using Lipofectamine 2000 and performed according to the manufacturer's instructions (Invitrogen). Cotransfections were verified by screening for two fluorescent proteins (e.g. CaM–RhoA(DP)–mRFP and ChR2-YFP).

5.4. Reagents used

ATP and UTP were from Fermentas while ionomycin, EDTA, CDZ, Y-27632 and ACh were from Sigma. CHO and HEK293 cells were stimulated with 10 μM ATP, HeLa cells were stimulated with 50 μM UTP and all cells were stimulated with 2 μM ionomycin. CHO cells were stimulated with 1 mM ACh prepared in PBS. Unless otherwise noted, imaging experiments took place in PBS supplemented with approximately 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Invitrogen). For experiments where Ca^{2+} influx was dependent on ChR2, this buffer was further supplemented with 10 mM $CaCl_2$. Cells that were pretreated with CDZ or Y-27632 were washed with PBS, placed in DMEM supplemented with 5% FBS and then incubated with 50 μM CDZ or 10 μM Y-27632 for at least 30 min. Immediately prior to stimulation and imaging, the buffer was replaced with new PBS and inhibitors. Fluo4-AM was purchased from Sigma and used as follows: Cell culture medium was removed and cells were washed twice with PBS. Cells were incubated with PBS containing 4 μM Fluo-4 AM and 0.0001% DMSO for 20 min. Cells were washed twice with PBS and then the 1 mM Ca^{2+} PBS buffer was added for imaging. After the transient was complete, ionomycin was added to determine F_{max} and then 2.5 mM EDTA was added to determine F_{min}.

5.5. Morphological data analysis

Blebs were counted if they were a mostly circular extension from the cell body. *p*-Values of morphological change from before and after stimulus experiments were calculated using the paired (dependent) Student's *t*-test. *p*-Values of morphological change across different conditions were calculated using the unpaired (independent) Student's *t*-test without assuming equal variances. *p*-Values less than 0.05 are generally considered statistically significant. In all cases for determining bleb retraction, the data is presented as the mean of at least 10 cells over at least 3 independent experiments and the error bars are the standard deviation.

Conflict of interest

Authors declare that there is no competing financial interest.

Author contributions

EM designed and carried out the experiments, analysed the data and wrote the manuscript. EP provided technical assistance, performed computational modeling and helped in writing the manuscript. KT conceived the initial idea, analysed the data, provided guidance and helped in writing the manuscript.

Acknowledgments

This work was supported by fellowships to EM and EP from the Natural Science and Engineering Research Council (NSERC) and grants to KT from the Canadian Institutes of Health Research (#81262).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceca.2010.08.009.

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