Published in final edited form as:

Biochemistry. 2016 November 22; 55(46): 6395–6405. doi:10.1021/acs.biochem.6b00908.

Regulation of a Coupled MARCKS–PI3K Lipid Kinase Circuit by Calmodulin: Single-Molecule Analysis of a Membrane-Bound Signaling Module

Brian P. Ziemba[†], G. Hayden Swisher[†], Glenn Masson[‡], John E. Burke^{‡,§}, Roger L. Williams[‡], and Joseph J. Falke^{*,†}

[†]Molecular Biophysics Program and Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215, United States

[‡]Laboratory of Molecular Biology, Medical Research Council, Cambridge CB2 0QH, U.K

Abstract

Amoeboid cells that employ chemotaxis to travel up an attractant gradient possess a signaling network assembled on the leading edge of the plasma membrane that senses the gradient and remodels the actin mesh and cell membrane to drive movement in the appropriate direction. In leukocytes such as macrophages and neutrophils, and perhaps in other amoeboid cells as well, the leading edge network includes a positive feedback loop in which the signaling of multiple pathway components is cooperatively coupled. Cytoplasmic Ca^{2+} is a recently recognized component of the feedback loop at the leading edge where it stimulates phosphoinositide-3-kinase (PI3K) and the production of its product signaling lipid phosphatidylinositol 3,4,5-trisphosphate (PIP_3). A previous study implicated Ca^{2+} -activated protein kinase C (PKC) and the phosphatidylinositol 4,5-bisphosphate (PIP_2) binding protein MARCKS as two important players in this signaling, because PKC phosphorylation of MARCKS releases free PIP_2 that serves as the membrane binding target and substrate for PI3K. This study asks whether calmodulin (CaM), which is known to directly bind MARCKS, also stimulates PIP_3 production by releasing free PIP_2 . Single-molecule fluorescence microscopy is used to quantify the surface density and enzyme activity of key protein components of the hypothesized Ca^{2+} –CaM–MARCKS– PIP_2 –PI3K– PIP_3 circuit. The findings show that CaM does stimulate PI3K lipid kinase activity by binding MARCKS and displacing it from PIP_2 headgroups, thereby releasing free PIP_2 that recruits active PI3K to the membrane and serves as the substrate for the generation of PIP_3 . The resulting CaM-triggered activation of PI3K is complete in seconds and is much faster than PKC-triggered activation, which takes minutes. Overall, the available evidence implicates both PKC and CaM in the coupling of Ca^{2+} and PIP_3

*Corresponding Author: falke@colorado.edu. Telephone: (303) 817-9814.

§J.E.B.: Department of Biochemistry and Microbiology, University of Victoria, P.O. Box 1700 STN CSC, Victoria, BC, Canada V8W 2Y2.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00908. Comparison of the effects of CaM and BSA on PI3K α kinase activity (Figure S1) and controls testing for effects of CaM on the membrane binding and 2D diffusion of PI3K α and GRP-PH (Figure S2) (PDF)

Notes

The authors declare no competing financial interest.

signals and suggests these two different pathways have slow and fast activation kinetics, respectively.

Graphical abstract

At the leading edge membrane of macrophages and neutrophils, a complex, tightly regulated signaling network maintains an actively ruffling leading edge and directs cell movement up attractant gradients.^{1–5} This signaling network requires the second messenger Ca^{2+} and the signaling lipid PIP_3 , as well as an array of signaling proteins recruited from the cytoplasm to the lipid bilayer surface of the leading edge membrane.^{6–10} Many of the signaling proteins involved in the leading edge network are master kinases that play central roles in diverse cell pathways. In all chemotaxing cells, the leading edge network senses the attractant gradient and defines the direction of cell movement. In specialized chemotaxing cells such as leukocytes, the leading edge network includes a positive feedback loop that maintains a stable, actively ruffling leading edge primed to detect an attractant gradient, even in the absence of an added attractant.^{5,8,11–13} Thus, the leading edge of macrophages and neutrophils remains ready to rapidly respond to new attractant gradients arising from sites of infection, tissue damage, or disease. Moreover, the positive feedback loop may play a central role in the cell compass by amplifying attractant signals in the regions of the leading edge membrane exposed to the highest attractant concentrations, thereby triggering local membrane and actin mesh remodelings that expand the leading edge up the gradient. Beyond leukocytes, some evidence indicates the positive feedback loop may also be found at the leading edge of other amoeboid cells.¹²

A number of essential components of the leukocyte leading edge positive feedback loop have been identified to date: PI3K and its substrate and product lipids PIP_2 and PIP_3 , respectively;^{1,3,5,8,11,14,15} F-actin;^{11,16,17} one or more Rho/Rac GTPases;^{10,15,18–20} Ca^{2+} ;^{1,6–8} and PKC.^{8,17,21,22} This feedback loop is essential for leukocyte chemotaxis, and remarkably, either stimulation or inhibition of a single component can activate or suppress the other loop components.^{1,8,23–25} Ca^{2+} and PKC are the two most recently identified loop components and have been shown to be essential to leading edge function in both macrophages and neutrophils.^{1,6,8,26,27}

MARCKS is a major substrate of PKC α , and other conventional PKCs, that becomes phosphorylated during leukocyte activation.^{17,28,29} Unphosphorylated MARCKS possesses a high affinity for anionic and PIP₂ lipids while this interaction is disrupted upon specific phosphorylation by Ca²⁺-activated PKC at multiple sites within its PIP₂ binding region, leading to PIP₂ release.^{30–33} Previous work has shown that MARCKS inhibits the activity of PI3K α by sequestering its target and substrate lipid PIP₂ and that such inhibition is reversed by Ca²⁺–PKC phosphorylation of MARCKS, where-upon the newly released PIP₂ recruits PI3K to the membrane and thereby increases the surface density of active lipid kinase, yielding faster net PIP₃ production.²⁶ Release of PIP₂ from MARCKS also enhances actin cytoskeleton–membrane interactions, because many actin binding proteins employ PIP₂ as a membrane anchor.^{34–38}

This study asks whether calmodulin, like PKC, can stimulate PI3K α activity and PIP₃ production by reversing the sequestration of PIP₂ by MARCKS. The ubiquitous, multi-functional, Ca²⁺-activated signaling protein calmodulin (CaM)³⁹ is known to be involved in macrophage and neutrophil chemotaxis.^{29,40,41} During a cytoplasmic Ca²⁺ signal, CaM binds four Ca²⁺ ions and docks to specific protein targets.^{42–45} Notably, MARCKS is a CaM target, and CaM tightly binds to its PIP₂ binding region,^{31,46–49} leading to the simple hypothesis that Ca²⁺–CaM, like Ca²⁺–PKC, may release sequestered PIP₂ molecules and activate PI3K-catalyzed PIP₃ production (Figure 1). Consistent with this hypothesis, several studies have demonstrated the importance of the Ca²⁺–CaM complex in cellular migration and chemotaxis, such that chelation of Ca²⁺ or inhibition of CaM abolishes cell polarization and migration.^{6,8,50–52}

This study also examines the ability of CaM to directly bind and regulate the ubiquitous α isoform of PI3K. Several instances of direct binding and regulatory interactions have been described between CaM and PI3K.^{53–55} Specifically, the p110 and p85 subunits of PI3K have been shown to form a complex with CaM, although it has not yet been ascertained which PI3K isoforms are capable of these interactions.

To address the ability of CaM to regulate PI3K α and to ascertain the mechanism of that regulation, this study employs a previously described single-molecule approach to quantifying the lipid kinase activity of PI3K α on a supported lipid bilayer in a simplified physiological system that approximates the concentrations of key cytoplasmic components.²⁶ This simplified system allows quantitative analysis of CaM and MARCKS regulation of lipid kinase activity in the absence of other CaM, MARCKS, and PI3K α binding proteins that complicate the analysis *in vivo*, thereby facilitating molecular studies of regulatory mechanisms involving just these three proteins. The findings reveal that CaM can dramatically stimulate PI3K α activity and PIP₃ production *in vitro* by removing MARCKS and releasing free PIP₂ to serve as the binding target and substrate of PI3K α . In contrast, the findings show that CaM has only a weak affinity for direct binding to PI3K α that yields minimal kinase stimulation of this PI3K isoform. Overall, the observed stimulation of PIP₃ signaling by Ca²⁺–CaM, together with that previously observed for Ca²⁺–PKC,²⁶ provides a molecular explanation for the link between Ca²⁺ signaling and PIP₃ signaling observed in the leading edge positive feedback loop of leukocytes, as evidenced by

the dramatic upregulation of PIP₃ observed at the leading edge of polarized macrophages during a Ca²⁺ signal.⁸

MATERIALS AND METHODS

Reagents

Synthetic dioleoyl phospholipids PS (phosphatidylserine; 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine), PIP₂ (1,2-dioleoyl-*sn*-glycero-3-phosphoinositol 4,5-diphosphate), and PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) were from Avanti Polar Lipids (Alabaster, AL). Alexa Fluor 555 C₂-maleimide (AF555) and CoverWell perfusion chambers were from Invitrogen (Carlsbad, CA). Glass supports were from Ted Pella, Inc. (Redding, CA). 2-Mercaptoethanol, ultrapure (>99%) BSA, ATP magnesium salt, and CoA trilithium salt were from Sigma (St. Louis, MO). Monophosphorylated peptide (pY) (sequence of RQIKIWFQNRRMKWKKSDGGpYMDMS) produced by TOCRIS Bioscience (Bristol, U.K.) contained the conserved sequence of the PDGFR Y740 phosphorylation site (SDGGpYMDMS). This peptide activated PI3K α to the same level as the bis-phosphorylated PDGFR-derived peptide we used previously (compare the results presented here with those in ref 26 and data not shown). Ultrapure (99%) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} was from Anatrace (Maumee, OH). Human MARCKS PIP₂ binding domain (MARCKS residues 151–175) was produced by SynBioSci Corp. (Livermore, CA) and includes an N-terminal cysteine residue added for probe labeling (N-CKKKKKRFSFKKSFKL-SGFSFKKNKK-C).

PI3K α Cloning and Expression

The PI3K α construct utilized in this study was generated by cloning the human PI3K p110 α catalytic and PI3K p85 α regulatory subunits into the pFastbacHT vector (Invitrogen), which encodes an N-terminal His₆ tag and a TEV protease cleavage site, and the pFastbac1 vector (Invitrogen), respectively, as previously described.⁵⁶ Subsequently, an 11-amino acid ybbR labeling peptide (sequence of DSLEFIASKLA)⁵⁷ was inserted at the N-terminus of the *Homo sapiens* PI3K p85 α regulatory subunit, generating an N-terminal enzymatic labeling tag. This construct was used to express the full length, functional p85 α /p110 α heterodimer (PI3K α) in *Spodoptera frugiperda* (*Sf 9*) insect cells, and the protein was purified as previously described.⁵⁶ Final PI3K α -containing fractions in PI3K storage buffer [20 mM HEPES (pH 7.4), 100 mM NaCl, and 5 mM DTT] were collected, concentrated to 11 μ M, and then snap-frozen in 20 μ L aliquots using liquid nitrogen.

The resulting PI3K α was tested for its specific lipid kinase activity in both bulk and single-molecule measurements, yielding measured turnover rates of 6.5 ± 0.4 (SEM for six triplicates) and 6.0 ± 0.1 (SEM for 20 triplicates) molecules of PIP₃ produced per enzyme molecule per minute, respectively. These turnover rates were indistinguishable from those measured previously for PI3K α in bulk and single-molecule assays, demonstrating that the purified enzyme was functional.

GRP1 PH Domain Cloning and Expression

A human GRP1 PH domain construct possessing an N-terminal ybbR enzymatic labeling tag was created and purified as previously described.⁵⁸ Final PH domain-containing fractions in GRP-PH storage buffer [50 mM TRIS (pH 7.5), 15 mM NaCl, and 2.5 mM CaCl₂] were collected, concentrated to 80 μ M, and then snap-frozen in 100 μ L aliquots using liquid nitrogen.

Labeling PI3K, the GRP1 PH Domain, and MARCKS with a Fluorophore

Recombinant PI3K α and GRP1-PH proteins were covalently modified with the fluorophore AF555 by the Sfp enzyme using a published protocol.^{58,59} Briefly, $\sim$ 2 μ M target protein was incubated with 2.5 μ M Alexa Fluor 555–CoA conjugate, 0.5 μ M Sfp, and 50 μ M Mg²⁺ in the storage buffer of that protein at room temperature for 60 min, except PI3K α , which was incubated for 30 min on ice. Excess fluorophore was removed by buffer exchange with storage buffer using Vivaspin concentrators (Sartorius Stedim, Göttingen, Germany) until the flow-through was not visibly colored by the AF555 fluorophore, and the final flow-through was checked for absorbance at 555 nm to ensure complete removal of the free label. The labeling efficiency and concentration of labeled protein were determined from the measured absorbances of AF555 and intrinsic tryptophan residues. The labeled protein at 11 μ M in its storage buffer was aliquoted and snap-frozen in 10 μ L aliquots using liquid nitrogen.

The MARCKS PIP₂ binding domain was labeled by incubating $\sim$ 1 μ M target peptide and 1.5 μ M AF555-maleimide in the presence of 1 μ M TCEP at room temperature for 1 h. The free fluorophore was removed from each MARCKS labeling reaction mixture via exchange with TIRF assay buffer (see below) using Amicon (Millipore, Billerica, MA) Ultra 3 kDa centrifugal filters.

Unlabeled Calmodulin Expression

A human calmodulin (CaM) construct possessing an N-terminal His₆ tag (Applied Biological Materials) was expressed in Rosetta2 *Escherichia coli* cells. TALON metal affinity resin (Clontech) was used to purify CaM from the cellular lysate, and dialysis was used to remove imidazole from the retained protein. Pure CaM protein was concentrated to $\sim$ 1.2 mM in CaM storage buffer [100 mM KCl, 20 mM HEPES (pH 6.9), 15 mM NaCl, 5 mM glutathione, 2.0 mM EGTA, 6.8 mM Ca²⁺, and 0.5 mM Mg²⁺] designed to occupy CaM with Ca²⁺ to prevent changes in free Ca²⁺ upon addition of CaM to the single-molecule experiment. Lastly, CaM was snap-frozen in liquid nitrogen and stored in 100 μ L aliquots at -80°C .

Fluorescent Calmodulin Cloning and Expression

Human untagged pKK233-hCaM was a gift from E. Strehler.⁶⁰ Site-directed mutagenesis was employed to introduce an individual N-terminal cysteine residue into this CaM expression plasmid, which was then transformed into Rosetta2 *E. coli* cells. The calmodulin expression and purification methodology was adapted from those published by Gopalakrishna and Anderson⁶¹ and Hayashi et al.⁶² Briefly, 5 mL of overnight transformed cells grown in LB broth supplemented with ampicillin and chloramphenicol was used to

seed 0.5 L of antibiotic-supplemented LB broth. A final isopropyl β -D-1-thiogalactopyranoside concentration of 0.4 mM was added to cells grown to an OD₆₀₀ of 0.5 to induce protein growth at 37 °C for 4 h. Harvested cells were suspended in 50 mM TRISHCl (pH 7.5) with 2 mM EDTA and 0.2 mM PMSF (40 mL per 1 L of growth). Cells were lysed by two cycles of probe sonication, and the lysate was centrifuged for 30 min at 37000g. The CaCl₂ concentration of the soluble lysate fraction was adjusted to 5 mM and passed over 4 mL of phenyl-sepharose CL4B resin (Sigma) that was equilibrated with 5 column volumes of 50 mM TRIS-HCl (pH 7.5) with 5 mM CaCl₂ and 0.1 M NaCl (equilibration buffer). The CaCl₂-supplemented lysate was passed through the equilibrated column twice and washed with 10 column volumes of (low calcium) equilibration buffer with 0.1 mM CaCl₂. The column was then washed with 10 column volumes of (high-salt, low-calcium) equilibration buffer with 0.5 M CaCl and 0.1 mM CaCl₂ and then returned to (low-calcium, low-salt) buffer with 0.1 M CaCl and 0.1 mM CaCl₂. Calmodulin was eluted with 50 mM TRIS-HCl (pH 7.5) with 1 mM EGTA and 0.1 M NaCl. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis revealed both N- and C-terminal cysteine calmodulin constructs to be 80–90% pure. A Micro Bio-Spin column with a P-6 gel (Bio-Rad) was used to exchange calmodulin constructs into 10 mM TRIS (pH 7.5) and 1 mM TCEP. Buffer-exchanged constructs were fluorescently labeled for 2 h at room temperature by combining 50 μ M calmodulin, 200 μ M Alexa Fluor 555 C₂-maleimide (AF555), 2 mM TCEP, and 1 mM CaCl₂ in a total volume of 55 μ L. To quench the unreacted fluorophore, 5 mM reduced glutathione was added to the reaction mixture. To remove the unreacted fluorophore, reaction mixtures were passed through P-6 gel columns equilibrated with 10 mM TRIS-HCl (pH 7.5) with 5 mM reduced glutathione, 1 mM TCEP, and 1 mM CaCl₂. Fluorescence analysis of purified proteins analyzed via SDS–PAGE revealed fluorescent calmodulin running in line with the 15 kDa molecular weight marker and accounted for ~95% of the fluorescent protein.

Supported Lipid Bilayer Preparation

Supported lipid bilayers were prepared from sonicated unilamellar vesicles (SUVs) as described previously.^{58,63} CHAPS (0.05%) was included in all experiments, as it was found to stabilize PI3K activity and did not increase the level of membrane binding or lipid kinase activity in the absence of pY.²⁶

TIRFM Measurements

TIRFM experiments were performed at 21.5 ± 0.5 °C on an objective-based TIRFM instrument as described previously.^{58,63} Supported bilayers were first washed with TIRF assay buffer [20 mM KCl, 20 mM HEPES (pH 6.9), 15 mM NaCl, 5 mM glutathione, 2.0 mM EGTA, 1.9 mM Ca²⁺, and 0.5 mM Mg²⁺, where this Ca²⁺/Mg²⁺ buffering system yields 10 μ M free Ca²⁺ and 0.5 mM free Mg²⁺] and then imaged before and after the addition of a concentrated mixture of BSA and CHAPS to final concentrations of 100 μ g/mL and 0.05%, respectively. After this addition, only a few dim, rapidly dissociating fluorescent contaminants were typically observed on the bilayer prior to protein addition and were easily eliminated from the data as described below. Occasionally, the contaminant level was excessive, and the reagents were remade. Following confirmation of minimal contamination, proteins and ATP (1 mM) were added as needed and equilibrated for 5 min.

Labeled or unlabeled proteins were thawed on ice and diluted into buffer containing stabilizers as needed and a low level of BSA to block sticky surfaces that could absorb the dilute proteins.⁶⁴ Aliquots of PI3K were diluted into stabilizing buffer that maximizes its stability [20 mM HEPES (pH 7.2), 125 mM NaCl, 10% glycerol, 4 mM TCEP, 0.05% CHAPS, and 100 $\mu\text{g mL}^{-1}$ BSA] until dilution into the TIRF chamber containing assay buffer (see above). Aliquots of CaM, MARCKS, and GRP1-PH were diluted into assay buffer as needed and then added to the chamber.

To minimize contributions from small numbers of immobile unfolded proteins, a bleach pulse power ~30-fold higher than that used for imaging was applied for ~10 s, and then the fluorescence was allowed to return to a steady state for at least 60 s before data were acquired. For each sample, a set of two to four movie streams were acquired at a frame rate of 20 frames/s, and a spatial resolution of 4.2 pixels/ μm on the home-built instrument, using NIS Elements Basic Research (Nikon, Melville, NY).

Single-Particle Tracking

As in our previous studies,^{58,63,65} diffusion trajectories of single protein molecules were tracked and quantitated using the Particle Tracker plugin for ImageJ,⁶⁶ yielding a per-frame quantitation of particle position and brightness. Then resulting data were then imported into Mathematica for further analysis. Only particles possessing fluorescence intensities within a defined range were included in the analysis, thereby eliminating bright fluorescent contaminants and/or protein aggregates and dim, nonprotein contaminants. Additional displacement-based exclusions removed immobile particles, rapidly dissociating particles, and overlapping tracks for which the particle identity was lost. All exclusions were described and validated previously.^{58,63,65}

Membrane Binding Assays

To quantify the average density of a given protein on the membrane surface in a given TIRF movie, the number of single-particle tracks (defined as described above) in a given field of view was determined for each movie frame and then averaged over all frames. For each of the three mobile fluorescent proteins, bleaching of individual tracks or the bulk population was not a major issue because the average residence time on the membrane prior to dissociation was short compared to the average bleach time. Thus, fluorescent proteins dissociate from the membrane before bleaching and are replaced by other fluorescent proteins from the bulk population that is predominantly outside the TIRF excitation field. The average residence time of mobile fluorescent proteins on the supported bilayer was 0.88 ± 0.02 s for MARCKS, 0.57 ± 0.02 s for PI3K, and 0.29 ± 0.01 s for the PH domain (error ranges are SEMs for 275 mean residence time determinations, each obtained from a movie with a duration of 5 s containing multiple mobile tracks). To measure the bleach rate, fluorescent proteins were immobilized on the supported bilayer by eliminating the blocking protein BSA from the sample, thereby allowing them to stick to unblocked membrane defects. The average time to bleach of these immobile fluorescent proteins was 6.7 ± 0.8 s for MARCKS, 3.9 ± 0.1 s for PI3K, and 4.9 ± 0.2 s for the PH domain (error ranges are SEMs for 60 mean bleach time determinations, each obtained from a movie with a duration of 5 s containing multiple immobile particles).

PI3K Kinase Assay

A single-molecule kinase assay developed for quantifying the specific activity of PI3K α was previously described.²⁶ To determine the PI3K α specific activity, first the average density of PI3K α was determined via the binding assay described above (with appropriate correction for the PI3K fluorescence labeling efficiency). Second, to count all single molecules of product PIP₃ produced by the PI3K lipid kinase reaction, a saturating concentration of the GRP-PH domain (300 pM) was employed to tag each PIP₃ molecule generated on the membrane surface with a fluorescent PH domain. To maintain constant levels of free ATP (1 mM), Mg²⁺ (0.5 mM), and Ca²⁺ (10 μ M) in all assays, both the TIRF assay buffer (see above) and the ATP stock (TIRF assay buffer containing 100 mM ATP and 82.5 mM Mg²⁺) were buffered with EGTA as defined by MaxChelator.⁶⁷

Statistics

Error bars represent standard errors of the mean for n means (where there are 12–47 means, and each mean is determined from three or four movies), except where indicated otherwise. Statistical significance was examined using the appropriate test; most commonly, the two-tailed t test was used to determine whether an event was statistically significant.

RESULTS

Physiologically Based Model System Employed for Single-Molecule Studies

To conduct single-molecule studies of the ability of CaM and MARCKS to regulate PI3K α bilayer interactions and lipid kinase activity, this work utilized a previously described *in vitro* model system that mimics the intracellular conditions at the inner leaflet of the plasma membrane during an active Ca²⁺ signaling event. This system directly quantifies the membrane binding and lipid kinase activity of PI3K α by counting (i) the number of single kinase molecules bound to a target-supported bilayer and (ii) the number of product PIP₃ lipid molecules produced by those enzyme molecules in a near-physiological setting on a membrane surface, where the concentrations of effectors, target lipids, and aqueous ions closely approximate cellular levels (Table 1). Supported lipid bilayers were comprised of PE, PS, DAG, and PIP₂ in a 73:24:2:1 ratio (mole percent), a simple lipid mixture providing the key background and signaling lipids of the plasma membrane inner leaflet needed for the activity of PI3K α and its Ca²⁺-modulated effectors, including MARCKS, CaM, and PKC. The PE/PS/DAG/PIP₂ bilayer was assembled on a glass support, with a thin intervening buffer layer between the glass and the lower membrane leaflet, while the upper leaflet was exposed to bulk buffer and to the effectors and proteins added to that buffer.

Engineering and Purifying Protein Constructs for Single-Molecule Fluorescence Studies

As previously described, full length PI3K α and a synthetic peptide representing the PIP₂ binding region of MARCKS (termed MARCKS peptide or MARCKS_p) were engineered so they could be labeled with a photostable fluorescent probe (Figure 2) for use in single-molecule TIRF experiments. The PI3K α construct possessed the ybbR labeling tag at the N-terminus of p85 and was enzymatically labeled with Alexa Fluor 555 under mild conditions to minimize effects on protein function.⁵⁷ The MARCKS peptide possessed an N-terminal

Cys residue that allowed efficient chemical labeling with Alexa Fluor. In all cases, the free, uncoupled fluorophore was removed by ultrafiltration.

Quantifying the Effects of Calmodulin on the Membrane Binding of the MARCKS Peptide and PI3K α

As detailed in our previous studies, a known concentration of a fluorescent protein was added to the bulk buffer above the supported bilayer, and single-molecule TIRF was employed to image membrane-bound fluorescent proteins slowly diffusing on the bilayer surface while ignoring the proteins in the aqueous phase (which diffuse >100-fold faster, too fast to image, because of the ~100-fold lower viscosity of the latter phase relative to the bilayer). Particle tracking software was employed to track and analyze the two-dimensional (2D) movements of each membrane-bound, freely diffusing fluorescent protein (see Materials and Methods). This approach allowed quantitation of the surface density and average 2D diffusion constant of each native, membrane-bound protein of interest, as well as identification and exclusion of fluorescent contaminants and immobile, improperly folded protein molecules.^{58,63}

Figure 3 shows representative single-molecule surface densities of the membrane-bound, fluorescent MARCKS peptide and PI3K α and analyzes the effects of the addition of nonfluorescent calmodulin on the surface densities of the MARCKS peptide and PI3K α populations. As observed previously,²⁶ MARCKS peptide and PI3K α both required PS and PIP₂ for maximal binding affinity. Consistent with accounts detailing the high-affinity complex between CaM and the MARCKS PIP₂ binding region,^{48,31} the addition of CaM bound and removed the majority of fluorescent MARCKS peptide from the supported bilayer (Figure 3). Because each MARCKS peptide is known to sequester up to four PIP₂ lipids, it follows that the addition of CaM significantly increased the availability of free PIP₂.

In the absence of the MARCKS peptide, full length, fluorescent PI3K α molecules were observed to bind to the supported bilayer, but PI3K binding was blocked when the lipid kinase was added to bilayers where PIP₂ was sequestered by the MARCKS peptide, as previously reported.²⁶ Here we observed that subsequent addition of CaM to this fluorescent PI3K α /MARCKS peptide system restored PI3K α membrane binding to levels approaching those observed in the absence of MARCKS, indicating that the PIP₂ released by binding of CaM to MARCKS was once again available as a target lipid for PI3K α membrane binding.

Effects of Calmodulin and the MARCKS Peptide on PI3K α Activity

To perform quantitative studies of PI3K α kinase regulation, we employed our recently developed single-molecule kinase assay.²⁶ In brief, as PI3K α phosphorylates its substrate lipid PIP₂ on the supported bilayer surface, each individual PIP₃ product produced is rapidly bound by a fluorescent GRP1 PH domain. The resulting single-molecule TIRF fluorescence permits direct detection and unambiguous identification of the PIP₃–PH domain complex by its 2D diffusion track and characteristic diffusion constant. Figure 4 shows that as the PI3K α reaction proceeded, increasing numbers of PIP₃ product molecules were detected via their

bound PH domain fluorophores, yielding the specific activity (turnover number) of the PI3K α molecule.

The single-molecule kinase assay was further used to investigate CaM–MARCKS regulation of PI3K α lipid kinase activity. As observed previously, the addition of the MARCKS peptide to the single-molecule PI3K α lipid kinase reaction mixture slowed the rate of production of PIP₃ >3-fold, indicating that MARCKS significantly downregulates PI3K α lipid kinase activity (Figure 4A). This decrease in kinase activity parallels the decreased level of binding of the membrane to PI3K α due to MARCKS sequestration of its PIP₂ target lipid (Figure 3).

Addition of CaM to the PI3K α /MARCKS peptide system yielded a highly significant and reproducible 4-fold increase in the net rate of PIP₃ production on the membrane surface (Figure 4A,B). This activity enhancement arose primarily from the aforementioned capture of the MARCKS peptide by CaM, the concomitant release of the MARCKS peptide from the membrane and the freeing of its sequestered PIP₂ lipids, and the restoration of PI3K α membrane binding to its pre-MARCKS level (Figure 3). Thus, the CaM-triggered increase in the lipid kinase activity of MARCKS-inhibited PI3K α is fully explained by the CaM-triggered increase in the level of binding of PI3K α to the target membrane (compare Figures 3 and 4). Within error, the specific activity of membrane-bound PI3K α is the same under all conditions (Figure 4C), confirming that the large differences in net PIP₃ production rates arise from differences in the surface density of membrane-bound PI3K α rather than from effects on its turnover rate.

As a control, the effect of CaM on PI3K α lipid kinase activity was also measured in the absence of the MARCKS peptide. Under these conditions, addition of 20 μ M CaM triggered only a small (<30%) increase in PI3K α kinase activity, at least 13-fold smaller than the increase observed in the presence of the MARCKS peptide (Figure 4B). A similar increase in kinase activity was observed when 20 μ M BSA was added instead of CaM, in the absence of the MARCKS peptide (Figure S1). A simple explanation for these small increases in PI3K α kinase activity is that the addition of background protein protects PI3K from nonspecific absorption to membrane defects or the walls of the experimental chamber. The low total concentration (2 nM) of PI3K α employed makes the system sensitive to such losses, and the addition of a blocking protein is known to reduce nonspecific absorption (see Materials and Methods and ref 64). Alternatively, previous studies have reported that CaM can bind directly to one or more PI3K isoforms, raising the possibility that CaM regulates PI3K α via a direct binding mechanism. To test this possibility, the binding of fluorescent CaM to PI3K α was studied but was found to be too weak to quantify under the achievable experimental conditions. The activating effect of CaM on PI3K α lipid kinase activity increased approximately linearly with CaM concentration (Figure S1), consistent either with CaM blocking of nonspecific PI3K α absorption or with direct binding of CaM to PI3K α with a very low affinity ($K_d > 20 \mu$ M). If CaM does bind directly to PI3K α , it has no detectable effect on the 2D diffusion of the lipid kinase on the bilayer surface (Figure S2). In either case, in a cellular setting, the weak activation of PI3K α by CaM in the absence of MARCKS is predicted to be negligible relative to the much larger PI3K α activation resulting from binding of CaM to MARCKS and the concomitant release of free PIP₂.

Time Scale of the Effects of CaM on MARCKS and PI3K α

The observed CaM removal of MARCKS from the membrane and the resulting membrane docking and activation of PI3K α were all rapid reactions compared to the slower kinetics previously observed for the PKC α –MARCKS–PI3K α system. Figure 5 shows that following the addition of Ca²⁺-activated CaM, within 10 s most of the fluorescent MARCKS molecules were removed from the supported bilayer surface and most of the membrane binding of the fluorescent PI3K α population was restored. Moreover, the rate of PI3K α -catalyzed PIP₃ production exhibited maximal stimulation within 1 min of CaM addition [where 1 min was the first rate measurement (Figure 3B)]. By contrast, the speed of the corresponding regulatory reactions was approximately 10-fold slower for the PKC α –MARCKS–PI3K α system (see Figure 5 and ref 26), because each PKC α molecule must phosphorylate many MARCKS molecules (the mole ratio MARCKS/PKC = 56, Table 1) to drive the MARCKS population off its PIP₃ target lipids.

DISCUSSION

Previous studies in polarized macrophages have shown that cellular Ca²⁺ signals dramatically stimulate PIP₃ production by PI3K α , particularly at the leading edge membrane where the positive feedback loop and other components of the leading edge signaling network are most active.⁸ The present *in vitro* studies demonstrate that Ca²⁺-activated CaM can stimulate PIP₃ production in a system comprised of CaM, the PIP₂ binding protein MARCKS, and PI3K α . These findings reveal a previously unknown, MARCKS-mediated molecular link between Ca²⁺–CaM signaling and PI3K α –PIP₃ signaling that helps explain the Ca²⁺-triggered enhancement of PIP₃ production at the leukocyte leading edge.

The MARCKS-mediated stimulation of PI3K α lipid kinase activity by Ca²⁺-activated CaM observed in this study is analogous to the previously described, MARCKS-mediated stimulation of PI3K α by Ca²⁺-activated PKC α .²⁶ In both studies, MARCKS was found to inhibit PI3K α lipid kinase activity by sequestering PIP₂, thereby blocking binding of PI3K α to its target membrane surface. Removal of MARCKS from PIP₂ on the membrane surface was found to restore binding of PI3K α to its target lipid, increasing the number of active, membrane-bound lipid kinase molecules and thus upregulating the production of PIP₃ signaling lipid.

The past and new findings indicate that either Ca²⁺-activated CaM or Ca²⁺-activated PKC α can displace MARCKS from PIP₂ lipids, driving an increased level of binding of PI3K to its target PIP₂ and lipid kinase activation. In this study, MARCKS is displaced from PIP₂ by the high-affinity binding of Ca²⁺-activated CaM to the PIP₂ binding region of MARCKS. By contrast, PKC α displaces MARCKS by phosphorylating and inactivating its PIP₂ binding sites. These two contrasting mechanisms yield significantly different activation and inactivation kinetics: binding of CaM to MARCKS occurs in seconds, while the PKC α protein kinase reaction that phosphorylates the MARCKS population takes minutes to complete at physiological levels of PKC α and MARCKS.

A simple model can explain why cellular pathways, including the positive feedback loop at the leading edge of leukocytes, could require both Ca^{2+} -CaM and Ca^{2+} -PKC α complexes to shape the time profile of PI3K α regulation via MARCKS. Because the direct binding of Ca^{2+} -CaM to MARCKS yields PI3K α activation during a Ca^{2+} signal significantly faster than the phosphorylation of MARCKS by PKC α , it is likely that the initial phase of PI3K α activation is dominated by binding of Ca^{2+} -CaM to MARCKS, yet MARCKS is one of the most abundant signaling proteins in cells ($\sim 10 \mu\text{M}$);^{32,68} thus, it is unlikely there is sufficient Ca^{2+} -CaM to fully occupy MARCKS even on a specific region of the plasma membrane given that (i) the total CaM concentration may be lower ($\sim 9 \mu\text{M}$) than the MARCKS concentration, (ii) the local Ca^{2+} stoichiometry at the plasma membrane may not be high enough to fully load the local CaM population except in the vicinity of open Ca^{2+} channels,^{69,70} and (iii) once loaded, Ca^{2+} -CaM can bind to a large and diverse pool of target proteins besides MARCKS.⁴²⁻⁴⁵ It follows that the rapid, partial activation of PI3K α by binding of CaM to MARCKS will be followed by a slower, more complete activation as PKC α phosphorylates MARCKS, further reducing the free, unphosphorylated MARCKS subpopulation responsible for sequestering PIP₂. Moreover, the phosphorylated MARCKS generated by PKC α is unavailable for PIP₂ sequestration until its covalent modifications are reversed by a phosphatase, while the MARCKS blocked by Ca^{2+} -CaM can rapidly be available for PIP₂ binding as soon as the noncovalent complex dissociates. Thus, the time scales of PI3K α activation and inhibition via modulation of MARCKS are both considerably faster for CaM than for PKC α , and the presence of both activators allows cells to generate PIP₃ signals of different temporal patterns and durations.

An alternative mechanism proposed for CaM stimulation of PI3K α lipid kinase activity, namely the direct binding of CaM to PI3K α ,⁵³⁻⁵⁵ appears to play a negligible role in the activation of PI3K α , at least relative to CaM-triggered, MARCKS-mediated stimulation of PI3K α . However, these findings do not rule out activation of other PI3K isoforms via direct binding of CaM to the lipid kinase. Further studies will be needed to determine whether specific PI3K isoforms are responsible for previous reports of direct, CaM-triggered stimulation of PIP₃ production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

Work was funded by National Institutes of Health (NIH) Grant R01 GM063235 (to J.J.F.), by MC Grant U10518430 (to R.L.W.), and NIH Grant T32 GM065103 (to G.H.S.).

ABBREVIATIONS

PI3K α

phosphoinositide-3-kinase isoform α

CaM

calmodulin

MARCKS

myristoylated alanine rich C kinase substrate

pY

PDGFR-related phosphotyrosine peptide

PKC α

protein kinase C isoform α

Sfp

phospho-pantethienyl-transferase

AF555

Alexa Fluor 555

PE

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine

PS

phosphatidylserine or 1,2-dioleoyl-*sn*glycero-3-phospho-L-serine

PI(4,5)P₂ or PIP₂

1,2-dioleoyl-*sn*glycero-3-phosphoinositol 4,5-diphosphate

PI(3,4,5)P₃ or PIP₃

1,2-dioleoyl-*sn*-glycero-3-phosphoinositol 3,4,5-triphosphate

diacylglycerol or DAG

1,2-dioleoyl-*sn*-glycerol

SUV

sonicated unilamellar vesicle

DTT

dithiothreitol

EGTA

ethylene glycol tetraacetic acid

TIRFM

total internal reflection fluorescence microscopy

SEM

standard error of the mean

References

1. Falke JJ, Ziemba BP. Interplay between phosphoinositide lipids and calcium signals at the leading edge of chemotaxing ameboid cells. *Chem Phys Lipids*. 2014; 182:73–79. [PubMed: 24451847]
2. Swaney KF, Huang CH, Devreotes PN. Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu Rev Biophys*. 2010; 39:265–289. [PubMed: 20192768]
3. Stephens L, Milne L, Hawkins P. Moving towards a better understanding of chemotaxis. *Curr Biol*. 2008; 18:R485–494. [PubMed: 18522824]
4. Wu D. Signaling mechanisms for regulation of chemotaxis. *Cell Res*. 2005; 15:52–56. [PubMed: 15686628]
5. Niggli V. Signaling to migration in neutrophils: importance of localized pathways. *Int J Biochem Cell Biol*. 2003; 35:1619–1638. [PubMed: 12962702]
6. Beerman RW, Matty MA, Au GG, Looger LL, Choudhury KR, Keller PJ, Tobin DM. Direct In Vivo Manipulation and Imaging of Calcium Transients in Neutrophils Identify a Critical Role for Leading-Edge Calcium Flux. *Cell Rep*. 2015; 13:2107–2117. [PubMed: 26673320]
7. Wei C, Wang X, Chen M, Ouyang K, Song LS, Cheng H. Calcium flickers steer cell migration. *Nature*. 2009; 457:901–905. [PubMed: 19118385]
8. Evans JH, Falke JJ. Ca²⁺ influx is an essential component of the positive-feedback loop that maintains leading-edge structure and activity in macrophages. *Proc Natl Acad Sci U S A*. 2007; 104:16176–16181. [PubMed: 17911247]
9. von Philipsborn A, Bastmeyer M. Mechanisms of gradient detection: a comparison of axon pathfinding with eukaryotic cell migration. *Int Rev Cytol*. 2007; 263:1–62. [PubMed: 17725964]
10. Kumar G, Ho CC, Co CC. Cell-Substrate Interactions Feedback to Direct Cell Migration along or against Morphological Polarization. *PLoS One*. 2015; 10:e0133117. [PubMed: 26186588]
11. Charest PG, Firtel RA. Feedback signaling controls leading-edge formation during chemotaxis. *Curr Opin Genet Dev*. 2006; 16:339–347. [PubMed: 16806895]
12. Artemenko Y, Lampert TJ, Devreotes PN. Moving towards a paradigm: common mechanisms of chemotactic signaling in Dictyostelium and mammalian leukocytes. *Cell Mol Life Sci*. 2014; 71:3711–3747. [PubMed: 24846395]
13. Foxman EF, Kunkel EJ, Butcher EC. Integrating conflicting chemotactic signals. The role of memory in leukocyte navigation. *J Cell Biol*. 1999; 147:577–588. [PubMed: 10545501]
14. Kolsch V, Charest PG, Firtel RA. The regulation of cell motility and chemotaxis by phospholipid signaling. *J Cell Sci*. 2008; 121:551–559. [PubMed: 18287584]
15. Maree AF, Grieneisen VA, Edelstein-Keshet L. How cells integrate complex stimuli: the effect of feedback from phosphoinositides and cell shape on cell polarization and motility. *PLoS Comput Biol*. 2012; 8:e1002402. [PubMed: 22396633]
16. Eckert RE, Neuder LE, Park J, Adler KB, Jones SL. Myristoylated alanine-rich C-kinase substrate (MARCKS) protein regulation of human neutrophil migration. *Am J Respir Cell Mol Biol*. 2010; 42:586–594. [PubMed: 19574534]
17. Nairn AC, Aderem A. Calmodulin and protein kinase C cross-talk: the MARCKS protein is an actin filament and plasma membrane cross-linking protein regulated by protein kinase C phosphorylation and by calmodulin. *Ciba Found Symp*. 1992; 164:145–154. discussion 154–161. [PubMed: 1395931]
18. Roberts AW, Kim C, Zhen L, Lowe JB, Kapur R, Petryniak B, Spaetti A, Pollock JD, Borneo JB, Bradford GB, Atkinson SJ, Dinauer MC, Williams DA. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity*. 1999; 10:183–196. [PubMed: 10072071]
19. Allen WE, Zicha D, Ridley AJ, Jones GE. A role for Cdc42 in macrophage chemotaxis. *J Cell Biol*. 1998; 141:1147–1157. [PubMed: 9606207]
20. Yang HW, Shin MG, Lee S, Kim JR, Park WS, Cho KH, Meyer T, Do Heo W. Cooperative activation of PI3K by Ras and Rho family small GTPases. *Mol Cell*. 2012; 47:281–290. [PubMed: 22683270]

21. Spitaler M, Cantrell DA. Protein kinase C and beyond. *Nat Immunol.* 2004; 5:785–790. [PubMed: 15282562]
22. Antal CE, Newton AC. Tuning the signalling output of protein kinase C. *Biochem Soc Trans.* 2014; 42:1477–1483. [PubMed: 25399557]
23. Zhang H, Sun C, Glogauer M, Bokoch GM. Human neutrophils coordinate chemotaxis by differential activation of Rac1 and Rac2. *J Immunol.* 2009; 183:2718–2728. [PubMed: 19625648]
24. Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE. Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J Immunol.* 2003; 170:2647–2654. [PubMed: 12594293]
25. Carnevale KA, Cathcart MK. Protein kinase C beta is required for human monocyte chemotaxis to MCP-1. *J Biol Chem.* 2003; 278:25317–25322. [PubMed: 12724308]
26. Ziemba BP, Burke JE, Masson G, Williams RL, Falke JJ. Regulation of PI3K by PKC and MARCKS: Single-Molecule Analysis of a Reconstituted Signaling Pathway. *Biophys J.* 2016; 110:1811–1825. [PubMed: 27119641]
27. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, Messing RO. Protein kinase C isozymes and the regulation of diverse cell responses. *Am J Physiol.* 2000; 279:L429–L438.
28. Rosen A, Keenan KF, Thelen M, Nairn AC, Aderem A. Activation of protein kinase C results in the displacement of its myristoylated, alanine-rich substrate from punctate structures in macrophage filopodia. *J Exp Med.* 1990; 172:1211–1215. [PubMed: 2212950]
29. Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature.* 1992; 356:618–622. [PubMed: 1560845]
30. Kim J, Blackshear PJ, Johnson JD, McLaughlin S. Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin. *Biophys J.* 1994; 67:227–237. [PubMed: 7918991]
31. Verghese GM, Johnson JD, Vasulka C, Haupt DM, Stumpo DJ, Blackshear PJ. Protein kinase C-mediated phosphorylation and calmodulin binding of recombinant myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein. *J Biol Chem.* 1994; 269:9361–9367. [PubMed: 8132675]
32. Gambhir A, Hangyas-Mihalyne G, Zaitseva I, Cafiso DS, Wang J, Murray D, Pentyla SN, Smith SO, McLaughlin S. Electrostatic sequestration of PIP2 on phospholipid membranes by basic/aromatic regions of proteins. *Biophys J.* 2004; 86:2188–2207. [PubMed: 15041659]
33. Sundaram M, Cook HW, Byers DM. The MARCKS family of phospholipid binding proteins: regulation of phospholipase D and other cellular components. *Biochem Cell Biol.* 2004; 82:191–200. [PubMed: 15052337]
34. Yarmola EG, Edison AS, Lenox RH, Bubb MR. Actin filament cross-linking by MARCKS: characterization of two actin-binding sites within the phosphorylation site domain. *J Biol Chem.* 2001; 276:22351–22358. [PubMed: 11294839]
35. Smith BA, Padrick SB, Doolittle LK, Daugherty-Clarke K, Correa IR Jr, Xu MQ, Goode BL, Rosen MK, Gelles J. Three-color single molecule imaging shows WASP detachment from Arp2/3 complex triggers actin filament branch formation. *eLife.* 2013; 2:e01008. [PubMed: 24015360]
36. Sohn RH, Chen J, Koblan KS, Bray PF, Goldschmidt-Clermont PJ. Localization of a binding site for phosphatidylinositol 4,5-bisphosphate on human profilin. *J Biol Chem.* 1995; 270:21114–21120. [PubMed: 7673143]
37. DiNubile MJ, Huang S. Capping of the barbed ends of actin filaments by a high-affinity profilin-actin complex. *Cell Motil Cytoskeleton.* 1997; 37:211–225. [PubMed: 9227851]
38. McLaughlin S, Wang J, Gambhir A, Murray D. PIP(2) and proteins: interactions, organization, and information flow. *Annu Rev Biophys Biomol Struct.* 2002; 31:151–175. [PubMed: 11988466]
39. Zhang M, Tanaka T, Ikura M. Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nat Struct Biol.* 1995; 2:758–767. [PubMed: 7552747]
40. Tano JY, Vazquez G. Requirement for non-regulated, constitutive calcium influx in macrophage survival signaling. *Biochem Biophys Res Commun.* 2011; 407:432–437. [PubMed: 21414290]

41. Sezzi ML, De Luca G, Materazzi M, Bellelli L. Effects of a calcium-antagonist (flunarizine) on cancer cell movement and phagocytosis. *Anticancer Res.* 1985; 5:265–271. [PubMed: 4015040]
42. Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB, Bax A. Solution structure of a calmodulin-target peptide complex by multidimensional NMR. *Science.* 1992; 256:632–638. [PubMed: 1585175]
43. Tidow H, Nissen P. Structural diversity of calmodulin binding to its target sites. *FEBS J.* 2013; 280:5551–5565. [PubMed: 23601118]
44. Peersen OB, Madsen TS, Falke JJ. Intermolecular tuning of calmodulin by target peptides and proteins: differential effects on Ca²⁺ binding and implications for kinase activation. *Protein Sci.* 1997; 6:794–807. [PubMed: 9098889]
45. Yap KL, Kim J, Truong K, Sherman M, Yuan T, Ikura M. Calmodulin target database. *J Struct Funct Genomics.* 2000; 1:8–14. [PubMed: 12836676]
46. Qin Z, Cafiso DS. Membrane structure of protein kinase C and calmodulin binding domain of myristoylated alanine rich C kinase substrate determined by site-directed spin labeling. *Biochemistry.* 1996; 35:2917–2925. [PubMed: 8608129]
47. Porumb T, Crivici A, Blackshear PJ, Ikura M. Calcium binding and conformational properties of calmodulin complexed with peptides derived from myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP). *Eur Biophys J.* 1997; 25:239–247. [PubMed: 9112755]
48. Yamauchi E, Nakatsu T, Matsubara M, Kato H, Taniguchi H. Crystal structure of a MARCKS peptide containing the calmodulin-binding domain in complex with Ca²⁺-calmodulin. *Nat Struct Biol.* 2003; 10:226–231. [PubMed: 12577052]
49. Glaser M, Wanaski S, Buser CA, Boguslavsky V, Rashidzada W, Morris A, Rebecchi M, Scarlata SF, Runnels LW, Prestwich GD, Chen J, Aderem A, Ahn J, McLaughlin S. Myristoylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. *J Biol Chem.* 1996; 271:26187–26193. [PubMed: 8824266]
50. Elferink JG, Deierkauf M, Riemersma JC. Involvement of calmodulin in granulocyte chemotaxis: the effect of calmodulin inhibitors. *Res Commun Chem Pathol Pharmacol.* 1982; 38:77–84. [PubMed: 7146623]
51. Gauthier ML, O'Day DH. Detection of calmodulin-binding proteins and calmodulin-dependent phosphorylation linked to calmodulin-dependent chemotaxis to folic and cAMP in *Dictyostelium*. *Cell Signalling.* 2001; 13:575–584. [PubMed: 11483410]
52. Verploegen S, van Leeuwen CM, van Deutekom HW, Lammers JW, Koenderman L, Coffier PJ. Role of Ca²⁺/calmodulin regulated signaling pathways in chemoattractant induced neutrophil effector functions. Comparison with the role of phosphatidylinositol-3 kinase. *Eur J Biochem.* 2002; 269:4625–4634. [PubMed: 12230575]
53. Joyal JL, Burks DJ, Pons S, Matter WF, Vlahos CJ, White MF, Sacks DB. Calmodulin activates phosphatidylinositol 3-kinase. *J Biol Chem.* 1997; 272:28183–28186. [PubMed: 9353264]
54. Fischer R, Julsgart J, Berchtold MW. High affinity calmodulin target sequence in the signalling molecule PI 3-kinase. *FEBS Lett.* 1998; 425:175–177. [PubMed: 9541031]
55. Nussinov R, Muratcioglu S, Tsai CJ, Jang H, Gursoy A, Keskin O. The Key Role of Calmodulin in KRAS-Driven Adenocarcinomas. *Mol Cancer Res.* 2015; 13:1265–1273. [PubMed: 26085527]
56. Hon WC, Berndt A, Williams RL. Regulation of lipid binding underlies the activation mechanism of class IA PI3-kinases. *Oncogene.* 2012; 31:3655–3666. [PubMed: 22120714]
57. Yin J, Lin AJ, Golan DE, Walsh CT. Site-specific protein labeling by Sfp phosphopantetheinyl transferase. *Nat Protoc.* 2006; 1:280–285. [PubMed: 17406245]
58. Knight JD, Lerner MG, Marciano-Velazquez JG, Pastor RW, Falke JJ. Single molecule diffusion of membrane-bound proteins: window into lipid contacts and bilayer dynamics. *Biophys J.* 2010; 99:2879–2887. [PubMed: 21044585]
59. Ziemba BP, Li J, Landgraf KE, Knight JD, Voth GA, Falke JJ. Single-molecule studies reveal a hidden key step in the activation mechanism of membrane-bound protein kinase C- α . *Biochemistry.* 2014; 53:1697–1713. [PubMed: 24559055]

60. Rhyner JA, Koller M, Durussel-Gerber I, Cox JA, Strehler EE. Characterization of the human calmodulin-like protein expressed in *Escherichia coli*. *Biochemistry*. 1992; 31:12826–12832. [PubMed: 1334432]
61. Gopalakrishna R, Anderson WB. Ca²⁺-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-Sepharose affinity chromatography. *Biochem Biophys Res Commun*. 1982; 104:830–836. [PubMed: 6803791]
62. Hayashi N, Matsubara M, Takasaki A, Titani K, Taniguchi H. An expression system of rat calmodulin using T7 phage promoter in *Escherichia coli*. *Protein Expression Purif*. 1998; 12:25–28.
63. Knight JD, Falke JJ. Single-molecule fluorescence studies of a PH domain: new insights into the membrane docking reaction. *Biophys J*. 2009; 96:566–582. [PubMed: 19167305]
64. Nair PM, Salaita K, Petit RS, Groves JT. Using patterned supported lipid membranes to investigate the role of receptor organization in intercellular signaling. *Nat Protoc*. 2011; 6:523–539. [PubMed: 21455188]
65. Ziemba BP, Knight JD, Falke JJ. Assembly of membrane-bound protein complexes: detection and analysis by single molecule diffusion. *Biochemistry*. 2012; 51:1638–1647. [PubMed: 22263647]
66. Sbalzarini IF, Koumoutsakos P. Feature point tracking and trajectory analysis for video imaging in cell biology. *J Struct Biol*. 2005; 151:182–195. [PubMed: 16043363]
67. Bers DM, Patton CW, Nuccitelli R. A practical guide to the preparation of Ca(2+) buffers. *Methods Cell Biol*. 2010; 99:1–26. [PubMed: 21035681]
68. McLaughlin S, Wang J, Gambhir A, Murray D. PIP(2) and proteins: interactions, organization, and information flow. *Annu Rev Biophys Biomol Struct*. 2002; 31:151–175. [PubMed: 11988466]
69. Parekh AB. Ca²⁺ microdomains near plasma membrane Ca²⁺ channels: impact on cell function. *J Physiol*. 2008; 586:3043–3054. [PubMed: 18467365]
70. Neher E. Usefulness and limitations of linear approximations to the understanding of Ca⁺⁺ signals. *Cell Calcium*. 1998; 24:345–357. [PubMed: 10091004]
71. Black DJ, Tran QK, Persechini A. Monitoring the total available calmodulin concentration in intact cells over the physiological range in free Ca²⁺. *Cell Calcium*. 2004; 35:415–425. [PubMed: 15003851]
72. Kakiuchi S, Yasuda S, Yamazaki R, Teshima Y, Kanda K, Kakiuchi R, Sobue K. Quantitative determinations of calmodulin in the supernatant and particulate fractions of mammalian tissues. *J Biochem*. 1982; 92:1041–1048. [PubMed: 7174634]
73. Sarpel G, Barp AN, Lubansky HJ, Omachi A. Erythrocyte phosphate content in Huntington's disease. *Neurosci Lett*. 1982; 31:91–96. [PubMed: 6214732]
74. Lodish, H., Berk, A., Zipursky, S., Matsudaira, P., Baltimore, D., Darnell, J. *Molecular and Cellular Biology*. 4. W. H. Freeman and Co; New York: 2000.
75. Ryschon TW, Rosenstein DL, Rubinow DR, Niemela JE, Elin RJ, Balaban RS. Relationship between skeletal muscle intracellular ionized magnesium and measurements of blood magnesium. *J Lab Clin Med*. 1996; 127:207–213. [PubMed: 8636650]
76. Garfinkel L, Altschuld RA, Garfinkel D. Magnesium in cardiac energy metabolism. *J Mol Cell Cardiol*. 1986; 18:1003–1013. [PubMed: 3537318]
77. Maughan D. Diffusible magnesium in frog skeletal muscle cells. *Biophys J*. 1983; 43:75–80. [PubMed: 6603873]
78. Hein MY, Hubner NC, Poser I, Cox J, Nagaraj N, Toyoda Y, Gak IA, Weisswange I, Mansfeld J, Buchholz F, Hyman AA, Mann M. A human interactome in 3 quantitative dimensions organized by stoichiometries and abundances. *Cell*. 2015; 163:712–723. [PubMed: 26496610]
79. Kim MS, et al. A draft map of the human proteome. *Nature*. 2014; 509:575–581. [PubMed: 24870542]
80. Martens L, Van Damme P, Van Damme J, Staes A, Timmerman E, Ghesquiere B, Thomas GR, Vandekerckhove J, Gevaert K. The human platelet proteome mapped by peptide-centric proteomics: a functional protein profile. *Proteomics*. 2005; 5:3193–3204. [PubMed: 16038019]
81. Milo R. What is the total number of protein molecules per cell volume? A call to rethink some published values. *BioEssays*. 2013; 35:1050–1055. [PubMed: 24114984]

Figure 1.

Working model for the hypothesized Ca^{2+} -CaM-MARCKS- PIP_2 -PI3K- PIP_3 circuit at the leading edge membrane of a chemotaxing macrophage (see text). Calmodulin (CaM), a peptide corresponding to the PIP_2 binding region of MARCKS (MARCKS_p), phosphoinositide-3-kinase (PI3K), and key effector cofactors and proteins are presented on the cytoplasmic leaflet of the leading edge membrane (blue and yellow bands representing the headgroup and acyl tail lipid monolayer regions, respectively). Ca^{2+} -occupied CaM is known to form a complex with the MARCKS peptide, which is proposed to release sequestered PI(4,5)P_2 (PIP_2). Each MARCKS peptide is known to sequester up to four PIP_2 lipids (labeled as $n\text{PIP}_2$ in the diagram). Newly released PIP_2 , which serves as both a membrane binding target and a substrate lipid for PI3K, activates PI3K lipid kinase activity yielding phospho-conversion of PIP_2 to the signaling lipid PI(3,4,5)P_3 (PIP_3). Downstream PIP_3 recruits several signaling proteins, including PDK1 and PKB/AKT1, to the leading edge membrane, where they participate in the signaling network that controls leading edge expansion up an attractant gradient.

Figure 2.

Domain structures of protein constructs employed in this study. The full length, heterodimeric construct of phosphoinositide-3-kinase isoform α (PI3K α) possesses an N-terminal six-His affinity purification tag on the p110 α catalytic subunit and an 11-residue, N-terminal enzymatic labeling tag on the p85 α regulatory subunit. The full length CaM construct is comprised of an N-terminal cysteine designated for maleimide labeling and dual EF-hand domains. The peptide construct of the PIP₂ binding domain (PBD) of the myristoylated alanine rich C kinase substrate (MARCKS) corresponds to MARCKS residues 151–175, preceded by an N-terminal cysteine as a labeling site.

Figure 3.

Single-molecule analysis of MARCKS_p and PI3K α membrane binding. (A) Single-molecule TIRF quantitation of fluorescent MARCKS_p bound to PE/PS/PIP₂ bilayers. (B) SM TIRF quantitation of binding of PI3K α to supported lipid bilayers. In both figures, components were at concentrations listed in Table 1. Shown are the average total numbers of molecules per TIRF field bound to bilayers comprised of a 3:1 PE:PS ratio with 1 mol % PIP₂. Each condition average was determined from at least 490 temporally isolated frames extracted from at least four separate movies during at least nine separate experiments. Error bars are standard errors of the mean. All measurements were taken at 21.5 ± 0.5 °C in 100 mM KCl, 20 mM HEPES (pH 6.9) (optimal pH for PI3K α activity), 15 mM NaCl, 5 mM glutathione, 2.0 mM EGTA, 1.9 mM Ca²⁺, 0.5 mM Mg²⁺, 1.0 mM Mg²⁺-ATP, 100 μ g mL⁻¹ BSA, and 0.05% CHAPS. Under these conditions, the EGTA/ATP/Ca²⁺/Mg buffering system yields 10 μ M free Ca²⁺ and 0.5 mM free Mg²⁺.

Figure 4.

Effects of the MARCKS peptide and CaM on PI3K α lipid kinase activity. Regulation of PI3K α lipid kinase activity quantified by the single-molecule TIRF assay, in which each new PIP₃ lipid is detected by binding by the fluorescent high-affinity PIP₃ sensor GRP1-PH domain protein. (A) Time course of production of PIP₃ by PI3K α , illustrating the effects of CaM and MARCKS_p on the net production of product PIP₃ molecules per TIRF field. The time course was initiated 1 min after the addition of CaM by adding PI3K α . (B) Slopes of the time courses in panel A, indicating that CaM recovers PI3K α lipid kinase activity lost due to inhibition by MARCKS_p. (C) Specific lipid kinase activity per membrane-bound PI3K α enzyme under the reaction conditions detailed in Figure 3. The specific lipid kinase activity was determined from the ratio of total kinase activity (B) to the density of bound kinase under each condition (Figure 3B). Within error, the specific activity of the membrane-bound PI3K α molecule is similar under all four conditions, indicating that large overall activity differences arise from different surface densities of membrane-bound PI3K α . Experimental conditions and statistical analyses are identical to those listed in Figure 3. Each condition average was determined from at least 490 temporally isolated frames extracted from at least four separate movies during at least nine separate experiments. Error bars are standard errors of the mean.

Figure 5.

Time course of the effects of CaM on MARCKS and PI3K α membrane binding, and comparison to PKC α . Single-molecule surface density of MARCKS and PI3K α measured as described in the legend of Figure 3 following addition of Ca²⁺-activated CaM or PKC α . The indicated curves are nonlinear least-squares best fits to a decreasing or increasing exponential model, respectively, where the fitted parameters are as follows. (A) Dissociation time course showing loss of membrane-bound MARCKS (total concentration of 17 μ M) after addition of 0.3 μ M PKC α (rate constant $k = 0.33 \pm 0.03 \text{ min}^{-1}$, and asymptote $C = 36.9 \pm 1.5\%$) or 20 μ M CaM ($k = 45 \text{ min}^{-1}$, and $C = 22 \pm 5\%$). (B) Association time course showing PI3K α (total concentration of 2 nM) binding to the membrane following addition of 0.3 μ M PKC α (rate constant $k = 1.3 \pm 0.3 \text{ min}^{-1}$, and asymptote $C = 83 \pm 4\%$) or 20 μ M CaM ($k = 8 \pm 7 \text{ min}^{-1}$, and $C = 96 \pm 15\%$). Experimental conditions and statistical analyses are identical to those listed in Figure 3. Each condition average was determined from at least 490 temporally isolated frames extracted from at least four separate movies during at least six separate experiments. Error bars are standard errors of the mean.

Table 1

Comparison of Intracellular Conditions with the Experimental Conditions Employed in Single-Molecule TIRF Measurements

	<i>in vivo</i> conditions	<i>in vitro</i> SM experiment
calmodulin	~9 μM ^{71,72,78}	20 μM
MARCKS	~10 μM ^{32,68}	17 μM
PI3K	~3–16 nM ^{78, a}	2 nM
ATP	1 mM ⁷³	1 mM
Na ⁺	12 mM ⁷⁴	15 mM
K ⁺	140 mM ⁷⁴	100 mM
free Mg ²⁺	0.5 mM ^{75–77}	0.5 mM
local Ca ²⁺ signal	1–10 μM ^{69,70}	10 μM
PKC	0.3 μM ^{79–81}	0.3 μM

^a Unpublished observation from N. Tsolakos, P. Hawkins, and L. Stephens of the Babraham Institute, Cambridgeshire, U.K.