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Engineering *de Novo* Membrane-Mediated Protein–Protein Communication Networks

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Supporting Information

ABSTRACT: Mechanical properties of biological membranes are known to regulate membrane protein function. Despite this, current models of protein communication typically feature only direct protein–protein or protein–small molecule interactions. Here we show for the first time that, by harnessing nanoscale mechanical energy within biological membranes, it is possible to promote controlled communication between proteins. By coupling lipid–protein modules and matching their response to the mechanical properties of the membrane, we have shown that the action of phospholipase A_2 on acyl-based phospholipids triggers the opening of the mechanosensitive channel, MscL, by generating membrane asymmetry. Our findings confirm that the global physical properties of biological membranes can act as information pathways between proteins, a novel mechanism of membrane-mediated protein–protein communication that has important implications for (i) the underlying structure of signaling pathways, (ii) our understanding of *in vivo* communication networks, and (iii) the generation of building blocks for artificial protein networks.

The broader literature contains a significant body of evidence that protein function in cells is often modulated by the physical properties of biological membranes. In particular, stored curvature elastic stress,[1] surface charge density,[2] curvature,[3] and bilayer asymmetry[4,5] have been shown to regulate protein stability,[6] folding,[7,8] and activity[9−11] via well-characterized coupling of proteins with lipid bilayers. In contrast to lipid–protein interactions, where there is specific recognition of the lipid moieties by the protein, the broad mechanism of membrane–protein interaction is one in which the protein can sense a physical property of the membrane and respond accordingly, regardless of the chemical composition of the membrane. While it is well established that lipid-derived second messengers facilitate protein communication by exploiting the chemical properties of a membrane, little is known about the role that the physical properties of membranes play in protein communication, and to date there have been no reports of protein A–membrane–protein B (PA-M-PB) networks. Although individual PA-M and M-PB modules have been characterized, combining them to build *de novo* higher-order networks has proven a difficult task. Modes of interaction between proteins and membranes are regulated by different global properties of the membrane. The requirement for optimal interaction between the two components will impose specific constraints on the magnitude of these properties. Using such modules as system components within *in vitro* systems imposes the requirement of coupling the mode, magnitude, and spatial dynamics of particular protein–membrane interactions. Here we report the first example of user-defined PA-M-PB communication, mediated by the physical properties of the lipid bilayer.

This PA-M-PB network consists of the secretory phospholipase A_2 ($sPLA_2$), the mechanosensitive channel of large conductance (MscL) mutated to enable chemically gated gain of function channels (G22C, F93W MscL),[12] and a host lipid bilayer composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) at a 1:1 ratio which has been shown to optimize chemical gating of MscL. This specific PA-M-PB network (Figure 1) will be denoted as the $sPLA_2$-M-MscL network.[13]

$sPLA_2$ is a small (~16 kDa), calcium-dependent enzyme that catalyzes lipid hydrolysis specifically at the sn-2 acyl bond of phospholipids, yielding the release of free fatty acid and lysophospholipids.[14] The vastly slower flip-flop rates of lysophospholipids compared with fatty acids result in the formation of asymmetric bilayers.[15,16] Interestingly, phospholipases such as $sPLA_2$ are found in elevated levels in cancers,[17] and it has been shown previously that their activity depends strongly on the physical state and the microstructure of the liposome.[18] At low PLA_2 concentrations (<1 μM) and low $CaCl_2$ concentrations, the structure of large unilamellar vesicles (LUVs) is not disrupted by hydrolysis.[18]

MscL is a mechanosensitive channel of *Escherichia coli* that opens in response to increases in membrane stress from extreme turgor and is involved in osmoregulation.[19] Physiological responses including touch and hearing in eukaryotes and chemotaxis and osmoregulation in prokaryotes are driven by modification of intrabilayer pressure gradients, a control mechanism that is known to regulate mechanosensitive channels.[20,21]

The chemically gated gain-of-function mutant of MscL containing the G22C substitution located in the transmembrane helix 1 (TM1) has been shown to activate upon addition of sulfhydryls such as trimethylammonium ethyl

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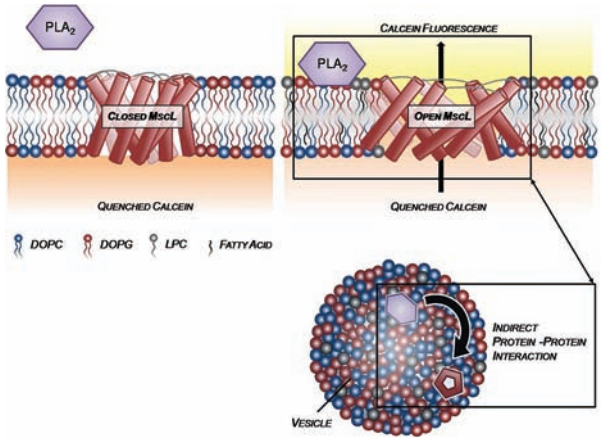

Figure 1. sPLA$_2$-M-MscL network. sPLA$_2$ hydrolyzes lipids at the sn-2 acyl bond in the DOPC:DOPG (1:1) bilayer, producing fatty acid and lysophospholipid. This results in membrane asymmetry as the rate of lysophospholipid flip-flop is orders of magnitude slower than that of fatty acids. Membrane asymmetry is sensed by MscL embedded in the bilayer, leading to activation of the sPLA$_2$-M-MscL network and release of the calcein contained within the vesicles.

methanethiosulfonate (MTSET), even in the absence of increases in bilayer pressure.[12]

Previous studies have shown that MscL channel opening is modulated by the asymmetric addition of lysophosphatidylcholine (LPC) to phosphatidylcholine (PC).[4] In effect, the sPLA$_2$-M and MscL-M systems represent two modules that generate or sense membrane asymmetry, respectively. By exploiting the fact that the rate of flip-flop of lysophospholipids is considerably slower than that of fatty acids, we have demonstrated that it is possible to couple the two P-M modules with sPLA$_2$-induced membrane asymmetry, promoting MscL opening in a predictable and controlled fashion.

The recombinant MscL channel was expressed, purified, and reconstituted in lipid vesicles in the presence of quenched (50 mM) calcein as described in the Supporting Information. MscL function was monitored using an adapted fluorescence-based assay,[22] which monitors the concentration-dependent changes in the fluorescence profile of calcein. As shown in Figure 1, upon MscL opening, calcein exits the vesicle through the MscL pore down a concentration gradient that is measured by an increase in fluorescence signal as calcein is diluted below its quenched concentration (>30 mM).[22]

This assay was employed to verify that reconstituted MscL channels were correctly folded and active by testing chemical (MTSET-induced) and mechanical (PLA$_2$-induced) gating sequentially or in parallel. Reconstituted samples treated with MTSET (Figure 2, solid and open black circles) showed a characteristic increase in fluorescence consistent with channel opening and release of calcein, confirming that MscL is correctly folded and active. MTSET added to liposomes without MscL had no effect on calcein leakage (data not shown), confirming that MTSET specifically causes MscL opening. Interestingly, addition of MTSET to previously mechanically gated channels (Figure 2, solid red squares) results in a further increase in fluorescence, suggesting that the molecular rearrangement through mechanical gating differs from that of chemical gating. It is suggested that PLA$_2$ operates via a scooting mechanism[23] whereby an enzyme molecule is tightly associated to a single vesicle and remains associated with that vesicle until all lipids are hydrolyzed. This may account for

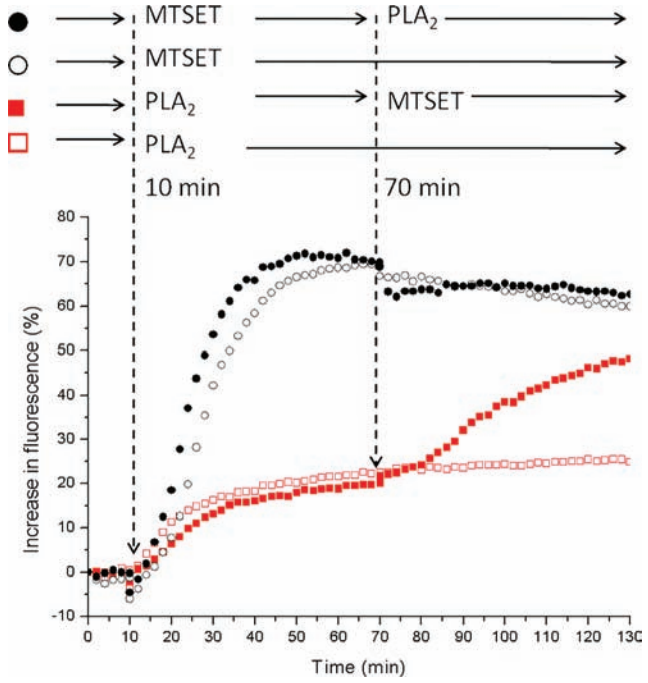

Figure 2. Chemical (1 mM MTSET) versus mechanical (10 nM PLA$_2$) activation of MscL reconstituted in DOPC:DOPG (1:1 w/w) liposomes. MscL activation is indicated by an increase in fluorescence caused by the dilution of quenched calcein as it exits the vesicle through the MscL pore: addition of (open red squares) PLA$_2$ (10 min), (solid red squares) PLA$_2$ (10 min) followed by addition of MTSET (70 min), (open black circles) MTSET (10 min), and (solid black circles) MTSET (10 min) followed by addition of PLA$_2$ (70 min). Data were normalized to the value at time = 0 min. The decrease in fluorescence at time = 10 and 70 min is caused by sample mixing.

the lower levels of calcein release (~60%) following mechanical (PLA$_2$-mediated) versus chemical (MTSET-mediated) activation of MscL at low PLA$_2$ concentrations (10 nM) and within this experimental time frame (<2 h).

Data presented in Figure 3 demonstrate activation of the sPLA$_2$-M-MscL network following addition of PLA$_2$ to preformed, calcein-containing DOPC:DOPG (1:1) vesicles reconstituted with MscL. A dose-dependent increase in fluorescence with respect to PLA$_2$ concentration was observed, demonstrating that the rate of calcein release via MscL is PLA$_2$ dependent. An increase in PLA$_2$ concentration is proposed to result in an increase in the rate of formation and levels of LPC in the outer bilayer leaflet, which in turn increases the level of asymmetry in the membrane and activates MscL through the sPLA$_2$-M-MscL network. Control experiments on calcein-containing liposomes without reconstituted MscL showed no calcein leakage upon addition of 10 nM PLA$_2$ within the experimental time frame (70 min). This demonstrates that the observed increase in fluorescence is a consequence of the protein coupling through the sPLA$_2$-M-MscL network and that the incorporation of LPC and fatty acid alone following PLA$_2$-mediated lipid hydrolysis (under these experimental conditions) does not result in calcein leakage. The apparent decrease in fluorescence observed in control samples (Figure 3, inset graph) is attributed to sample settling over the experimental time frame.

To confirm that PLA$_2$-M-MscL network activation is driven by changes in membrane asymmetry following an increase in LPC resulting from PLA$_2$ lipid hydrolysis and not by a direct

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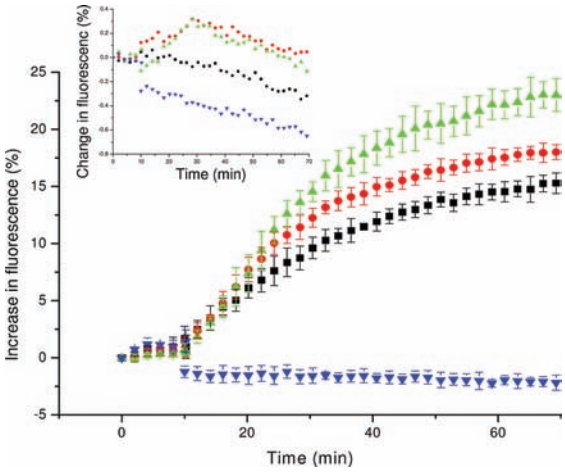

Figure 3. Concentration-dependent changes in calcein fluorescence following activation of MscL reconstituted in DOPC:DOPG (1:1 w/w) liposomes upon addition of PLA_2: (blue down-triangles) 0, (black squares) 0.16, (green triangles) 1.26, and (red circles) 10.00 nM concentrations of PLA_2 were added to proteoliposomes reconstituted with MscL at 10 min. Inset graph: control experiments of the same concentrations of PLA_2 added to liposomes in the absence of MscL at 10 min. Data were normalized to the first reading following addition of PLA_2 (time = 10 min). Errors are shown as the standard deviation of three measurements.

interaction between PLA_2 and MscL, control experiments were made using non-PLA_2-hydrolyzable lipid substrates in place of the PLA_2 lipid substrate DOPC. As shown in Figure 4 (solid red squares), proteoliposomes consisting solely of diether PC (100% 1,2-di-*O*-(9*Z*-octadecenyl)-*sn*-glycero-3-phosphocholine), which lack the target site (sn-2 acyl bond) for PLA_2 action, did not mediate MscL channel opening upon PLA_2 exposure. In contrast, diester PC proteoliposomes facilitated protein communication through the PLA_2-M-MscL network (solid black circles).

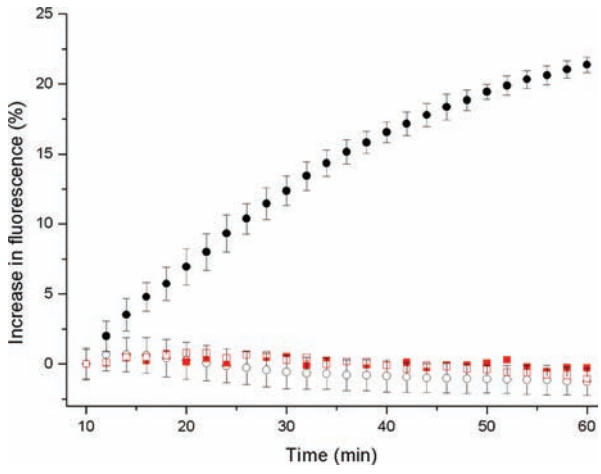

Figure 4. PLA_2 (10 nM)-induced MscL activation in (solid black circles) 100% DOPC and (solid red squares) 100% diether PC liposomes. Control experiments on liposomes without MscL consisting of (open black circles) 100% DOPC and (open red squares) 100% diether PC. Data were normalized to the first reading following addition of 10 nM PLA_2 (time = 10 min). Errors are shown as the standard deviation of three measurements.

Recent models for lipid biosynthesis and protein communication pathways have postulated frameworks that couple biochemical and biophysical features of the network.[24,25] These models predict and rely upon the presence of feedback between membranes and proteins driven by PA-M-PB type networks (and higher order networks) that increase the robustness of the steady state of the host biological system. To date, PA-M-PB networks have not been validated experimentally, and examples of membrane−protein interactions have been restricted to binary lipid bilayer−protein couples (PA-M).[11,26] The $sPLA_2$-M-MscL network represents the first experimental example of one of the key links in such regulatory frameworks: the ability for proteins to communicate directly through biological membranes. We have demonstrated that, by modifying the level of membrane asymmetry, we are able to control information flow through the $sPLA_2$-M-MscL network, either turning it off completely or fine-tuning the extent of communication between $sPLA_2$ and MscL.

Under these conditions, membrane-mediated protein−protein communication can be predictably controlled. Our work demonstrates that it is possible to reconstruct complex biological signaling pathways involving cytoplasmic proteins, membrane proteins, and the membrane itself *in vitro* by using logical engineering principles in tandem with well-characterized P-M binary modules. This will also drive the design and assembly of *de novo* networks and rewiring of existing pathways. In the future, such bottom-up approaches will enable the systematic characterization of biological signaling networks with respect to biomechanics and bioenergetics, allowing experimentalists to screen for and identify direct protein−protein and lipid−protein as well as membrane-mediated protein interactions. In addition, the robust nature of the $sPLA_2$-M-MscL network demonstrates that it is possible to hijack and manipulate component parts of a cell's machinery and re-route their function by design, a process which speaks to the future ambitions of *in vitro* synthetic biology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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