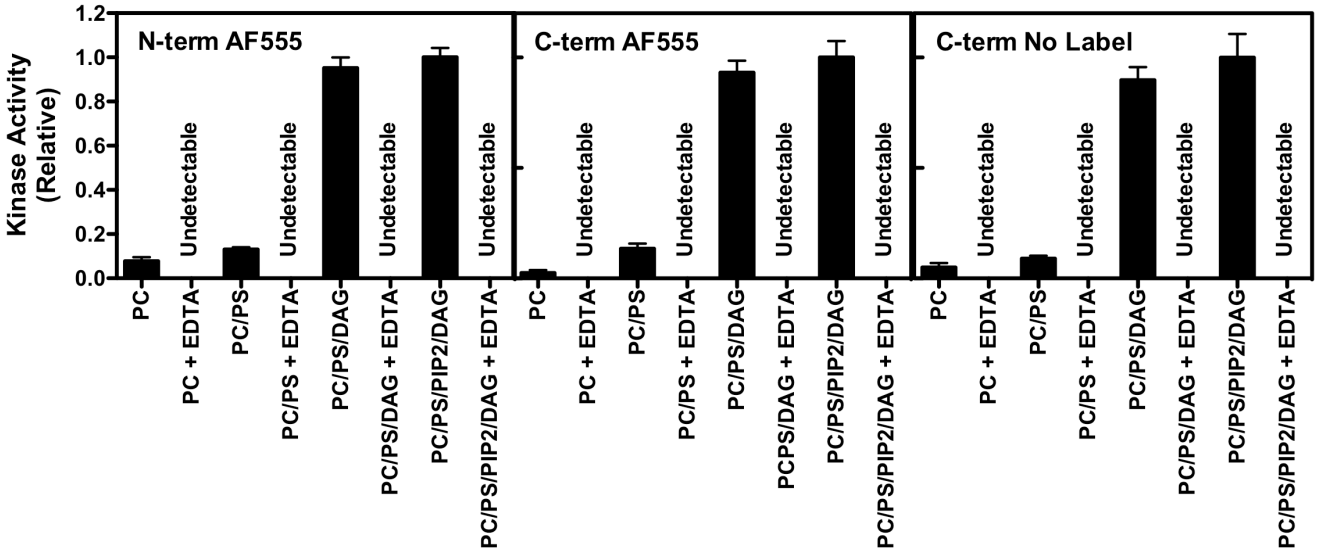
Supplemental Figure S1

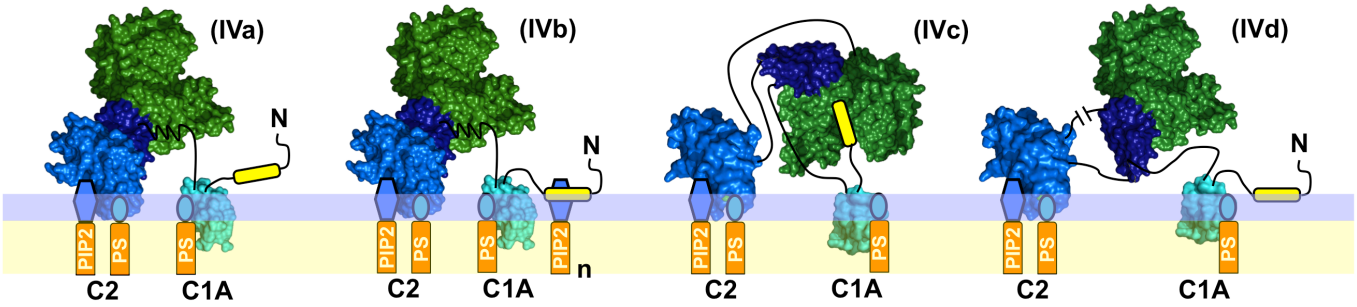
Effect of fluorescent label and its location on PKCα kinase activity and its regulation.



Total kinase activity of fluorescently-labeled and unlabeled PKCα was measured by the modified PepTag (Promega) assay described in Fig. 3 and Methods. The indicated lipid bilayer compositions (Table 1) were used to activate the enzyme, while PKC-specific phosphorylation of the PepTag target peptide was quantitated by electrophoresis. Each condition was repeated 2 times in duplicate (n = 4). Free Ca^{2+} was 6 μM in a physiological buffer (Methods), except in conditions labeled "EDTA" that contained 0.5 mM additional EDTA to lower the free Ca^{2+} to 0.5 nM, thereby testing whether Ca^{2+} regulation was functioning properly. In all experiments T = 22°C ± 0.5°C.

Supplemental Figure S2

Alternate Models of the Predominant Intermediate (IV)



Alternative molecular views of the newly discovered intermediate (IV), all consistent with the single molecule diffusion results since all states share the same C1A and C2 contacts with the bilayer. Additional studies are required to ascertain whether the working model (Fig. 10A) or an alternative model (perhaps one of these) best represent the slowly diffusing, predominant pre-DAG intermediate (IV) on the membrane surface.

Supplemental Table S1.

LC-MS/MS analysis of diglycerides in lipid mixtures to test for DAG contamination.

	DAG
	Concentration
3:1 PC:PS (+) 2% PIP_2	<0.5 ng/µl
3:1 PC:PS (+) 2% DAG	82.1 ng/µl
3:1 PC:PS (+) 2% DAG, 2% PIP_2	63.1 ng/µl
PIP_2 lipid	<0.5 ng/µl

The results indicate that the lipid mixtures employed herein did not contain detectable levels of diacylglycerol (DAG) except in samples to which DAG was added. **Methods**: The presence of DAGs was determined essentially as previously published using normal phase HPLC chromatography and tandem mass spectrometry (115). Briefly, a known aliquot of the liposome preparation (typically 5 uL in volume containing 0.32 µg of phospholipid) was diluted with distilled water (1 mL) to which 290 pmol of 1,2-DAG(20:0/20:0-d_5) internal standard was added and samples thoroughly mixed. An extraction solvent of 1:1 methyl tertiary butyl ether (MTBE)/hexane (1 mL) was added and sonicated. After centrifugation for 3 minutes at 3,000 RPM, the upper (organic) layer removed and a second extraction performed. The organic layers were combined then taken to dryness under vacuum. The dried extract was dissolved in dichloromethane (400 µL) and then converted to the urethane derivative using 2,4-difluorophenyl isocyanate (200 µg) and dimethylamino-pyridine (200 µg) heated with the sample extract at 60^{o}C for 30 minutes. After drying the sample with a gentle stream of nitrogen at ambient temperature, the extract was dissolved in MTBE/hexane (4/100; v/v). Normal phase chromatographic separations were carried out using a Phenomenex 50 x 2.1mm, 1.7m HILIC column. Solvent A was 100% isooctane, solvent B was 1:1 MTBE/isooctane. Solvent B at injection was held at 7% for one minute then programed up to 25% over the next 4 minutes. Solvent B was then increased to 85% over the next 2.5 minutes and held there for 4 minutes. A modifying solvent was added to the effluent, post-column at .05 ml/minute and consisted of 10mM ammonium acetate in 5%/95% (v/v) water in acetonitrile. The 1,2- and 1,3-DAG species were identified and quantitated using a neutral loss scan (190.3 Da, loss of the urethane derivative and ammonia) using a 4000 QTrap tandem quadrupole mass spectrometer (AB Sciex, Thornhill, Ontario, Canada) scanned from 600 to 1200 daltons at a scan rate of 3 scans per second.

Reference: Leiker, T. J., Barkley, R. M., and Murphy, R. C. (2011) Analysis of Diacylglycerol Molecular Species in Cellular Lipid Extracts by Normal-Phase LC-Electrospray Mass Spectrometry, Int J Mass Spectrom 305, 103-109.

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Supplemental Table S2.

Multi-state analysis, proposed membrane contacts, and predicted D for each construct-bilayer pairing.

	Measured *D* (Fractional Population) [a]				Predicted *D* Based on New Model [b]				Proposed Membrane Contacts [c]			
	PC/PS	PC/PS/ PIP2	PC/PS/ DAG	PC/PS/ PIP_2/DAG	PC/PS	PC/PS/ PIP2	PC/PS/ DAG	PC/PS/ PIP_2/DAG	PC/PS	PC/PS/ PIP2	PC/PS/DAG	PC/PS/ PIP_2/DAG
lipid	2.58 ± 0.11 (100%)	2.81 ± 0.12 (100%)	2.63 ± 0.03 (100%)	2.56 ± 0.10 (100%)	—	—	—	—	—	—	—	—
C1A (state 1)	1.63 ± 0.26 (29 ± 2%)	1.63 ± 0.65 (42 ± 7%)	1.39 ± 0.10 (30 ± 2%)	1.13 ± 0.15 (37 ± 4%)	—	—	—	—	$C1A_s$	$C1A_s$	$C1A_s$	$C1A_s$
C1A (state 2)	0.15 ± 0.02 (71 ± 2%)	0.08 ± 0.01 (58 ± 7%)	0.14 ± 0.02 (70 ± 2%)	0.08 ± 0.01 (63 ± 4%)	—	—	—	—	$C1A_d$	$C1A_d$	$C1A_d$	$C1A_d$
C1B (state 1)	0.66 ± 0.27 (61 ± 5%)	0.46 ± 0.15 (65 ± 4%)	0.99 ± 0.22 (55 ± 5%)	0.65 ± 0.12 (58 ± 11%)	—	—	—	—	$C1B_s$	$C1B_s$	$C1B_s$	$C1B_s$
C1B (state 2)	0.20 ± 0.12 (39 ± 5%)	0.06 ± 0.01 (35 ± 4%)	0.12 ± 0.04 (44 ± 5%)	0.07 ± 0.02 (42 ± 11%)	—	—	—	—	$C1B_d$	$C1B_d$	$C1B_d$	$C1B_d$
C2	1.45 ± 0.06 (100%)	1.07 ± 0.02 (100%)	1.41 ± 0.09 (100%)	1.00 ± 0.12 (100%)	—	—	—	—	$C2_s$	$C2_d$	$C2_s$	$C2_d$
C1A-C1B (state 1)	0.42 ± 0.11 (47 ± 4%)	0.34 ± 0.06 (39 ± 1%)	0.42 ± 0.07 (35 ± 3%)	0.11 ± 0.01 (45 ± 4%)	0.47 ± 0.19	0.36 ± 0.18	0.58 ± 0.13	0.07 ± 0.01	$C1A_s$ + $C1B_s$	$C1A_s$ + $C1B_s$	$C1A_s$ + $C1B_s$	$C1A_d$ + $C1B_s$
C1A-C1B (state 2)	0.11 ± 0.02 (53 ± 4%)	0.08 ± 0.02 (61 ± 1%)	0.11 ± 0.02 (65 ± 3%)	0.03 ± 0.01 (55 ± 4%)	0.12 ± 0.04	0.07 ± 0.02	0.12 ± 0.02	0.04 ± 0.01	$C1A_d$ + $C1B_s$	$C1A_d$ + $C1B_s$	$C1A_d$ + $C1B_s$	$C1A_d$ + $C1B_d$
C1A-C1B-C2 (state 1)	0.89 ± 0.23 (44 ± 4%)	0.29 ± 0.05 (45 ± 2%)	0.35 ± 0.04 (45 ± 8%)	0.29 ± 0.07 (61 ± 3%)	0.77 ± 0.08	0.28 ± 0.04	0.70 ± 0.07	0.29 ± 0.01	$C2_s$ + $C1A_s$	$C2_d$ + $C1A_s$ + $C1B_s$	$C2_s$ + $C1A_s$	$C2_d$ + $C1A_s$ + $C1B_s$
C1A-C1B-C2 (state 2)	0.13 ± 0.02 (56 ± 4%)	0.09 ± 0.02 (55 ± 2%)	0.10 ± 0.01 (55 ± 8%)	0.08 ± 0.01 (39 ± 3%)	0.14 ± 0.03	0.06 ± 0.01	0.06 ± 0.02	0.04 ± 0.01	$C2_s$ + $C1A_d$	$C2_d$ + $C1A_d$ + $C1B_s$	$C2_s$ + $C1A_d$ + $C1B_d$	$C2_d$ + $C1A_d$ + $C1B_d$
C1B-C2 (state 1)	1.63 ± 0.05 (58 ± 5%)	1.53 ± 0.19 (44 ± 4%)	1.46 ± 0.06 (65 ± 3%)	0.93 ± 0.07 (47 ± 7%)	1.45 ± 0.06	1.07 ± 0.02	1.41 ± 0.09	1.00 ± 0.12	$C2_s$	$C2_d$	$C2_s$	$C2_d$
C1B-C2 (state 2)	0.18 ± 0.01 (42 ± 5%)	0.20 ± 0.02 (66 ± 4%)	0.26 ± 0.03 (35 ± 3%)	0.12 ± 0.01 (53 ± 7%)	0.45 ± 0.02	0.32 ± 0.01	0.11 ± 0.04	0.07 ± 0.03	$C2_s$ + $C1B_s$	$C2_d$ + $C1B_s$	$C2_s$ + $C1B_d$	$C2_d$ + $C1B_d$
FL PKC (state 1)	0.59 ± 0.07 (71 ± 2%)	0.60 ± 0.16 (56 ± 4%)	0.52 ± 0.03 (76 ± 3%)	0.40 ± 0.07 (44 ± 7%)	0.76 ± 0.08	0.64 ± 0.05	0.69 ± 0.07	0.53 ± 0.08	$C2_s$ + $C1A_s$	$C2_d$ + $C1A_s$	$C2_s$ + $C1A_s$	$C2_d$ + $C1A_s$
FL PKC (state 2)	0.08 ± 0.01 (29 ± 2%)	0.10 ± 0.02 (44 ± 4%)	0.07 ± 0.01 (24 ± 3%)	0.08 ± 0.01 (56 ± 7%)	0.13 ± 0.03	0.07 ± 0.01	0.06 ± 0.02	0.04 ± 0.01	$C2_s$ + $C1A_d$	$C2_d$ + $C1A_d$	$C2_s$ + $C1A_d$ + $C1B_d$	$C2_d$ + $C1A_d$ + $C1B_d$

a Diffusion constants determined by single molecule TIRF analysis of at least 2500 diffusion tracks from 5 experiments ($n \geq 5$). Multicomponent diffusion constants ($\mu m^2/s$) for all constructs except lipid and C2 domain determined by a 2-component Rayleigh fit of each protein-lipid dataset. In the cases of lipid and C2 domain, the data were best fit to a 1-component distribution indicating one predominant speed for the diffusing species. In all experiments, free Ca^{2+} was 6 μM in a physiological buffer, T = 22°C ± 0.5°C.

b Predicted difusion constants ($\mu m^2/s$) for multidomain constructs determined by Equation 1. Component-matched (state 1 or state 2) diffusion constants of individual domains proposed to contact bilayer in the predicted dominant intermediate were combined to generate predicted diffusion constants for multidomain regulatory constructs.

c Proposed PKC domain regulatory domain membrane contacts in each protein-membrane combination assessed by SM TIRF. In each row, expected domain contacts with membrane and relative degree of protein insertion per domain are listed. Domain subscripts indicate degree of insertion ($_s$ = shallow, $_d$ = deep).