C2 Domains from Different Ca²⁺ Signaling Pathways Display Functional and Mechanistic Diversity†

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ABSTRACT: The ubiquitous C2 domain is a conserved Ca²⁺-triggered membrane-docking module that targets numerous signaling proteins to membrane surfaces where they regulate diverse processes critical for cell signaling. In this study, we quantitatively compared the equilibrium and kinetic parameters of C2 domains isolated from three functionally distinct signaling proteins: cytosolic phospholipase A₂-α (cPLA₂-α), protein kinase C-β (PKC-β), and synaptotagmin-IA (Syt-IA). The results show that equilibrium C2 domain docking to mixed phosphatidylcholine and phosphatidylserine membranes occurs at micromolar Ca²⁺ concentrations for the cPLA₂-α C2 domain, but requires 3- and 10-fold higher Ca²⁺ concentrations for the PKC-β and Syt-IA C2 domains ([Ca²⁺]₀ = 4.7, 16, 48 μM, respectively). The Ca²⁺-triggered membrane docking reaction proceeds in at least two steps: rapid Ca²⁺ binding followed by slow membrane association. The greater Ca²⁺ sensitivity of the cPLA₂-α domain results from its higher intrinsic Ca²⁺ affinity in the first step compared to the other domains. Assembly and disassembly of the ternary complex in response to rapid Ca²⁺ addition and removal, respectively, require greater than 400 μs for the cPLA₂-α domain, compared to 13 ms for the PKC-β domain and only 6 ms for the Syt-IA domain. Docking of the cPLA₂-α domain to zwitterionic lipids is triggered by the binding of two Ca²⁺ ions and is stabilized via hydrophobic interactions, whereas docking of either the PKC-β or the Syt-IA domain to anionic lipids is triggered by at least three Ca²⁺ ions and is maintained by electrostatic interactions. Thus, despite their sequence and architectural similarity, C2 domains are functionally specialized modules exhibiting equilibrium and kinetic parameters optimized for distinct Ca²⁺ signaling applications. This specialization is provided by the carefully tuned structural and electrostatic parameters of their Ca²⁺ and membrane-binding loops, which yield distinct patterns of Ca²⁺ coordination and contrasting mechanisms of membrane docking.

During signal transduction in eukaryotic cells, elevation of the second messenger Ca²⁺ directs diverse fundamental events such as cell division, motility, and contraction, activation of numerous intracellular pathways, secretion of bio-active compounds, and programmed cell death (1). The biochemical events underlying these processes are controlled by the spatial and temporal properties of Ca²⁺ transients generated as local puffs or sparks, or propagated as global spikes or waves. These transients are highly specialized and differ dramatically between cell types. For instance, at the active presynaptic zones of neuronal cells, brief localized transients exceeding 100 μM Ca²⁺ direct the release of neurotransmitters via vesicle fusion with the plasma membrane, thus resulting in transmission of signals to postsynaptic neurons on the millisecond time scale (2–4). Smaller Ca²⁺ oscillations that reach peaks of 1–10 μM Ca²⁺ pass through specialized regions of cardiac cells up to 10 times per second, thereby directing the contraction and relaxation phases of the heart beat (5). In nonexcitable cells, micromolar Ca²⁺ waves of lower frequency and longer duration, lasting from seconds to hours, transmit Ca²⁺ signals throughout the cytoplasm and nucleus (6). Given the diversity of cellular Ca²⁺ signals, it is reasonable to postulate that Ca²⁺-regulated proteins will exhibit highly specialized equilibrium and kinetic Ca²⁺-binding parameters that are “tuned” to match the levels and time scales of specific Ca²⁺ transients (7, 8).

The C2 domain is a conserved membrane-targeting motif present in a multitude of Ca²⁺-regulated signaling proteins (9–11). The C2 domain and the EF-hand motif of the calmodulin superfamily are the two most frequently occurring Ca²⁺ sensors: for example, the C. elegans, D. melanogaster, and H. sapiens genomes contain at least 61, 57, and 126 C2 domain proteins, respectively, while the same genomes contain at least 58, 92, and 144 EF-hand proteins, respectively (12). Prototypical C2 domains bind Ca²⁺ during a cytoplasmic transient and trigger protein docking to a specific intracellular membrane (9–11). Subsequently, one or more associated domains modulate signaling by membrane-bound
receptors, kinases, GTPases, and regulatory lipids. There are six known functional families of proteins containing C2 domains: (i) kinases that phosphorylate membrane protein targets; (ii) phospholipid-modifying enzymes that generate or inactivate lipid-derived second messengers; (iii) vesicle targeting and fusion proteins; (iv) GTPase-activating proteins; (v) ubiquitination enzymes that target membrane proteins for degradation; and (vi) proteins that form transmembrane pores (9–11). Whereas prototypical C2 domains bind Ca\(^{2+}\) and dock to phospholipid membranes, other C2
domains dock to membrane protein targets or fail to exhibit Ca\(^{2+}\) regulation (9–11), suggesting a broad range of functional specialization in the C2 domain superfamily. Even prototypical C2 domains have been hypothesized to employ different mechanisms of membrane docking (61).

To elucidate the conserved and specialized features of prototypical C2 domains, it is essential to directly compare the equilibrium and kinetic parameters of C2 domains isolated from functionally distinct signaling proteins. Figure 1A illustrates the conserved \(\beta\)-sandwich architectures of three representative C2 domains of known structure (28, 29, 43) selected for the present study due to their importance as Ca\(^{2+}\) sensors in different pathways and cellular environments. (i) The C2A domain of synaptotagmin-I (Syt-IA)\(^{1}\) is one of the Ca\(^{2+}\) sensors responsible for triggering synaptic vesicle fusion and neurotransmitter release at neuronal synapses (13–15). (ii) The C2 domain of protein kinase C (PKC-\(\beta\)) helps drive the Ca\(^{2+}\)-activated docking of this anionic lipid-dependent Ser/Thr kinase to the plasma membrane during regulation of numerous pathways (16–18). (iii) The C2 domain of cytosolic phospholipase A\(_2\) (cPLA\(_2\)-\(\alpha\)) binds Ca\(^{2+}\) and targets this protein primarily to nucleary and reticular membranes (19–23), where its enzymatic domain hydrolytically releases arachidonic acid to initiate the inflammatory response blocked by aspirin and ibuprofen (24–26). Previous equilibrium and kinetic studies of these C2 domains have provided much useful information, but have utilized different buffer, ionic, pH, and temperature conditions, contrasting membrane compositions, and in some cases docking site mutations to incorporate spectroscopic probes (30, 47, 61, 64). Such variations prevent quantitative comparisons of C2 domain activation parameters and mechanisms. The present comparison indicates that these three C2 domains differ in their Ca\(^{2+}\) affinity, kinetics, and stoichiometry, as well as in membrane affinity, kinetics, and headgroup selectivity. These results suggest that C2 domains are functionally specialized modules adapted in different cellular contexts to respond to distinct Ca\(^{2+}\) signals.

**MATERIALS AND METHODS**

**Reagents.** The lipids used were: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (phosphatidylcholine, PC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (phosphatidylserine, PS), both from Avanti Polar Lipids, and N-(5-dimethylaminonaphthalene-1-sulfonoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (dansyl-PE, dPE) from Molecular Probes. Small unilamellar phospholipid vesicles were prepared by sonication. Solutions and plasticware were calcified as described (27).

**Purification of C2 Domains.** C2 domains of Syt-IA and PKC-\(\beta\) were expressed as glutathione S-transferase (GST) fusion proteins and purified away from GST after cleavage with thrombin as described (28, 29). The cPLA\(_2\)-\(\alpha\) C2 domain was affinity-purified from refolded protein as described (30). Protein concentrations were determined using the tyrosinate method (30).

**Fluorescence Spectroscopy of Equilibrium Processes.** Fluorescence spectroscopy was carried out as described (30) on an SLM 48000S fluorescence spectrometer at 25 °C in standard assay buffer composed of 100 mM KCl, 20 mM N-(2-hydroxyethyl)piperazine-N\(^{-}\)-2-ethanesulfonic acid (HEPES), pH 7.4, and 5 mM diithothreitol (DTT). Excitation and emission slit widths were 4 and 8 nm, respectively. Because of differences in the quantum yields of the C2 domains, the concentrations of the domains necessary to achieve similar fluorescence signals varied, ranging from 0.1 to 1.0 \(\mu\)M. Where necessary, fluorescence signals were corrected for inner filter effects as described (31).

Fluorescence resonance energy transfer (FRET) was employed to measure the Ca\(^{2+}\) dependence of membrane docking as described (30). C2 domain was mixed with target vesicles of PS–PC–dPE (mole percent 47.5%:47.5%:5%; 250 \(\mu\)M total phospholipid), and PC–dPE or PS–dPE (95%; 100 \(\mu\)M phospholipid). The Ca\(^{2+}\)-dependent change in the protein-to-membrane FRET (\(\Delta F\)) was subjected to nonlinear, least-squares analysis using a modified Hill equation:

\[
\Delta F = \Delta F_{\text{max}} \left( \frac{x^H}{([\text{Ca}^{2+}]_{1/2}^H + x^H)} \right)
\]

where \(\Delta F_{\text{max}}\) represents the calculated maximal fluorescence change, \(x\) is the free Ca\(^{2+}\) concentration, \(H\) represents the Hill coefficient, and \([\text{Ca}^{2+}]_{1/2}\) represents the free Ca\(^{2+}\) concentration that induces half-maximal fluorescence change. \(\Delta F\) values were normalized to \(\Delta F_{\text{max}}\) to allow comparisons. For the cPLA\(_2\)-\(\alpha\) C2 domain, \(\Delta F_{\text{max}}\) values from experiments involving PC–dPE vesicles were used to normalize \(\Delta F\) values from PS–dPE. Likewise, for the PKC-\(\beta\) and Syt-IA domains, \(\Delta F_{\text{max}}\) values from experiments involving PS–dPE were used for PC–dPE. Fluorescence experiments such as these do not quantitatively determine the Hill coefficient for Ca\(^{2+}\)-triggered binding, since the individual binding events in a multistep reaction may yield different microscopic fluorescence changes. Although the Hill coefficient is quantitatively related to the number of bound ligands only in a cooperative system of identical sites (32), for proteins that bind two Ca\(^{2+}\) ions, observed Hill coefficients that exceed 1.4 in fluorescence experiments provide strong evidence for positive cooperativity (33).

Phospholipid titrations were carried out using PS–PC–dPE vesicles (47.5%:47.5%:5%) in the presence of 1 mM excess Ca\(^{2+}\) over ethylendiaminetetraacetic acid (EDTA) in standard buffer as described (30). The Ca\(^{2+}\)-dependent change in the protein-to-membrane FRET (\(\Delta F\)) was analyzed using an equation describing the binding to a single population of independent sites:

\[
\Delta F = \Delta F_{\text{max}} \left( \frac{x}{K_D + x} \right)
\]

where \(x\) is the concentration of phospholipid and \(K_D\) represents the apparent membrane dissociation constant in the presence of 1 mM free Ca\(^{2+}\). Intrinsinc tryptophan fluorescence was monitored in standard assay buffer as described (30). Emission spectra were recorded from 300 to 400 nm. The Ca\(^{2+}\) dependence of the

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1 Abbreviations: cPLA\(_2\)-\(\alpha\), cytosolic phospholipase A\(_2\)-\(\alpha\); PKC-\(\beta\), protein kinase C-\(\beta\); Syt-I, synaptotagmin-I; PC, phosphatidylcholine; PS, phosphatidylserine; dansyl-PE, N-(5-dimethylaminonaphthalene-1-sulfonoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; GST, glutathione S-transferase; FRET, fluorescence resonance energy transfer; DTT, diithothreitol; HEPES, N-(2-hydroxyethyl)piperazine-N\(^{-}\)-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.
intrinsic fluorescence change was measured by recording the emission at the fluorescence maximum (325 nm for cPLA₂-α and 340 nm for PKC-β), and fluorescence changes were analyzed by eq 1. When present, PS–PC vesicles (50%/50%; 250 μM phospholipid) were used.

The effect of ionic strength on membrane docking was tested by incubating C2 domains with PS–PC–dPE vesicles (47.5%/47.5%/5%; 100 μM phospholipid) in standard assay buffer lacking KCl but containing 1 mM EDTA. All tested by incubating C2 domains with PS–PC–dPE (47.5%/47.5%/5%; 100–250 μM phospholipid) in standard buffer containing 1.9 M Na₂SO₄ and 340 nm for PKC-β.

stopped fluorescence changes were corrected for any small direct effect of NaCl, Ca²⁺, and EDTA on vesicle fluorescence. After recording the initial dPE emission (F₀), 1 mM excess Ca²⁺ was added, and dPE emission (F_Ca²⁺) was recorded. Ionic strength was increased by reciprocal dilution with a like sample prepared in a high concentration of NaCl; dPE emission was recorded (F_NaCl) after each increase in NaCl. Finally, excess EDTA was added, and dPE emission was recorded (F_EDTA). Assuming that the C2 domain–membrane ternary complex is formed from a simple bimolecular interaction between the Ca²⁺-occupied C2 domain and an excess of phospholipid, then the apparent membrane dissociation constant (K_D) equals the product of the total lipid concentration and the ratio of free to bound C2 domain (34). The concentrations of free and bound C2 domain were approximated by the differences F_Ca²⁺ – F_NaCl and F_NaCl – F₀, respectively, to calculate K_D at each concentration of NaCl. Slopes of log [NaCl] plotted against log K_D were determined by linear regression to estimate the minimal number of electrostatic interactions involved in the domain–membrane complex based on polyelectrolyte theory (35).

The ability of Na₂SO₄ to promote membrane docking was carried out essentially as before (36). C2 domains were added to vesicles of PS–PC–dPE (47.5%/47.5%/5%; 100 μM phospholipid) in standard buffer containing 1.9 M Na₂SO₄ and 5 mM EDTA. The resulting FRET signal was compared to the FRET signal arising from membrane docking induced by Ca²⁺ in the absence of Na₂SO₄.

Stopped-Flow Fluorescence Spectroscopy. Stopped flow was carried out essentially as described (30) in standard assay buffer at 25 °C on an Applied Photophysics model 17MV stopped-flow apparatus. The dead-time of the instrument was determined to be 1.5 ms using a control reaction as described (37).

The membrane docking reaction was triggered by stopped-flow mixing a solution containing C2 domain (0.5–2 μM, all concentrations prior to mixing), vesicles of PS–PC–dPE (47.5%/47.5%/5%; 500 μM phospholipid), and trace amounts of EDTA (2 μM) with an equal volume of Ca²⁺ (400 μM). The resulting protein-to-membrane FRET time course reveals the approach to equilibrium of the docking reaction. Omission of either the C2 domain or Ca²⁺ eliminated the observed FRET increase upon mixing (data not shown). The dissociation reaction was triggered by stopped-flow mixing a solution of C2 domain (1–8 μM), Ca²⁺ (100 μM), and vesicles of PS–PC–dPE (47.5%/47.5%/5%; 250 μM) with an equal volume of EDTA (5 mM). The resulting protein-to-membrane FRET time course reveals the irreversible dissociation of C2 domains from membranes. Omission of either the C2 domain or Ca²⁺ eliminated the observed decrease upon mixing (data not shown). Alternatively, the dissociation reaction was initiated by stopped-flow mixing C2 domain (4 μM), Ca²⁺ (100 μM), and PS–PC vesicles (50%/50%; 250 μM) with an equal volume of the fluorescent Ca²⁺ chelator Quin-2 (200 μM). Vesicles were omitted in experiments designed to measure Ca²⁺ release from the free C2 domain. The Quin-2 fluorescence signal monitored the irreversible release of Ca²⁺ ions from the C2 domain. Omission of the C2 domain eliminated the Quin-2 fluorescence increase observed after mixing (data not shown). Stoichiometry was determined at pH 7.0 by comparing fluorescence amplitudes (see below) to a standard curve (30). At this pH, all three C2 domains tested were fully membrane-bound at 100 μM Ca²⁺ (data not shown), which was not the case at pH 7.4 (see Figure 2).

The fluorescence signals, F(t), were analyzed using both mono- and biexponential equations:

\[
F(t) = \Delta F(1 - e^{-kt}) + C
\]

\[
F(t) = \Delta F_1(1 - e^{-k_{1t}}) + \Delta F_2(1 - e^{-k_{2t}}) + C
\]

where k, k₁, and k₂ are rate constants, ΔF, ΔF₁, and ΔF₂ are the fluorescence amplitudes, and C is the intrinsic voltage offset of the stopped-flow experiment. F(t) values in all time courses were normalized to ΔF to allow comparison.

RESULTS

The isolated Syt-IA, PKC-β, and cPLA₂-α C2 domains were expressed in E. coli and purified to homogeneity. Use of the isolated domains prevented interference by other Ca²⁺- or membrane-binding motifs present in the full-length proteins, thereby facilitating comparison of the intrinsic C2 domain Ca²⁺-activation and membrane docking parameters. Moreover, each of the isolated domains has been shown by crystallographic and NMR studies to be monomeric (28, 29, 38–45), obviating the artificial interdomain cooperativity that could arise in membrane docking experiments involving domains coupled either to multiple sites on affinity beads or to the dimer-forming GST fusion partner (46, 47). To facilitate quantitative comparison of the isolated domains, all experiments were carried out at 25 °C in 100 mM KCl, 20 mM HEPES, pH 7.4, 5 mM DTT unless otherwise noted. Membranes contained an equimolar mixture of the two predominant physiological lipids, phosphatidylcholine (PC) and phosphatidylserine (PS), unless otherwise noted.

Equilibrium Ca²⁺ Dependence of Membrane Docking. The Ca²⁺ dependence of membrane docking by these C2 domains was measured using fluorescence resonance energy transfer (FRET) (Figure 2). This assay detects the transfer of energy from intrinsic tryptophan donors in the C2 domain to trace amounts of the acceptor dansyl-phosphatidylethanolamine (dPE), incorporated into synthetic phospholipid vesicles composed of phosphatidylcholine (PC) and phosphatidylserine (PS), upon formation of the domain–membrane complex. Nonlinear least-squares analysis of the data using the Hill equation (eq 1) yielded the [Ca²⁺]₅₀ values and Hill coefficients (H) summarized in Table 1. These data allow the first direct comparison of equilibrium Ca²⁺ sensitivities for the docking of monovalent C2 domains to membranes under identical conditions. In the presence of 250 μM total phospholipid (mole percent 47.5% PC, 47.5% PS, 5% dPE), the apparent Hill coefficients all significantly exceeded 1.4,
Syt-IA C2 domains. For the cPLA2 - Ca\(^2+\) PKC-\(\alpha\) where the C2 domain is 3-fold more sensitive to Ca\(^2+\) docking varied 10-fold, ranging from 4.7 \(\pm\) 1.0 \(\mu\)M with 1 mM free Ca\(^2+\) - the FRET assay was nonperturbing (Figures 3 and 4; Table 1). Thus, the membrane-docking equilibrium of the cPLA2 - the FRET results and indicating the dPE acceptor used in

Table 1: Equilibrium Parameters of Ca\(^2+\) and Membrane Binding to C2 Domains

<table>
<thead>
<tr>
<th>C2 domain</th>
<th>([\text{Ca}^2+]_{1/2} ) ((\mu)M)</th>
<th>(H)</th>
<th>([\text{Ca}^2+]_{1/2} ) ((\mu)M)</th>
<th>(H)</th>
<th>intrinsic Ca(^2+) binding</th>
<th>membrane binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPLA2-(\alpha)</td>
<td>4.7 (\pm) 0.8</td>
<td>1.7 (\pm) 0.2</td>
<td>4.1 (\pm) 0.3</td>
<td>1.8 (\pm) 0.1</td>
<td>14 (\pm) 2</td>
<td>1.7 (\pm) 0.2</td>
</tr>
<tr>
<td>PKC-(\beta)</td>
<td>16 (\pm) 4</td>
<td>1.6 (\pm) 0.1</td>
<td>10 (\pm) 3</td>
<td>2.0 (\pm) 0.2</td>
<td>39 (\pm) 2</td>
<td>1.6 (\pm) 0.1</td>
</tr>
<tr>
<td>Syt-IA</td>
<td>48 (\pm) 12</td>
<td>2.0 (\pm) 0.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(\alpha\) Results represent the average (\(\pm\)SEM) of 3–4 independent steady-state fluorescence experiments. \([\text{Ca}^2+]_{1/2}\) and \(H\) represent the best-fit midpoints and apparent Hill coefficient for Ca\(^2+\) titrations (eq 1). \(K_d\) is the best-fit apparent dissociation constant for phospholipid binding to a population of independent binding sites (eq 2). Experimental conditions: 25 °C; 100 mM KCl, 20 mM HEPES, pH 7.4, 5 mM DTT, ND, not determined.

\(\beta\) Determined in the presence of PS–PC–dPE vesicles (47.5%:47.5%:5%; 250 \(\mu\)M phospholipid), as in Figure 2. \(\gamma\) Determined in the presence of PS–PC vesicles (50%:50%; 250 \(\mu\)M phospholipid), as in Figure 4. \(\delta\) Determined in the presence of 1 mM free Ca\(^2+\) using PS–PC–dPE vesicles (47.5%:47.5%:5%), as in Figure 5.

![Figure 2](image)

**Figure 2**: Ca\(^2+\) threshold for membrane docking by C2 domains. Ca\(^2+\) was titrated into solutions containing the cPLA2-\(\alpha\) (filled circles), PKC-\(\beta\) (open circles), and Syt-IA (open squares) C2 domains and synthetic vesicles composed of PS–PC–dPE (47.5%:47.5%:5%). Membrane docking was measured by monitoring protein-to-membrane FRET. Solid lines indicate the best-fit of the resulting normalized FRET signals to the modified Hill equation (eq 1). Experimental conditions: 25 °C; 100 mM KCl, 20 mM HEPES, pH 7.4, 5 mM DTT, and 250 \(\mu\)M phospholipid. Results are summarized in Table 1.

indicating that membrane docking is triggered by the binding of multiple Ca\(^2+\) ions with positive cooperativity (see Materials and Methods). The \([\text{Ca}^2+]_{1/2}\) values for membrane docking varied 10-fold, ranging from 4.7 \(\mu\)M for the cPLA2-\(\alpha\) C2 domain to 16 \(\mu\)M for PKC-\(\beta\) and 48 \(\mu\)M for Syt-IA C2 domains. For the cPLA2-\(\alpha\) and PKC-\(\beta\) C2 domains, we obtained similar \([\text{Ca}^2+]_{1/2}\) values (4.1 and 10 \(\mu\)M, respectively) in separate experiments monitoring docking-induced changes to intrinsic Trp fluorescence, confirming the FRET results and indicating the dPE acceptor used in the FRET assay was nonperturbing (Figures 3 and 4; Table 1). Thus, the membrane-docking equilibrium of the cPLA2-\(\alpha\) C2 domain is 3-fold more sensitive to Ca\(^2+\) than that of the PKC-\(\beta\) domain and is 10-fold more sensitive than that of the Syt-IA domain.

Assuming that a given C2 domain rapidly binds multiple Ca\(^2+\) ions before docking to the membrane surface (this assumption is justified below), Ca\(^2+\)-triggered membrane docking may be depicted by a two-step mechanism:

**Scheme 1**

**Step I**: \(C2 + n\text{Ca} \rightleftharpoons C2\cdot\text{Ca}_n\)

**Step II**: \(C2\cdot\text{Ca}_n + \text{PL} \rightleftharpoons C2\cdot\text{Ca}_n\cdot\text{PL}\)

where \(n\) represents the Ca\(^2+\) stoichiometry, \(\text{PL}\) represents phospholipid membrane, and \(C2\cdot\text{Ca}_n\cdot\text{PL}\) represents the C2 domain–membrane ternary complex. Thermodynamic principles require that an observed \([\text{Ca}^2+]_{1/2}\) value for assembly of the ternary complex depends on both (i) the intrinsic Ca\(^2+\) affinity of the free C2 domain (Step I) and (ii) the membrane affinity of the Ca\(^2+\)-occupied domain (Step II). To deconvolute these factors, the intrinsic Ca\(^2+\) and membrane affinities were determined, allowing the first quantitative comparison of such parameters for native C2 domains.

**Equilibrium Binding of Ca\(^2+\) to the Free C2 Domain.** Ca\(^2+\) binding to free C2 domains was measured by monitoring Ca\(^2+\)-induced changes in the intrinsic tryptophan fluorescence, since tryptophan emission has been used to detect environmental changes in the cPLA2-\(\alpha\) C2 domain that are directly coupled to Ca\(^2+\) binding (30, 48, 49). The emission spectrum of the cPLA2-\(\alpha\) C2 domain was characterized by a blue-shifted emission maximum (\(\lambda_{max} \sim 325\) nm) arising from its sole buried tryptophan, Trp71 (Figure 3A). Ca\(^2+\) binding to the free cPLA2-\(\alpha\) C2 domain increased its fluorescence emission intensity by approximately 15%. By contrast, the emission spectrum of the PKC-\(\beta\) C2 domain was red-shifted (\(\lambda_{max} \sim 340\) nm), suggesting that emission of its three solvent-exposed tryptophans, Trp245, Trp247, and Trp274, dominates over that from the one buried tryptophan, Trp233, which occupies the position corresponding to Trp71 in the cPLA2-\(\alpha\) C2 domain (Figure 3B). Nevertheless, Ca\(^2+\) binding to the free PKC-\(\beta\) C2 domain also affected its fluorescence emission, increasing its intensity by approximately 30% and causing a slight blue shift. The fluorescence emission spectrum of the Syt-IA domain was also characterized by a red-shifted maximum (\(\lambda_{max} \sim 340\) nm) due to the presence of its sole, solvent-exposed tryptophan, Trp259 (Figure 3C). In agreement with a previous study (30), its intrinsic fluorescence was insensitive to ligand binding.

The Ca\(^2+\) dependences of these fluorescence increases were measured in order to directly compare the intrinsic Ca\(^2+\) affinities of the cPLA2-\(\alpha\) and PKC-\(\beta\) C2 domains (Figure 4). The resulting \([\text{Ca}^2+]_{1/2}\) values (Table 1) indicate that the free cPLA2-\(\alpha\) C2 domain cooperatively binds Ca\(^2+\) with higher affinity ([Ca\(^2+\)]\(_{1/2}\) = 14 \(\mu\)M) than the PKC-\(\beta\) domain ([Ca\(^2+\)]\(_{1/2}\) = 39 \(\mu\)M), where each of these \([\text{Ca}^2+]_{1/2}\) values represents an average affinity for the binding of multiple Ca\(^2+\) ions. For comparison, NMR and optical measurements have shown that the free Syt-IA C2 domain binds Ca\(^2+\) with multiple apparent dissociation constants ranging from 60 \(\mu\)M to >1 mM (38, 40, 41, 47). Thus, both the free cPLA2-\(\alpha\)
PKC-β C2 domains bind Ca\(^{2+}\) with considerably higher affinity than the free Syt-IA domain.

**Equilibrium Docking of the Ca\(^{2+}\)-Occupied C2 Domain to Membranes.** The membrane affinity of each Ca\(^{2+}\)-occupied C2 domain was measured by the FRET assay (Figure 5), yielding the apparent phospholipid dissociation constants (\(K_D\)) in the presence of 1 mM Ca\(^{2+}\) (Table 1). These \(K_D\) values varied approximately 7-fold, ranging from 3.4 \(\mu\)M for PKC-β to 24 and 19 \(\mu\)M for the cPLA\(_2\)-R and Syt-IA C2 domains, respectively. Therefore, the greater Ca\(^{2+}\) sensitivity of the cPLA\(_2\)-R C2 domain relative to PKC-β in membrane docking is due to the higher intrinsic affinity of the free cPLA\(_2\)-α C2 domain for Ca\(^{2+}\), whereas the lesser Ca\(^{2+}\) sensitivity of the Syt-IA domain is due to a combination of both its reduced intrinsic Ca\(^{2+}\) affinity and its lower affinity for phospholipid membranes. The latter findings are surprising, especially given the similar structures, headgroup selectivities, and docking mechanisms exhibited by the PKC-β and Syt-IA membrane docking loops (see below).

**Kinetics of Ca\(^{2+}\) Binding and Membrane Docking.** The kinetics of the Ca\(^{2+}\)-triggered membrane docking reaction were analyzed in vitro by stopped-flow fluorescence spectroscopy. To probe the kinetics of ternary complex formation, Ca\(^{2+}\) was mixed with a solution of C2 domain and phospholipid vesicles while monitoring membrane docking via protein-to-membrane FRET. This experiment measures a complex, multistep approach to equilibrium involving the binding of multiple Ca\(^{2+}\) ions and docking to the membrane surface. The resulting time courses exhibited simple, monoeXponential behavior (Figure 6, eq 3). Such simple behavior for a multistep approach to equilibrium is consistent with the existence of a reversible, rate-determining step that controls the kinetics of ternary complex formation and dissociation. Nonlinear best-fit analysis yielded the apparent, pseudo-first-order rate constants of ternary complex formation (\(k_{\text{form}}\)) summarized in Table 2. These data allow the first quantitative comparison of rate constants for the reactions of native C2 domains with membranes. In the presence of Ca\(^{2+}\) (200 \(\mu\)M) and phospholipid (250 \(\mu\)M; mole percent 47.5% PS, 47.5% PC, 5% dPE), equilibration of ternary complex formation was slowest for cPLA\(_2\)-R, intermediate for PKC-β, and fastest for the Syt-IA domain. Different relative rates (PKC-β < cPLA\(_2\)-α < Syt-IA) were reported in an independent study where membrane binding was monitored by tryptophan emission (47), which also detects the rapid binding of Ca\(^{2+}\) to the free cPLA\(_2\)-α C2 domain and thus may artifactually increase the apparent rate of ternary complex formation (30). Our results indicate that the apparent time constants for ternary complex formation (\(\tau_{\text{form}} = 1/k_{\text{form}}\)) varied nearly 10-fold, ranging from 4.3 ms for Syt-IA and 5.6 ms for PKC-β to 38 ms for the cPLA\(_2\)-α domain. Notably, at physiological Ca\(^{2+}\) concentrations, the
membrane docking equilibrium is reached considerably more quickly for the Syt-IA and PKC-β C2 domains than for the cPLA₂-α domain.

In principle, because the reaction is an approach to equilibrium, the observed kinetics of ternary complex formation depend on the association and dissociation rate constants determine step is the release of Ca²⁺ from the bound ternary complex, or the release of the Ca²⁺-bound apo protein. Overall, the reaction provides the first quantitative comparison of rate constants for the dissociation of native C2 domains from membranes. The time courses observed for the Syt-IA and PKC-β domains were monoexponential (Figure 7A, eq 3), indicating the existence of a rate-determining step during the multistep disassembly of the ternary complex. It is not yet clear whether this rate-determining step is the release of Ca²⁺ from the membrane-bound ternary complex, or the release of the Ca²⁺-occupied...
The resulting sequential two-step process is depicted by the dissociation rate constants $k_{\text{diss}}$ and $k_{\text{diss2}}$, measured by rapidly mixing a solution of C2 domain and PS–PC–dPE vesicles (47.5%:47.5%:5%) with a solution of Ca$^{2+}$ (200 μM final), as in Figure 6. The rate constant for C2 domain dissociation from membranes, measured by mixing a solution of C2 domain, Ca$^{2+}$, and phospholipid vesicles (PS–PC–dPE, 50%:50%:50%) with a solution of the fluorescent Ca$^{2+}$ chelator Quin-2, as in Figure 7B. The dissociation rate constants $k_{\text{diss}}$ and $k_{\text{diss2}}$ were extracted as in the previous footnote. 

### Table 2: Kinetic Parameters of C2 Domain Activation and Deactivation Time Courses

<table>
<thead>
<tr>
<th>C2 domain</th>
<th>$k_{\text{diss}}$ (s$^{-1}$)</th>
<th>$k_{\text{diss2}}$ (s$^{-1}$)</th>
<th>Stoichiometry (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPLA$_2$-α</td>
<td>26 ± 1 (6)</td>
<td>14 ± 1 (6)</td>
<td>1.0 ± 0.2 (5)</td>
</tr>
<tr>
<td>PKC-β</td>
<td>180 ± 10 (6)</td>
<td>132 ± 1 (12)</td>
<td>30 ± 1 (10)</td>
</tr>
<tr>
<td>Syt-IA</td>
<td>230 ± 20 (7)</td>
<td>520 ± 20 (12)</td>
<td>3.1 ± 0.3 (9)</td>
</tr>
</tbody>
</table>

### Scheme 2A

$$k_{\text{diss}}$$

C2·Ca$_n$·PL $\xrightarrow{k_{\text{diss}}} C2 + n\text{Ca} + \text{PL}$

where $k_{\text{diss}}$ is the measured first-order rate constant. Membrane dissociation was most rapid for the Syt-IA C2 domain ($k_{\text{diss}} = 520$ s$^{-1}$) and was considerably slower for the PKC-β domain ($k_{\text{diss}} = 132$ s$^{-1}$) (Table 2). In contrast to the monoeXponential kinetics observed for the Syt-IA and PKC-β domains (Figure 7A), the rate constant for C2 domain dissociation from membranes, measured by mixing a solution of C2 domain, Ca$^{2+}$, and phospholipid vesicles (PS–PC–dPE, 47.5%:47.5%:5%) with a solution of the fluorescent Ca$^{2+}$ chelator Quin-2, as in Figure 7B. The dissociation rate constants $k_{\text{diss}}$ and $k_{\text{diss2}}$ were extracted as in the previous footnote.

### Scheme 2B

$$k_{\text{diss1}}$$

C2·Ca$_2$·PL $\xrightarrow{k_{\text{diss1}}} C2 + 2\text{Ca} + \text{PL}$

Thus, during the slow decay of the cPLA$_2$-α ternary complex, one of the Ca$^{2+}$ ions dissociates before the domain is released from the membrane. Overall, the results indicate that the effective lifetimes of the membrane-bound state following removal of free Ca$^{2+}$ vary considerably, ranging from 1.9 and 7.6 ms for the Syt-IA and PKC-β C2 domains, respectively, to 400 ms for the cPLA$_2$-α domain. Since this experiment measures the average time for dissociation of the domain from the membrane after removal of the Ca$^{2+}$ signal, it measures the duration that the C2 domain "remembers" the Ca$^{2+}$ signal. Thus, the duration of Ca$^{2+}$ memories for C2 domains from different signaling proteins varies over several hundred milliseconds: the Syt-IA and PKC-β C2 domains have considerably shorter Ca$^{2+}$ memories than the cPLA$_2$-α domain.

The time course of Ca$^{2+}$ release from C2 domains was measured in order to determine the number of Ca$^{2+}$ ions in the domain—membrane ternary complex and the degree to which their release is correlated with disassembly of the complex. Recent evidence suggests that the Ca$^{2+}$-binding loops of the C2 domain directly contact the membrane surface (42, 44, 45, 47, 49, 50, 52, 57). As a result, bound Ca$^{2+}$ ions could become trapped within the protein—membrane interface until the ternary complex dissociates (30). To monitor Ca$^{2+}$ release from the free or membrane-bound C2 domain, the fluorescent Ca$^{2+}$ chelator Quin-2 was mixed by stopped flow with the Ca$^{2+}$-occupied C2 domain; then the appearance of free Ca$^{2+}$ was detected as an increase of the Quin-2 fluorescence (Figure 7B). Quin-2 binds free Ca$^{2+}$ within the instrument dead-time (58) and does not add a lag time to the kinetics. The number of Ca$^{2+}$ ions released per domain was determined by comparing the fluorescence signal to a standard curve. While the dissociation of two Ca$^{2+}$ ions from the free cPLA$_2$-α C2 domain is slow enough to be quantified ($k = 110$ s$^{-1}$, ref (30)), the dissociation of Ca$^{2+}$ from the free C2 domains of SytI-A and PKC-β is too rapid to be measured by stopped flow ($k \gg 700$ s$^{-1}$) (data not shown). By contrast, Ca$^{2+}$ release from all three premade C2 domain—membrane complexes was readily detected, thereby enabling the first measurement of Ca$^{2+}$ stoichiometries for membrane-bound SytI-A and PKC-β C2 domains, and of their rate constants for Ca$^{2+}$ release. Two Ca$^{2+}$ ions were released from the cPLA$_2$-α ternary complex, whereas three ions were released from the PKC-β and SytI-A complexes (Table 2). In each case, there was a relatively close agreement between the rate constants for the release of Ca$^{2+}$ and dissociation of C2 domains from membranes (Table 2), indicating that membrane dissociation is kinetically linked to Ca$^{2+}$ dissociation. Since Ca$^{2+}$ dissociation from each ternary complex is at least 1 order of magnitude slower than dissociation from the corresponding free C2 domain, these findings support the proposal that the membrane surface occludes the Ca$^{2+}$-binding sites in all three ternary complexes.

### Mechanism of Ca$^{2+}$-Triggered Membrane Docking

Given that the three C2 domains exhibit significantly different Ca$^{2+}$ thresholds for membrane docking, different kinetics of membrane association and dissociation, and different Ca$^{2+}$ stoichiometries, it is possible that the fundamental forces used by these domains to bind membranes could differ as well.
A recent study hypothesized that different C2 domains may utilize contrasting mechanisms of membrane docking (61). To further test this idea, the phospholipid headgroup selectivities of the cPLA2-α, PKC-β, and Syt-IA C2 domains were probed in the FRET assay by replacing the equimolar PS–PC mixture in vesicles with pure PS or PC (Figure 8A,B). Ca2+ binding triggered the docking of both the PKC-β and Syt-IA C2 domains to anionic PS but not zwitterionic PC, whereas the cPLA2-α C2 domain docked to PC but not PS. Similarly, the PKC-β and Syt-IA C2 domains exhibited Ca2+-triggered docking to other anionic phospholipids, including phosphatidylglycerol, phosphatidic acid, and phosphatidylinositol (data not shown), in agreement with previous studies on the PKC-α (55) and Syt-IA (57, 59, 60) C2 domains. By contrast, the cPLA2-α C2 domain failed to dock to these anionic membranes [data not shown and ref (36)]. The effect of ionic strength on membrane docking by these C2 domains was tested to determine the importance of electrostatic interactions (50, 57, 61). Ca2+-dependent docking of the PKC-β and Syt-IA domains, but not the cPLA2-α domain, to membranes containing a PS–PC mixture was inhibited by increasing ionic strength (Figure 9). Furthermore, where $K_D$ again represents the apparent dissociation constant for phospholipid binding (see Materials and Methods), plots of log $K_D$ vs log [NaCl] for PKC-β and Syt-IA were linear between 150 and 700 mM NaCl (data not shown). The resulting slopes allow calculation of the effective number of charges involved (35) and indicate a minimum of three to four ionic interactions between the PKC-β and Syt-IA C2 domains and the anionic lipid surface.

The ability of Na$_2$SO$_4$ to induce membrane docking was determined in order to directly test the importance of hydrophobic interactions in membrane docking. Na$_2$SO$_4$, which strengthens hydrophobic interactions (36), promoted Ca2+-independent docking of the cPLA2-α C2 domain, but not the PKC-β and Syt-IA domains, to PS–PC vesicles (Figure 10). These results support the hypothesis that membrane docking by the cPLA2-α C2 domain is stabilized mainly through hydrophobic interactions with the membrane, whereas the docking of the Syt-IA or PKC-β C2 domain is stabilized primarily by electrostatic interactions between the heads of the C2 domains.
DISCUSSION

Our results demonstrate that the C2 domains of cPLA2-α, PKC-β, and Syt-IA can be selectively triggered to dock to membranes by Ca²⁺ concentrations that vary over a 10-fold range, from approximately 5 to 50 μM Ca²⁺. These in vitro [Ca²⁺]₅₀ values likely represent upper limits to the values that would be obtained in vivo, where intracellular membranes also contain a mixture of PS and PC but are present in much higher local concentrations. Most importantly, the results predict that these three C2 domains will exhibit significantly different Ca²⁺ activation thresholds in vivo, such that the relative Ca²⁺ concentrations needed for intracellular membrane docking are lowest for cPLA2-α, intermediate for PKC-β, and highest for the Syt-IA domain. The relatively low Ca²⁺ threshold of the cPLA2-α C2 domain suggests that it is well adapted for low-amplitude Ca²⁺ signals and does not require significant assistance in membrane binding from other domains. By contrast, the higher Ca²⁺ threshold of the PKC-β C2 domain is consistent with the proposal that activation of the full-length enzyme requires assistance from other domains, including the docking of its regulatory C1 domain to membrane-partitioned diacylglycerol and phosphatidylserine (62, 63). The high Ca²⁺ threshold of the Syt-IA domain, on the other hand, suggests that it is best adapted for high-amplitude Ca²⁺ signals such as those generated at presynaptic plasma membranes (3, 4).

Figure 1A illustrates the similar architectures of the cPLA2-α, PKC-β, and Syt-IA C2 domains, each of which is a sandwich of two four-stranded, antiparallel β-sheets. The three Ca²⁺-binding loops lie at one end of the β-sandwich where they also serve as the primary membrane docking surface (42, 44, 45, 47, 49, 50, 52, 57). Figure 1B summarizes the observed Ca²⁺ stoichiometries of the membrane-bound C2 domains. Notably, these loops bind different numbers of Ca²⁺ ions: the cPLA2-α C2 domain binds two Ca²⁺ ions in solution, in crystals, and while bound to membranes (30, 43–45), whereas the PKC-β C2 domain binds two to three Ca²⁺ ions in solution and in crystals (29, 38), and the Syt-IA C2 domain binds from one to three Ca²⁺ ions in crystals and in solution (28, 38, 41). Our results provide the first direct evidence that the latter domains bind three or more Ca²⁺ ions when docked to target membranes. At least in part, the differing Ca²⁺ sensitivities of the three C2 domains are likely to arise from specialized modifications of the four potential Ca²⁺-binding sites (I–IV) on their Ca²⁺-binding loops. For example, the higher Ca²⁺ affinity of the free cPLA2-α domain could stem from the fact that it binds only two Ca²⁺ ions in sites I and II, while the Syt-IA and PKC-β C2 domains bind three Ca²⁺ ions in sites II, III, and IV (29). Alternatively, the lower Ca²⁺ affinity of the Syt-IA and PKC-β domains could stem from the higher density of positive charges on their Ca²⁺-binding loops, which may destabilize the Ca²⁺–protein complex by repulsion between like charges.

The Ca²⁺-binding loops are also specialized to generate different types of protein–membrane interactions. Figures 1B and 1C provide a close-up view of the Ca²⁺-binding loops and summarize four different mechanisms that could contribute to membrane docking, respectively. Figure 1C presents four major interactions that could stabilize membrane docking: (i) direct inner sphere coordination of the bound Ca²⁺ ions by anionic phospholipid headgroup oxygens, (ii) other types of electrostatic interactions between the Ca²⁺–protein complex and the membrane surface, (iii) recognition of one or more headgroups by a specific binding site, and (iv) nonspecific hydrophobic interactions between protein side chains and the membrane interior. Figure 1B indicates that the Ca²⁺-binding loops of the cPLA2-α domain possess numerous solvent-exposed hydrophobic side chains that account for the hydrophobic nature of membrane docking by this C2 domain, whereas the Ca²⁺-binding loops of the Syt-IA and PKC-β C2 domains appear to utilize multiple basic side chains and a third Ca²⁺ ion to drive electrostatic membrane docking. Our results emphasize the importance of hydrophobic interactions in the docking of cPLA2-α C2

FIGURE 9: Role of electrostatic interactions in membrane docking by C2 domains. The indicated C2 domains and vesicles of PS–PC–dPE (47.5%:47.5%:5%) were incubated in standard assay buffer containing 1.9 M Na₂SO₄ and 5 mM EDTA (filled bars) or 2 mM CaCl₂ and 1 mM EDTA (open bars). Membrane docking was measured by monitoring protein-to-membrane FRET. The resulting FRET signals were corrected for small effects of NaCl on dPE emission. The NaCl concentration was raised incrementally, and the resulting FRET signal was normalized to the initial value. Following the NaCl additions, excess EDTA was added (dashed line) to demonstrate the reversibility of cPLA2-α C2 domain binding to membranes. Experimental conditions: 25 °C; 20 mM HEPES, pH 7.4, 5 mM DTT, and 100 μM phospholipid.

FIGURE 10: Role of hydrophobic interactions in membrane docking by C2 domains. The indicated C2 domains and vesicles of PS–PC–dPE (47.5%:47.5%:5%) were incubated in standard assay buffer containing 1.9 M Na₂SO₄ and 5 mM EDTA (filled bars) or 2 mM CaCl₂ and 1 mM EDTA (open bars). Membrane docking was measured by monitoring protein-to-membrane FRET. The resulting FRET signals were corrected for small effects of NaCl on dPE emission. The NaCl concentration was raised incrementally, and the resulting FRET signal was normalized to the initial value. Following the NaCl additions, excess EDTA was added (dashed line) to demonstrate the reversibility of cPLA2-α C2 domain binding to membranes. Experimental conditions: 25 °C; 20 mM HEPES, pH 7.4, 5 mM DTT, and 250 μM phospholipid.

anionic lipid headgroups and the Ca²⁺–protein complex (36, 40, 41, 49, 50, 53–55, 57, 61).
domain to its preferred zwitterionic lipids and electrostatic interactions in the docking of PKC-β and Syt-IA C2 domains to their preferred anionic lipids (36, 40, 41, 49, 50, 53–55, 57, 61). The different lipid preferences of C2 domains may allow their activation parameters, including Ca²⁺ affinity, to be tuned by the varying lipid compositions of membranes in different regions of the cell.

Overall, the present results indicate that membrane docking triggered by Ca²⁺ progresses via two distinct steps (Scheme 1): the C2 domain first loads rapidly with Ca²⁺ ions, thereby triggering the slower membrane docking step. Rapid Ca²⁺ removal results in disassembly of the C2 domain ternary complexes, whereupon bound Ca²⁺ ions trapped at the protein–membrane interface are released as the domain dissociates from the membrane (Schemes 2A and 2B). A recent crystal structure of the ternary complex formed by the PKC-α C2 domain, Ca²⁺ ions, and phosphatidylinerine (56) directly supports this model. These findings further support the proposal that the Ca²⁺-binding loops interact directly with the membrane surface, as well as the hypothesis that one or more Ca²⁺ ions are directly coordinated both by protein and by headgroup oxygens in the ternary complex.

Importantly, the kinetics of membrane docking by C2 domains are tuned to different time scales, resulting in domain activation–inactivation cycles that vary in duration. This cycle is initiated by rapid Ca²⁺ addition that triggers membrane association and is terminated by rapid Ca²⁺ removal. A full cycle (1/κ_{on} + 1/κ_{off}) requires approximately 6 and 13 ms for the Syt-IA and PKC-β C2 domains, respectively, but at least 400 ms for the cPLA₂-α C2 domain. The comparatively short cycle times of the Syt-IA and PKC-β C2 domains are consistent with their roles as rapid on/off switches in the Ca²⁺ triggering of synaptic events and membrane protein phosphorylation, respectively. The longer cycle time of the cPLA₂-α domain provides a longer memory after a Ca²⁺ signal ends, thereby ensuring a minimal number of enzyme turnovers for each activation event. Additionally, the longer time constant could be used to damp out fluctuating Ca²⁺ signals, whereas the short time constants of the Syt-IA and PKC-β C2 domains would ensure that these domains faithfully track Ca²⁺ transients. It follows that rapid changes in the local cytoplasmic Ca²⁺ concentration will cause different C2 domains to dock to membranes on time scales that differ by tens of milliseconds or more, which could result in stepwise activation of signaling components.

We further propose that the different lifetimes observed for C2 domains arise directly from their contrasting membrane-binding mechanisms. For example, the rapid formation and dissociation kinetics of the Syt-IA and PKC-β ternary complexes (C2-Ca₄-PL) are consistent with C2 domain docking to the headgroup layer of the membrane surface via multiple nonspecific electrostatic interactions that are rapidly formed and broken. By contrast, the slow formation and dissociation of the cPLA₂ ternary complex (C2-Ca₄-PL) is consistent with the hypothesis that this C2 domain may undergo more extensive hydrophobic penetration into the membrane (43, 49, 52–54, 61), which raises the transition state barrier for the docking reaction and slows both membrane docking and release. Thus, there is considerable heterogeneity in the mechanism of membrane docking, indicating that each C2 domain uses a specialized combination of the components summarized in Figure 1C to stabilize the docking interaction. Finally, the role played by Ca²⁺ in initiating membrane docking is not fully understood. In addition to its established role in triggering electrostatic changes in the membrane binding surface of the Syt-IA C2 domain (40, 41, 57), Ca²⁺ may provide strong energetic contributions via direct interactions with phospholipid headgroups (56) or by triggering changes to protein conformation or dynamics.

In summary, the activation parameters of C2 domains are highly specialized for their biological context. The equilibrium and kinetics of Ca²⁺ activation, membrane docking, and inactivation are carefully optimized so that the C2 domain will act in concert with other signaling protein domains, thereby yielding a sensor that is activated by the appropriate Ca²⁺ threshold concentration and that exhibits the optimal activation and inactivation response times. The observed diversity of Ca²⁺ activation parameters enables C2 domains to function in different intraprotein and intracellular environments. Such diversity also suggests that pathways utilizing multiple C2 domains could exhibit stepwise activation and inactivation of components, thereby triggering complex sequences of events in a carefully controlled manner. Finally, the present findings illustrate that comparative studies of related proteins provide important insights that complement and extend the information provided by genomic analysis of protein families.

REFERENCES