Roles of calcium ions in the membrane binding of C2 domains

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The C2 domain is a membrane-targeting domain found in many cellular proteins involved in signal transduction or membrane trafficking. The majority of C2 domains co-ordinate multiple Ca\(^{2+}\) ions and bind the membrane in a Ca\(^{2+}\)-dependent manner. To understand the mechanisms by which Ca\(^{2+}\) mediates the membrane binding of C2 domains, we measured the membrane binding of the C2 domains of group IV cytosolic phospholipase A\(_2\) (cPLA\(_2\)) and protein kinase C-\(\alpha\) (PKC-\(\alpha\)) by surface plasmon resonance and lipid monolayer analyses. Ca\(^{2+}\) ions mainly slow the membrane dissociation of cPLA\(_2\)-C2, while modulating both membrane association and dissociation rates for PKC-\(\alpha\)-C2. Further studies with selected mutants showed that for cPLA\(_2\), a Ca\(^{2+}\) ion bound to the C2 domain of cPLA\(_2\) induces the intramembrane conformational change that leads to the membrane penetration of the C2 domain whereas the other Ca\(^{2+}\) is not directly involved in membrane binding. For PKC-\(\alpha\), a Ca\(^{2+}\) ion induces the inter-domain conformational changes of the protein and the membrane penetration of non-C2 residues. The other Ca\(^{2+}\) ion of PKC-\(\alpha\)-C2 is involved in more complex interactions with the membrane, including both non-specific and specific electrostatic interactions. Together, these studies of isolated C2 domains and their parent proteins allow for the determination of the distinct and specific roles of each Ca\(^{2+}\) ion bound to different C2 domains.

Key words: monolayer, surface plasmon resonance.

INTRODUCTION

The agonist-induced subcellular targeting of protein is an important process in cell signalling and regulation. Recently, the targeting of peripheral proteins [e.g. protein kinases C (PKCs), phospholipases and lipid kinases] by Ca\(^{2+}\) and lipid second messengers, including diacylglycerol and phosphoinositides, has received much attention as an important mechanism of cell signalling. It has been shown that the subcellular targeting of peripheral proteins is driven by a small set of membrane-targeting domains, including PKC conserved 1 (C1), PKC conserved 2 (C2) and pleckstrin homology domains. The C2 domain has been identified in many cellular proteins involved in signal transduction or membrane trafficking [1–3]. The majority of C2 domains bind the membrane in a Ca\(^{2+}\)-dependent manner and thereby play an important role in Ca\(^{2+}\)-dependent membrane targeting of peripheral proteins. Structural analyses of multiple Ca\(^{2+}\)-dependent membrane-binding C2 domains have shown that they share a common fold of an eight-stranded anti-parallel \(\beta\)-sandwich connected by variable loops and that three Ca\(^{2+}\)-binding loops located at one side of the domain bind multiple Ca\(^{2+}\) ions [4–11]. Structural [4–11] and binding [12–14] analyses have also determined the calcium-binding stoichiometry, geometry and affinity for various C2 domains. Although several roles have been proposed for C2-bound Ca\(^{2+}\) ions, the exact roles of individual Ca\(^{2+}\) ions in membrane targeting of C2 domains have yet to be determined. The C2 domains of group IV cytosolic phospholipase A\(_2\) (cPLA\(_2\)) and PKC-\(\alpha\), both of which bind two Ca\(^{2+}\) ions, exhibit significant differences in their calcium-binding loop structures [9,10]. A recent study also showed that the C2 domains of cPLA\(_2\) and PKC-\(\beta\), which is similar to PKC-\(\alpha\), have different Ca\(^{2+}\) affinities in both the presence and absence of phospholipids [15]. Thus the C2 domains of cPLA\(_2\) and PKC-\(\alpha\) serve as an excellent model to explore different roles of C2-bound Ca\(^{2+}\) ions. In this study, we measured the Ca\(^{2+}\)-dependent membrane binding of C2 domains, their respective C2 domains and mutants by surface plasmon resonance (SPR) analysis and by phospholipid monolayer technique. The results provide new molecular insights into the distinct mechanisms by which Ca\(^{2+}\) ions drive the membrane binding of C2 domains.

EXPERIMENTAL

Materials

1,2-Di-O-hexadecyl-sn-glycero-3-phosphocholine (DHPC) was from Sigma. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) and 1,2-sn-dioleoylglycerol (DOG) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and used without further purification. Phospholipid concentrations were determined by phosphate analysis [16]. The Liposofast microextruder and 100 nm polycarbonate filters were from Avestin (Ottawa, Ontario, Canada). Fatty acid-free BSA was from Bayer (Kankakee, IL, U.S.A.). Triton X-100 was from Pierce Chemical Co. (Rockford, IL, U.S.A.). Restriction endonucleases and other enzymes for molecular biology were from either Boehringer Mannheim or New England Biolabs (Beverly, MA, U.S.A.). CHAPS and octyl glucoside were from Sigma and Fisher Scientific, respectively. Pioneer L1 sensor chip was from Biacore AB (Piscataway, NJ, U.S.A.).

Mutagenesis and protein expression

The isolated C2 domain of cPLA\(_2\) and mutants therein as well as the whole cPLA\(_2\) and respective mutants of the C2 domain were expressed and purified as described previously [17]. PKC-\(\alpha\) and mutants of the C2 domain were expressed as described previously [18,19]. The isolated C2 domain of PKC-\(\alpha\) was expressed and

Abbreviations used: cPLA\(_2\), group IV cytosolic phospholipase A\(_2\); DHPC, 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine; DOG, 1,2-sn-dioleoylglycerol; PKC, protein kinase C; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; PS, phosphatidylserine; SPR, surface plasmon resonance.

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purified as described in [20]. Mutants of the isolated C2 domain of PKC-α were generated in the following fashion. Mutations were generated by the overlap-extension PCR [21] using pVL1392-PKC-α plasmid as a template [19]. Briefly, four primers, including two complementary oligonucleotides introducing a desired mutation and two additional oligonucleotides complementary to the 5′-end of the C2 domain (residue 154) and the 3′-end (residue 284) of the PKC-α gene, respectively, were used for PCR performed in a DNA thermal cycler (PerkinElmer) using Pfu DNA polymerase (Stratagene). Two DNA fragments overlapping at the mutation site were first generated and purified on an agarose gel. These two fragments were then annealed and extended to generate an entire C2-domain gene containing a desired mutation, which was further amplified by PCR. The product was subsequently purified on an agarose gel, digested with NcoI and XhoI, and subcloned into the pET21d vector digested with the same restriction enzymes. The mutagenesis was verified by DNA sequencing using a T7 Sequenase kit (United States Biochemical, Cleveland, OH, U.S.A.). Mutants were expressed and purified using the same protocol as for the wild-type C2 domain [20].

**Monolayer measurements**

Surface pressure (π) of solution in a circular Teflon trough (4 cm diameter × 1 cm deep) was measured using a Wilhelmy plate attached to a computer-controlled Cahn electrobalance (model C-32) as described previously [18]. Phospholipid solution (5–10 μl) in ethanol/hexane (1:9, v/v) or chloroform was spread on to 10 ml of subphase (20 mM Tris/HCl, pH 7.5, containing 0.1 M KCl and 0.1 mM CaCl2) to form a monolayer with a given initial surface pressure (π0). The subphase was stirred continuously at 60 rev./min with a magnetic stir bar. Once the surface pressure reading of monolayer had been stabilized (after ≈ 5 min), the protein solution (typically 30 μl) was injected into the subphase through a small hole drilled at an angle through the wall of the trough, and the change in surface pressure (Δπ) was measured as a function of time. Typically, the Δπ value reached a maximum after 35 min. The maximal Δπ value depended on the protein concentration and reached a saturation value (e.g. at [PKC-α] ≥ 1 μg/ml). Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed Δπ represented a maximal value. The Δπ versus π0 plots were constructed from these measurements [22].

**SPR measurements**

The preparation of the vesicle-coated Pioneer L1 sensor chip was described in detail elsewhere [23,24]. The sensor surface was coated with DHPC vesicles for cPLA2 and POPC/POPS (7:3) vesicles (with or without 1% DOG) for PKC-α. In control experiments, the fluorescence intensity of the flow buffer after rinsing the sensor chip coated with DHPC or POPC/POPS vesicles incorporating 10 mM 5-carboxyfluorescein (Molecular Probes) was monitored to confirm that the vesicles remained intact on the chip. All experiments for cPLA2 were performed with a control cell in which a second sensor surface was coated with BSA. For all PKC experiments the control cell was coated with 100%, POPC, for which PKC-α and its C2 domain have extremely low affinity. The drift in signal for both sample and control flow cells was allowed to stabilize below 0.3 resonance unit/min before any kinetic experiments were performed. All kinetic experiments were performed at 24 °C with a flow rate of 60 μl/min. A high flow rate was used to circumvent mass transport effects. The association was monitored for 90 s and dissociation for 4 min. The immobilized vesicle surface was then regenerated for subsequent measurements using 10 μl of 50 mM NaOH (see Figure 1). In all measurements, the vesicle surface could also be regenerated by treatment with 0.1 mM EGTA, demonstrating the specific and reversible nature of the binding. The regeneration solution was passed over the immobilized vesicle surface until the SPR signal reached the initial background value before protein injection (Figure 1). For data acquisition, five or more different concentrations (typically within a 10-fold range above or below the Kd of each protein were used. After each set of measurements, the entire immobilized vesicles were removed by injection of 25 μl of 40 mM CHAPS, followed by 25 μl of octyl glucoside at 5 μl/min, and the sensor chip was re-coated with a fresh vesicle solution for the next set of measurements. Note that in the presence of 0.1 mM EGTA, cPLA2 or PKC-α or their respective C2 domains displayed no appreciable change in the SPR response beyond the refractive index change. All data were evaluated using BIAevaluation 3.0 software (Biacore). For each trial, the signal was corrected against the control surface response in order to eliminate any refractive index changes due to buffer change. Furthermore, the derivative plot was used to monitor potential mass transport effects. Once these factors were checked for each set of data, the association and dissociation phases of all sensograms were globally fitted to a 1:1 Langmuir binding model: [protein + vesicle] ⇌ protein + vesicle. The association phase was analysed using the equation:

\[ R = \frac{[k_a C/(k_i C + k_d)] R_{max} (1 - e^{-((k_a C + k_d)/d) t})}{d} + R_I \]

where \( R_I \) is the refractive index change, \( R_{max} \) is the theoretical binding capacity, \( C \) is the analyte concentration, \( k_a \) is the association rate constant, and \( k_d \) is the dissociation rate constant and
Results of C2 domain calcium ions

Roles of C2 domain calcium ions

In Figure 3, Ca2+-phospholipid hydrolysis during binding measurements. As shown hydrolysable DHPC for cPLA2 to optimize the membrane binding. The selection of non-

RESULTS

Effects of Ca2+ ions on membrane association and dissociation

Our previous structure-function studies on the Ca2+-co-ordinating residues of cPLA2 [17] and PKC-ζ [19] suggested that two Ca2+ ions (CA1 and CA2; see Figure 2) in these C2 domains are involved in distinct processes. To elaborate on these studies, we first measured the effects of Ca2+ (1 μM–1 mM) on the membrane association (kₐ) and dissociation rate constants (k_d) of the two C2 domains by SPR analysis. We recently showed that the SPR analysis allows for the determination of the specific roles of individual residues of peripheral proteins in their membrane binding [23]. A typical sensorgram for C2-domain phospho-

Role of each Ca2+ ion in membrane association and dissociation

To investigate these aspects further, we then measured the effects of mutations of specific Ca2+-co-ordinating residues of the two C2 domains on their membrane binding. For these studies, we employed the mutants of both isolated C2 domains and whole proteins. For PKC-ζ, three residues involved in co-ordination to CA1 (Asp95) and CA2 (Asp136) and both (Asp136) were mutated. Similarly, three residues involved in co-ordination to CA1 (Asp95), CA2 (Asp136) and both (Asp136) were mutated for cPLA2. By means of SPR analysis, we measured the membrane binding of these proteins as a function of calcium concentration in the range 1 μM–1 mM. The kₐ and k_d values at 0.1 mM Ca2+ are listed in Table 1. At this Ca2+ concentration, the largest differences were observed between the wild-type and most of the mutants. For cPLA2, the mutation of CA1 ligand Asn95 to Ala only modestly changed kₐ and k_d for both isolated C2 domain and holoenzyme, but the effect on kₐ was slightly larger. Aside from the fact that Asn is a weaker ligand than Asp, this suggests that CA1 is not essential for the membrane binding of cPLA2. In contrast, the mutation of CA2 ligand Asp136 to Asn resulted in a 3-fold decrease in kₐ and a much larger 10–20-fold increase in kₐ for both isolated domain and holoenzyme. This result indicates

Figure 2 Schematic representations of Ca2+-binding loops of cPLA2 and PKC-ζ

Calcium ligands and two calcium ions (CA1 and CA2) are illustrated. For PKC-ζ-C2, a PS head group co-ordinated to CA1 is also shown. CBR1–CBR3 indicate three calcium-binding loops.
that CA2 is critically involved in hydrophobic and/or specific interactions. In view of our previous work on cPLA2-C2, it is likely that CA2 induces the conformational change that leads to the exposure of aliphatic and aromatic side chains in the calcium-binding loops and membrane penetration [17]. Because the effects of the D93N mutation on cPLA2 and cPLA2-C2 were essentially the same, the conformational changes should be confined within the C2 domain. The mutation of bidentate ligand Asp to Asn also primarily affected $k_a$; however, the degree of change was smaller than that observed for D93N, underscoring the hypothesis that Asp is not as critical as Asp in CA2 coordination and that CA1 is not essential for the membrane binding of cPLA2-C2.

For PKC-ε-C2, the mutation of a CA1 ligand, Asp, to Asn lowered $k_a$ by 2.9-fold and raised $k_b$ by 1.4-fold. The X-ray structure of PKC-ε-C2–Ca$^{2+}$–phosphatidylserine (PS) complex showed that CA1 directly co-ordinates PS [9]. Our data suggest that CA1 (and presumably its co-ordination to PS) largely contributes to the initial non-specific membrane adsorption of PKC-ε-C2. Interestingly, the same mutation led to a larger 4.9-fold increase in $k_a$ for the PKC-ε holoenzyme while showing a similar 3.6-fold decrease in $k_b$. Thus for the whole PKC-ε molecule CA1 is more likely to be involved in hydrophobic and/or specific interactions. This notion is also consistent with our previous finding that the PS co-ordination to the C2 domain of PKC-ε induces the membrane penetration of its C1 domain, thereby resulting in hydrophobic interactions [20]. Thus the case with cPLA2, the putative conformational changes caused by CA1 should be long-range (i.e. interdomain conformational changes). The mutation of a CA2 ligand Asp to Asn had little effect on $k_a$ and $k_b$ for PKC-ε-C2, suggesting that CA2 might not play an essential role in the membrane binding of the isolated C2 domain. Again, however, the same mutation showed a much larger 6.1-fold increase in $k_a$ (with only a 1.5-fold decrease in $k_b$) for the holoenzyme, indicating that CA2 induces the interdomain conformational changes. Similarly, the differential mutational effects of bidentate ligand Asp on PKC-ε-C2 and the holoenzyme can be accounted for by the interdomain conformational changes.

### Effects of Ca$^{2+}$ ions on membrane penetration

To corroborate the notion that CA1/CA2 of PKC-ε-C2 and CA2 of cPLA2-C2 induce inter- and intra-domain conformational

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$k_a$ (M$^{-1}$·s$^{-1}$)</th>
<th>$k_b$ (s$^{-1}$)</th>
<th>$K_s$ (M$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>cPLA2-C2</td>
<td>(3.0 ± 0.6) $\times$ 10$^3$</td>
<td>(3.3 ± 0.7) $\times$ 10$^{-3}$</td>
<td>(1.1 ± 0.3) $\times$ 10$^{-6}$</td>
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<tr>
<td>cPLA2-C2/D40N</td>
<td>(1.6 ± 0.4) $\times$ 10$^3$</td>
<td>(2.7 ± 0.5) $\times$ 10$^{-2}$</td>
<td>(1.7 ± 0.5) $\times$ 10$^{-7}$</td>
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<td>cPLA2-C2/D93N</td>
<td>(1.8 ± 0.2) $\times$ 10$^3$</td>
<td>(6.3 ± 0.6) $\times$ 10$^{-3}$</td>
<td>(3.5 ± 0.5) $\times$ 10$^{-8}$</td>
</tr>
<tr>
<td>cPLA2 holoenzyme</td>
<td>(9.4 ± 0.8) $\times$ 10$^3$</td>
<td>(3.6 ± 0.5) $\times$ 10$^{-2}$</td>
<td>(3.8 ± 0.8) $\times$ 10$^{-7}$</td>
</tr>
<tr>
<td>PKC-ε-C2</td>
<td>(2.9 ± 0.6) $\times$ 10$^3$</td>
<td>(1.7 ± 0.4) $\times$ 10$^{-3}$</td>
<td>(5.9 ± 0.2) $\times$ 10$^{-9}$</td>
</tr>
<tr>
<td>PKC-ε-C2/D193N</td>
<td>(1.7 ± 0.5) $\times$ 10$^3$</td>
<td>(1.3 ± 0.2) $\times$ 10$^{-2}$</td>
<td>(7.6 ± 2.0) $\times$ 10$^{-8}$</td>
</tr>
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<td>PKC-ε-C2/D248N</td>
<td>(2.5 ± 0.5) $\times$ 10$^3$</td>
<td>(3.4 ± 0.6) $\times$ 10$^{-3}$</td>
<td>(1.4 ± 0.4) $\times$ 10$^{-7}$</td>
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<tr>
<td>PKC-ε-C2/D254N</td>
<td>(1.1 ± 0.8) $\times$ 10$^3$</td>
<td>(2.9 ± 0.2) $\times$ 10$^{-2}$</td>
<td>(2.6 ± 0.8) $\times$ 10$^{-7}$</td>
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<td>PKC-ε holoenzyme</td>
<td>(3.2 ± 0.7) $\times$ 10$^3$</td>
<td>(4.6 ± 0.1) $\times$ 10$^{-3}$</td>
<td>(1.4 ± 0.3) $\times$ 10$^{-8}$</td>
</tr>
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<td>PKC-ε-C2/D193N</td>
<td>(1.1 ± 0.2) $\times$ 10$^3$</td>
<td>(1.6 ± 0.5) $\times$ 10$^{-3}$</td>
<td>(6.0 ± 1.2) $\times$ 10$^{-8}$</td>
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<tr>
<td>PKC-ε-C2/D248N</td>
<td>(6.0 ± 0.9) $\times$ 10$^3$</td>
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<td>PKC-ε-C2/D254N</td>
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<td>(5.0 ± 0.3) $\times$ 10$^{-3}$</td>
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<td>PKC-ε/C2</td>
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</tr>
<tr>
<td>PKC-ε/C2/D254N</td>
<td>(1.0 ± 0.3) $\times$ 10$^3$</td>
<td>(7.3 ± 0.9) $\times$ 10$^{-4}$</td>
<td>(7.3 ± 2.3) $\times$ 10$^{-10}$</td>
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Figure 4 Penetration of PKC-α-C2 and mutants into a POPC/POPS (7:3) monolayer

About 40 μg of PKC-α-C2 or its mutants were injected into 10 ml of subphase (10 mM Hepes, 0.1 M KCl and 0.1 mM Ca^{2+}, pH 7.4) under the POPC/POPS (7:3) monolayer at a given $\pi_0$. $\Delta\pi$ was then plotted as a function of $\pi_0$. Proteins were PKC-α-C2 (○) and its mutants D193N (▼), D248N (▲) and D254N (■). PKC-α-C2 (○) in the presence of 0.1 mM EGTA in the subphase is also shown.

Figure 5 Penetration of PKC-α and mutants into a POPC/POPS (7:3) monolayer

About 15 μg of each PKC-α was injected into 10 ml of subphase (see Figure 4) under the POPC/POPS (7:3) monolayer at a given $\pi_0$. $\Delta\pi$ was then plotted as a function of $\pi_0$. Proteins include wild-type PKC-α (●) and the D193N (▼), D248N (▲) and D254N (■) mutants.

changes, respectively, we performed monolayer-penetration studies of the proteins. In these measurements, a phospholipid monolayer of a given initial surface pressure $\pi_0$ was spread over a constant area and the change in surface pressure ($\Delta\pi$) was monitored after the injection of the protein into the subphase

Figure 6 Penetration of cPLA$_2$-C2 and mutants into a DHPC monolayer

About 40 μg of each cPLA$_2$-C2 was injected into 10 ml of subphase (see Figure 4) under the DHPC monolayer at a given $\pi_0$. $\Delta\pi$ was then plotted as a function of $\pi_0$. Proteins were wild-type cPLA$_2$-C2 (●) and the mutants C2-D40N (■), C2-N65A (▼) and C2-D93N (▲). cPLA$_2$-C2 (○) in the presence of 0.1 mM EGTA in the subphase is also shown.

Figure 7 Penetration of cPLA$_2$ and mutants into a DHPC monolayer

About 30 μg of each cPLA$_2$ was injected into 10 ml of subphase (see Figure 4) under the DHPC monolayer at a given $\pi_0$. $\Delta\pi$ was then plotted as a function of $\pi_0$. Proteins were wild-type cPLA$_2$ (●) and the D40N (■), N65A (▼) and D93N (▲) mutants.

[25]. First, we measured the monolayer penetration of PKC-α-C2 and mutants. As shown in Figure 4, Ca^{2+} had a significant effect on the monolayer penetration of PKC-α-C2. In the presence of 0.1 mM Ca^{2+}, PKC-α-C2 and its mutants D193N and D254N all showed a comparable degree of monolayer penetration under the

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same conditions. Only D248N exhibited noticeably reduced monolayer penetration. In contrast, both D248N and D254N mutants of the full-length PKC-ζ protein showed large drops in monolayer penetration compared with the wild type, whereas D193N exhibited slightly lower monolayer penetration (see Figure 5). These results are consistent with the notion that two Ca$^{2+}$ ions bound to PKC-ζ, CA2 in particular, are involved in inducing interdomain conformational changes that lead to the membrane penetration of protein (i.e. C1 domain) [20,24]. We then measured the monolayer penetration of cPLA$_2$-C2 and mutants. As illustrated in Figure 6, Ca$^{2+}$ had a dramatic effect on the monolayer penetration of cPLA$_2$-C2, again demonstrating the critical role of Ca$^{2+}$ in inducing intradomain conformational changes and membrane penetration. In the presence of 0.1 mM Ca$^{2+}$, D40N and D93N mutants of cPLA$_2$-C2 showed large decreases in monolayer penetration whereas the N65A mutant showed only modestly reduced monolayer penetration. Wild-type and mutants of the cPLA$_2$ holoenzyme showed a similar pattern (Figure 7). Thus it is evident that CA2 of cPLA$_2$-C2 induces the intradomain conformational changes that lead to membrane penetration of the C2 domain.

**DISCUSSION**

Our previous studies on the C2 domains of PKC-ζ [19] and cPLA$_2$ [17] suggested that two Ca$^{2+}$ ions have distinct roles in membrane binding and enzyme activation. More specifically, it was proposed that CA1 of PKC-ζ is mainly involved in membrane binding, whereas CA2 contributes not only to membrane binding but also to enzyme activation [9,19]. For cPLA$_2$, CA2 was proposed to be involved in inducing conformational changes to expose hydrophobic residues and enhance hydrophobic membrane binding and CA1 was suggested to be involved in proper membrane orientation of cPLA$_2$ and activation [17]. The present SPR and monolayer analyses on both isolated C2 domains and full-length proteins corroborate much of these hypotheses and, more importantly, provide important molecular details about Ca$^{2+}$-induced intra- and inter-domain conformational changes. SPR and monolayer studies on cPLA$_2$ and its C2 domain clearly show that CA2 plays a primary role in the membrane binding of the protein by inducing intradomain conformational change and ensuing membrane penetration. Resulting hydrophobic interactions will elongate the residence time of the membrane-bound C2 domain. CA1 is not critically involved in membrane binding itself but might play some role in the activation of cPLA$_2$ on the membrane surface [17]. Comparable effects of mutations on cPLA$_2$ and its isolated C2 domain also imply that the membrane binding of cPLA$_2$ is driven mainly by the Ca$^{2+}$-dependent membrane interactions of its C2 domain.

In comparison with cPLA$_2$, which contains a single membrane-targeting domain, the roles of Ca$^{2+}$ ions in the membrane binding of PKC-ζ with three membrane-targeting domains (i.e. two tandem C1 domains and a C2 domain) appear to be more complex. The monolayer penetration of PKC-ζ-C2 in the presence and absence of Ca$^{2+}$ shows that Ca$^{2+}$ ions exert a much less dramatic effect on the membrane penetration of this C2 domain that has lower intrinsic membrane-penetrating power than cPLA$_2$-C2 (see Figures 4 and 6). Evidently, Ca$^{2+}$ ions affect both non-specific membrane adsorption and specific interactions/hydrophobic interactions for PKC-ζ. CA1 is clearly involved in the non-specific membrane contact for both PKC-ζ and its isolated C2 domain. Based on the X-ray structure of the PKC-ζ-C2-Ca$^{2+}$-PS complex [9], this is more likely to be achieved by direct co-ordination of CA1 to anionic phospholipids in the membrane (e.g. PS) than by the CA1-induced change in surface electrostatic potential. The same Ca$^{2+}$ is not critically involved in specific and/or hydrophobic membrane interactions for the isolated C2 domain, but makes a significant contribution to these interactions for the full-length PKC-ζ. This can be interpreted by at least two different mechanisms. First, CA1–PS co-ordination might induce the specific interdomain conformational changes that lead to the membrane penetration of non-C2 residues and hydrophobic interactions. This model is supported by our earlier studies on PKC-ζ activation [20,24]. Second, CA1 might be able to co-ordinate PS more specifically in the presence of non-C2 residue(s). This model is consistent with the previous finding that the PS specificity of PKC-ζ is much more pronounced for the full-length PKC-ζ than for its isolated C2 domain [20]. The modest effect of the D193N mutation on the monolayer penetration of PKC-ζ favours the latter model; however, more studies are needed to distinguish these hypothetical models. The role of CA2 in the membrane binding of PKC-ζ is clearly to induce the interdomain conformational changes and membrane penetration. Although the characterization of these conformational changes is beyond the scope of this investigation, it is tempting to propose, based on our previous studies [20,24], that the conformational changes involve the movement of C1 domain(s) that eventually leads to its membrane penetration and enzyme activation. In summary, these studies demonstrate that the combination of SPR and monolayer analyses of isolated C2 domains and their parent proteins allows the determination of the distinct and specific roles of each Ca$^{2+}$ ion bound to the C2 domain. The application of this methodology to other C2 domains will lead to the better understanding of the mechanisms of Ca$^{2+}$-induced membrane binding and cellular targeting of C2 domains.

This work was supported by National Institutes of Health grants GM52598 and GM39987. W. C. is an Established Investigator of the American Heart Association. We thank Sudipto Das for his help in the purification of cPLA$_2$ and its mutants.

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Received 18 June 2001/31 July 2001; accepted 28 August 2001