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 (cPLA₂) and protein kinase C- α (PKC- α) by surface plasmon resonance and lipid monolayer analyses. Ca^{2+} ions mainly slow the membrane dissociation of cPLA₂-C2, while modulating both membrane association and dissociation rates for PKC- α -C2. Further studies with selected mutants showed that for cPLA₂ a Ca^{2+} ion bound to the C2 domain of cPLA₂ induces the intra-

domain 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- α , 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- α -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 β -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 (cPLA₂) and PKC- α , 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 cPLA₂ and PKC- β , which is similar to PKC- α , have different Ca^{2+} affinities in both the presence and absence of phospholipids [15]. Thus the C2 domains of cPLA₂ and PKC- α 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 cPLA₂, PKC- α , 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 cPLA₂ and mutants therein as well as the whole cPLA₂ and respective mutants of the C2 domain were expressed and purified as described previously [17]. PKC- α and mutants of the C2 domain were expressed as described previously [18,19]. The isolated C2 domain of PKC- α was expressed and

Abbreviations used: cPLA₂, 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 *Nco*I and *Xho*I, 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 $\times$ 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 μ l) 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 Ca^{2+}) to form a monolayer with a given initial surface pressure (π_0). 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 $\approx$ 5 min), the protein solution (typically 30 μ l) 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 ($\Delta\pi$) was measured as a function of time. Typically, the $\Delta\pi$ value reached a maximum after 35 min. The maximal $\Delta\pi$ value depended on the protein concentration and reached a saturation value (e.g. at [PKC- α] $\geq$ 1 μ g/ml). Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed $\Delta\pi$ represented a maximal value. The $\Delta\pi$ versus π_0 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 μ l/min. A high flow rate was used to circumvent mass transport effects. The association was monitored for 90 s

Figure 1 Sensorgram for PKC- α -C2 binding to POPC/POPS (7:3)

PKC- α -C2 (90 μ l, 50 nM) in 10 mM Hepes, 0.1 M NaCl, 0.5 mM Ca^{2+} and 5% glycerol, pH 7.4, was injected (a) over a POPC (control) surface and a POPC/POPS (7:3) surface to monitor the real-time binding. The data were then corrected for change of refractive index by subtracting the response to the POPC surface to yield the sensorgram shown. After the end of protein injection (b), dissociation was monitored for 250 s before the injection port was washed (c). Then, 10 μ l of 50 mM NaOH was injected (d) to completely regenerate the lipid surface. RU, resonance units.

and dissociation for 4 min. The immobilized vesicle surface was then regenerated for subsequent measurements using 10 μ l 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 K_d) of each protein were used. After each set of measurements, the entire immobilized vesicles were removed by injection of 25 μ l of 40 mM CHAPS, followed by 25 μ l of octyl glucoside at 5 μ l/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 sensorgrams were globally fitted to a 1:1 Langmuir binding model: [protein + vesicle] $\leftrightarrow$ protein + vesicle. The association phase was analysed using the equation:

$$R = [k_a C / (k_a C + k_d)] R_{\max} [1 - e^{-(k_a C + k_d)(t - t_0)}] + RI$$

where RI is the refractive index change, $R_{\max}$ is the theoretical binding capacity, C is the analyte concentration, k_a is the association rate constant, k_d is the dissociation rate constant and

t_0 is the start time of data fitting. The dissociation phase was analysed using the equation:

$$R = R_0 e^{-k_d(t-t_0)}$$

where R_0 is the response at the start of data fitting. Then, the curve-fitting efficiency was checked by residual plots and ϕ^2 . The dissociation constant (K_d) was then calculated from the equation:

$$K_d = k_d/k_a$$

RESULTS

Effects of Ca^{2+} ions on membrane association and dissociation

Our previous structure-function studies on the Ca^{2+} -co-ordinating residues of cPLA₂ [17] and PKC- α [19] suggested that two Ca^{2+} 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 Ca^{2+} (1 μM –1 mM) on the membrane association (k_a) 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 phospholipid binding at a given Ca^{2+} concentration is shown in Figure 1. DHPC, POPC/POPS (7:3) and POPC/POPS/DOG (69:30:1) were used for cPLA₂ species, PKC- α -C2 and PKC- α , respectively, to optimize the membrane binding. The selection of non-hydrolysable DHPC for cPLA₂ (and cPLA₂-C2) was to prevent phospholipid hydrolysis during binding measurements. As shown in Figure 3, Ca^{2+} showed strikingly different effects on the membrane binding of the two C2 domains: Ca^{2+} primarily influenced the k_d value for cPLA₂-C2, whereas it modulated both k_a and k_d to comparable degrees for PKC- α -C2. For cPLA₂-C2, a 1000-fold increase in Ca^{2+} led to an 11-fold increase in k_a and a much larger 60-fold decrease in k_d . For PKC- α -C2, however, it resulted in a 37-fold increase in k_a and a 36-fold decrease in k_d . Our previous study [23] indicated that non-specific electrostatic

interactions primarily accelerate the association of protein to anionic membrane surfaces, whereas hydrophobic interactions and specific interactions, whether electrostatic interactions or hydrogen bonds, mainly slow the membrane dissociation. For membrane-protein interactions, hydrophobic interactions typically involve a conformational change of the protein to expose hydrophobic protein residues, which then penetrate into the hydrophobic core of the membrane. Thus it appears that the primary role of Ca^{2+} ions bound to the cPLA₂-C2 is to enhance either hydrophobic or specific interactions. On the other hand, PKC- α -C2-bound Ca^{2+} ions seem to be involved in both non-specific electrostatic interactions and hydrophobic/specific interactions.

Role of each Ca^{2+} ion in membrane association and dissociation

To investigate these aspects further, we then measured the effects of mutations of specific Ca^{2+} -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- α , three residues involved in co-ordination to CA1 (Asp¹⁹³), CA2 (Asp²⁵⁴) and both (Asp²⁴⁸) were mutated. Similarly, three residues involved in co-ordination to CA1 (Asn⁶⁵), CA2 (Asp⁹³) and both (Asp⁴⁰) 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 k_a and k_d values at 0.1 mM Ca^{2+} are listed in Table 1. At this Ca^{2+} concentration, the largest differences were observed between the wild-type and most of the mutants. For cPLA₂, the mutation of CA1 ligand Asn⁶⁵ to Ala only modestly changed k_a and k_d for both isolated C2 domain and holoenzyme, but the effect on k_d 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 Asp⁹³ to Asn resulted in a $\approx$ 3-fold decrease in k_a and a much larger 10–20-fold increase in k_d for both isolated domain and holoenzyme. This result indicates

Figure 2 Schematic representations of Ca^{2+} -binding loops of cPLA₂ and PKC- α

Calcium ligands and two calcium ions (CA1 and CA2) are illustrated. For PKC- α -C2, a PS head group co-ordinated to CA1 is also shown. CBR1–CBR3 indicate three calcium-binding loops.

Figure 3 Effects of calcium on membrane association and dissociation rates of PKC- α -C2 (A) and cPLA₂-C2 (B)

Relative rate constants indicate the ratios of k_a (○) and k_d (●) values to those at 1 μM Ca^{2+} , respectively. L1 sensor chips coated with DHPC vesicles and POPC/POPS (7:3) were used for cPLA₂-C2 and PKC- α -C2, respectively. All measurements were performed in 10 mM Hepes, pH 7.4, containing 0.1 M NaCl. Absolute values of k_a and k_d at 1 μM Ca^{2+} were: $k_a = (6.4 \pm 0.7) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_d = (8.6 \pm 0.9) \times 10^{-2} \text{ s}^{-1}$ for cPLA₂-C2 and $k_a = (8.6 \pm 0.9) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_d = (1.7 \pm 0.2) \times 10^{-1} \text{ s}^{-1}$ for PKC- α -C2.

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 Asp⁴⁰ to Asn also primarily affected k_d : however, the degree of change was smaller than that observed for D93N, underscoring the hypothesis that Asp⁴⁰ is not as critical as Asp⁹³ in CA2 co-

Table 1 Binding parameters for Ca^{2+} ligand mutants of cPLA₂, PKC- α and their C2 domains

Parameters represent means $\pm$ S.D. from three to five determinations. All measurements were performed with 0.1 M NaCl and 0.1 mM Ca^{2+} . DHPC was used for cPLA₂ species. POPC/POPS/DOG (69:30:1) and POPC/POPS (7:3) were used for PKC- α and its C2 domains, respectively.

Enzymes	k_a ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_d (s^{-1})	K_d (M)
cPLA ₂ -C2			
cPLA ₂ -C2	$(3.0 \pm 0.6) \times 10^5$	$(3.3 \pm 0.7) \times 10^{-3}$	$(1.1 \pm 0.3) \times 10^{-8}$
cPLA ₂ -C2/D40N	$(1.6 \pm 0.4) \times 10^5$	$(2.7 \pm 0.5) \times 10^{-2}$	$(1.7 \pm 0.5) \times 10^{-7}$
cPLA ₂ -C2/N65A	$(1.8 \pm 0.2) \times 10^5$	$(6.3 \pm 0.6) \times 10^{-3}$	$(3.5 \pm 0.5) \times 10^{-8}$
cPLA ₂ -C2/D93N	$(9.4 \pm 0.8) \times 10^4$	$(3.6 \pm 0.5) \times 10^{-2}$	$(3.8 \pm 0.8) \times 10^{-7}$
cPLA ₂ holoenzyme			
cPLA ₂	$(2.9 \pm 0.6) \times 10^5$	$(1.7 \pm 0.4) \times 10^{-3}$	$(5.9 \pm 0.3) \times 10^{-9}$
cPLA ₂ /D40N	$(1.7 \pm 0.5) \times 10^5$	$(1.3 \pm 0.2) \times 10^{-2}$	$(7.6 \pm 2.0) \times 10^{-8}$
cPLA ₂ /N65A	$(2.5 \pm 0.5) \times 10^5$	$(3.4 \pm 0.6) \times 10^{-3}$	$(1.4 \pm 0.4) \times 10^{-8}$
cPLA ₂ /D93N	$(1.1 \pm 0.8) \times 10^5$	$(2.9 \pm 0.2) \times 10^{-2}$	$(2.6 \pm 0.8) \times 10^{-7}$
PKC- α -C2			
PKC- α -C2	$(3.2 \pm 0.7) \times 10^5$	$(4.6 \pm 0.1) \times 10^{-3}$	$(1.4 \pm 0.3) \times 10^{-8}$
PKC- α -C2/D193N	$(1.1 \pm 0.2) \times 10^5$	$(6.6 \pm 0.5) \times 10^{-3}$	$(6.0 \pm 1.2) \times 10^{-8}$
PKC- α -C2/D248N	$(8.0 \pm 0.9) \times 10^4$	$(1.6 \pm 0.4) \times 10^{-2}$	$(2.0 \pm 0.6) \times 10^{-7}$
PKC- α -C2/D254N	$(3.1 \pm 0.6) \times 10^5$	$(5.0 \pm 0.5) \times 10^{-3}$	$(1.6 \pm 0.3) \times 10^{-8}$
PKC- α holoenzyme			
PKC- α	$(1.5 \pm 0.5) \times 10^6$	$(1.2 \pm 0.3) \times 10^{-4}$	$(8.0 \pm 3.0) \times 10^{-11}$
PKC- α /D193N	$(4.2 \pm 0.3) \times 10^5$	$(5.9 \pm 0.5) \times 10^{-4}$	$(1.4 \pm 0.2) \times 10^{-9}$
PKC- α /D248N	$(4.9 \pm 0.9) \times 10^5$	$(1.5 \pm 0.2) \times 10^{-3}$	$(3.1 \pm 0.6) \times 10^{-9}$
PKC- α /D254N	$(1.0 \pm 0.3) \times 10^6$	$(7.3 \pm 0.9) \times 10^{-4}$	$(7.3 \pm 2.3) \times 10^{-10}$

ordination and that CA1 is not essential for the membrane binding of cPLA₂-C2.

For PKC- α -C2, the mutation of a CA1 ligand, Asp¹⁹³, to Asn lowered k_a by 2.9-fold and raised k_d by 1.4-fold. The X-ray structure of PKC- α -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- α -C2. Interestingly, the same mutation led to a larger 4.9-fold increase in k_d for the PKC- α holoenzyme while showing a similar 3.6-fold decrease in k_a . Thus for the whole PKC- α 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- α induces the membrane penetration of its C1 domain, thereby resulting in hydrophobic interactions [20]. Thus unlike 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 Asp²⁵⁴ to Asn had little effect on k_a and k_d for PKC- α -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_d (with only a 1.5-fold decrease in k_a) for the holoenzyme, indicating that CA2 induces the interdomain conformational changes. Similarly, the differential mutational effects of bidentate ligand Asp²⁴⁸ on PKC- α -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- α -C2 and CA2 of cPLA₂-C2 induce inter- and intra-domain conformational

Figure 4 Penetration of PKC- α -C2 and mutants into a POPC/POPS (7:3) monolayer

About 40 μ g 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 π_0 . $\Delta\pi$ was then plotted as a function of π_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 μ g of each PKC- α was injected into 10 ml of subphase (see Figure 4) under the POPC/POPS (7:3) monolayer at a given π_0 . $\Delta\pi$ was then plotted as a function of π_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 π_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 cPLA₂-C2 and mutants into a DHPC monolayer

About 40 μ g of each cPLA₂-C2 was injected into 10 ml of subphase (see Figure 4) under the DHPC monolayer at a given π_0 . $\Delta\pi$ was then plotted as a function of π_0 . Proteins were wild-type cPLA₂-C2 (●) and the mutants C2-D40N (■), C2-N65A (▼) and C2-D93N (▲). cPLA₂-C2 (○) in the presence of 0.1 mM EGTA in the subphase is also shown.

Figure 7 Penetration of cPLA₂ and mutants into a DHPC monolayer

About 30 μ g of each cPLA₂ was injected into 10 ml of subphase (see Figure 4) under the DHPC monolayer at a given π_0 . $\Delta\pi$ was then plotted as a function of π_0 . Proteins were wild-type cPLA₂ (●) 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

same conditions. Only D248N exhibited noticeably reduced monolayer penetration. In contrast, both D248N and D254N mutants of the full-length PKC- α 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- α , 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 cPLA₂-C2 and mutants. As illustrated in Figure 6, Ca^{2+} had a dramatic effect on the monolayer penetration of cPLA₂-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 cPLA₂-C2 showed large decreases in monolayer penetration whereas the N65A mutant showed only modestly reduced monolayer penetration. Wild-type and mutants of the cPLA₂ holoenzyme showed a similar pattern (Figure 7). Thus it is evident that CA2 of cPLA₂-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- α [19] and cPLA₂ [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- α is mainly involved in membrane binding, whereas CA2 contributes not only to membrane binding but also to enzyme activation [9,19]. For cPLA₂, 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 cPLA₂ 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 cPLA₂ 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 cPLA₂ on the membrane surface [17]. Comparable effects of mutations on cPLA₂ and its isolated C2 domain also imply that the membrane binding of cPLA₂ is driven mainly by the Ca^{2+} -dependent membrane interactions of its C2 domain.

In comparison with cPLA₂, which contains a single membrane-targeting domain, the roles of Ca^{2+} ions in the membrane binding of PKC- α 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- α -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 cPLA₂-C2 (see Figures 4 and 6). Evidently, Ca^{2+} ions affect both non-specific membrane adsorption and specific interactions/hydrophobic interactions for PKC- α . CA1 is clearly involved in the non-specific membrane contact for both PKC- α and its isolated C2 domain. Based on the X-ray structure of the PKC- α -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- α . 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- α 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- α is much more pronounced for the full-length PKC- α than for its isolated C2 domain [20]. The modest effect of the D193N mutation on the monolayer penetration of PKC- α favours the latter model: however, more studies are needed to distinguish these hypothetical models. The role of CA2 in the membrane binding of PKC- α 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 GM53987. W. C. is an Established Investigator of the American Heart Association. We thank Sudipto Das for his help in the purification of cPLA₂ and its mutants.

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