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Synergistic effect of Pb^{2+} and PIP_2 on C2 domain-membrane interactions

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Abstract

Ca^{2+} -responsive C2 domains are peripheral membrane modules that target their host proteins to anionic membranes upon binding Ca^{2+} ions. Several C2-domain containing proteins, such as Protein Kinase C (PKC) isoenzymes, have been identified as molecular targets of Pb^{2+} , a known environmental toxin. We demonstrated previously that the C2 domain from PKC α (C2 α) binds Pb^{2+} with high affinity and undergoes membrane insertion in the Pb^{2+} -complexed form. The objective of this work was to determine the effect of phosphatidylinositol-4,5-bisphosphate (PIP_2) on the C2 α - Pb^{2+} interactions. Using Nuclear Magnetic Resonance (NMR) experiments, we show that Pb^{2+} and PIP_2 synergistically enhance each other's affinity to C2 α . Moreover, the affinity of C2 α to PIP_2 increases upon progressive saturation of the metal-binding sites. Combining the NMR data with the results of protein-to-membrane Förster Resonance Energy Transfer (FRET) and vesicle sedimentation experiments, we demonstrate that PIP_2 can influence two aspects of C2 α - Pb^{2+} -membrane interactions: the affinity of C2 α to Pb^{2+} , and the association of Pb^{2+} with the anionic sites on the membrane. Both factors may contribute to the toxic effect of Pb^{2+} resulting from the aberrant modulation of PKC α activity. Finally, we propose a mechanism for Pb^{2+} outcompeting Ca^{2+} from the membrane-bound C2 α .

Keywords

C2 domain; Protein Kinase C; phosphatidylinositol-4,5-bisphosphate; PIP_2 ; peripheral membrane protein; protein-membrane interactions; $Pb(II)$; Pb toxicity; NMR; protein-to-membrane FRET

INTRODUCTION

Conserved homology 2 (C2) domains are found in ~100 known eukaryotic host proteins that play key roles in signal transduction and membrane trafficking.¹ C2 domains, many of which function as Ca^{2+} sensors within their host proteins, are independently folded structural and functional modules of ~130 amino acids.^{2–4} The binding of two or more Ca^{2+} ions^{5–8} drives the association of Ca^{2+} -responsive C2 domains with lipid membranes, where they recognize the head group of phosphatidylserine (PtdSer) and other anionic phospholipids.^{8–12} The role of metal ions in promoting the C2-membrane association was proposed to be three-fold:¹ (i) modulation of the C2 electrostatic potential;^{13,14} (ii) direct

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Supporting Information Available. Full NMR spectra of C2 α in three states of metal ligation at varying concentrations of Ca^{2+} - PIP_2 ; fluorescence control experiments and Ca^{2+} / Pb^{2+} competition results; ICP measurements of membrane-bound Pb^{2+} ; cryoelectron microscopy images of LUV suspensions; superposition of C2 α structures; Hahn-Echo relaxation data; and estimation of C2 α affinities to metal ions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

coordination of the anionic lipid head group(s),¹⁵ and (iii) facilitation of the inter-domain rearrangement in multi-domain proteins.^{16,17}

C2 domains can interact with Pb^{2+} , a potent environmental toxin that acts as a Ca^{2+} surrogate in the context of specific molecular targets.¹⁸ Interaction of Pb^{2+} with several Ca^{2+} -dependent C2 domain-containing proteins has been implicated in the disruption of neurotransmitter release,¹⁹ which is a hallmark of Pb^{2+} poisoning. One of the notable examples is synaptotagmin 1, a membrane-associated Ca^{2+} sensor protein that is localized to the synaptic vesicles. The Ca^{2+} -sensing function of synaptotagmin 1 is carried out by two C-terminal C2 domains: C2A and C2B. Pb^{2+} was found to be ~1000-fold more potent than Ca^{2+} in driving the association of synaptotagmin 1 with lipid membranes; in addition, Pb^{2+} ions disrupted the interactions between synaptotagmin 1 and its molecular partner, syntaxin.²⁰

Another example is conventional Protein Kinase C isoenzymes (cPKCs) that comprise α , βI , βII , and γ isoforms.^{21,22} The stimulatory effect of PKCs on the neurotransmitter release has been attributed to the phosphorylation of ion channels and neuronal proteins involved in synaptic vesicle dynamics.²³ It has also been demonstrated that activation of PKC increases the size of the readily releasable vesicle pool and the rate with which it is replenished.²⁴ The kinase function of cPKCs is activated upon protein translocation to the plasma membrane in response to binding two second messengers: Ca^{2+} and diacylglycerol. A single C2 domain is responsible for the Ca^{2+} -induced activation of cPKCs. Following the original report of Pb^{2+} modulating the cPKC activity,²⁵ a number of studies demonstrated that conventional cPKCs are activated by picomolar-to-nanomolar and partially inhibited by micromolar concentrations of Pb^{2+} .^{26–28} Experiments on the recombinant PKC α isoform provided evidence for the existence of three Pb^{2+} interaction sites.²⁸ Recently, our laboratory reported that the high-affinity Pb^{2+} interaction site resides on the C2 domain of PKC α .⁶

Despite the well-documented effect of Pb^{2+} on the membrane-dependent activity of C2 domain-containing proteins, the molecular mechanism of Pb^{2+} action at the membrane is not fully understood. The objective of this work was to determine the influence of a lipid second messenger, phosphatidylinositol-4,5-bisphosphate (PIP₂), on the interactions of C2 domains with Pb^{2+} .

PIP₂ is involved in the regulation of a variety of cellular processes^{29,30} including synaptic vesicle trafficking.³¹ PIP₂ is selectively enriched in the cytoplasmic leaflet of the plasma membrane, where it occurs at a molar concentration of 1–3%. The significance of PIP₂ as a modulator of C2-membrane interactions became apparent upon detection of its specific binding to the C2 domains from conventional PKC isoforms,^{32,33} synaptotagmin 1,^{34,35} and rabphilin-3A.^{36,37} The presence of PIP₂ in PtdSer-containing lipid membranes increases the Ca^{2+} affinity of C2 domains^{33,38–40} and makes them responsive to Ca^{2+} concentrations that are typically associated with the Ca^{2+} spike during signaling. The role of PIP₂ as a sole determinant of PKC α localization to plasma membrane is still being debated.^{38,41} However, there is general agreement that the presence of PIP₂ in the membrane results in the decrease of the C2 α off-rate and, as a result, the increased residency time of the protein on the plasma membrane.^{38,39}

To determine the influence of PIP₂ on the C2- Pb^{2+} interactions, we used the C2 domain from PKC α (C2 α) as a paradigm for Ca^{2+} -responsive C2 domains. In one of the first studies of the C2 α -PIP₂ system, Corbalán-García et al.³² demonstrated that C2 α could bind to membrane-embedded PIP₂ even in the absence of the PtdSer component, albeit with a lower affinity. Another unexpected finding was that the association of C2 α and PIP₂ occurred even in the absence of Ca^{2+} . The specific region of C2 α involved in PIP₂ interactions,

which is distinct from the PtdSer binding site, was identified using mutagenesis.^{32,42} A subsequent determination of the crystal structure of the ternary C2 α -Ca²⁺-PIP₂ complex revealed the molecular details of the PIP₂ site.⁴³ Figure 1 shows the structure of the complex with all functional regions highlighted. The tip of the C2 α domain comprises three calcium-binding loops (CBLs). Two of them, CBL1 and CBL3, coordinate calcium ions. EPR studies identified CBL1 and CBL3 as the protein segments that penetrate the lipid headgroup region of the PtdSer-containing membranes.⁴⁴ The PIP₂ binding site is mostly formed by two beta-strands, β 3 and β 4. A total of six residues are involved in the interactions with the oxygen atoms of the three phosphate groups in PIP₂: Y195, K197, K209, K211, W245, and N253. The three lysines that belong to strands β 3 and β 4 – K197, K209, and K211 – are part of the region that has been referred to in the literature as the “ β -groove”, “polybasic cluster”, or “lysine-rich cluster”. We will use the term lysine-rich cluster in this paper.

Recently, our laboratory has characterized the interactions of C2 α with Pb²⁺ in the presence and absence of PtdSer-containing model membranes.⁶ We determined the crystal structure of Pb²⁺-complexed C2 α , established that C2 α has two Pb²⁺-binding sites with a 2,000-fold difference in binding affinities, and demonstrated that Pb²⁺ can outcompete Ca²⁺ from C2 α in the presence of PtdSer-containing model membranes. In this work, we employed Nuclear Magnetic Resonance (NMR) spectroscopy, protein-to-membrane Förster Resonance Energy Transfer (FRET), and vesicle sedimentation experiments to determine the effect of PIP₂ on the C2 α -Pb²⁺ and C2 α -Pb²⁺-membrane interactions. Our findings not only shed light on the molecular mechanism of C2 α -Pb²⁺-PIP₂ interactions, but also illustrate the contribution of PIP₂ to Pb²⁺ interfering with the Ca²⁺-dependent function of C2 α .

EXPERIMENTAL PROCEDURES

Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), L- α -phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (bp-PIP₂), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (dansyl-PE) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Short chain (C4) D-myo-phosphatidylinositol-(4,5)-bisphosphate (C4-PIP₂) used for NMR-detected titrations was purchased from Echelon Bioscience (Salt Lake City, UT). The ion-chelating resin, Chelex 100 (Sigma-Aldrich), was used to treat all buffers to remove residual divalent metal ions.

C2 α over-expression and purification

Over-expression and purification of C2 α was carried out as previously described.⁶ Uniformly ¹⁵N-enriched ([U-¹⁵N]) C2 α was prepared using the method of Marley et al.⁴⁵ De-calcification of C2 α was accomplished by carrying out the last purification step in the presence of 0.1 mM ethylenediaminetetraacetic acid (EDTA). EDTA was subsequently removed by several rounds of solvent exchange into a decalcified 10 mM MES buffer at pH 6.0 and 100 mM KCl.

Binding of C4-PIP₂ to C2 α monitored by NMR

The binding of C4-PIP₂ to [U-¹⁵N] C2 α was monitored using NMR-detected titration experiments. All experiments were carried out at 25 °C on a Varian Inova spectrometer operating at a ¹H Larmor frequency of 600 MHz (14.1 T). A series of ¹⁵N-¹H Heteronuclear Single Quantum Coherence (HSQC) spectra were collected for C2 α in three different states of metal ligation: apo, Ca²⁺-bound, and single Pb²⁺-bound, at increasing concentrations of C4-PIP₂.

The Ca^{2+} -bound state, $\text{C2}\alpha\cdot\text{Ca}_2$, was prepared by adding Ca^{2+} to a final concentration of 2.5 mM to the 100 μM apo $\text{C2}\alpha$. The single Pb^{2+} -bound $\text{C2}\alpha$, $\text{C2}\alpha\cdot\text{Pb}$, was prepared by adding Pb^{2+} to a final concentration of 100 μM to the 100 μM apo- $\text{C2}\alpha$. We observed significant precipitation of C4-PIP_2 at Pb^{2+} concentrations that are required to generate the $\text{C2}\alpha\cdot\text{Pb}_2$ state. This prevented us from carrying out the C4-PIP_2 titration experiments on the $\text{C2}\alpha\cdot\text{Pb}_2$ complex. To determine the affinity of Pb^{2+} to the second $\text{C2}\alpha$ site in the presence of saturating C4-PIP_2 , we titrated Pb^{2+} into the preformed $\text{C2}\alpha\cdot\text{Pb}\cdot\text{PIP}_2$ complex. The concentration of C4-PIP_2 in the mixture was 1.5 mM. The NMR buffer for all experiments was 10 mM MES (pH 6.0), 100 mM KCl, 8% D_2O and 0.02% NaN_3 . The C4-PIP_2 stock solution was standardized using phosphate quantification assay.⁴⁶ NMR data were processed with NMRPipe⁴⁷ and analyzed using Sparky.⁴⁸

Site-specific chemical shift perturbations of $\text{C2}\alpha$ due to ligand binding were calculated according to the following equation:

$$\Delta = [\Delta\delta_H^2 + (0.152\Delta\delta_N)^2]^{1/2} \quad \text{Eq. (1)}$$

where $\Delta\delta_H$ and $\Delta\delta_N$ are the residue-specific chemical shift changes between the two states that are being compared, and 0.152 is the scaling factor.⁴⁹

Binding curves were constructed by plotting the change in $\text{C2}\alpha$ chemical shifts as a function of the total ligand concentration. The dissociation constants, K_d , were determined by globally fitting the binding curves with a single-site binding equation:⁵⁰

$$\Delta\delta = (\Delta\delta_{\text{PL}}/2P_0)[K_d + P_0 + L_0 - ((K_d + P_0 + L_0)^2 - 4P_0L_0)^{1/2}] \quad \text{Eq. (2)}$$

where $\Delta\delta$ is a combined residue-specific change in chemical shift at total ligand concentration L_0 ; P_0 is the total protein concentration; and $\Delta\delta_{\text{PL}}$ is the residue-specific chemical shift difference between the complexed and free protein species.

Association of $\text{C2}\alpha$ with PIP_2 -containing membranes measured by FRET

Protein-to-membrane FRET experiments were carried out using the ISS Phoenix spectrofluorometer. The Trp residues of $\text{C2}\alpha$ and the dansyl-PE lipid component of the large unilamellar vesicles (LUVs) were used as the donor and acceptor, respectively. The lipid composition of LUVs was POPC/POPS/bp- PIP_2 /dansyl-PE with a molar ratio of 61:30:2:7. Fluorescence emission spectra of the sample containing 0.5 μM $\text{C2}\alpha$ and LUVs (150 μM total lipid concentration) were collected at increasing concentrations of the metal ion, Ca^{2+} or Pb^{2+} . The spectra were corrected by subtracting the fluorescence of the reference sample that contained all components but $\text{C2}\alpha$. The change in FRET intensity at 505 nm generated upon $\text{C2}\alpha$ membrane binding, ΔF , relative to the maximum attainable change, ΔF_{max} , was plotted as a function of the total metal concentration. Data were fitted with the following equation:

$$\Delta F/\Delta F_{\text{max}} = \frac{[M^{2+}]^H}{[M^{2+}]^H + [M^{2+}]_{1/2}^H} \quad \text{Eq. (3)}$$

where H is the Hill coefficient and $[M^{2+}]_{1/2}$ is the metal concentration required for half-maximum binding.

Calculations of the protein electrostatic potential

The calculations were carried out using the Adaptive Poisson-Boltzmann Solver (APBS)⁵¹ implemented through the Visual Molecular Dynamics (VMD)⁵² plugin. The PDB files were prepared for electrostatics calculations using the PDB2PQR software package.⁵³ The parameterization of the PIP₂ ligand produced a net charge of -4 , which is consistent with the available experimental evidence.⁵⁴ Pb²⁺ ions were parameterized with a Born radius of 2.17 Å.⁵⁵ Figures showing the surface-mapped electrostatic potential were generated with VMD.

RESULTS

C2α binds PIP₂ in all three states of metal ligation

To determine the effect of Pb²⁺ on the C2α-PIP₂ interactions, we carried out a series of NMR-detected titrations of C2α with a water-soluble C4-PIP₂. We made use of the single high-affinity Pb²⁺-binding site on C2α and prepared the C2α·Pb state by mixing the equimolar quantities of protein and metal ion. To provide the reference for our Pb²⁺ data and determine the general role of divalent metal ions in modulating the affinity of C2α to PIP₂, we also tested the binding of C4-PIP₂ to the apo- and Ca²⁺-complexed forms of C2α. ¹H-¹⁵N HSQC spectra were collected on the [U-¹⁵N] C2α that was prepared in three states of metal ligation: apo, Ca²⁺-saturated (C2α·Ca₂), and single Pb²⁺-bound (C2α·Pb), with C4-PIP₂ concentrations ranging from 0 to 1.5 mM.

The results are presented in Figure 2. Panels A, B, and C are the expansions of the ¹H-¹⁵N HSQC spectra that illustrate the response of the C2α N-H resonances (or cross-peaks) to increasing concentrations of C4-PIP₂. The amino-acid identities of the C2α cross-peaks were determined previously using a set of triple-resonance NMR assignment experiments.⁶ Full spectra are shown in Figure S1 of the Supporting Information. In all three states of metal ligation, the binding of C4-PIP₂ to C2α falls into the exchange regime that is fast on the NMR chemical-shift timescale. In this regime, the cross-peak positions change smoothly as a result of increasing ligand concentration. Based on the absolute value of chemical shift changes, Ca²⁺- and Pb²⁺-complexed forms of C2α show a more pronounced response to the C4-PIP₂ binding than the apo-form.

Chemical shift perturbations that are experienced by C2α residues between 0 and 1–1.5 mM C4-PIP₂ were calculated using Eq. 1. In all cases, we used the chemical shifts of the appropriate metal-ligated form as a reference state. Panels D, E, and F in Figure 2 show the color-coded Δ values mapped onto the corresponding C2α crystal structures: apo (3RDJ),⁶ Ca²⁺-complexed (1DSY),¹⁵ and Pb²⁺-complexed (3TWY).⁶ In the latter, only the Pb²⁺ ion that occupies the high-affinity C2α site is shown. The protein region affected by the C4-PIP₂ binding is not limited to the lysine-rich cluster, but also includes the calcium-binding loops.

This effect is further illustrated in Figure 3 by plotting the Δ values as a function of primary structure. Three C2α regions – irrespective of the metal-ligation state – experience chemical shift perturbation upon C4-PIP₂ binding: the lysine-rich cluster located on the β3–β4 segment; CBL1, which immediately precedes the β3–β4 region; and CBL3, which is distant from the β3–β4 region in the primary structure but is close to it in the tertiary structure. The smaller effect of PIP₂ binding on the apo state of C2α is evident from the magnitude of chemical shift perturbations. Given that the backbone conformation of the C2α·Ca²⁺-PIP₂ complex is close to that of the apo-form (the rmsd for the backbone atoms is 0.40 Å), the chemical-shift perturbations are likely due to the changes in the electrostatic properties and/or conformational dynamics of the protein. The chemical shift perturbation pattern is very similar for C2α·Ca₂ and C2α·Pb (Figures 3B and 3C), indicating that a full complement of metal ions is not a stringent requirement for the C2α-PIP₂ interactions.

Pb²⁺ enhances the affinity of C2α to PIP₂

To obtain the affinities of C2α to C4-PIP₂, we constructed binding curves for all N-H groups whose combined ¹⁵N and ¹H chemical shift changed by more than 0.05 in the course of the titration. The data are presented in Figure 4 for 2 representative residues: K197 and Q210. Each panel in Figure 4 shows the residue-specific binding curves for three different states of metal ligation: apo C2α, C2α·Pb, and C2α·Ca₂.

In the apo C2α, the binding curves for all but two PIP₂-responsive residues could be globally fitted with Eq. 2, which yielded a K_d of 1130 ± 38 μM. These data indicate that, in the absence of metal ions and lipid membranes, C2α is a low-affinity PIP₂ binding module. Having a single Pb²⁺ bound to the high-affinity C2α site increases the affinity of the protein to PIP₂. The global fit of the C2α·Pb binding curves produced a K_d of 198 ± 2 μM. The C2α·Ca₂ state showed a more complex response to PIP₂. To facilitate the analysis of binding data, we separated the 38 responsive residues into three groups based on their titration behavior. Group 1 comprises 27 members that either belong to the β3–β4 region or are in its immediate vicinity. Group 1 data can be well fitted with the single-site binding equation to obtain a K_d of 48 ± 1 μM (Figure 4). Groups 2 and 3 are further described in the “Interplay between Ca²⁺ and PIP₂” section.

In summary, the affinity of metal ion-complexed C2α to PIP₂ is significantly enhanced compared to that of the apo-form. The K_d for the C2α·Pb-PIP₂ complex falls in between the K_d values of the C2α·PIP₂ and C2α·Ca₂-PIP₂ complexes. This effect – the 4–6 fold decrease in K_d upon progressive saturation of C2α metal-binding sites – appears to depend mostly on the number of bound metal ions (and hence the overall charge of the protein).

Figure 5 highlights the contributions of individual metal-binding sites of C2α to PIP₂ binding. For this analysis, we chose the sub-stoichiometric concentration of C4-PIP₂ – 80 μM – to avoid the saturation regime, where the effect of the increased PIP₂ affinity would not be discernible. The residues chosen for the chemical shift perturbation analysis belong to the β3–β4 region. The residues from this region respond weakly if at all to metal ions but show significant perturbations in response to PIP₂ binding, as illustrated in Figure 5A (blue, purple, and orange spectra). The binding of the second Pb²⁺ ion to C2α results in the shift of cross-peaks that is solely due to the enhancement of the C2α affinity to PIP₂ (green spectrum). The spectra of Pb²⁺- (green) and Ca²⁺-complexed (grey) C2α in the presence of 80 μM PIP₂ are virtually identical for the β3–β4 region, which is not the case for the CBLs.

The results of the chemical shift perturbation analysis is shown in Figure 5B. For the Pb²⁺ data, there are two processes that contribute to the observed C2α chemical shift change: the binding of PIP₂ to the C2α·Pb state (orange bars) and the binding of additional PIP₂ upon filling the second Pb²⁺-binding site (green bars). For many residues in the β3–β4 region, the sum of these two contributions is virtually identical to the Δ values obtained for the PIP₂ binding to the C2α·Ca₂ state (grey bars). This “additive” behavior illustrates that the enhancement of the C2α affinity to PIP₂ brought about by metal ions does not depend on the identity of metal ions, at least for the Ca²⁺ and Pb²⁺ pair.

PIP₂ enhances the affinity of C2α to Pb²⁺

The next step was to determine the binding affinities of C2α to Pb²⁺ in the presence of saturating PIP₂. The Pb²⁺ affinities of two C2α metal-binding sites, (1) and (2), differ 2,000-fold. The K_d for the high-affinity site (1) was determined in our previous study by isothermal titration calorimetry (ITC).⁶ However, the quantities of C4-PIP₂ required to produce the PIP₂-saturated state of apo C2α make the ITC experiments cost-prohibitive in this case. Instead, we determined the K_d for the low-affinity Pb²⁺ site (2) in the presence of

saturating PIP₂. This value, $K_{d2}^{Pb,PIP}$, was then used to calculate the dissociation constant for the first site, $K_{d1}^{Pb,PIP}$ (*vide infra*).

We prepared the C2 α -Pb-PIP₂ complex by combining the equimolar amounts of C2 α and Pb²⁺, followed by the addition of C4-PIP₂ to the final concentration of 1.5 mM. According to the binding data of Figure 4, under these conditions, the C2 α -Pb-PIP₂ complex is the dominant protein species in solution. The titration of the C2 α -Pb-PIP₂ complex with Pb²⁺ was monitored using ¹⁵N-¹H HSQC experiments. We identified the residues that responded to Pb²⁺(2) binding and constructed a total of 8 binding curves. All of them were fitted globally with Eq. 2, which produced a $K_{d2}^{Pb,PIP}$ of $17 \pm 1 \mu\text{M}$. An example of the binding curve for D248 is shown in Figure 6A. For comparison, on the same graph, we plotted the D248 data for Pb²⁺(2) binding to the C2 α -Pb complex in the absence of PIP₂. The K_d for this process, $129 \pm 4 \mu\text{M}$, was determined in our previous work.⁶ These data indicate that the pre-bound PIP₂ results in the 8-fold increase of the C2 α affinity to Pb²⁺(2).

To understand the effect of Pb²⁺(2) binding on the PIP₂-complexed C2 α , we carried out chemical shift perturbation analysis for the pair of PIP₂ complexes, C2 α -Pb₂-PIP₂ and C2 α -Pb-PIP₂, with the latter used as a reference state. The results are shown in Figure 6B. It is evident that the binding of Pb²⁺(2) to C2 α mostly affects CBL3, and has little effect on CBL1 or the lysine-rich cluster.

Combining the data obtained in this and previous work,⁶ we constructed a full scheme of thermodynamic equilibria present in the C2 α , Pb²⁺, and PIP₂ system. We have experimentally determined the dissociation constants for the five out of seven binding equilibria, as indicated in Figure 7. Using simple thermodynamic considerations, we were able to calculate the two remaining dissociation constants. $K_{d1}^{Pb,PIP}$ for the reaction of Pb²⁺(1) dissociation from the C2 α -Pb-PIP₂ complex is 12 nM; this represents a ~6-fold increase in the Pb²⁺(1) affinity compared to the PIP₂-lacking system. $K_d^{PIP,Pb(2)}$ for the reaction of PIP₂ dissociation from the C2 α -Pb₂-PIP₂ complex is 26 μM , which corresponds to a ~43-fold increase in PIP₂ affinity compared to the apo C2 α . In summary, our binding data clearly illustrate that Pb²⁺ and PIP₂ significantly enhance each other's interactions with C2 α .

Interplay between Ca²⁺ and PIP₂

The NMR response of C2 α -Ca₂ to PIP₂ binding was rather complex, which prompted us to separate the responsive residues into three groups. The 27 residues of Group 1 – the largest – belong to the lysine-rich cluster and adjacent protein regions (*vide supra*). The residues of Groups 2 and 3 could be fit with K_d values that are either above or below the K_d values that were obtained for the residues of Group 1. The corresponding binding curves and the table of K_d values are given in Figure S2. Figures 8A and 8B show the NMR-detected binding curves for D248 and N253 as representative members of Groups 2 and 3, respectively.

There are six residues in Group 2: D248, T250, D254, F255, G257, and Q280. Four of them: D248, T250, D254, and F255 belong to the CBL3 or its hinges, as shown in Figure 8C. Group 3 with five members: D187, G190, L191, Y195, and N253 maps onto the CBL1 region of C2 α (Figure 8C, blue) with the exception of Y195 and N253, which reside on the β 3 strand and CBL3, respectively.

There could be two possible explanations for the differences in the apparent PIP₂ dissociation constants. First, if the conformational dynamics of the PIP₂- and Ca²⁺-complexed C2 α is different from that of the Ca²⁺-complexed C2 α , this difference could contribute to the observed chemical shift changes during the PIP₂ titration. Second, an additional binding process that affects the backbone chemical shifts of specific protein

segments and manifests itself as an apparent change in the C2 α affinity to PIP₂ could be present in the system. This additional process is likely to be the binding of the third Ca²⁺ ion to the PIP₂-complexed C2 α . In the crystal structure of the PIP₂-complexed C2 α , Ca²⁺(3) is coordinated by the carboxyl oxygens of D254 and D248, the sidechain oxygen of T251, and the backbone carbonyl oxygen of R252. We know from our previous work that, in the absence of PIP₂, the Ca²⁺(3) site is not populated under the conditions of our NMR experiments. However, the binding of PIP₂ to the C2 α ·Ca₂ complex may enhance the affinity of C2 α to Ca²⁺(3) in much the same way it enhances the affinity of C2 α ·Pb·PIP₂ to Pb²⁺(2) (*vide supra*). As a result, some protein residues may show the titration behavior that essentially reflects the two binding processes that involve PIP₂ and Ca²⁺(3). As a final note, the heterogeneity in the apparent K_d values for Group 2 and 3 residues is specific to the C2 α -Ca²⁺ system where Ca²⁺ is in 25-fold excess with respect to protein, and is not observed in the C2 α ·Pb complex, where there is no excess of metal ions.

A similar behavior with respect to PIP₂ binding was reported for another domain C2 domain, the C2A domain of rabphilin-3A.³⁷ Those data were interpreted as the existence of an additional – albeit weaker – PIP₂ binding site.

Pb²⁺ promotes the association of C2 α with PIP₂-containing membranes

Our next step was to determine the effect of Pb²⁺ on the interactions between C2 α and PIP₂ embedded into lipid bilayers. This was accomplished using protein-to-membrane FRET experiments. The tryptophan residues of C2 α served as a donor. Dansyl-PE, a fluorescent lipid that was incorporated into the LUVs, served as an acceptor. The LUVs were composed of POPC/POPS/bp-PIP₂/dansyl-PE with molar ratios of 61:30:2:7.

To provide the baseline for Pb²⁺ experiments, we first measured the protein-to-membrane FRET in the absence of metal ions. We determined that, in addition to our standard decalcification procedures applied to C2 α and buffers, the LUV preparations had to be decalcified as well to eliminate sub-micromolar concentrations of contaminating Ca²⁺ that were sufficient to promote the C2 α -membrane association. The metal titration experiments shown in Figure 9A were carried out with decalcified lipids. In these experiments, we titrated the divalent metal ion into the mixture of C2 α and LUVs containing 2% PIP₂, and monitored the increase in the intensity of the dansyl emission band at 505 nm. The Ca²⁺-driven protein-membrane binding curve was fitted with Eq. 3, which produced [Ca²⁺]_{1/2}=0.7 ± 0.1 μ M and H_{Ca}=1.5 ± 0.1. This represents a ~7-fold decrease in the [Ca²⁺]_{1/2} concentration compared to that obtained for the PIP₂-lacking membranes.

Similar to Ca²⁺, the addition of Pb²⁺ ions to the mixture of apo C2 α and PIP₂-containing LUVs resulted in a systematic increase in the protein-to-membrane FRET, albeit with a lower overall FRET efficiency. Fitting the Pb²⁺-driven membrane-binding curve produced [Pb₂₊]_{1/2}=1.3 ± 0.1 μ M and H_{Pb}=2.1 ± 0.1. This represents a ~5-fold increase in the apparent C2 α affinity to Pb²⁺ compared to the PtdSer-containing PIP₂-lacking LUVs.⁶ Overall, our results show that the ability of Pb²⁺ and Ca²⁺ to drive protein-membrane associations is comparable, and that the presence of PIP₂ in the membrane increases the affinity of C2 α to metal ions several-fold.

To characterize the competitive behavior of Pb²⁺ with respect to Ca²⁺, we prepared the ternary C2 α -Ca²⁺-LUV complex by adjusting the final concentration of Ca²⁺ to 10 μ M. We titrated the complex with Pb²⁺, while monitoring the intensity of the dansyl emission band. A steady decrease in FRET efficiency was observed (Figure 9B) with increasing Pb²⁺ concentration. Neither Pb²⁺ nor Ca²⁺ appreciably quench the dansyl fluorescence, as shown in Figure S3. Moreover, the maximum attainable FRET efficiency in the Pb²⁺-driven membrane association experiments is typically ~40–60% of that attainable in Ca²⁺-driven

experiments. This is illustrated in the inset of Figure 9B with the FRET spectra of metal-free (black), Pb^{2+} -containing (green), and Ca^{2+} -containing (blue) C2 α -LUV. Given these properties of the C2 α -LUV-metal ion system, we interpreted the decrease in FRET efficiency in the competition experiments as Pb^{2+} gradually displacing Ca^{2+} from the protein. A plateau region – indicative of full Ca^{2+} displacement by Pb^{2+} – is reached at the Pb-to-Ca ratio of 1.0.

The results of the reverse experiment, in which we attempted to outcompete Pb^{2+} with Ca^{2+} , are shown in Figure S4. In agreement with our previous observations, we detected a steady increase in FRET efficiency with increasing Ca^{2+} concentration, until it reached a plateau value at the Ca-to-Pb ratio of ~ 10 . A comparison of the competition data demonstrates that Pb^{2+} is more effective than Ca^{2+} in displacing the competing metal ion from C2 α in the presence of PIP_2 -containing membranes. In vivo, the specifics of the $\text{Pb}^{2+}/\text{Ca}^{2+}$ competition behavior would depend on the relative and absolute bio-available concentrations of these metal ions.

We have previously shown that, in addition to mediating the protein-membrane interactions, Pb^{2+} itself could associate with PtdSer-containing LUVs.⁶ To test the influence of PIP_2 on this process, we combined vesicle sedimentation assays with Inductively Coupled Plasma (ICP) detection of Pb^{2+} in the pellet and supernatant fractions. No protein was added to the samples. We explored two total lipid concentration regimes: 1.5 mM and 150 μM (as used in FRET experiments of Figure 9). The results are summarized in Figures S5 and S6 of the Supporting Information. For comparison, we also included the data obtained in our previous work on the Pb^{2+} association with PIP_2 -lacking vesicles.

The ICP data clearly demonstrate that the presence of PIP_2 in the LUVs increases the fractional population of the membrane-associated Pb^{2+} . This trend is especially prominent for the “high-lipid” regime of 1.5 mM, in which, depending on the total Pb^{2+} concentration, 60 to 90% of total Pb^{2+} is membrane-associated. Under conditions of our FRET experiments (150 μM total lipids and, e.g., 2.2 μM total Pb^{2+}), 76% of Pb^{2+} is in the supernatant fraction for the PIP_2 -lacking, PtdSer-containing vesicles; this value decreases to 33% in the presence of 2% PIP_2 . This binding equilibrium effectively reduces the amount of Pb^{2+} available for C2 α and would have an effect of over-estimating the $[\text{Pb}^{2+}]_{1/2}$ value from the data shown in Figure 9A.

DISCUSSION

The experiments described in this manuscript were designed to determine the influence of PIP_2 on the C2 α - Pb^{2+} interactions, with the ultimate objective of gaining insight into the molecular mechanism of Pb^{2+} interference with the Ca^{2+} -dependent function of C2 domains. There are three “checkpoints” where Pb^{2+} interference can occur: (1) interaction with the membrane-free C2 via the high-affinity Pb^{2+} -binding site(s) of the protein; (2) modulation of the metal-dependent C2-membrane association; and (3) direct interaction with the anionic membrane-binding sites. In our previous studies of the ternary system composed of C2 α , Pb^{2+} ions, and PtdSer-containing LUVs, we determined that (1) and possibly (3) are the most likely routes. Given the significant role of PIP_2 in C2 α localization and membrane binding, we sought to determine the mutual effect of PIP_2 and Pb^{2+} on their respective interactions with C2 α . Along with the Pb^{2+} -specific data, we obtained PIP_2 -binding information for the apo and Ca^{2+} -complexed states of C2 α . Taken together, this information enabled us to dissect the contribution of individual metal-binding sites to the PIP_2 binding and evaluate the competitive behavior of Pb^{2+} with respect to Ca^{2+} .

Apo C2 α is a low-affinity PIP₂ binding module

Using NMR-detected titration of apo C2 α with a soluble C4-PIP₂, we identified protein regions whose electronic environment changed as a result of PIP₂ binding. The chemical shift perturbation data of Figure 3 show that this region is not limited to the lysine-rich cluster, but also involves three calcium-binding loops: CBL1, CBL2, and CBL3. The β -strands flanking CBL3, β 6 and β 7, are also affected by PIP₂. Superposition of the apo- and ligand-bound C2 α crystal structures revealed very few differences in the overall domain conformation including the functional regions: the pairwise RMSD values for the backbone atoms do not exceed 0.5 Å (Figure S8). This means that large chemical shift perturbations upon PIP₂ binding are most likely due to the changes in the electrostatic environment and/or conformational dynamics of the CBLs rather than in their average conformation.

The affinity of C2 α to PIP₂ is rather weak – 1.13 mM – in the absence of metal ions. The NMR-detected binding curves could be fit well with Eq. 2, indicating the presence of a single PIP₂ binding site on the apo C2 α . These data are in agreement with the results of previous studies on the GST-fused C2 domains³² and the SPR study of the C2-membrane interactions.³⁸ The metal-independent association of C2 α with PIP₂-containing membranes was not detectable under the conditions of our FRET experiments (Figure 9).

How prevalent are the metal-ion-independent interactions with PIP₂ among the C2 domains? Both C2 domains of rabphilin-3A can interact with inositol-1,4,5-triphosphate, a head group of PIP₂, in Ca²⁺-independent manner.³⁷ Calcium-independent association with PIP₂-containing membranes was reported for the C2B domains of synaptotagmin 1^{35,56} and synaptotagmin 9.⁵⁷ The physiological significance of the apo C2-PIP₂ interaction, as formulated for the C2B domain of synaptotagmin 1, is to increase the speed of response of the host protein to Ca²⁺ by generating the protein state that is already pre-bound to the membrane.³⁵

Progressive saturation of metal-binding sites increases the affinity of C2 α to PIP₂

The 2000-fold difference in C2 α affinities to Pb²⁺(1) and Pb²⁺(2) enabled us to prepare the state of C2 α with the metal ion bound to site (1) only. This state, C2 α -Pb, along with the apo C2 α and C2 α -Ca₂, was used to determine the contribution of individual metal sites to PIP₂ binding. The equilibria of Figure 7 indicate that the affinity of C2 α to PIP₂ increases several-fold upon progressive saturation of metal binding sites. The K_d is reduced 6-fold for C2 α -Pb-PIP₂ compared to C2 α -PIP₂, from 1.13 mM to 198 μ M. The K_d is further reduced 8-fold for C2 α -Pb₂-PIP₂ compared to C2 α -Pb-PIP₂, from 198 μ M to 26 μ M. Expressed in terms of free energies, the $\Delta(\Delta G^0)$ contribution of the first and second Pb²⁺ ions to the C2 α -PIP₂ binding reactions are –1.7RT and –2.0RT, respectively. We conclude that metal ions make individual and comparable contribution to the energetics of the PIP₂ binding to C2 α .

The increase in the C2 α affinity to PIP₂ does not appear to depend on the nature of the metal ion. We reached this conclusion by comparing the dissociation constants of PIP₂ from the C2 α -Pb₂-PIP₂ and C2 α -Ca₂-PIP₂ complexes. The K_d values are similar – 48 μ M and 26 μ M – for the Ca²⁺ and Pb²⁺ complexes, respectively.

The ability of Ca²⁺-dependent C2 domains to associate with anionic lipids and protein partners in response to binding Ca²⁺ has been attributed to the “electrostatic switch” mechanism.^{3,12,13,58} The basic premise of this mechanism is that the binding of Ca²⁺ ions increases the electrostatic potential of C2 domains and hence its attraction to the negatively charged interacting partner. Figure 10 shows the calculated electrostatic potential of C2 α mapped onto the crystal structures. The apo C2 α has a negatively charged metal binding site formed by the aspartate residues, and a positively charged lysine-rich cluster (panel A). The

interaction of PIP₂ with the lysine-rich cluster results in the decrease of the electrostatic potential of the complex (panel B). In contrast, Pb²⁺ (or Ca²⁺) binding to C2α increases the electrostatic potential of the complex and its attraction to the negatively charged PIP₂. The individual contributions of Pb²⁺ ions are illustrated in panels (C) and (D). Given that metal binding to C2α does not appreciably change the conformational dynamics of the β3–β4 segment (Figure S9), we conclude that the modulation of the C2α affinity to PIP₂ by metal ions is mostly electrostatic in nature.

PIP₂ increases the affinity of C2α to Pb²⁺

Pre-bound PIP₂ enhances the affinity of C2α to metal ions. In the presence of saturating PIP₂, the affinity of C2α to Pb²⁺(1) increased 6-fold, from 67 to 12 nM. Similarly, the binding affinity of C2α to Pb²⁺(2) increased 8-fold, from 129 to 17 μM. Our data lend support to the TAMA (“target activated messenger affinity”) mechanism that was proposed by Falke’s laboratory to explain how C2 domains, while being poor Ca²⁺ sensors in membrane-free environment, are able to respond to micromolar concentrations of Ca²⁺ in the cell.³⁹ In the context of TAMA, membrane-embedded PIP₂ is the target and the metal ion is the messenger. The proximity (or pre-binding) of C2α to PIP₂-containing membranes makes C2α more responsive to low concentrations of metal ions by increasing its binding affinity. This behavior has an immediate relevance to Pb²⁺ being a Ca²⁺ surrogate, because the presence of PIP₂ in the membranes will effectively sensitize C2α to the bioavailable concentrations of Pb²⁺.

Pb²⁺ facilitates the association of C2α with PIP₂-containing lipid membranes

We used protein-to-membrane FRET experiments to probe the Pb²⁺-dependent association of C2α with the PIP₂-containing membranes. Similar to Ca²⁺, we observed a steady increase in FRET efficiency as a function of metal ion concentration. In the Ca²⁺-driven experiments, the incorporation of 2% PIP₂ into the membranes decreased the [Ca²⁺]_{1/2} value from 5 μM to 0.7 μM, or ~7-fold. Our data are in general agreement with the results of the previous SPR work³⁸ (4-fold decrease in Ca²⁺ K_d) and FRET studies with multi-component mimics of the plasma membrane³⁹ (20-fold decrease in [Ca²⁺]_{1/2}). In the Pb²⁺-driven experiments, the incorporation of 2% PIP₂ into the membranes decreased the [Pb²⁺]_{1/2} value from 7 μM to 1.3 μM, or ~5-fold. This value is probably underestimated by a factor of ~2–3, because the sequestration of Pb²⁺ by the PIP₂- and PtdSer-containing membranes reduces the amount of Pb²⁺ available to C2α. Figures S3 and S4 illustrate how the presence of membrane-embedded PIP₂ enhances the interactions of Pb²⁺ with anionic membranes. In summary, the presence of PIP₂ in the membranes results in the enhancement of the C2α affinity to divalent metal ions by roughly one order of magnitude.

Pb²⁺ outcompetes Ca²⁺ in the presence of lipid membranes

The ability of Pb²⁺ to compete with Ca²⁺ for protein binding sites is what makes Pb²⁺ a potent environmental toxin. To assess the competitive behavior of Pb²⁺ in the context of C2α function, we carried out a Pb²⁺/Ca²⁺ competition experiment in the presence of PIP₂-containing membranes. As shown in Figure 9B, the displacement of Ca²⁺ from the ternary C2α–Ca²⁺–LUV complex by Pb²⁺ can be readily monitored because of the differences in the maximum attainable FRET efficiency. Our data indicate that the equal population of Pb²⁺- and Ca²⁺-bound C2α species is reached at [Pb²⁺]/[Ca²⁺] of 0.3. This corresponds to Pb²⁺ concentration of 3.0 μM, which is ~2-fold smaller than the value obtained in our previous experiments with the PIP₂-lacking PtdSer-containing lipid membranes.⁶

Given the comparable ability of Pb²⁺ and Ca²⁺ to promote the association of C2α with lipid membranes, how does Pb²⁺ displace Ca²⁺? We speculate that the displacement occurs through the interaction of Pb²⁺ with the high-affinity site on C2α (Figure 11). The Ca²⁺-

bound form of C2 α exists in equilibrium between membrane-bound and membrane-free states (reaction 1). By itself, C2 α is a low-affinity Ca²⁺-binding module, which makes the exact Ca²⁺ ligation state of membrane-free C2 α difficult to ascertain. The interaction of Pb²⁺ with a single high-affinity site displaces Ca²⁺ from the protein (reaction 2), including the second metal-binding site. This occurs because saturating the high-affinity Pb²⁺(1) site on the C2 α decreases the affinity of the Ca²⁺ ion to the second site to ~13 mM,⁶ thereby precluding the formation of the mixed Pb²⁺/Ca²⁺ species.

The concentration of free Ca²⁺ in the cytosol is ~100 nM and 1 μ M in the resting and stimulated states, respectively.⁵⁹ The concentration of bioavailable Pb²⁺ is picomolar-to-nanomolar.⁶⁰ Given that the C2 α affinity to Pb²⁺(1) exceeds its affinity to Ca²⁺ by more than 1,000-fold, the formation of the C2 α ·Pb species in the cell is plausible.

Further fate of the C2 α ·Pb species depends on the bioavailable Pb²⁺ concentration. According to our FRET data, the high affinity of Pb²⁺(1) to C2 α does not translate into high affinity of the C2 α ·Pb complex to the lipid membranes. The Hill coefficient of 2 that we obtained from the fit of the Pb²⁺-driven FRET membrane-binding curve implies that the binding of the second Pb²⁺ ion to C2 α is required for protein-membrane association (reaction 3). The affinities of C2 α to Pb²⁺(2) and Ca²⁺ are comparable in the membrane-free form of the protein, which in turn results in the comparable ability of these two ions to drive the C2 α -membrane association (Figure 9A). If the concentration of Pb²⁺ in the cell is not sufficient to promote the association of C2 α with membranes, Pb²⁺ binding may still activate PKC α by inducing the inter-domain rearrangement within the regulatory domain of the enzyme. In either scenario, the presence of Pb²⁺(1) at the high-affinity C2 α site will interfere with the normal catalytic activity of PKC α by making the enzyme unresponsive to Ca²⁺ activation during the signaling event.

As a final note, the saturation of one Pb²⁺ site is not sufficient for the C2 α to undergo membrane association under the conditions of our experiment. However, given the proposed mechanistic roles of individual Ca²⁺ sites³⁹ and our NMR results on PIP₂ binding to the C2 α ·Pb complex, we cannot completely exclude the possibility that, at high local concentrations of anionic lipids, such as PtdSer and PIP₂, the association of C2 α ·Pb with membranes may in fact occur. This situation can arise upon the interaction of PKC α with its substrates, such as MARCKS (myristoylated alanine-rich C kinase substrate), that laterally sequesters PIP₂ molecules⁶¹ and releases them upon phosphorylation by PKC or interactions with calmodulin.

CONCLUSIONS

Based on the results of our work, we conclude that PIP₂ can influence two aspects of C2 α -Pb²⁺-membrane interactions: the affinity of C2 α to Pb²⁺, and the association of Pb²⁺ with the anionic sites on the membrane. Both factors may play a role in the aberrant activation of PKC α and thus contribute to the toxic effect of Pb²⁺. Divalent metal ions and PIP₂ enhance each other's affinity to C2 α primarily through modulation of the protein's electrostatic properties. This behavior – which is independent of the nature of the divalent metal ion – demonstrates the synergy between two cofactors that are required for the C2 α function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The structure of the C2α-Ca²⁺-PIP₂ complex (PDB ID 3GPE)⁴³ showing the details of the PIP₂ interaction site. CBL1, CBL2, and CBL3 are the Ca²⁺-binding loops.

Figure 2.

Expansions of ^{15}N - ^1H HSQC spectra of 100 μM apo C2 α (A), C2 α ·Ca₂ (B), and C2 α ·Pb (C) at different concentrations of C4-PIP₂. The arrows point in the direction of increasing C4-PIP₂ concentration, which varies from 0 to 1.5 mM for apo-C2 α and C2 α ·Pb; and from 0 to 1 mM for C2 α ·Ca₂. Site-specific Δ values were calculated according to Eq. 1, color-coded, and mapped onto the structures of apo (D), Ca²⁺-complexed (E), and Pb²⁺-complexed (F) C2 α . The side-chains of K197, K199, K209, and K211 indicate the position of the lysine-rich cluster. Prolines and the N-terminal residue T156, for which NMR data are not available, are shown in grey.

Figure 3.

Chemical shift perturbation Δ due to PIP_2 binding plotted as a function of primary structure for apo C2 α (A), C2 α ·Ca $_2$ (B), and C2 α ·Pb (C). Calcium-binding loops – CBL1 (residues 182–193), CBL2 (residues 216–222), and CBL3 (residues 246–253) are highlighted in gray. The $\beta 3$ – $\beta 4$ region (residues 194–211) that directly interacts with PIP_2 is highlighted in yellow.

Figure 4. C4-PIP₂ binding curves for K197 (A) and Q210 (B) in apo-C2 α (triangles), C2 α ·Ca₂ (circles), and C2 α ·Pb (squares). Solid lines represent the fits to Eq. 2. $\Delta\delta$ is the absolute value of the chemical shift change in ppm. The table summarizes the PIP₂ dissociation constants determined for three states of metal ligation.

Figure 5.

Individual contributions of C2 α metal sites to PIP₂ binding. (A) The expansions of the ¹H-¹⁵N HSQC spectra for four C2 α residues from the β 3- β 4 region. (B) The chemical shift perturbations Δ for the β 3- β 4 region. Δ values were calculated according to Eq. 1 using the final and initial states listed in the table.

Figure 6.

PIP₂ increases the affinity of C2α to Pb²⁺(2). (A) D248 binding curves for the titration of C2α·Pb·PIP₂ (open circles) and C2α·Pb (filled circles) complexes with Pb²⁺(2). $\Delta\delta$ values are normalized to the maximum observed change, $\Delta\delta_{\max}$. (B) Chemical shift perturbation analysis of the C2α·Pb₂·PIP₂ using C2α·Pb·PIP₂ as a reference state. The β3/β4 and CBL3 regions are highlighted in grey and yellow, respectively.

Figure 7. Thermodynamic binding equilibria and dissociation constants in the C2 α , Pb²⁺, and PIP₂ system. The constants calculated from the experimentally measured K_d values are highlighted for the Pb²⁺ (cyan) and PIP₂ (yellow) dissociation reactions.

Figure 8.

(A) NMR-detected PIP₂ binding curves for D248 from Group 2 ($K_d = 24 \pm 1 \mu\text{M}$) and N253 ($K_d = 175 \pm 12 \mu\text{M}$) from Group 3. (C) Group 2 (red) and Group 3 (blue) residues mapped onto the crystal structure of Ca²⁺- and PIP₂-complexed C2 α (PDB ID: 3GPE).

Figure 9.

(A) Normalized protein-to-membrane FRET as a function of total Ca^{2+} and Pb^{2+} concentration in the presence of POPC/POPS/bp-PIP₂/dansyl-PE (61:30:2:7) LUVs. The error bars were calculated as standard deviations in triplicate experiments. (B) Decrease in FRET efficiency as a result of Ca^{2+} displacement with Pb^{2+} . The Ca^{2+} concentration was kept constant at 10 μM while the Pb^{2+} concentration was varied. The dashed vertical line marks the $[Pb^{2+}]/[Ca^{2+}]$ ratio of 0.3, at which about half of the protein is in the Pb^{2+} -bound form. For comparison, the inset shows the FRET spectra of the C2 α -LUV system at zero metal ion concentration (black), 10 μM Ca^{2+} (blue), and 20 μM Pb^{2+} (green).

Figure 10.

Electrostatic potential mapped onto the surface of (A) apo C2 α , (B) C2 α ·PIP $_2$, (C) C2 α ·Pb, and (D) C2 α ·Pb $_2$. The color-coding corresponds to the electrostatic potential ranging from $-6k_bT/e$ (red) to $+6k_bT/e$ (blue). Structures in (A) and (B) were generated using the PIP $_2$ -bound C2 α (3GPE), and structures in (C) and (D) were generated using the Pb $^{2+}$ -complexed C2 α (3TWY).

Figure 11.
 $\text{Pb}^{2+}/\text{Ca}^{2+}$ competition through the interaction with the $\text{C2}\alpha$ high-affinity metal-binding site.