Trinuclear Calcium Site in the C2 Domain of PKCa/γ Is Prone to Lithium Attack

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ABSTRACT: Lithium (Li+) is the first-line therapy for bipolar disorder and a candidate drug for various diseases such as amyotrophic lateral sclerosis, multiple sclerosis, and stroke. Despite being the captivating subject of many studies, the mechanism of lithium’s therapeutic action remains unclear. To date, it has been shown that Li+ competes with Mg2+ and Na+ to normalize the activity of inositol and neurotransmitter-related signaling proteins, respectively. Furthermore, Li+ may co-bind with Mg2+-loaded adenosine or guanosine triphosphate to alter the complex’s susceptibility to hydrolysis and mediate cellular signaling. Bipolar disorder patients exhibit abnormally high cytosolic Ca2+ levels and protein kinase C (PKC) hyperactivity that can be downregulated by long-term Li+ treatment. However, the possibility that monovalent Li+ could displace the bulkier divalent Ca2+ and inhibit PKC activity has not been considered. Here, using density functional theory calculations combined with continuum dielectric methods, we show that Li+ may displace the native dication from the positively charged trinuclear site in the C2 domain of cytosolic PKCa/γ. This would affect the membrane-docking ability of cytosolic PKCa/γ and reduce the abnormally high membrane-associated active PKCa/γ levels, thus downregulating the PKC hyperactivity found in bipolar patients.

INTRODUCTION

An in-depth understanding of the therapeutic mechanism of lithium (Li+), a first-line drug for bipolar disorder and a candidate drug for treating stroke and chronic neurodegenerative diseases such as amyotrophic lateral sclerosis and multiple sclerosis, is of importance and widespread interest, as evidenced by its numerous ongoing clinical trials. Various mechanisms have been proposed for the highly complex therapeutic actions of this simple monocation with only two electrons. They suggest that lithium’s effects are mostly related to its interactions with native cations, especially Na+ and Mg2+, in various signaling proteins such as inositol monophosphatase (IMPase) or polyphosphate 1-phosphatase (IPPase), guanine nucleotide-binding proteins (G-proteins), and glycosyn synthase kinase-3β (GSK3β) as well as organic cofactors such as adenosine triphosphate (ATP) or guanosine triphosphate (GTP). We had proposed that lithium’s multifaceted effects can be unified by considering its therapeutic actions as a free Li+ monocation and as phosphate-bound anionic complexes acting on multiple targets. Bound lithium can co-bind with Mg2+ to adenosine/guanosine triphosphate (ATP/GTP) to form [NTP–Mg–Li]2+ anions (N = A or G), which can activate or inhibit the host protein depending on the shape of the nucleoside triphosphate (NTP)-binding pocket. Free Li+, on the contrary, may displace allosteric Na+ bound by ≤2 protein ligands in buried sites of certain G-protein-coupled receptors (GPCRs) because compared to Na+, the smaller Li+ has higher charge density and can form stronger interactions with the few ligands in the solvent-inaccessible metal-binding site. This enhances the stability of the inactive receptor conformation, preventing the receptor from relaying the signal to the respective G-protein. Free Li+ may also displace Mg2+ in enzymes containing highly cationic Mg2+-binding sites such as IMPase, IPPase, and GSK3β, thus attenuating hyperactive phosphatidylinositol and GSK3β signaling in bipolar patients. Besides Na+ and Mg2+, how lithium competes with another important biogenic cation in the cell, namely, Ca2+, remains enigmatic. This is vital, as bipolar patients exhibit abnormally high intracellular Ca2+ levels, which correlate with the increased membrane-associated protein kinase C (PKC) activity. Notably, membrane-associated PKCγ isozymes have been found to be elevated in cortices of bipolar subjects versus controls. Protein kinase C (PKC) consists of a family of Ser/Thr kinases that mediate a myriad of cellular processes including immune responses, cellular growth, apoptosis, release of hormones/neurotransmitters,
gene expression, neuronal excitability, mitochondrial dysfunction, and oxidative stress. PKC contains an N-terminal regulatory region comprising C2 and/or C1 domains and a C-terminal catalytic region involved in binding ATP and substrate. Its activation requires translocation from the cytosol to the membrane, and second messengers, Ca\(^{2+}\) and/or diacylglycerol (DAG), depending on the isoenzyme: conventional/classical PKCs (α, β, γ, δ) require both Ca\(^{2+}\) and DAG for activation, novel PKCs (ζ, ι, ι', β', θ, μ, ν, ε, ρ, σ, φ, χ, χ', ψ, ω) need only DAG, whereas atypical PKCs (ζ, ι) do not use any cofactor. Under resting conditions, the basal Ca\(^{2+}\) concentration (∼10\(^{-7}\) M) is insufficient to activate PKC, which remains in the cytosol. However, increasing the cytosolic Ca\(^{2+}\) concentration induces the translocation of cytosolic autoinhibited latent PKC to the membrane-associated active form, as Ca\(^{2+}\) binding to the C2 domain enhances electrostatic interactions with the plasma membrane. The fact that Ca\(^{2+}\) binding increases the affinity of PKC for negatively charged lipids is evidenced by the lower dissociation constant of Ca\(^{2+}\) from membrane-bound PKC (K\(_D\) ∼ 7 × 10\(^{-7}\) M) compared with the K\(_D\) from soluble PKC (∼3 × 10\(^{-7}\) M). Docking of the C2 domain to membrane phospholipids then initiates the activation sequence of the kinase. Hence, the abnormally high intracellular Ca\(^{2+}\) levels in bipolar patients would increase the ratio of membrane-associated active PKC to cytosolic inactive PKC, resulting in PKC hyperactivity.

Several experimental studies have shown that chronic (i.e., long-term) Li\(^+\) treatment attenuates translocation of cytosolic PKC to the membrane and mitigates PKC hyperactivity. They showed that PKCa responds to long-term lithium treatment: PKCa activity in the frontal cortex and hippocampus, brain areas implicated in mood disorders, was reduced after chronic lithium administration. However, the molecular mechanism of lithium’s inhibition of PKC hyperactivity remains unclear. It has been postulated that by displacing Mg\(^{2+}\) in IMPase, lithium reduces IMPase activity and thus myoinositol levels, which in turn decreases Ca\(^{2+}\) levels and PKC activity. However, the competition between Ca\(^{2+}\) and Li\(^{+}\) in PKC, to our knowledge, has not been considered as a mode of PKC enzyme inhibition. Previous studies did not consider the possibility that Li\(^{+}\) could displace Ca\(^{2+}\) likely because the two cations differ not only in charge but also in size: the ionic radius of Li\(^{+}\) (0.76 Å) is significantly smaller than that of Ca\(^{2+}\) (1.00 Å).

Our aim is to determine if the Ca\(^{2+}\) sites in the C2 domains of conventional PKCs are vulnerable to Li\(^{+}\) substitution. We focus on conventional PKCs since they require Ca\(^{2+}\) for activation unlike other isoenzymes. First, we searched the Protein Data Bank (PDB) for all PKC C2 domain structures. This yielded the PDB structures of PKCa (3GPE, 2.0 Å), PKCβ (1A2S, 2.7 Å), and PKCγ (2UZP, 2.0 Å) C2 domains, which reveal trinuclear Ca\(^{2+}\) sites. Based on these PDB structures, we modeled trinuclear Ca\(^{2+}\) sites and computed the free energy, Δ\(HG\), for replacing the native Ca\(^{2+}\) bound to PKC denoted as [Ca\(^{2+}\)-PKC] by Li\(^{+}\), i.e.,

\[
\text{[Li}^+\text{-aq]} + \text{[Ca}^{2+}\text{-PKC]} \rightarrow \text{[Li}^+\text{-PKC]} + \text{[Ca}^{2+}\text{-aq]}
\]

In eq 1, [Ca\(^{2+}\)/Li\(^{+}\)-aq] denotes heptahydrated Ca\(^{2+}\) or tetrahydrated Li\(^{+}\) with three water molecules in the second shell (see the Methods) in an aqueous medium characterized by a dielectric constant of ε = 78, whereas [Ca\(^{2+}\)/Li\(^{+}\)-PKC] denotes the cation bound to PKC ligands in the polynuclear metal-binding site characterized by an effective ε ranging from 4 for buried sites to 30 for more exposed sites. A positive Δ\(HG\) value implies a Ca\(^{2+}\)-selective site, whereas a near-zero or negative value implies that Ca\(^{2+}\) may be replaced by Li\(^{+}\). The results herein reveal that the relatively buried trinuclear Ca\(^{2+}\) site with a net positive charge in PKCa/γ is susceptible to Li\(^{+}\) attack, whereas that with zero net charge is immune to Li\(^{+}\) substitution. Our results suggest a novel mechanism for lithium’s inhibition of PKC activity: by outcompeting Ca\(^{2+}\) for the trinuclear Ca\(^{2+}\) site in the C2 domain of cytosolic PKCa/γ, Li\(^{+}\) decreases the abnormally high ratio of membrane-associated PKCa/γ to cytosolic PKCa/γ, which in turn helps to normalize PKC enzyme activity.

### METHODS

#### Geometry Optimization of Ca\(^{2+}\) Sites

The models for PKCa, PKCβ, and PKCγ were derived from PDB structures 3GPE, 1A2S, and 2UZP (no citation available), respectively. The initial models included the complete side chains of coordinating residues D187, D193, D246, D248, D254, and S/T251 (and E281 for PKCβ) as well as the backbone of residues M186, W247, and R252. We also added the entire R252/R219 residue in the outer shell of these initial models. Among various combinations of different density functionals and basis sets, the M06-2X/6-311++G(d,p) combination was found to be the most efficient at reproducing the geometries of ultrahigh-resolution
structures containing Ca\textsuperscript{2+} or Li\textsuperscript{+}. Hence, this combination was used to optimize the geometries of the model Ca\textsuperscript{2+} sites employing the polarizable continuum model (PCM) implemented in the Gaussian 09 program.\textsuperscript{53} During geometry optimization, the coordinates of all C\textsubscript{α} atoms were frozen to take into account the constraints imposed by the protein matrix. Having obtained the constrained optimized structures, we replaced each Ca\textsuperscript{2+} ion in the trinuclear site with Li\textsuperscript{+} and the system was reoptimized with the frozen C\textsubscript{α} atoms. In accord with their observed experimental hydration numbers,\textsuperscript{54−56} hydrated Ca\textsuperscript{2+} and Li\textsuperscript{+} were modeled as hepta- and tetrahydrated, respectively, but three water molecules were added in the second shell of Li\textsuperscript{+} so that the number of water molecules is the same as that for hydrated Ca\textsuperscript{2+}. Vibrational frequencies were computed using the M06-2X/6-311++G(d,p) method for each optimized structure to ensure that no imaginary frequency was present. Due to the prohibitive computational cost, the smaller 6-311++G(d) basis set without the p-functions on hydrogens was employed for computing the frequencies of the larger models containing an Arg residue in the outer shell.

**Computing Ca\textsuperscript{2+} → Li\textsuperscript{+} Exchange Free Energies.** The experimental solution free energies, ΔΔG\textsubscript{80}, for replacing Ca\textsuperscript{2+} by Li\textsuperscript{+} in nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA), 5.3 and 10.5 kcal/mol,\textsuperscript{57} respectively, were used to select an optimal method to compute the electronic energies in the condensed phase, E\textsubscript{elec}. After geometry optimization using the same method for the model Ca\textsuperscript{2+} sites, single-point energy calculations with the Solvation Model based on Density (SMD) solvation model\textsuperscript{58} were performed using 88 methods, namely, 22 density functionals (BMK, BP86, B-LYP, B3-LYP, B3-LYP+GD3, B3PW91, B97-2, ωB97-XD, M05, M06, M06-2X, M06-L, M11, M11-L, MN12-L, MN12-SX, MPW1K, M06-2X/6-311++G(d,p)-optimized structures with the frozen C\textsubscript{α} atoms (transparent spheres) are shown on the left. ΔΔG\textsubscript{ε} free energies as a function of effective dielectric constant ε for replacing native Ca\textsuperscript{2+} by Li\textsuperscript{+} in each site are shown on the right.
N12, N12-SX, PBE0, S-VWN, and TPSS), each combined with four basis sets (6-31+G(2d,p), 6-31+G(3dp), 6-311+G(2d,p), and 6-311+G(3dp)). The single-point $E_{\text{elec}}$ energies were then used to compute the probability of each structure according to the Boltzmann distribution at a given temperature and the Boltzmann-weighted $\Delta G^\epsilon$ for replacing Ca$^{2+}$ by Li$^+$ in NTA and EDTA

$$\Delta G^\epsilon = \Delta E_{\text{elec}} + \Delta E_{\text{therm}} - T \Delta S^\epsilon$$

(2)

The results in Figure S1 show that M11/6-31+G(2d,p) overestimated the experimental numbers similarly, by $+0.7$ kcal/mol for NTA and $+0.6$ kcal/mol for EDTA. In contrast, the MN12-L functional with the larger 6-31+G(3dp) basis set underestimated the experimental numbers by different amounts ($+0.3$ kcal/mol for NTA and $+0.8$ kcal/mol for EDTA). Hence, based on the constrained optimized structures, single-point M11/6-31+G(2d,p) calculations were used to compute $\Delta E_{\text{elec}}$ energies in eq 2 with the SMD solvation model. The vibrational frequencies were used to compute the change in the thermal energy ($\Delta E_{\text{therm}}$) and vibrational entropy ($\Delta S^\epsilon$). These contributions (involving vibrational frequencies) to the Ca$^{2+} \rightarrow$ Li$^+$ free energies (eq 2) in the model Ca$^{2+}$ sites mostly fall within $\pm 1$ kcal/mol, so their inclusion/omission does not alter the main trends/conclusions found herein.

**Simulation Protocol.** To estimate the distances between charged outer-shell arginines and the Ca$^{2+}$ ions, we ran eight 20 ns simulations of the entire C2 domain of PKCa starting from the X-ray structure PDB 3GPE by using the CHARMM36 force field at a temperature of 300 K with the NAMD 2.12 program. The only histidine of PKCa was found to be deprotonated by Propka 3.1. Long-range electrostatic forces were treated using the particle mesh Ewald method with a grid spacing of 1 Å and a nonbonded cutoff of 12 Å. The nonbonded interactions were updated every 1 fs. Each system was solvated in a cubic box of TIP3P water molecules with an edge length of $\sim 65$ Å. The solvated system was neutralized with eight Cl$^-$ ions. All bonds to hydrogen atoms were constrained with the SHAKE algorithm.

We first performed three equilibration runs of 100 000 steps each. Harmonic restraints placed on the backbone and Ca$^{2+}$ ions and side chains were gradually reduced during equilibration and completely removed during the production dynamics. A time step of 2 fs was used except for the first equilibration run in which 1 fs was used. The root-mean-square deviation (RMSD) of all backbone atoms in each of the eight simulations from the starting X-ray structure ranged between 0.80 and 1.14 Å with an average RMSD over the eight simulations of $\sim 1$ Å. The simulations also maintained the coordination geometry of the Ca$^{2+}$ ions seen in the X-ray structure.

**RESULTS**

Based on the PDB structures of the C2 domains of PKCa (3GPE), PKCa$^{\beta}$ (1A25), and γ (2UZP), we modeled trinuclear Ca$^{2+}$ sites and optimized them using M06-2X/6-311+G(d,p) with the frozen Ca$^{2+}$ atoms (see the Methods). The resulting Ca$^{2+}$ sites geometries PKCa$^{\beta}$ (Figure 1a,b) and PKCa (Figure S2) maintain the X-ray geometries with backbone root-mean-square deviations (RMSD) of 0.09–0.16 Å. We oriented the trinuclear site such that the three dications form an inverted triangle and labeled the Ca$^{2+}$ on the left, center, and right as I, II, and III, respectively. The PKCa/γ crystal structures show that Cal and CalII are each coordinated to four Asp$^-$/side chains and a backbone carbonyl, yielding a net charge of $-2$ in each site, whereas CalIII is bound by two Asp$^-$ side chains and two neutral protein ligands (a Ser/Thr hydroxyl group and a backbone carbonyl), yielding a neutral site. In each Ca$^{2+}$ site, one of the Asp$^-$ residues binds bidentately to the dication. Notably, D248 binds bidentately to the central Cal II and monodentately to the other two dications, thus connecting all three Ca$^{2+}$ ions. In addition, D248 and D246 connect Cal and CalII, whereas D254 bridges CalI and CalII. Altogether, the three dications are bound to five aspartates and three neutral backbones, yielding an overall charge of $+1$ e for the trinuclear site in PKCa/γ. The crystal structure of PKC$^{\beta}$ (1A25) shows an additional sixth acidic residue (E281) that binds Cal II and Cal III, yielding an overall neutral trinuclear Ca$^{2+}$ site (Figure 1b).

| Q$^\epsilon$ (e) | ion | first-shell ligands | % SASA$^a$ | # of HBs$^a$ |
|-----------------|-----|---------------------|------------|-------------|
| −2              | Cal | D187 D187 D193 D246 | 0.8 1.6    | 3 1         |
| −2              | Cal | M186 D187 D246    | 2.2 2.7    | 1 1         |
| 0               | Cal | D248 T251 R252     | 7.3 7.7    | 0 0         |

$^a$Net charge Q of the cation and its ligands in a given site. A repeated Asp indicates bidentate coordination; superscript “b” indicates coordination to the backbone; those in bold are retained upon Ca$^{2+}$ → Li$^+$ substitution. Note that in PKCa, T251 is replaced by S251. After Ca$^{2+}$ → Li$^+$ substitution, Ca$^{2+}$ remains heptacoordinated except when Li$^+$ replaces Cal, Cal II becomes octacoordinated. The average relative solvent-accessible surface area (SASA) of the metal-ligating side chain or backbone atoms. The number of hydrogen bonds (HBs) to Ca$^{2+}$ ligands in the crystal structures of PKCa (3GPE) and PKCa$^{\gamma}$ (2UZP).

### Monoclonic Trinuclear Ca$^{2+}$ Site in PKCa/γ Is Prone to Li$^+$ Substitution

Each Ca$^{2+}$ in the optimized structure of the native trinuclear site in PKCa/γ was substituted by Li$^+$ and the resulting optimized structures were used to compute the $\Delta G^\epsilon$(Ca$^{2+}$ → Li$^+$) free energies for replacing Ca$^{2+}$ with Li$^+$ in PKCa (Figure 2) and PKCa (Figure S3). The mean $\Delta G^\epsilon$(Ca$^{2+}$ → Li$^+$) free energies are indicated by a color gradient going from gold (Ca$^{2+}$/Li$^+$-selective) to turquoise (Li$^+$/Ca$^{2+}$-selective). They indicate that the three Ca$^{2+}$ sites exhibit different vulnerabilities to Li$^+$ displacement: sites I and II with positive $\Delta G^\epsilon$(Ca$^{2+}$ → Li$^+$) values seem to be more Ca$^{2+}$/Li$^+$-selective than site III where $\Delta G^\epsilon$(Ca$^{2+}$ → Li$^+$) is negative ($\sim -4$ kcal/mol). Thus, the monoclonic trinuclear Ca$^{2+}$ site in PKCa/γ may be vulnerable to Li$^+$ substitution if site III is buried ($\epsilon = 4$).

Interestingly, although the Cal and Cal II sites in PKCa/γ have the same ligand composition and net charge (four Asp$^-$ and one backbone), the Ca$^{2+}$ → Li$^+$ substitution resulted in different coordination ligands, modes, and geometries in these two sites. When Li$^+$ replaced CalI, all protein Ca$^{2+}$ ligands (D187, D193, D246, W247, and D248) were retained in the first shell, except that D187 switched its coordination mode from bidentate when Cal-bound to monodentate when Li$^+$-bound (Table 1). The presence of five strong charge-donating protein ligands in site I favored the better charge-accepting cation, Ca$^{2+}$. In contrast to site I, two Ca$^{2+}$ ligands (D187 and M186) were lost upon CalI → Li$^+$ substitution and D248, which was bidentately bound to CalI, became monodentately bound to Li$^+$, enabling Li$^+$ to adopt its preferred coordination number of 4. The loss of three charge-donating ligating atoms in site II reduced ligand → cation charge transfer and repulsion among the ligating O atoms.
surrounding the small Li\(^+\) in a low-dielectric environment. Hence, if sites I and II are buried, the Ca\(^{II} \rightarrow \) Li\(^+\) substitution is not as disfavored (\(\Delta \Delta G^\circ = 2 - 3\) kcal/mol) as the Ca\(^{I} \rightarrow \) Li\(^+\) substitution (\(\Delta \Delta G^\circ = 5 - 6\) kcal/mol) in PKCa/\(\gamma\) (Figures 2 and S3). The fact that Li\(^+\) bound by four Asp\(^-\) in site I is strongly disfavored, which is in line with the previous finding that the maximum number of carboxylates bound to a buried monovalent cation would likely not exceed three.\(^{67}\)

**Second-Shell Arginines Promote Li\(^+\)/Ca\(^{2+}\) Selectivity.** The X-ray structures of PKCa (3GPE)\(^{50}\) and PKCa/\(\gamma\) (2UZP) show three Arg/Lys side chains, namely, R252, R249, and R216 (PKCa) or K219 (PKCa/\(\gamma\)), in the outer shell of the trinuclear Ca\(^{2+}\) site. Such positively charged side chains in the second shell would be expected to favor the less positively charged cation, i.e., monovalent Li\(^+\) rather than divalent Ca\(^{2+}\). However, the computational cost to optimize all charged residues in the outer shell in addition to the three cations, five aspartates, and three backbones comprising the PKCa trinuclear site would be prohibitive. Hence, we opted to first evaluate the effect of the closest outer-shell arginine on the Ca\(^{2+} \rightarrow \) Li\(^+\) substitution free energies. To obtain an estimate of how far each Ca\(^{2+}\) is from these outer-shell Arg residues, we carried out eight 20 ns molecular dynamics (MD) simulations starting from the X-ray structure of PKCa (3GPE)\(^{50}\) (see the Methods). The radial distributions of the distances from an outer-shell Arg C\(_{\zeta}\) atom to each Ca\(^{2+}\) in the trinuclear site computed from the MD simulations (Figure S4) exhibit peaks in agreement with the respective distances seen in the crystal structure (3GPE),\(^{50}\) except for the peak of the R252(C\(_{\zeta}\))–CaII distribution (13 Å), which is greater than the corresponding X-ray distance (10.3 Å).

Since the MD simulations show that the outer-shell arginines are free to move, and R216 is closest to the PKCa trinuclear site, we derived the trinuclear Ca\(^{2+}\) site of PKCa including R216 from the respective X-ray structure (3GPE) and optimized it

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**Figure 3.** Ca\(^{2+}\) versus Li\(^+\) competition in the PKCa trinuclear Ca\(^{2+}\) sites in the presence of outer-shell R216. M06-2X/6-311++G(d,p)-optimized structures with the frozen C\(_{\alpha}\) atoms (transparent spheres) are shown on the left. \(\Delta \Delta G^\circ\) free energies as a function of \(\varepsilon\) for replacing native Ca\(^{2+}\) by Li\(^+\) in each site are shown on the right.
with the frozen Cα atoms. The distance between CaI/II/III (7.5 Å, 11.2 Å, or 13.4 Å) in the M06-2X/6-311+G(d,p) optimized structure is in line with the peak of the respective distribution from the MD simulations (∼8.5, ∼11.5, or ∼13.5 Å). We then replaced each Ca2+ in the optimized structure with Li+ and optimized the resulting structures. The mean Ca2+/Li+ ΔΔG free energies derived from the Ca2+/Li+-bound optimized structures confirm that the presence of outer-shell Arg+ promotes Li+ substitution in buried sites (∼4): the ΔΔGε(CaII→Li+) free energies in sites I, II, and III (Figure 3) are all negative and are more favorable than those in the absence of outer-shell R216 (Figure 2) by 7.1, 4.6, and 3.5 kcal/mol, respectively.

As the ΔΔGε free energies in Figures 2 and 3 show that the trinuclear Ca2+ site in PKCα becomes Ca2+-selective when exposed to the solvent, we computed the average percentage relative solvent-accessible surface areas (SASAs) of the metal-ligating aa residues in each Ca2+ site in the X-ray structures. The relative SASA values (Table 1) indicate that sites I and II are relatively buried, whereas site III is less buried. Since R252 is coordinated to CaIII via its backbone carbonyl oxygen (Figure 1a), could the inclusion of its positively charged guanidinium side chain enable Li+ to displace CaIII even if it is not fully buried?

To address this possibility, we optimized the Ca2+ and Li+-bound trinuclear site of PKCα in the presence of the entire R252 residue and computed the Ca2+/Li+ ΔΔG free energies. The results in Figure 4 show that outer-shell positively charged R252 residue and computed the Ca2+→Li+ ΔΔGε free energies in site III is quite negative for ε ranging from 4 to 20. It is also negative for buried site II (ΔΔGε(CaII→Li+) = −5.4 kcal/mol). The ΔΔGε free energies in Figures 3 and 4 were obtained assuming that the outer-shell Arg side chain was free to reorient during geometry optimization. This may result in unrealistic Arg

Figure 4. Ca2+ versus Li+ competition in the PKCα trinuclear Ca2+ sites in the presence of outer-shell R252. M06-2X/6-311++G(d,p)-optimized structures with the frozen Cα atoms (transparent spheres) are shown on the left. ΔΔGε free energies as a function of ε for replacing native Ca2+ by Li+ in each site are shown on the right.
side chain reorientations upon Ca\textsuperscript{2+} → Li\textsuperscript{+} substitution. Hence, we also computed ΔΔ\textit{G}\textsubscript{ε} for Li\textsuperscript{+} replacing the central Ca\textsuperscript{2+} in the PKCa trinuclear site with outer-shell Arg (R216, R249, or R252) whose C\text{β}, C\text{γ}, and C\text{δ} atoms were frozen during geometry optimization. The results in Figure S5 show that outer-shell Arg, if oriented as seen in the crystallographic structure, rendered the relatively buried site II vulnerable to Li\textsuperscript{+} attack (ΔΔ\textit{G}\textsubscript{4} = −3 to −4.5 kcal/mol). Altogether, the results suggest that with three positively charged Arg/Lys side chains in the outer shell, the cationic trinuclear Ca site in PKCa/γ may be vulnerable to Li\textsuperscript{+} substitution.

**Li\textsuperscript{+} Cannot Displace Native Ca\textsuperscript{2+} in a Trinuclear Ca\textsuperscript{2+} Site with Zero Net Charge.** In the crystal structure of PKCβ (1A2S),\textsuperscript{51} the trinuclear Ca\textsuperscript{2+} site is neutral because of an extra coordinating ligand, Glu281, provided by the second asymmetric unit in the structure. While it is unclear if E281 is a bona\textsuperscript{fide} metal ligand, it is nevertheless interesting to assess how this additional acidic residue affects the susceptibility of the neutral trinuclear Ca\textsuperscript{2+} site to Li\textsuperscript{+} attack. Thus, we optimized the Ca\textsuperscript{2+} or Li\textsuperscript{+}-bound structure of the trinuclear site in PKCβ with the frozen Ca\textsubscript{α} atoms and then computed the mean Ca\textsuperscript{2+} → Li\textsuperscript{+} ΔΔ\textit{G}\textsubscript{ε} free energies. The results in Figure 5 are quite positive over the entire dielectric range studied (4 ≤ ε ≤ 30), indicating that a neutral trinuclear Ca\textsuperscript{2+} site would be immune to Li\textsuperscript{+} attack.

**DISCUSSION**

Our calculations predict that the relatively buried monocationic trinuclear Ca\textsuperscript{2+} site in PKCa/γ may be vulnerable to Li\textsuperscript{+} substitution. Lithium’s displacement of Ca\textsuperscript{2+} in PKCa/γ is aided by its therapeutic concentration ((0.6–1.2) × 10\textsuperscript{−3} M), which is several orders higher than the intracellular Ca\textsuperscript{2+} concentration (∼10\textsuperscript{−7}–10\textsuperscript{−5} M), and its increased accumulation after long-term...

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**Figure 5.** Ca\textsuperscript{2+} versus Li\textsuperscript{+} competition in a neutral trinuclear Ca\textsuperscript{2+} site. Each ion in the optimized structure of the native trinuclear Ca\textsuperscript{2+} site was substituted by Li\textsuperscript{+} and the resulting M06-2X/6-311++G(d,p)-optimized conformations with the frozen C\textsubscript{α} atoms (transparent spheres) are displayed on the left. ΔΔ\textit{G}\textsubscript{ε} free energies as a function of ε for replacing native Ca\textsuperscript{2+} by Li\textsuperscript{+} in each site are shown on the right.
lithium treatment.3 Indeed, Li+, at therapeutic concentrations, has been shown to interact with conventional PKCs, whereas other monovalent cations such as Rb+ and Cs+ are not able to do so even at an elevated concentration of 8 mM.39 By displacing Ca2+ from the C2 domain of cytosolic PKCa/γ, Li+ may affect its membrane-docking ability and reduce the abnormally high membrane-associated PKCa/γ levels found in the frontal cortex of bipolar brains. Our results are consistent with several experimental studies showing that (i) Li+ inhibits the translocation of cytoplasmic PKC to membranes4,3,3,34,41–43,68,69 and (ii) long-term Li+ treatment resulted in a reduction of membrane-associated PKC activity.28,35,40 Since some of these experimental studies have been carried in animals, it is noteworthy the first-shel ligands (five aspartates and one Ser/Thr) and the three second-shell arginines of the PKCa trinuclear site are conserved in human, rat, bovine, mouse, and rabbit C2 domain sequences in the PFAM database (accession code PF00168).

The results herein suggest a novel mode of lithium’s therapeutic mechanism: Li+ may reduce the PKCa/γ hyperactivity seen in bipolar patients by outcompeting Ca2+ for the C2 domain of cytosolic PKCa/γ. As PKCa/γ activity affects the activities of several Ca2+ regulators such as phospholipase C,40 by "normalizing" PKCa/γ hyperactivity, Li+ may also normalize the activity of control mechanisms affecting intracellular Ca2+ handling.3 Our findings may also be relevant for other human diseases such as cardiac disease,11 cancer,12 diabetes,12 and mood disorders13 that have been associated with dysregulation of PKCs.

For future studies, it would be interesting to perform more genome-wide association studies to reveal novel risk genes for lithium-responsive bipolar disorder—those coding for metalloproteins such as calcium channels could then be subject to calculations along the lines presented in this and previous works.34–25 The strategy presented in this work can be used to predict if other Ca2+-bound C2 domains74 or Ca2+ proteins other than C2 domains are susceptible to Li+ substitution. This would help to unravel the key factors (e.g., the type of inner- and outer-shell ligands or the solvent exposure/flexibility of the Ca2+ site) that enable Li+ to displace Ca2+ in proteins.

**ASSOCIATED CONTENT**

*Supporting Information* The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02882.

Choice of the protocol for energy calculations (Figure S1); optimized structures of the model trinuclear Ca2+ site in PKCy (Figure S2); Ca2+ versus Li+ competition for PKCy models (Figure S3); radial distributions of the outer-shell R216/R249/R252 Cα—Ca2+ distances (Figure S4); and Ca2+ versus Li+ competition for site II with the outer-shell R216/R249/R252 side chain C atoms constrained in the X-ray conformation (Figure S5) (PDF)

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