Cytosolic phospholipase A2 (cPLA2) catalyzes release of arachidonic acid from membranes following translocation to Golgi and endoplasmic reticulum. In response to an intracellular calcium concentration (\([Ca^{2+}]_i\)) increase, the C2 domain binds Ca\(^{2+}\) and brings the catalytic domain into proximity with its phospholipid substrate. Because membrane residence is important in the regulation of cPLA2 activity, we explored the contributions of the C2 and catalytic domains in mediating membrane residence using an imaging approach in live cells with fluorescent protein chimeras of cPLA2. The isolated cPLA2 C2 domain associated with Golgi membranes rapidly in proportion to the [Ca\(^{2+}\)]\(_i\), allowing for its use as a [Ca\(^{2+}\)]\(_i\) indicator. cPLA2 association with Golgi was slower than the isolated C2 domain in response to a [Ca\(^{2+}\)] increase. After [Ca\(^{2+}\)] decrease, cPLA2 remained associated with membrane in a Ca\(^{2+}\)-independent fashion whereas C2 domain rapidly dissociated. Ca\(^{2+}\)-independent membrane association was greatly reduced by mutation of Trp 464, located at the membrane-exposed face of the catalytic domain, to Gly or Ala. Mutation of Trp 464 to Phe supported Ca\(^{2+}\) binding neutralizes the negative charge at the tips of the Ca\(^{2+}\) binding loops, allowing hydrophobic residues in the C2 domain to insert into the lipid surface and stabilize membrane association (5, 6, 15–17). Disruption of either the Ca\(^{2+}\) binding residues or the hydrophobic residues responsible for membrane penetration results in the loss of membrane binding in vitro (5, 6) and the failure of membrane association in vivo (9, 18). Deletion of the C2 domain also prevents membrane association in vivo (8, 18).

Because of the central roles of Ca\(^{2+}\) and the C2 domain in membrane binding, there have been several studies examining [Ca\(^{2+}\)] requirements in vitro. Two early reports investigating full-length and isolated C2 domain binding have reported Ca\(^{2+}\) requirements in the range of 100–300 nM for cPLA2 membrane association in vitro (4, 19). However, most in vitro studies have used the isolated C2 domain and have reported a minimum [Ca\(^{2+}\)] of between 2 and 11 μM for membrane binding (6, 16, 20–22). Diverse experimental conditions make comparisons between the in vitro studies difficult to interpret, but the magnitude of the [Ca\(^{2+}\)] predicted for binding from most of the in vitro studies is higher than would be expected in vivo for a cytosolic protein that responds to agonist-mediated [Ca\(^{2+}\)] changes. An investigation of membrane binding in vivo reported that the [Ca\(^{2+}\)] requirement for full-length and isolated C2 domain membrane association to be ~120 nM for Golgi and ~250 nM for ER membranes (8).

Investigation of full-length cPLA2 binding in vivo has demonstrated that, following Ca\(^{2+}\)-dependent membrane association, the full-length cPLA2 remains associated with membranes in a Ca\(^{2+}\)-independent manner (8, 23). This suggests a possible role for the catalytic domain, or cooperativity between the C2 and catalytic domains in prolonging membrane residence time. Although studies have evaluated dissociation kinetics of the cPLA2 C2 domain in vitro (20, 21), there are few agents (1, 2). The lipid second messenger AA functions directly to regulate a number of cellular processes and is the precursor for a variety of other lipid mediators, including prostaglandins and leukotrienes (3). Because cPLA2 substrates are membrane phospholipids, a fundamental regulatory mechanism of cPLA2 lies in controlling its access to membrane.
Association of cPLA₂ with Golgi

in vitro studies to date that have evaluated the contribution of the catalytic domain in Ca²⁺-independent association with membrane (24). We have used imaging of fluorescent protein-cPLA₂ chimeras to investigate further the role of the cPLA₂ catalytic domain in regulating membrane association and residence time in living cells.

MATERIALS AND METHODS

Fluorescent Protein Constructs—For imaging studies, DNAs encoding wild-type human cPLA₂ and the cPLA₂ C2 domain (residues 1–149 and 17–145, respectively; GenBank™ accession no. M72393) were cloned into the vector pEGFP-C3 (Clontech) to create pEGFP-cPLA₂, pEGFP-cPLA₂-C2, as previously described (8). pECFP-cPLA₂-W464G, pECFP-cPLA₂-W464A, and pECFP-cPLA₂-W464F were made from pECFP-cPLA₂ by site-directed mutagenesis (Stratagene). pECFP-GT, a Golgi marker encoding the N-terminal 82 residues of β1,4-galactosyltransferase, was purchased from Clontech. The pEFYP plasmid used in this study was constructed from pEFYP-C1 (Clontech) by introducing a Q70M mutation by site directed mutagenesis (Stratagene) to improve its qualities (25). Different color fluorescent protein constructs were produced by interchanging NheI/BsrGI fragments encoding the background-corrected average pixel values for an expression of FP-tagged wild-type and mutant proteins at the predicted molecular weights was confirmed by immuno blot of protein lysates from MDCK cells transfected with each construct. A band at ~135 kDa was observed in protein lysates from cells expressing pECFP-cPLA₂-W464A, pECFP-cPLA₂-W464G, and pECFP-cPLA₂-W464F, which migrated at the expected molecular weight as pECFP-cPLA₂ (data not shown). We have previously demonstrated that pECFP-cPLA₂-C2 is expressed at the predicted molecular weight in MDCK cells (8). All constructs were confirmed by sequencing.

Cell Culture—MDCK cells obtained from ATCC were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 mg/ml glucose in 5% CO₂ at 37 °C. Subconfluent cells (1 × 10⁶ cells/cm²) were transfected with the relevant plasmid(s) using FuGene 6 (Roche Molecular Biochemicals) in Dulbecco’s modified Eagle medium containing 2.0% bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 mg/ml glucose following the protocol from the manufacturer.

Microscopy of Fluorescent Proteins—Transfected MDCK cells grown on glass-bottomed culture dishes (MatTek) were washed with and incubated in Hanks’ balanced salt solution additionally buffered with 1 mM probenecid and imaged after a 30-min incubation for equilibration with [Ca²⁺]i was lower than the [Ca²⁺] in cytosol before imaging. The process of de-esterification of the Fura-2 AM. Single-cell imaging was performed on the Olympus system described above, but using a 40× 1.35 numeric aperture oil immersion objective and a Fura2/EGFP dichroic mirror and emission filter (Chroma). Fura2/EGFP image sets were illuminated at 340, 380, and 465 nm at the time intervals specified. The [Ca²⁺]i increase, was expressed as the ratio of the background-corrected Fura2 fluorescence at 340 and 380 nm (26).

 résultats

Expression and Localization of cPLA₂ in MDCK Cells—To determine the intracellular membrane to which cPLA₂ translocates in response to increases in [Ca²⁺]i, live cells, EYFP-cPLA₂-C2 was co-expressed with ECFP-GT, which localizes to medial and trans-Golgi cisternae (27), and imaged in time-lapse in response to [Ca²⁺]i mobilization. In response to 5 μM ATP, EYFP-cPLA₂-C2 moved from the cytosol to juxtanuclear membranes (Fig. 1A), and co-localized with ECFP-GT (Fig. 1, B and C). This shows that cPLA₂ associates prominently with the Golgi in living cells and extends previous immunofluorescence findings in fixed and permeabilized MDCK cells (8).

cPLA₂ and the cPLA₂ C2 Domain Have Equivalent Ca²⁺ Sensitivities for Membrane Association—In previous experiments, we correlated [Ca²⁺]i with the extent of EGFP-tagged cPLA₂ (EGFP-cPLA₂) or EYFP-tagged cPLA₂ C2 domain (EYFP-cPLA₂-C2) association with Golgi and ER membranes and found the [Ca²⁺] threshold for association with Golgi to be ~120 and ~250 nM for ER and nuclear membrane (8). The subcellular localization of the C2 and full-length cPLA₂ constructs also appeared similar. However, we were unable to directly compare membrane association of both constructs simultaneously at any given [Ca²⁺]i, or directly compare targeting in the same cell. Here, we directly compared membrane association of EYFP-cPLA₂-C2 and ECFP-cPLA₂ co-expressed in cells under calcium clamp conditions, where [Ca²⁺]i was increased in discrete steps and the chimeras imaged. At [Ca²⁺]i, less than 50 nM (resting level), fluorescence from both constructs was distributed in the cytoplasm (Fig. 1, D and H). EYFP-cPLA₂-C2 is also evident in the nucleoplasm, whereas

The background-corrected average pixel values were determined for ROI cell, the integrated fluorescence from the ROI cell, the threshold for association of EYFP-cPLA2C2 and ECFP-cPLA 2 co-expressed in the same cell. Here, we directly compared membrane association at static [Ca²⁺]i. (Figs. 1 and 8), a small volume of 100 μM CaCl₂ (~20–25 μl, depending on the experiment) was added to 1 ml of the ionomycin/EH buffer covering the cell, the buffer was mixed, and the [Ca²⁺]i allowed to establish an equilibrium with [Ca²⁺]i, for >5 min before imaging. The process of CaCl₂ addition, in increments of 2–3 μl, mixing, equilibration, and imaging was repeated for each [Ca²⁺]i, step. As previously reported (8), this treatment results in incremental increases in [Ca²⁺]i, in the physiological range of ~10–600 nM that remain stable for >10 min; however, the [Ca²⁺]i was lower than the [Ca²⁺]i, at equilibrium.

For time-lapse experiments to determine kinetics of membrane association and residence (Figs. 2, 3, 4 (A and B), and 7), cells were incubated in EH buffer and permeabilized with ionomycin as above. [Ca²⁺]i increases were stimulated by exchanging the buffer over the cells with 1 ml of EH buffer containing 2.5 mM CaCl₂ and 10 μM ionomycin at the times indicated. To decrease [Ca²⁺]i, 1 ml of EH buffer was added at the times indicated.

For some time-lapse experiments (Figs. 4 (C and D) and 9), cells were not pre-incubated in EH buffer and the internal Ca²⁺ stores remained intact. In these experiments, [Ca²⁺], was increased by addition of 100 μl of 10 μM ionomycin in EH to 900 μl of EH buffer over the cells, which resulted in Ca²⁺ release from internal stores. The [Ca²⁺]i was subsequently decreased by chelation of [Ca²⁺]i, by the EH buffer in the bath. This second treatment resulted in a faster, higher amplitude [Ca²⁺]i increase, but the results obtained were not different from the experiments where exogenous CaCl₂ was added to store-depleted cells.

calcium imaging—MDCK cells grown on glass-bottomed dishes were washed with HHBSS containing 1 mM probenecid and incubated with 5 μM Fura2-AM (Calbiochem) in HHBSS, 1 mM probenecid, and 0.1% MeSO for 45 min at 37 °C. Cells were then washed with HHBSS containing 1 mM probenecid and imaged after a 30-min incubation for

Results

Expression and Localization of cPLA₂ in MDCK Cells—To determine the intracellular membrane to which cPLA₂ translocates in response to increases in [Ca²⁺]i, live cells, EYFP-cPLA₂-C2 was co-expressed with ECFP-GT, which localizes to medial and trans-Golgi cisternae (27), and imaged in time-lapse in response to [Ca²⁺]i mobilization. In response to 5 μM ATP, EYFP-cPLA₂-C2 moved from the cytosol to juxtanuclear membranes (Fig. 1A), and co-localized with ECFP-GT (Fig. 1, B and C). This shows that cPLA₂ associates prominently with the Golgi in living cells and extends previous immunofluorescence findings in fixed and permeabilized MDCK cells (8).
ECFP-cPLA2 is excluded because of differences in protein size, as we have previously shown (8).

At each step increase in [Ca\(^{2+}\)], roughly equivalent fractions of EYFP-cPLA2C2 and ECFP-cPLA2 associated with Golgi (Fig. 1, panels E and F and panels I and J). Because cPLA2 associates with ER and nuclear membranes at [Ca\(^{2+}\)] greater than ~250 nM, the [Ca\(^{2+}\)], in images in panels E and F and panels I and J were judged to be below 250 nm. In Fig. 1 (G and K), [Ca\(^{2+}\)], is above 250 nM, as evidenced by fluorescence from both constructs on the nuclear envelope (white arrowhead) as well as Golgi (black arrowhead). Although the ER is not resolved in panels G and K as it is in panel A, we have shown previously that isolated C2 and full-length cPLA2 translocate to the nuclear membrane and ER at the same [Ca\(^{2+}\)], (8). EYFP-cPLA2C2 and ECFP-cPLA2 localized to the same intracellular membranes as shown in the merged image (Fig. 1L). These observations using dual imaging of cells co-expressing full-length cPLA2 and the isolated C2 domain directly confirm that the C2 domain provides targeting specificity and that the isolated C2 domain and full-length cPLA2 have equivalent Ca\(^{2+}\) sensitivities.
cPLA2C2 as a \([Ca^{2+}]_i\) Sensor—Because of the essential role of \([Ca^{2+}]_i\) in mediating cPLA2 association, we investigated using cPLA2C2 as a \([Ca^{2+}]_i\) sensor to monitor \([Ca^{2+}]_i\) changes. C2 domains have been used successfully as biosensors sensitive to \([Ca^{2+}]_i\) change (28). Based on our earlier work, translocation to Golgi, but not ER, occurred at \([Ca^{2+}]_i\) of 125–250 nM (8). By titrating the \([Ca^{2+}]_e\) in the extracellular buffer against the extent of EYFP-cPLA2C2 translocation (Fig. 1), we determined the \([Ca^{2+}]_e\) that reproducibly elicited association of cPLA2C2 with Golgi, but not ER (i.e. produced \([Ca^{2+}]_i\) of 125–250 nM).

Cells expressing EGFP-cPLA2C2 and loaded with Fura2 were placed in calcium clamp conditions, and exogenous CaCl2 was added at 7.5 s after initiation of imaging (between the first and second image set) to increase \([Ca^{2+}]_i\), and EGTA was added at 247.5 s to decrease \([Ca^{2+}]_i\). Representative images from a time-lapse recording show transient association of EGFP-cPLA2C2 at the area of the Golgi (Fig. 2 and Supplemental Movie 2A (available in the on-line version of this article)). The images show that a fraction of the cellular EGFP-cPLA2C2 associated with Golgi and that the association was limited primarily to Golgi, indicating that the \([Ca^{2+}]_i\) did not exceed \(250 \text{ nM}\) in the cell. Graphical analysis of EGFP-cPLA2C2 and ratioed Fura2 fluorescence in the area of the Golgi (EGFP-cPLA2C2Golgi and Fura2Golgi) with respect to time (Fig. 2B) shows that after the \([Ca^{2+}]_i\) exceeded the threshold for membrane association, EGFP-cPLA2C2Golgi increased at a similar rate as the Fura2Golgi. After addition of EGTA, both EGFP-cPLA2C2Golgi and Fura2Golgi decreased at similar rates until initial values were re-established. These results demonstrate that cPLA2 C2 domain association with Golgi membranes is coincident with changes in \([Ca^{2+}]_i\).

Contribution of the cPLA2 Catalytic Domain in Membrane Association and Residence Time—The results above show that the isolated cPLA2 C2 domain functions well as a \([Ca^{2+}]_i\) indicator in a physiological \([Ca^{2+}]_i\) range. This allowed for imaging studies to examine the relative contributions of the C2 and catalytic domains in regulating membrane association and residence time by co-expressing the cPLA2 C2 domain and full-length cPLA2, and directly comparing association and dissociation using the C2 domain as a monitor of \([Ca^{2+}]_i\) changes. For these experiments, it was necessary to utilize a system where \([Ca^{2+}]_i\) changes in cells were induced by sequential addition of exogenous CaCl2 and exogenous CaCl2 was added at 7.5 s after initiation of imaging (between the first and second image set) to increase \([Ca^{2+}]_i\), and EGTA was added at 247.5 s to decrease \([Ca^{2+}]_i\). Representative images from a time-lapse recording show transient association of EGFP-cPLA2C2 at the area of the Golgi (Fig. 2A and Supplemental Movie 2A (available in the on-line version of this article)). The images show that a fraction of the cellular EGFP-cPLA2C2 associated with Golgi and that the association was limited primarily to Golgi, indicating that the \([Ca^{2+}]_i\) did not exceed \(250 \text{ nM}\) in the cell. Graphical analysis of EGFP-cPLA2C2 and ratioed Fura2 fluorescence in the area of the Golgi (EGFP-cPLA2C2Golgi and Fura2Golgi) with respect to time (Fig. 2B) shows that after the \([Ca^{2+}]_i\) exceeded the threshold for membrane association, EGFP-cPLA2C2Golgi increased at a similar rate as the Fura2Golgi. After addition of EGTA, both EGFP-cPLA2C2Golgi and Fura2Golgi decreased at similar rates until initial values were re-established. These results demonstrate that cPLA2 C2 domain association with Golgi membranes is coincident with changes in \([Ca^{2+}]_i\).

**Fig. 3.** Membrane association and residence of cPLA2 and the isolated cPLA2 C2 domain. CaCl2 was added at 7.5 s after initiation of imaging and EGTA at 217.5 s to cells co-expressing ECFP-cPLA2 (A) and EYFP-cPLA2C2 (B) under calcium clamp conditions. C, graphical analysis of ECFP-cPLA2 and EYFP-cPLA2C2 fluorescence from the area of the Golgi (ECFP-cPLA2Golgi and EYFP-cPLA2C2Golgi, inset, black line). ECFP-cPLA2Golgi and EYFP-cPLA2C2Golgi are graphed on different axes to highlight the difference in time courses. D, early time points (30–120 s) from the data in C are graphed on the same axis to directly compare rates of translocation of ECFP-cPLA2Golgi and EYFP-cPLA2C2Golgi. Results are characteristic of 17 cells analyzed in 15 independent experiments performed on different days.
mobilize intracellular Ca\(^{2+}\), the cPLA\(_2\) C2 domain associates first with both Golgi and ER, because of the high local [Ca\(^{2+}\)]\(_i\) surrounding the ER immediately after stimulation, then dissociates from ER and remains associated with Golgi exclusively (8). However, unlike the cPLA\(_2\) C2 domain, the full-length cPLA\(_2\) associates primarily with Golgi in response to ATP or thapsigargin (8). The quantitative method we developed to compare translocation rates requires that full-length cPLA\(_2\) and the isolated C2 domain associate with membranes in the same area of the cell, and so precluded use of ATP or thapsigargin as Ca\(^{2+}\) mobilizing agonists.

Cells expressing ECFP-cPLA\(_2\) and EYFP-cPLA\(_2\)C2 were placed in calcium clamp conditions, where intracellular Ca\(^{2+}\) stores were depleted and cells were made permeant to [Ca\(^{2+}\)]\(_i\) with ionomycin. Representative frames from a time-lapse recording of such a cell shows association of ECFP-cPLA\(_2\) and EYFP-cPLA\(_2\)C2 with Golgi at the times indicated in response to addition of CaCl\(_2\) to the bath at 7.5 s after initiation of imaging (Fig. 3 (A and B) and Supplemental Movie 3AB (available in the on-line version of this article)). Addition of EGTA at 217.5 s resulted in the reduction of EYFP-cPLA\(_2\)C2 fluorescence at Golgi, but had little effect on the retention of ECFP-cPLA\(_2\) at Golgi. Image sequences of another experiment in which the EGTA was added at 255 s, but where the C2 domain translocated to ER and Golgi, is provided in Supplemental Movie 1 (available in the on-line version of this article).

Graphical analysis shows that, in response to the [Ca\(^{2+}\)]\(_i\) increase, EYFP-cPLA\(_2\)C2 fluorescence at Golgi (EYFP-cPLA\(_2\)C2\(_{\text{Golgi}}\)) increased more rapidly than ECFP-cPLA\(_2\) fluorescence at Golgi (ECFP-cPLA\(_2\)Golgi) (Fig. 3C). Detailed analyses of increases in EYFP-cPLA\(_2\)C2\(_{\text{Golgi}}\) and ECFP-cPLA\(_2\)Golgi in response to a buffered [Ca\(^{2+}\)]\(_i\) increase reveals that the rate of association of EYFP-cPLA\(_2\)C2 with Golgi is initially linear and slows after 2.5 min, whereas association of ECFP-cPLA\(_2\) with Golgi continues to increase up to 4 min (Fig. 3C). Because ECFP-cPLA\(_2\) and EYFP-cPLA\(_2\)C2 are imaged in the same cell, and the \(F/F_0\) is directly proportional to the fraction of total cellular fluorescent protein at Golgi (see “Materials and Methods”), ECFP-cPLA\(_2\)Golgi and EYFP-cPLA\(_2\)C2\(_{\text{Golgi}}\) may be compared directly (Fig. 3D). A comparison of the initial rates of EYFP-cPLA\(_2\)C2\(_{\text{Golgi}}\) and ECFP-cPLA\(_2\)Golgi graphed on the same y axis shows a greater initial rate of association for EYFP-cPLA\(_2\)C2 than for ECFP-cPLA\(_2\). We do not believe that the presence of isolated C2 domain is responsible for the relatively slower association of full-length cPLA\(_2\) either by chelating available Ca\(^{2+}\) or by out competing full-length for membrane binding sites for the following reasons. First, membrane association of both full-length and C2 cPLA\(_2\) reach a plateau, as shown in the calcium clamp experiments (Fig. 1) and earlier work (8), so the extent of their translocation is proportional to [Ca\(^{2+}\)]\(_i\), and equivalent fractions of both full-length and C2 cPLA\(_2\) associate with Golgi at a given [Ca\(^{2+}\)]\(_i\) (Fig. 1). The earlier plateau of the C2 domain suggests that it establishes an equilibrium with [Ca\(^{2+}\)]\(_i\) before full-length cPLA\(_2\), and the con-
Golgi after C2 association rate slows, suggesting that available binding sites are also not a limiting factor.

EYFP-cPLA2C2Golgi returned to baseline levels ~90 s after addition of EGTA (Fig. 3C). In contrast, ECFP-cPLA2Golgi remained elevated and was at ~75% of the peak value 8 min after EGTA addition, indicating that prolonged membrane retention mediated by the catalytic domain is Ca\(^{2+}\)-independent. Ca\(^{2+}\)-independent membrane retention of full-length cPLA2 was consistently observed in all experiments. Often, full-length cPLA2 was clearly observed to be associated with Golgi membranes for >30 min after dissociation of the isolated C2 domain. Because of cell-specific differences in translocation resulting from differences between rates of [Ca\(^{2+}\)]\(_i\) increase (after Ca\(^{2+}\) addition) and decrease (after EGTA addition) and between the final [Ca\(^{2+}\)]\(_i\) values reached before EGTA addition, we were not able to derive meaningful errors for fluorescence intensity change for translocation. Additional analyses of two cells treated as above are provided in Supplemental Fig. 1 (available in the on-line version of this article).

Differences in association and dissociation rates of the full-length cPLA2 and the C2 domain were consistently observed, even under conditions when initial association rates differed as a result of differences in the rate of [Ca\(^{2+}\)]\(_i\) increase. For example, in Ca\(^{2+}\) store-depleted cells (Fig. 4A), the rates of initial membrane association after Ca\(^{2+}\) addition (Fig. 4B) were much slower than the rates of membrane association in Ca\(^{2+}\) store-replete cells treated with ionomycin (Fig. 4, C and D). In the latter, the rate of full-length cPLA2 association with Golgi was initially rapid, but slowed considerably after a few seconds and was slightly slower than that of the isolated C2 domain (Fig. 4D). The very rapid initial association of full-length cPLA2 is most likely the result of a short-lived, high magnitude local [Ca\(^{2+}\)]\(_i\) increase caused by ionomycin-mediated release of Ca\(^{2+}\) from the intact stores, highlighting the role of local [Ca\(^{2+}\)]\(_i\) in translocation of cPLA2, as we have previously noted (8).

Similar results to those above were obtained in control experiments where the fluorescent tags on the FP-cPLA2 and FP-cPLA2C2 chimeras were switched (data not shown). In other control experiments using cells co-expressing ECFP- and EYFP-tagged C2 domain, rates of membrane association and dissociation were similar (data not shown). Rapid dissociation of C2 domain and slow dissociation of full-length cPLA2 was also observed in experiments where EYFP-cPLA2 C2 and ECFP-cPLA2 fluorescence were associated with both Golgi and ER after Ca\(^{2+}\) addition, indicating that the [Ca\(^{2+}\)]\(_i\) was increased to greater than 250 nM (Supplemental Movie 1 and Supplemental Fig. 1 (A and B), available on-line).

These results demonstrate that the catalytic domain both retards Ca\(^{2+}\)-mediated association of cPLA2 with membrane and mediates a prolonged, Ca\(^{2+}\)-independent membrane residence.

Membrane Residence in Response to Oscillations in [Ca\(^{2+}\)]\(_i\)—

Repetitive increases in [Ca\(^{2+}\)]\(_i\) ([Ca\(^{2+}\)]\(_i\) oscillations) may represent a normal physiological state in cells, and many cell responses and enzymes are sensitive to variations in the frequency of Ca\(^{2+}\) oscillations (29–32). To investigate the effect of Ca\(^{2+}\) oscillations on the cPLA2 C2 domain, cells expressing EGFPC-pla2C2 were loaded with Fura2 and stimulated with 5 \(\mu\)M ATP, and the EGFP-cPLA2C2Golgi and Fura2Golgi monitored over time. ATP elicited a sharp spike in Fura2Golgi which was followed by oscillations of diminishing magnitude that persisted for ~5 min (Fig. 5A). EGFP-cPLA2C2Golgi temporally followed the Fura2Golgi and was proportional to the magnitude of the Fura2Golgi. These results demonstrate that the cPLA2 C2 domain association with Golgi is sensitive to rapid changes in [Ca\(^{2+}\)]\(_i\), and proportional to [Ca\(^{2+}\)]\(_i\).
From the results above, we expected that full-length cPLA$_2$ would accumulate at Golgi in response to $\text{[Ca}^{2+}]_i$ oscillations because of its slow dissociation rate. To investigate this hypothesis, cells co-expressing ECFP-cPLA$_2$ and EYFP-cPLA$_2$C$_2$ were stimulated with 5 $\mu$M ATP and the ECFP and EYFP fluorescence at Golgi monitored. As shown in Fig. 5B, both EYFP-cPLA$_2$C$_2$Golgi and ECFP-cPLA$_2$Golgi increased in response to $\text{[Ca}^{2+}]_i$ increase. Because of the different scaling of the y-axis in Fig. 5B, ECFP-cPLA$_2$Golgi appears to increase before EYFP-cPLA$_2$C$_2$Golgi in response to an ATP-induced $\text{[Ca}^{2+}]_i$ increase. However, when compared directly, EYFP-cPLA$_2$C$_2$Golgi increases at a faster rate than ECFP-cPLA$_2$Golgi (Fig. 5C), as is observed in response to an ionomycin-induced $\text{[Ca}^{2+}]_i$ increase (Figs. 3 and 4).

Whereas the EYFP-cPLA$_2$C$_2$Golgi declined to base-line levels between the initial $\text{[Ca}^{2+}]_i$ increase and the first $\text{Ca}^{2+}$ oscillation, the ECFP-cPLA$_2$Golgi remained elevated (Fig. 5B), although there was a slight decrease in fluorescence as we have noted in response to ATP and thapsigargin previously (8). The period between the initial increase and the first oscillation (Fig. 5B) is greater than that observed in Fig. 5A because of the cell-to-cell heterogeneity observed in $\text{[Ca}^{2+}]_i$, mobilization responses to ATP (33). To illustrate the differences between the behavior between the isolated C2 domain and the full-length protein, we chose an example where the fluorescence of the isolated C2 domain returned to base-line level between oscillations.

In response to the $\text{[Ca}^{2+}]_i$ increase associated with the first oscillation, EYFP-cPLA$_2$C$_2$Golgi increased to less than one half of the level observed in response to the initial $\text{[Ca}^{2+}]_i$ increase (Fig. 5B). However, ECFP-cPLA$_2$Golgi increased to nearly the same level as in response to the initial $\text{[Ca}^{2+}]_i$ increase. This result highlights the effect of the catalytic domain on prolonging membrane residence in response to physiological $\text{[Ca}^{2+}]_i$ signals.

**Trp$^{464}$ Mediates $\text{Ca}^{2+}$-independent Membrane Residence of cPLA$_2$**—To investigate the mechanism for $\text{Ca}^{2+}$–independent membrane residence of full-length cPLA$_2$, we next examined its structural characteristics. The crystal structure of cPLA$_2$ reveals a helical region on the membrane binding face of the catalytic domain, previously identified as helix Fd, which constitutes part of the catalytic domain “cap” structure (Fig. 6A) (7). Trp$^{464}$ orients outward at the top of helix Fd (Fig. 6B). The residues associated with this helical structure and the placement of the tryptophan are evolutionarily conserved in all sequenced cPLA$_2$ proteins (Fig. 6C). Because Trp residues...
are known to play critical roles in the interfacial binding of other PLA2 isoforms (34), we investigated the role of Trp 464 in Ca\(^{2+}\)-independent membrane residence by treating cells expressing cPLA2C2 and a cPLA2W464G mutant in calcium clamp conditions with CaCl\(_2\) and EGTA, using the same protocol as in Fig. 3. Representative images from time-lapse imaging (Fig. 7, A and B) show that, in response to CaCl\(_2\) addition, ECFP-cPLA2W464G and EYFP-cPLA2C2 both associated with Golgi. However, in contrast to full-length cPLA2, ECFP-cPLA2W464G rapidly dissociated from Golgi and ER following EGTA addition at 217.5 s (Fig. 7B). Graphical analysis shows that following addition of EGTA, ECFP-cPLA2W464G relocated into the Golgi (ECFP-cPLA2W464G and EYFP-cPLA2C2, inset, black line). These results suggest that Trp 464 in the catalytic domain is important in mediating Ca\(^{2+}\)-independent membrane association.

Shorter residence time of the cPLA2 W464G mutant on Golgi than the C2 domain was surprising and suggested that the [Ca\(^{2+}\)]\(_i\) requirement for W464G association was greater than for the isolated C2 domain. To test this possibility, cells expressing ECFP-cPLA2W464G and EYFP-cPLA2C2 were placed in calcium clamp conditions and subjected to step increases in [Ca\(^{2+}\)]\(_i\), as shown in Fig. 1 (D–K). Images show that at low [Ca\(^{2+}\)]\(_i\), which is comparable with resting [Ca\(^{2+}\)]\(_i\), or lower, the distributions of ECFP-cPLA2W464G and EYFP-cPLA2C2 were similar except for the nucleoplasmic distribution of the C2 domain (Fig. 8, A and D). Following step increases in [Ca\(^{2+}\)]\(_i\), EYFP-cPLA2C2 associated with Golgi and ER membranes (Fig. 8B), indicating that the [Ca\(^{2+}\)]\(_i\) exceeded 250 nM, but ECFP-cPLA2W464G remained cytoplasmic (Fig. 8E). This suggests that the cPLA2W464G mutant requires a higher [Ca\(^{2+}\)]\(_i\) for membrane association (>250 nM) than does wild-type cPLA2 (>120 nM). At still higher [Ca\(^{2+}\)]\(_i\) (Fig. 8, C and F), ECFP-cPLA2W464G associated with Golgi and ER. The merged image of ECFP-cPLA2W464G and EYFP-cPLA2C2 at the highest [Ca\(^{2+}\)]\(_i\) shows similar distributions of fluorescence (Fig. 8G), indicating that targeting of ECFP-cPLA2W464G is not altered.

**Phe Can Substitute for Trp in Mediating Ca\(^{2+}\)-independent Membrane Residence**—To determine whether another aromatic residue, Phe, can substitute for Trp in mediating Ca\(^{2+}\)-independent membrane residence, a cPLA2W464F mutant was constructed and assayed. Representative images from time-lapse analysis of cells, expressing ECFP-cPLA2W464F and EYFP-

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**FIG. 7.** Mutation of Trp\(^{464}\) diminishes Ca\(^{2+}\)-independent membrane residence. Cells co-expressing ECFP-cPLA2W464G (A) and EYFP-cPLA2C2 (B) were placed in calcium clamp conditions, and CaCl\(_2\) was added at 7.5 s after initiation of imaging. EGTA was added at 217.5 s. C, graphical analysis of ECFP-cPLA2W464G fluorescence from the area of the Golgi (ECFP-cPLA2W464G and EYFP-cPLA2C2, inset, black line).
cPLA2C2 and treated with ionomycin, show that both constructs associated with Golgi, but, although the EYFP-cPLA2C2 dissociated, the ECFP-cPLA2W464F remained with Golgi (Fig. 9, A and B), similar to what is observed using wild-type cPLA2 (Fig. 3A). Graphical analysis of ECFP-cPLA2W464FGolgi and EYFP-cPLA2C2Golgi (Fig. 9C) showed that the W464F mutant displayed a Ca2+-independent association with Golgi, similar to what was observed with wild-type cPLA2 (Fig. 4C). This result suggests that Phe can substitute for Trp in mediating Ca2+-independent membrane residence.

**DISCUSSION**

cPLA2 is a critical enzyme in the release of AA, an important lipid second messenger, and the precursor for biologically active oxygenated metabolites. Spatial and temporal regulation of cPLA2 has been ascribed to the properties of the C2 domain, which functions as the targeting and calcium-sensing module of the protein, and we have confirmed these roles for the C2 domain in the work presented here. The surprising result is the unexpected role of the catalytic domain in regulating cPLA2 residence time on membrane, in addition to its role in catalysis of membrane phospholipids.

For signaling proteins that associate with membranes for function, such as the C2 domain-containing PKCs (α, β1, βI, and γ), PLCδ, and cPLA2, regulation of membrane residence is crucial. A recent analysis of C2 domain translocation characteristics identified two discrete modes of signaling based on the persistence of C2 domain membrane residence as a function of temporal Ca2+ signals (28). Transient association of C2 domain on membrane in response to Ca2+ increase (transient association with membranes) in response to Ca2+ influx was termed an “integrating” signaling mode. Our present work shows that, in the case of cPLA2, the inherently differentiating response of the C2 domain in response to transient [Ca2+]i increase (transient association with membranes) is modified to an integrating response by the Ca2+-independent membrane association mediated by Trp464 in the catalytic domain. Modification of membrane residence by adjoining domains is not unique to cPLA2; PKC membrane residence mediated by the C2 domain is modified by C1 domain association with diacylglycerol in the membrane (35), although the effect of the PKC C1 domain mediating Ca2+-independent membrane association is more subtle than that observed here by the cPLA2 catalytic domain. Because of the role of the catalytic domain in maintaining membrane residence revealed here, membrane-binding properties of full-length cPLA2 cannot be implied by the properties of the isolated C2 domain (36). This is exemplified by the experiments investigating membrane binding in response to Ca2+ oscillations, where the isolated C2 domain returned to base-line levels during the period between [Ca2+]i spikes, but the full-length cPLA2 did not.

In the case of cPLA2, Ca2+-independent membrane residence in MDCK cells does not lead to an integrated catalytic response. In vivo experiments temporally correlating cPLA2 on membrane with AA release have shown that release ceases within minutes after stimulation (8), although cPLA2 remains associated with membranes, suggesting that the enzyme inactivates after a period of catalysis. In vitro experiments have...
also demonstrated this apparent “inactivation” of cPLA2 (24). cPLA2 also becomes “trapped” on negatively charged lipid bilayers and trapping may be caused in part by product accumulation (37). It is also possible that trapping may be the result of a Ca\(^{2+}\)-independent association of the catalytic domain with anionic membranes (24). It has been suggested that trapping of cPLA2 may be responsible for this apparent inactivation because of local substrate depletion. Experiments with a catalytically inert cPLA2 S228A mutant or in the presence of pyridine-1, a specific cPLA2 inhibitor that blocks AA release in MDCK cells (38), showed no effect on membrane residence in our system (data not shown), suggesting that catalytic activity or product formation does not play a role in Ca\(^{2+}\)-independent membrane residence. Thus, trapping in vitro and the Ca\(^{2+}\)-independent membrane residence in vivo may result from different mechanisms. We have also shown that Ca\(^{2+}\)-mediated association of cPLA2 with membrane is not in itself sufficient for optimal catalytic activity (39). Although the mechanism is unknown, these studies demonstrate an additional level of catalytic regulation aside from membrane residence. The results from the experiment investigating membrane residence in response to Ca\(^{2+}\) oscillations elicited by low concentrations of ATP also suggest that additional factors may be important in cPLA2 regulation. Should cells in tissues exhibit physiological Ca\(^{2+}\) oscillations, a fraction of cPLA2 may be constitutively associated with membranes, but the small amount of dissociation/reeassociation may lead to a more prolonged low level AA release.

Phosphorylation is a major regulator of cPLA2 activity (1, 2), and phosphorylation of cPLA2 on Ser505 by mitogen-activated protein kinases, including p42/p44 extracellular signal-regulated kinases and p38, and on Ser727 by mitogen-activated protein kinase-interacting kinase I are important in regulating cPLA2-mediated AA release in vivo (40-42). However, the mechanism by which phosphorylation of these sites regulates cPLA2 is not clear. We have shown that the rates of association with Golgi of cPLA2 S505A and S727A mutants are equivalent to wild type and that the mutants target the same membranes as wild type in MDCK cells (39). We found that cPLA2 S505A and S727A mutations also exhibited Ca\(^{2+}\)-independent membrane residence (data not shown). Likewise, incubation with the MEK1 inhibitor U0126, which results in down-regulation of extracellular signal-regulated kinase activity and inhibition of AA release, failed to affect membrane residence in MDCK cells (data not shown).

The imaging approach utilized here allowed us to investigate, in a physiological setting, the contributions of the C2 and catalytic domains in membrane association and residence. Direct comparison of full-length cPLA2 and the isolated C2 domain in the same cell allowed us to control for the otherwise confounding problems of cell-to-cell heterogeneity and for cell-to-cell differences in the rate and magnitude of [Ca\(^{2+}\)] increase, which both influence membrane association. Using the C2 domain as a [Ca\(^{2+}\)] indicator was critical in evaluating membrane residence because we found considerable heterogeneity in the response of [Ca\(^{2+}\)] to EGTA in the calcium clamp.
experiments. Often, high concentrations of EGTA reduced the [Ca\(^{2+}\)]\(i\), in some cells, but failed to reduce the [Ca\(^{2+}\)]\(i\), in adjoining cells or in other cells in the same field as measured by Fura2 fluorescence. Thus, use of the C2 domain allowed for the monitoring of [Ca\(^{2+}\)]\(i\), as well as a comparison for membrane association and residence.

In the co-expression experiments presented here, the slower association of cPLA2 with Golgi is readily observed. This slower association of cPLA2, compared with the isolated C2 domain, may account for our previous observation that full-length cPLA2 translocation is limited to Golgi in response to stimulation by agonists that elicit a rapid [Ca\(^{2+}\)]\(i\) transient, such as ATP or thapsigargin, but that the isolated C2 domain translocates to ER, Golgi, and nuclear membrane in response to the same agonists (8). Release of Ca\(^{2+}\) from internal stores results in a much faster rate of full-length association than does extracellular influx in store-depleted cells, suggesting that the local high [Ca\(^{2+}\)]\(i\), may be important in mediating translocation, as we have previously suggested (8). Interestingly, in response to agonists stimulating such local [Ca\(^{2+}\)]\(i\), increases, such as ATP and thapsigargin, a fraction of full-length cPLA2 dissociates from Golgi in concert with the initial few seconds of [Ca\(^{2+}\)]\(i\), decrease, suggesting that there may be a certain residence time required for the Ca\(^{2+}\)-independent membrane association, as has been previously suggested (23). Many reports have demonstrated that hydrophobic residues in the calcium binding loops of the cPLA2 C2 domain partially or fully insert into lipid membranes (5, 6, 15, 16, 43) (Fig. 10). Our results suggest that Trp\(^{464}\) on the interfacial binding surface of the catalytic domain may also penetrate into the lipid bilayer when brought into proximity by the binding of the C2 domain while [Ca\(^{2+}\)]\(i\), is elevated, and that this penetration may be largely responsible for Ca\(^{2+}\)-independent residence after [Ca\(^{2+}\)]\(i\), declines (Fig. 10). Membrane association via the C2 domain is perhaps critical in the correct orienting of the catalytic domain and in providing the energy for Trp\(^{464}\) insertion as the cPLA2 catalytic domain by itself fails to bind membrane in live cells (8, 18). Generally, Trp plays an important role in interfacial binding of proteins to membranes as a result of its high affinity for the membrane-water interface (44). More specifically, Trp on the interfacial binding surfaces of other PLA2 isoforms is important in binding and activity (34). Mutagenesis studies of cobra venom PLA2 demonstrate that aromatic residues on the binding surface are critical for monolayer penetration and activity (45). Similarly, a single Trp in the membrane-binding surface of human group V PLA2 is critical for penetration and activity (46). In both cobra venom and group V PLA2 proteins, the Trp determines enzyme preference for zwitterionic phospholipids such as phosphatidylcholine (PC) (45, 46). The importance of interfacial Trp in PC preference and in membrane binding is exemplified by experiments where addition of a Trp residue in human group IIa PLA2 greatly increased affinity for and hydrolysis of PC vesicles and cell membranes (47). Interfacial Trp has also been implicated in binding of bacterial phosphoinositide-specific phospholipase C to membranes (48). Although the C2 domain is responsible for targeting and also shows preference for zwitterionic phospholipids (6, 15, 17, 49), the interfacial binding of the catalytic domain may provide a secondary mechanism of PC preference.

The W464G mutant required a higher [Ca\(^{2+}\)]\(i\), than the isolated C2 domain for membrane association. This may be the result of the presence of other residues in the catalytic domain that decrease the overall binding provided by the C2 domain. Possibly surface-exposed glutamic acid residues in the catalytic domain “lid” that covers the cPLA2 active site (7) (Fig. 10) provide electrostatic repulsive forces that repel the catalytic domain from the membrane surface when Trp\(^{464}\) is mutated. Mutation of glutamic acid residues in the lid increases the affinity of cPLA2 for anionic membranes, and it has been proposed that repulsive forces between the lid and the membrane surface are required for moving the lid aside to expose the active site (50). Possibly tethering of the catalytic domain by Trp\(^{464}\) acts a fulcrum for this proposed electrostatic repulsion that exposes the active site.

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