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New Concepts

Regulation of Cytosolic Phospholipase A_2 in a New Perspective: Recruitment of Active Monomers from an Inactive Clustered Pool

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ABSTRACT: $cPLA_2$ plays a key role in many signal transduction cascades by hydrolyzing arachidonic acid from membrane phospholipids. Tight control of $cPLA_2$ activity by a number of regulatory mechanisms is essential to its cellular function. We recently described the localization of $cPLA_2$ in clusters in fibroblasts and now propose that these clusters reflect a localized inactive pool from which active monomers can be recruited to keep $cPLA_2$ activity under control on the subcellular level. Using an electron microscopic in vitro approach, we show that $cPLA_2$ monomers, but not the clusters, bind to membranes in a Ca^{2+}-dependent manner. This binding is accompanied by hydrolytic activity. The present data combined with our previous observation of a relative abundance of clusters over monomers in fixed fibroblasts [Bunt, G., de Wit, J., van den Bosch, H., Verkleij, A., and Boonstra, J. (1997) *J. Cell Sci. 110*, 2449−2459] gives rise to a concept of $cPLA_2$ regulation in which small amounts of active monomers are recruited to fulfill their function upon stimulation. This is in contrast to processes described for inflammatory cells, where a substantial part of the cytoplasmically localized $cPLA_2$ translocates to the perinuclear region upon stimulation to become active. Small-scale regulation of $cPLA_2$ by the proposed cluster−monomer cycle allows local and strictly confined control of $cPLA_2$ activity, apparently necessary for its cellular role in fibroblasts.

Cytosolic phospholipase A_2 ($cPLA_2$),[1] the 85 kDa PLA_2 family member, plays a crucial role in many signal transduction cascades by its preferential release of arachidonic acid from the *sn*-2 position of membrane phospholipids. Arachidonic acid is essential in various cellular responses such as cell growth (*12*, *20*), inflammation (*9*), platelet activation (*14*, *17*), and cytotoxicity (*5*, *6*). Arachidonic acid acts directly as a second messenger (*10*, *22*) or indirectly in the form of bioactive metabolites when converted into eicosanoids.

Ca^{2+} and Ser^{505} phosphorylations by MAPK are the most prominent regulatory factors of $cPLA_2$ activity. Submicromolar Ca^{2+} concentrations induce a translocation and binding of $cPLA_2$ to membranes, thereby allowing access to its substrate (*4*, *25*). The membrane binding event is mediated by the N-terminal Ca^{2+}-dependent phospholipid binding domain (CalB or C2 domain) (*15*, *16*, *18*). Phosphorylation on Ser^{505} by $p42^{MAPK}$ enhances the hydrolyzing activity (*13*). Regardless of the presence of several regulation mechanisms in cells, localized control of $cPLA_2$ action is a prerequisite for an efficient and adequate cellular response. The ruling concept on localized $cPLA_2$ action primarily originates from observations in inflammatory cells where a substantial pool of $cPLA_2$ translocates from the cytosol to the perinuclear region to become active (*8*, *19*), consistent with its role in the eicosanoid production. Recently, we reported on the presence of $cPLA_2$ in clusters in Her14 fibroblasts indepen-

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[1] Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; MLV, multilamellar vesicle; SAPC, 1-stearoyl-2-arachidonoylphosphatidylcholine; DAG, diacylglycerol; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EM, electron microscopy; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PC, phosphatidylcholine.

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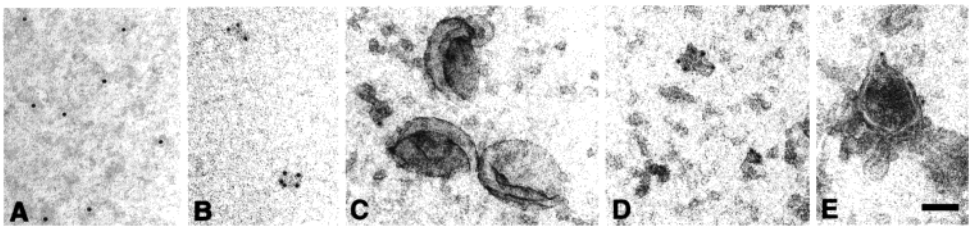

FIGURE 1: Detection of $cPLA_2$ clusters and monomers in cellular fractions of serum-exposed Her14 fibroblasts: (A) 60−100 kDa Superose 12 gel filtration fraction; (B) 200000*g* supernatant; (C−E) 200000*g* particulate fraction. Monomers were found attached to membranes whereas clusters were not. Bar = 125 nm.

dent of the stimulation condition of the cells (*2*). Clusters of $cPLA_2$ were found in the cytosol nearby all organellar membranes with the exception of the Golgi system. $cPLA_2$ clusters were also found in A431 cells, rat mesangial cells, CHO, and neuroblastoma cells. The clustered localization of $cPLA_2$ might reflect a different, more localized, regulation mechanism in cell types with primary functions other than eicosanoid synthesis.

Although $cPLA_2$ in intact fibroblasts occurs mainly in the form of clusters (*2*), most of the cellular $cPLA_2$ can be obtained as monomers in a 60−100 kDa fraction when homogenates of these cells are subjected to gel filtration chromatography (*23*) apparently as a result of massive dissociation of the clusters. To explore a role of the $cPLA_2$ clusters, their membrane binding characteristics were compared to monomeric $cPLA_2$ by an electron microscopic in vitro approach. A model of $cPLA_2$ regulation incorporating the obtained findings will be presented in which $cPLA_2$ clusters represent an inactive pool from which active monomeric enzyme is recruited that can be targeted to membranes upon cellular stimulation.

MATERIALS AND METHODS

Preparation of Cellular Fractions. Her14 fibroblasts, mouse 3T3(0) fibroblasts stably overexpressing the human EGF receptor, were cultured in DMEM supplemented with 7.5% FCS in a humidified atmosphere of 7% CO_2 at 37 °C. Subconfluent cultures were used in all experiments. Particulate and cytosolic fractions (200000*g*) were obtained as described previously (*2*). Partial purification of $cPLA_2$ monomers by Superose 12 gel filtration was performed essentially as described in ref *23*.

Preparation of MLV's Consisting of Stearoylarachidonoylphosphatidylcholine (SAPC). ^{14}C-Labeled (Amersham, Buckingshire, U.K.) or unlabeled (Sigma) SAPC were dried from the organic solvent under nitrogen and resuspended at a concentration of 2 μM in 20 mM Tris, pH 8.5, for $cPLA_2$ activity measurements or at 2 mM PBS for use in electron microscopic studies. The suspension was vortexed with a glass pearl until all lipid was dispersed. Size-homogeneous MLV's were obtained by three freeze−thaw cycles in an acetone−dry ice bath. The MLV's were used for experiments within several hours.

$cPLA_2$ Activity Assay. $cPLA_2$ activity was measured in vitro by the release of arachidonic acid from S[^{14}C]APC as described previously (*21*) with minor modifications. Routinely, the assay incubation mixture contained 0.2 M Tris-HCl, pH 8.5, 1 mM Ca^{2+}, 4 μM SAPC, and 2 μM DAG in a final volume of 200 μL, and incubation was performed for 10 min at 37 °C. For activity measurements under electron microscopic conditions, S[^{14}C]APC MLV's were used as substrate vesicles. The assay was performed for 90 min at room temperature using PBG [0.5% BSA, 0.1% (w/v) cold water fish gelatin 45% in PBS], 1 mM Ca^{2+}, and 2 μM SAPC.

Electron Microscopic Detection of $cPLA_2$. Formvar−carbon-coated nickel grids were placed on drops containing cellular material or synthetic substrate vesicles for 30 min at room temperature, enabling material adherence to the grids. After a brief wash in PBS, the material was fixed in 1% glutaraldehyde in PBS for 10 min. Next, the grids were twice incubated on 100 mM glycine in PBS for 5 min. Blocking was performed twice on PBG for 10 min. Endogenous $cPLA_2$ in cellular fractions was detected with the $cPLA_2$ monoclonal antibody sc-454 (Santa Cruz) at a concentration of 0.15 μg/mL in PBG for 1 h at room temperature. In $cPLA_2$−membrane binding studies, the grids were incubated with antibody preincubated with a 100-fold molar excess of $cPLA_2$, to avoid detection of endogenous $cPLA_2$, in the presence of either 1 mM Ca^{2+} or 1 mM EGTA for 1 h at room temperature. Complex formation of the antibody with $cPLA_2$ was performed by gentle rotation in PBG for 1.5 h at room temperature. The grids were extensively washed on drops of PBG and incubated with 10 nm gold-labeled protein A (1:15) (Aurion, Wageningen, The Netherlands) for 1 h. Next, the fractions were washed with PBG and PBS, postfixed with 1% glutaraldehyde in PBS for 5 min, and washed with distilled water. Fractions were contrasted and embedded by incubation with 0.3−0.5% uranyl acetate in 1.6% methylcellulose for 5 min and air-dried. Fractions were viewed on a CM10 or E420 Philips electron microscope at 80−100 kV.

RESULTS

An electron microscopic immunogold detection method was developed which allows discrimination of $cPLA_2$ monomers and clusters and at the same time provides information on the membrane binding characteristics of these forms. This detection method is based on the highly specific and single epitope recognition of $cPLA_2$ present in cellular fractions by sc-454 combined with the 1:1 binding of protein A to sc-454 (*1*, *2*). Cluster−monomer discrimination is illustrated by the detection of $cPLA_2$ monomers as isolated gold particles in the 60−100 kDa gel filtration chromatography fraction of Her14 (Figure 1A) and the detection of $cPLA_2$ clusters in the cytosol equivalent of the cells (200000*g* supernatant) (Figure 1B). Grids coated with PBG did not reveal labeling. Examination of the 200000*g* cytosolic (Figure 1B) and particulate fractions (Figure 1C−E) of serum-exposed Her14 fibroblasts revealed the predominant presence of $cPLA_2$ in clusters in both fractions. Only a minor fraction was detected as monomers in both the cytosolic and the

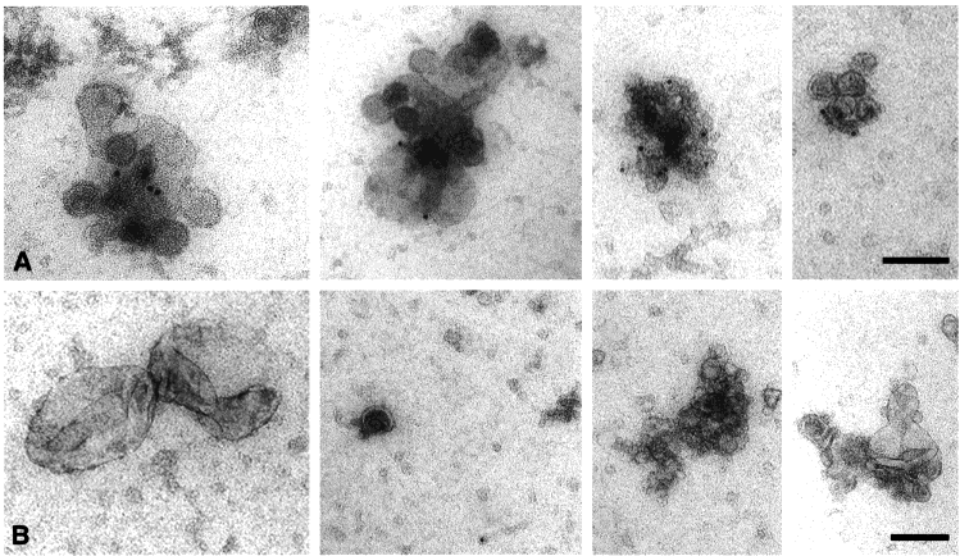

FIGURE 2: $cPLA_2$ monomers bind Ca^{2+} dependently to cellular membranes. Her14 homogenates were incubated with $cPLA_2$ monomers complexed with the $cPLA_2$ antibody sc-454 (ratio 100:1) to avoid detection of endogenous $cPLA_2$. Label was found exclusively at membranes when 1 mM Ca^{2+} was present (A) and was absent in the presence of 1 mM EGTA (B). Bars = 200 nm.

particulate fractions. $cPLA_2$ clusters present in the particulate fraction were not associated to membrane structures (Figure 1C,D), while occasionally monomers were detected at membranes (Figure 1E). Despite the presence of 1 mM Ca^{2+}, clusters were also found unattached to membranes in a $cPLA_2$-enriched fraction obtained by gradient centrifugation of a Her14 membrane lysate.

To study the Ca^{2+} dependency of the membrane binding by $cPLA_2$ monomers, cellular homogenates were coated to the grid and $cPLA_2$ monomers were added since endogenously they could only be detected in minor amounts. $cPLA_2$ monomers were introduced as a sc-454−$cPLA_2$ complex (with a 100-fold molar excess of $cPLA_2$ monomers) to avoid detection of endogenous $cPLA_2$ by the monoclonal antibody. Label was found exclusively at membranous structures when the sc-454−$cPLA_2$ mixture was added in the presence of Ca^{2+} (Figure 2, panel A). In contrast, the membrane labeling was lost when EGTA was present (Figure 2, panel B).

The Ca^{2+}-dependent membrane binding by monomers is consistent with the known characteristics of active $cPLA_2$. However, $cPLA_2$ could simply become immobilized on membranes without having implications for its activity. To test whether the membrane binding of monomers as observed by immunoelectron microscopy is related to activity, activity measurements were performed under EM assay conditions with MLV's composed of SAPC as substrate. When MLV's were coated to the grid and incubated with $cPLA_2$−sc-454 complexes, labeling was found at MLV's only when incubated in the presence of Ca^{2+} (Figure 3A) and was absent from MLV's in the presence of EGTA (Figure 3B). Activity measurements performed under the same conditions showed Ca^{2+}-dependent activity of $cPLA_2$ monomers toward S[^{14}C]-APC MLV's (Figure 3C). However, uncomplexed $cPLA_2$ monomers were used in the activity assays whereas in the EM study $cPLA_2$ is present as antibody complexes. Addition of a 10-fold molar excess of the antibody sc-454 to $cPLA_2$ monomers had no detrimental effect on the activity, as measured by the standard protocol (Figure 3D). Consequently, $cPLA_2$−sc-454 complexes contribute to the activity measured.

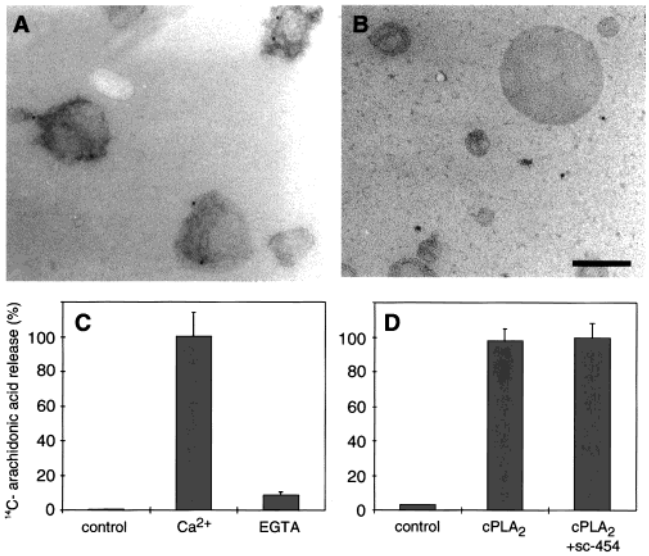

FIGURE 3: Ca^{2+}-dependent membrane binding by $cPLA_2$ monomers as observed with EM is interrelated with hydrolytic activity. $cPLA_2$ monomers bind Ca^{2+} dependently to SAPC MLV's [(A) 1 mM Ca^{2+}, (B) 1 mM EGTA] and exhibit Ca^{2+}-dependent arachidonic acid release toward S[^{14}C]APC MLV's under electron microscopic conditions (C). Addition of a 10-fold molar excess of sc-454 had no detrimental effect on activity (D). Bar = 200 nm.

DISCUSSION

Since monomers revealed Ca^{2+}-dependent membrane binding capacity as well as Ca^{2+}-dependent arachidonic acid hydrolysis whereas clusters did not exhibit characteristic active $cPLA_2$ behavior toward membranes, we propose that the clusters represent a pool of inactive $cPLA_2$ from which active $cPLA_2$ monomers can be recruited.

Previously, we showed in cryosections of Her14 cells that $cPLA_2$ is preferentially present in clusters in fixed cells. No reallocation of $cPLA_2$ from or by these clusters was observed upon stimulation (*2*). This suggests that in vivo arachidonic acid hydrolysis upon stimulation primarily results from a small fraction of active monomeric $cPLA_2$ in these cells. Although the in vitro assay primarily provides qualitative

information on the membrane binding characteristics of the $cPLA_2$ forms, the predominance of clusters over monomers was also observed in cellular fractions and is pronounced. Furthermore, in agreement with our previous observations of an unchanged clustered localization upon stimulation of cells with EGF and/or A23187 and artificially induced extreme Ca^{2+} conditions (*2*), we have not observed a substantial shift in cluster−monomer occurrence in the in vitro approach under the same conditions. However, this does not exclude the small-scale involvement of Ca^{2+} and phosphorylation in activation of $cPLA_2$ by cluster−monomer regulation. Integration of the Ca^{2+} and phosphorylation aspects into our cluster−monomer model leads us to propose the following sequence of events. Given the in vivo observation in Her14 fibroblasts that phosphorylation has to precede an increase in Ca^{2+} for maximal activation (*21*), we suggest that phosphorylation of clustered $cPLA_2$ first causes the local release of small amounts of monomers upon stimulation. The released phosphorylated monomers subsequently translocate to nearby located membranes due to a rise in intracellular calcium to become active. Notably, a role for phosphorylation in release of active $cPLA_2$ from clusters might provide a more effective tool in the regulation of in vivo arachidonic acid release than its 2−3-fold increase of the intrinsic activity as measured in vitro. Moreover, it allows a more selective and localized $cPLA_2$ activation than can be achieved by elevated intracellular Ca^{2+} concentrations.

Initial studies on the formation of clusters from $cPLA_2$ monomers revealed that they are unable to spontaneously form clusters. An additional component must therefore be responsible for $cPLA_2$ cluster formation and concomitant inactivation in cells. Characterization of the clusters is hampered by the fact that they have not yet been successfully isolated due to their instability. Furthermore, the clusters have no definite structure and are inhomogeneous in size, explaining their presence in both soluble and particulate fractions. Annexins and their binding proteins, in particular annexin I and the S100C lipid binding protein p11, seem obvious clustering candidates since they have been suggested to inhibit $cPLA_2$ activity by direct interaction (*11*, *24*). However, microscopical colocalization studies on $cPLA_2$ and annexins I, II, and V did not reveal a correlation of the staining pattern of both proteins in clusters in A431 cells (unpublished results). Therefore, the option of annexins as inhibitory clustering partners is unlikely. Other possible cluster constituents are lipids. In vitro kinetic studies showed that upon hydrolysis $cPLA_2$ becomes entrapped and inactivated on vesicles containing considerable (>10%) amounts of hydrolysis products (*7*). In analogy to the kinetic in vitro studies, the cellular $cPLA_2$ clusters may represent entrapped and therefore inactivated $cPLA_2$ on vesicles. Additional support comes from kinetic data suggesting that a high curvature of vesicles, with a diameter of 25 nm, may induce an enzyme state of $cPLA_2$ which does not support activity (*3*). The diameter of these vesicles is in the range of the size of $cPLA_2$ clusters (25−50 nm) found in cryosections and subcellular fractions. In cells, the release of the arachidonic acid and lysoPC by the initial hydrolytic action of $cPLA_2$ monomers at membranes might lead to the formation of vesicles on which $cPLA_2$ becomes entrapped and inactivated as "clusters".

Small-scale regulation of $cPLA_2$ activity by the proposed cluster−monomer model is considered to be of particular importance for cell functioning. If upon stimulation all cellular $cPLA_2$ would be activated and become aligned on membranes, destruction of cellular membranes would follow from the formation of high amounts of lysogenic products, i.e., arachidonic acid and lysophospholipids. Activity of $cPLA_2$ must therefore be strictly regulated and locally confined as a protective mechanism for cell viability.

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