Ca²⁺-induced changes in the secondary structure of a 60 kDa phosphoinositide-specific phospholipase C from bovine brain cytosol

Carmen HERRERO, Maria E. CORNET, Carmen LOPEZ, Pedro G. BARRENO, Angel M. MUNICIO and Jorge MOSCAT*

Medicina y Cirugia Experimental, Hospital General "Gregorio Marañon", Dr. Espuerto 46, 28007 Madrid, Spain

The purification to homogeneity of a 60 kDa phosphoinositide-specific phospholipase C from bovine brain cytosol is reported here. This enzyme exhibits the same properties, in terms of response to Ca²⁺, as does the cytosolic activity in a variety of cell types. We show here that Ca²⁺ does not appear to modulate the binding of the enzyme to the substrate, but induces dramatic changes in its secondary structure. Therefore we suggest that a decrease in the α -helix content of this enzyme correlates with its ability to be activated by Ca²⁺.

INTRODUCTION

Hydrolysis of inositol-containing phospholipids has been shown to be involved in the control of a wide variety of cellular functions through the generation of a number of putative second messengers [1–3]. The activation of the phosphodiesteratic hydrolysis of PtdInsP₂ by a receptor-mediated stimulus generates diacylglycerol, a cofactor for protein kinase C [4], and inositol 1,4,5-trisphosphate [5]. The latter controls cytosolic Ca²⁺ concentrations through the release of this ion from its non-mitochondrial stores (for reviews, see [6] and [7]). The proposal of the existence of a relationship between phosphoinositides and extracellular Ca²⁺ influx [3] has recently been supported by the discovery by Irvine & Moor [8,9] of the possible role of inositol 1,3,4,5-tetrakisphosphate, which is generated by the action of an inositol 1,4,5-trisphosphate 3-kinase [10], in the control of the entry of Ca²⁺ into the cell after the discharge of inositol 1,4,5-trisphosphate-sensitive pools. Although the roles of the different products of the phosphoinositide cycle are being progressively unveiled, the mechanisms whereby phospholipase C (PLC) is activated are still unclear. In this regard it has to be borne in mind that, once cells are disrupted and cytosolic and membrane fractions separated, two sorts of PLC activities can be detected: membrane-bound and cytosolic. In membranes, a receptor-activatable activity exists which is stimulated in the presence of GTP or its non-hydrolysable analogues [11–27]. By using exogenous substrate a cytosolic activity can be detected which hydrolyses PtdIns in a Ca²⁺-dependent fashion [27–29]. The phosphodiesteratic breakdown of PtdInsP₂ by cytosolic activity is also stimulated by Ca²⁺ and membrane destabilization [27,30]. More recently it has been reported that cytosolic PLC activity can be stimulated to attack PtdInsP₂, but not PtdIns, by GTP and its non-hydrolysable analogues [31–34]. This permits one to

speculate that a cytosolic G-protein could mediate in the attachment of the cytosolic enzyme to its substrate according to the general model proposed by Rodbell and co-workers whereby translocation of G-proteins might be a novel way to regulate cytosolic enzymes [35]. In this regard it has been suggested that the mechanism through which Ca²⁺ activates the cytosolic activity may be mediated by enzyme translocation [36] in an analogous way to what has been shown for protein kinase C [37]. A number of phosphoinositide-specific PLCs (PI-PLC) of cytosolic origin have been purified to homogeneity from several tissues [38–44]. No agreement seems to exist about the relationship between the different species isolated either from different sources or from a given tissue; whether or not these different species also play different roles has not yet been established. The aim of the present investigation was to study further the mechanisms of activation of cytosolic PI-PLC. Our data seem to indicate that Ca²⁺ is not necessary for the binding of cytosolic PLC to the lipid vesicle, but induces dramatic changes in the enzyme secondary structure which may be responsible for Ca²⁺-induced activation.

MATERIALS AND METHODS

Cell lines

Swiss 3T3 fibroblasts (passage 123) were purchased from Flow Laboratories and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-bovine serum, penicillin (100 units/ml)/streptomycin (100 µg/ml) and 2 mM-L-glutamine. Pig aorta endothelial cells were isolated and cultured as previously described [45].

Assay for PI-PLC

PLC was routinely assayed either with L-3-phosphatidyl[2-³H]inositol (Amersham International; sp. radio-

Abbreviations used: PAGE polyacrylamide-gel electrophoresis; PLC, phospholipase C; PI-PLC, phosphoinositide-specific phospholipase C; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdInsP₂, phosphatidylinositol 4,5-bisphosphate; PtdIns, phosphatidylinositol.

* To whom reprint requests should be addressed. Present address: Laboratory of Cellular and Molecular Biology, National Cancer Institute, NIH, Building 37, Room 1E24, Bethesda, MD 20892, U.S.A.

activity 10–15 Ci/mmol) or phosphatidyl[1,2-³H]inositol 4,5-bisphosphate (New England Nuclear; sp. radioactivity 3.6 Ci/mmol) in the following assay buffer: 100 mM-NaCl, 1 mM-EGTA, 12 mM-GSH, 50 mM-Hepes/NaOH, pH 5.5. Incubations were carried out for 10 min at 37 °C. PtdIns (100 μ M) was added pure to the reaction tube, whereas PtdInsP₂ (60 μ M) was mixed with a 10-fold excess of PtdEtn/PtdCho (2:3 molar ratio) mixture. Free Ca²⁺ concentrations were adjusted by using Ca²⁺/EGTA buffers and taking into account pH, ionic composition of the media and the association constant of EGTA as described previously [46]. A Ca²⁺ concentration of 0 was obtained by the addition of no Ca²⁺ to EGTA-containing buffer [46].

Preparation of high-speed supernatants

For preparation of high-speed-supernatant (cytosol) fractions from cultures, cells were scraped with a rubber policeman in Ca²⁺- and Mg²⁺-free EGTA-containing Dulbecco's phosphate-buffered saline and centrifuged at 10000 *g* for 15 min at 4 °C. Afterwards, cells were resuspended in assay buffer and sonicated (three 30 s pulses) while suspended in an ice bath. Cell homogenates were centrifuged at 100000 *g* for 60 min at 4 °C, and the cytosolic fraction was prepared.

For preparation of bovine brain cytosol fraction, 1 kg of calf brain was minced, rinsed twice with 0.9% NaCl and then homogenized by ten strokes in a glass/Teflon Potter homogenizer in 0.25 M-sucrose in buffer A (0.1 mM-dithiothreitol 0.1 mM-phenylmethanesulphonyl fluoride, 1 mM-EGTA, 50 mM-Tris/HCl, pH 7.4). The homogenate was centrifuged twice at 20000 *g* for 15 min at 4 °C. Supernatants were collected and centrifuged at 100000 *g* for 60 min at 4 °C. High-speed supernatants were decanted and stored at –70 °C.

Purification of PI-PLC

DEAE-cellulose anion-exchange chromatography. Cytosol (6 g of protein) was divided into six aliquots, and each was loaded on a DEAE-cellulose column (4 cm $\times$ 20 cm) that was previously equilibrated in buffer A. DEAE-cellulose was washed with 3 bed vol. of buffer A, followed by a 1500 ml linear NaCl gradient (0–0.6 M) in buffer A; 10 ml fractions were collected, and their *A*₂₈₀ and enzyme activity was measured. A single peak of activity was detected, eluted at approx. 0.3 M-NaCl.

Heparin-Sepharose chromatography. Active fractions were pooled, dialysed against buffer A and loaded on a heparin-Sepharose column (2.5 cm $\times$ 9 cm) that was previously equilibrated in buffer A. The column was washed with 10 bed vol. of buffer A, followed by a 500 ml linear NaCl gradient (0–1 M) in buffer A. Again, a single peak of activity was obtained, eluted at approx. 0.4 M-NaCl.

Phenyl-Sepharose chromatography. Active fractions from heparin-Sepharose columns were dialysed overnight against buffer A, followed by a 2 h dialysis against 3 M-KCl in buffer A. They were then loaded on a phenyl-Sepharose column (1.5 cm $\times$ 8 cm) previously equilibrated in 3 M-KCl in buffer A. The column was washed with 150 ml of buffer A, followed by 150 ml of an inverse linear KCl gradient (3–0 M) in buffer A. Active fractions were eluted neither in the wash nor in the inverse-gradient zone of the chromatography. PI-PLC activity

was eluted from the resin with 50% (v/v) ethylene glycol in buffer A.

Sephadex G-200 chromatography. Active fractions from the above step were pooled and dialysed against buffer A. Samples were concentrated 15-fold by using Minicon Macrosolute concentrators (Amicon, Lexington, MA, U.S.A) and loaded on a Sephadex G-200 column (2 cm $\times$ 100 cm) previously equilibrated in buffer A.

DEAE anion-exchange h.p.l.c. Active fractions from the above step were dialysed against buffer A, concentrated and injected on to a 7.5 mm $\times$ 75 mm Spherogel-TSK DEAE-5PW column (Beckman), which was equilibrated in buffer A. Samples were run at a flow rate of 1 ml/min, washed for 30 min with buffer A and eluted with a linear gradient of 0–1 M-NaCl in buffer A, 1 min fractions were collected and their PI-PLC activity was measured. A single peak of activity was observed.

Hydroxyapatite h.p.l.c. Active fractions from the above step were concentrated as described above, dialysed against buffer A' [10 mM-K₂HPO₄, 30 mM-KCl, 10% (v/v) glycerol, 1 mM- β -mercaptoethanol, pH 7.2] and injected on to a 7.8 mm $\times$ 100 mm Bio-Gel HPHT column (Bio-Rad) that was previously equilibrated in buffer A' at a flow rate of 1 ml/min. Samples were washed for 30 min with buffer A' and eluted with a linear gradient from 100% of buffer A' to 100% of buffer B (130 mM-K₂HPO₄, 30 mM-KCl, 10% glycerol, 1 mM- β -mercaptoethanol, pH 7.2) during 30 min; 1 ml fractions were collected and PI-PLC activity was measured.

Circular-dichroism spectra

C.d. spectra of the samples were measured in a Jobin Yvon mark III dichrograph fitted with a 250 W xenon lamp at 0.5 mm/s scanning speed. Millipore (5 μ m pore diameter)-filtered solutions of proteins were studied in the concentration range 0.2–0.4 mg/ml in 0.005 cm-optical-path cells. C.d. results are reported in terms of $[\theta]$, the mean residue ellipticity, in units of degrees $\cdot$ cm² $\cdot$ dmol⁻¹. The values of mean residue ellipticities were calculated on the basis of 110 amino acid residues in the enzyme.

Ca²⁺-binding assay

Ca²⁺ binding to PI-PLC was quantified by equilibrium dialysis as described previously [47].

Polyacrylamide-gel electrophoresis

SDS/PAGE was performed as described by Laemmli [48]. Samples were dissolved in SDS sample buffer (0.1 M-Tris/HCl, pH 6.8, 30% glycerol and 2% SDS) either in the presence or in the absence of 10 mM- β -mercaptoethanol, followed by heating at 100 °C for 5 min before loading on gels.

Protein determination

Protein concentration was determined by the Bradford method by using a commercially available kit (Bio-Rad).

RESULTS

Cytosolic PI-PLC activity

High-speed supernatants (cytosol) from bovine brain,

Swiss 3T3 fibroblasts and pig aorta endothelial cells were prepared, and PI-PLC activity was determined. This activity is able to hydrolyse both pure PtdIns and PtdInsP₂ when the latter is mixed with a phospholipid mixture that mimics a membrane bilayer (results not shown). This is in good agreement with our previously published results [27]. The exact mechanism whereby Ca²⁺ stimulates PLC is not known. One possibility is that Ca²⁺ favours the binding of the enzyme to its substrate; this is in keeping with the suggestion that Ca²⁺ induces the translocation of PI-PLC from the cytosol [36]. Alternatively, Ca²⁺ may induce a conformational change in the enzyme, which in turn induces its activation. To test these possibilities, we first undertook the purification of this cytosolic PI-PLC activity from bovine brain. This source was chosen because a greater amount of biological material is available for purification and because the brain cytosol PI-PLC activity behaved identically with that in the other cell systems investigated.

Purification of PI-PLC

Chromatography of cytosolic fractions from bovine brain on DEAE-cellulose yielded a peak of PLC activity (results not shown). Active fractions were collected, dialysed and further purified as described in the Materials and methods section. In all the purification steps, PLC appeared as one peak, and other enzyme species, if present, were not detected with our assay. It should be noted that the enzymic assay used to detect active fractions is the same as was used to characterize the enzyme in the cytosolic crude preparations (see above and ref. [27]). Chromatography of the activity on Sephadex G-200 indicates that the enzyme shows a molecular mass under native conditions of 180 kDa. When the enzyme was analysed by SDS/PAGE under reducing conditions, it appeared as a single band migrating at 60 kDa, indicating that the enzyme was purified to homogeneity. The protein under native conditions appears to be a trimer of 60 kDa monomers, which are not bound through disulphide bridges, since the enzyme shows the same molecular mass on SDS/PAGE under non-reducing conditions (results not shown).

Effect of Ca²⁺ on purified PI-PLC

Ca²⁺ was a potent stimulus for purified PI-PLC

Table 1. Effect of Ca²⁺ on PI-PLC activity

Purified PI-PLC activity was assayed at different Ca²⁺ concentrations at either pH 5.4 or 7.4 as described in the Materials and methods section. Results are expressed as $\mu\text{mol}/\text{min}$ per mg of protein and are means $\pm$ S.D. of three independent experiments with incubations in duplicate.

[Ca ²⁺]	Enzyme activity ($\mu\text{mol}/\text{min}$ per mg of protein)	
	pH 5.4	pH 7.4
0 nM	2.0 $\pm$ 0.4	1.8 $\pm$ 0.4
80 nM	5.0 $\pm$ 0.2	2.1 $\pm$ 0.1
300 nM	5.6 $\pm$ 0.3	2.3 $\pm$ 0.2
8 μM	7.3 $\pm$ 0.4	2.5 $\pm$ 0.1
80 μM	8.2 $\pm$ 0.3	2.5 $\pm$ 0.1

Fig. 1. Effect of Ca²⁺ on PI-PLC binding to PtdIns vesicles

Purified PI-PLC was incubated in the presence of PtdIns (100 μM) vesicles for 10 min at 37 °C either in the presence or in the absence of Ca²⁺ (80 μM). Afterwards, vesicles were pelleted at 100 000 *g* for 15 min, and PI-PLC content was measured in supernatants (S) and pellets (P) by SDS/PAGE. This is a representative experiment of four with identical results.

activity (Table 1, and refs. [27] and [31]). To test whether the effect of Ca²⁺ is to promote the binding of the enzyme to the phospholipid vesicle, the following experiment was performed. Purified enzyme was incubated either in the absence or in the presence of 80 μM -Ca²⁺, after which vesicles were centrifuged and pellet and supernatant fractions were isolated. The presence of the 60 kDa PI-PLC was determined in both supernatants and pellets by SDS/PAGE. Results shown in Fig. 1 indicate that PI-PLC is bound to the substrate irrespective of the presence of Ca²⁺. When the experiment was performed with bovine serum albumin instead of PI-PLC, no binding of the protein to the phospholipid vesicle was detected. Furthermore, the enzyme was unable to bind to a PtdCho/PtdEtn vesicle in the absence of substrate. This argues against the theory of enzyme translocation as an essential step in the activation of cytosolic PI-PLC by Ca²⁺. From these results we can speculate that the enzyme, under physiological conditions, is bound to the substrate and that the rise in Ca²⁺ concentration triggers its hydrolysis. One possibility is that Ca²⁺ induces a conformational change, which in turn activates the enzyme. To test this, changes in the secondary structure of purified PI-PLC were estimated by using c.d. Fig. 2 shows that at pH 5.4 Ca²⁺ induces dramatic changes in the secondary structure of the enzyme. These changes are dose-dependent (Fig. 2, inset). C.d. spectra of the enzyme in the absence of Ca²⁺ showed that the secondary structure of the enzyme was 38 % α -helix, 28 % β -bend and 34 % random coil, as determined in accordance with Chen *et al.* [49]. The addition of 80 μM -Ca²⁺ decreased the α -helix proportion to 26 %, whereas the percentages of β -bend and random coil increased to 38 % and 36 % respectively. Interestingly, increased Ca²⁺ gives a dramatic activation of PI-PLC at pH 5.4, but induces little or no changes in this activity at pH 7.4 (see Table 1). Binding experiments at both pH values clearly indicate that this different sensitivity of the enzyme to the Ca²⁺ effect at pH 5.4 and 7.4 cannot be accounted for by a different Ca²⁺ binding to the enzyme (results not shown). Also, it is noteworthy that the purified enzyme is bound to the substrate irrespective of the pH (results not shown). Thus it is possible that the non-activation of the enzyme at pH 7.4 by Ca²⁺ could be due to the existence at this pH of a 'resistant state' in the enzyme that would not allow Ca²⁺-induced changes in the secondary structure. Fig. 3 clearly shows that this may be the case,

Fig. 2. Effect of Ca^{2+} on the secondary structure of PI-PLC

C.d. spectra of PI-PLC were recorded at pH 5.4 at different Ca^{2+} concentrations: 0 (●); 80 nM (▲); 300 nM (△); 7.5 μM (○); 80 μM (■). Inset: changes in $[\theta]$ at 220 nm with the concentration of Ca^{2+} . This is a representative experiment of four with identical results.

since no alterations in c.d. spectra of the enzyme are detected on addition of Ca^{2+} at pH 7.4. This further strengthens our suggestion about the importance of secondary-structure changes in PI-PLC activation by Ca^{2+} . Also it is noteworthy that the enzyme showed identical spectra at pH 7.4 and 5.4 in the absence of Ca^{2+} .

DISCUSSION

We report here the isolation and purification of a 60 kDa PI-PLC from bovine brain cytosol that behaves identically, in terms of response to Ca^{2+} , with the activity determined in crude cytosolic preparations from different origins (i.e. Swiss 3T3 fibroblasts, macrophages, pig aorta endothelial cells and bovine brain). This enzyme is activated by Ca^{2+} at pH 5.4, but not at pH 7.4, and is bound to the phospholipid vesicle irrespective of the presence of Ca^{2+} and independently of the pH. The fact that Ca^{2+} does not modulate the ability of the enzyme to bind its substrate strongly argues against the hypothesis of the existence of a Ca^{2+} -induced translocation of PI-PLC as a key step in the activation of this cytosolic activity [36]. However, a correlation seems to exist between Ca^{2+} -induced enzyme activation and changes in

Fig. 3. Effect of Ca^{2+} on the secondary structure of PI-PLC

C.d. spectra of PI-PLC were recorded at pH 7.4 and at 0 (●) and 80 μM (△) Ca^{2+} . This is a representative experiment of four with identical results.

secondary structure measured by c.d. Thus Ca^{2+} is unable to induce changes in the secondary structure of the enzyme at the same pH (i.e. 7.4) at which activation by Ca^{2+} of the PI-PLC does not occur; but at pH 5.4, Ca^{2+} induces both structural changes and enzyme activation. Taken together, these results strongly suggest that changes in the secondary structure of the enzyme are actually important for enzyme activation by Ca^{2+} .

A number of cytosolic PI-PLCs have been purified from several mammalian origins [38–44]. The relationship between the different PI-PLCs so far isolated is not clear, although it appears that, at least in some situations, proteolysis may account for part of this heterogeneity [50,51]. Hofmann & Majerus [40] and Bennet & Crooke [44] have reported the purification to complete homogeneity of a cytosolic PI-PLC from sheep seminal-vesicular glands and guinea-pig uterus respectively. This enzyme has a molecular mass of 62 kDa [40] or 65 kDa [44]. Our enzyme showed on SDS/PAGE under either reducing or non-reducing conditions a molecular mass of 60 kDa. Therefore it appears that the enzyme described here may be similar to those described previously [40,44]. However, it is worth noting that this is the first report of the isolation of a 60 kDa PI-PLC from bovine brain. The relationship between this enzyme and the recently described 143–150 kDa [41,42] and 88 kDa [43] species isolated also from bovine brain deserves further investigation. On the other hand, contrary to what was found by Hofmann & Majerus [40] and by Bennet & Crooke [44], the PI-PLC described here does not show the same molecular mass when it is analysed by SDS/PAGE or by Sephadex G-200 chromatography. This might indicate that the enzyme described here may be able to polymerize

or aggregate under native conditions; the non-involvement of disulphide bridges in the formation of the trimer is confirmed by the fact that the protein behaves identically in SDS/PAGE under reducing or non-reducing conditions. The aggregation state of the enzyme may, on the other hand, be important for the control of PI-PLC activity, as has been shown for other enzymes [52,53]. On this regard it is noteworthy that the activity of the PI-PLC described here is inhibited when the protein is concentrated, suggesting that the aggregation state of the enzyme might actually control its activity (J. Moscat, C. Herrero & M. E. Cornet, unpublished work). Interestingly, when the enzyme is diluted (which correlated with increased activity) a change in the secondary structure of the enzyme similar to that produced by Ca^{2+} is detected (J. Moscat, C. Herrero & M. E. Cornet, unpublished work). This strongly suggests that the aggregation state of the enzyme might control its activity through changes in the secondary structure, lending further support to the notion that alterations in the secondary structure of this enzyme may be important for its activation not only by Ca^{2+} (Figs. 2 and 3) but possibly also by other stimulants.

Three independent groups [31–34] claim that PI-PLC in a crude cytosolic fraction is activated by GTP and/or its non-hydrolysable analogues. This suggests that an unidentified cytosolic G-protein may be involved in the regulation of cytosol PI-PLC. When either the purified 60 kDa cytosol PLC described here or crude cytosol preparations from bovine brain, fibroblasts, pig aorta endothelial cells or macrophages were incubated in the presence of GTP and/or its non-hydrolysable analogues (guanosine 5'-[γ -thio]triphosphate or guanosine 5'-[β - γ -imido]triphosphate), no modification of the activity of this enzyme was detected (J. Moscat, C. Herrero & M. E. Cornet, unpublished work). Enzyme activation was not observed even at high concentrations of nucleotides (200 μM). On some occasions very high concentrations of GTP (1 mM) stimulated PI-PLC in cytosolic extracts, but this effect was reproducible neither in all the crude preparations nor in the purified enzyme. Furthermore, this effect was also mimicked by ATP, ruling out the possibility of a G-protein mediating cytosol PI-PLC activity. On the other hand, the protein product of the *ras* oncogene, p21, has GTP-binding properties and has been suggested to be involved in the regulation of PI-PLC activity [54,55]. When pure bacterially expressed Ha-ras p21 proteins, either normal or transformant, were incubated with either the 60 kDa pure enzyme or crude cytosolic preparations, no effect was observed on the enzyme activity either in the absence or in the presence of GTP or its non-hydrolysable analogues (results not shown). This is in keeping with our more recently published data showing that increased diacylglycerol in Ha-ras transformants is produced by a constitutively activated PtdCho- and PtdEtn-specific PLC rather than a PI-PLC [56].

In summary, the results presented here strongly suggest that a 60 kDa PI-PLC from bovine brain cytosol, which shows the same properties as the cytosolic activity described in a number of cell systems, is permanently bound to its substrate irrespective to the concentration of Ca^{2+} . Ca^{2+} -induced activation of this enzyme appears to be mediated by a conformational change, implying a decrease in the content of residues in α -helix.

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