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Divalent cation-induced changes in conformation of protein kinase C

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Using physical techniques, circular dichroism and intrinsic and extrinsic fluorescence, the binding of divalent cations to soluble protein kinase C and their effects on protein conformation were analyzed. The enzyme copurifies with a significant concentration of endogenous Ca^{2+} as measured by atomic absorption spectrophotometry, however, this Ca^{2+} was insufficient to support enzyme activity. Intrinsic tryptophan fluorescence quenching occurred upon addition to the soluble enzyme of the divalent cations, Zn^{2+}, Mg^{2+}, Ca^{2+} or Mn^{2+}, which was irreversible and unaffected by monovalent cations (0.5 M NaCl). Far ultraviolet (200–250 nm) circular dichroism spectra provided estimations of secondary structure and demonstrated that the purified enzyme is rich in α-helices (42%) suggesting a rather rigid structure. At Ca^{2+} or Mg^{2+} concentrations similar to those used for fluorescence quenching, the enzyme undergoes a conformational transition (42–24% α-helix, 31–54% random structures) with no significant change in β-sheet structures (22–26%). Maximal effects on 1 μM enzyme were obtained at 200 μM Ca^{2+} or 100 μM Mg^{2+}, the divalent cation binding having a higher affinity for Mg^{2+} than for Ca^{2+}. The Ca^{2+}-induced transition was time-dependent, while Mg^{2+} effects were immediate. In addition, there was no observed energy transfer for protein kinase C with the fluorescent Ca^{2+}-binding site probe, terbium(III). This study suggests that divalent cation-induced changes in soluble protein kinase C structure may be an important step in in vitro analyses that has not yet been detected by standard biochemical enzymatic assays.

1. Introduction

The Ca^{2+}/phospholipid-dependent phosphotransferase, protein kinase C (PKC), is considered to be involved in a variety of biological processes including extracellular signal transduction, tumor promotion and cellular differentiation [2]. Previous models for activation suggested that a potential role for Ca^{2+} was to bind to the inactive cytosolic PKC species inducing a conformational change promoting association of the enzyme with the membrane [1–3]. However, examination of the primary sequence of PKC deduced from cDNA clones does not indicate any region typical for other Ca^{2+}-binding proteins, such as calmodulin or the Ca^{2+} (and phospholipid)-binding proteins, suggesting that if Ca^{2+} binds to the enzyme it is not at a typical Ca^{2+}-binding site [4,5]. In addition, Mg^{2+} is necessary for both optimal phosphorylation activity and binding of PKC to phorbol esters [6]. Thus, Mg^{2+} is required not only for the formation of the substrate, Mg · ATP, but also for maintenance of the correct enzyme conformation, such that it will optimally bind to its lipid activator. Interestingly, the binding of Mg^{2+} to the Ca^{2+}/calmodulin-dependent protein kinase is considered to be the initial priming step which may play a role in subsequent activation [7].

In this study, we examine conformational changes in secondary structure of soluble PKC, induced by divalent cations, employing circular dichroism and intrinsic and extrinsic fluorescence

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analyses. Aside from providing experimental information on PKC secondary structure, we also provide data on the specificity of these divalent cation-induced changes in the concentration of soluble PKC.

2. Materials and methods

Male Wistar rat brains (6 weeks old) were the source for purification of 'cytosolic' or soluble PKC. A modified purification procedure of Huang et al. [20] was used [8]. Soluble PKC was extracted with high concentrations of divalent cation chelators (EGTA, 10 mM; EDTA, 2 mM). The purification procedure was performed with buffers containing EGTA and EDTA (0.5 mM). Typically, 1 mg of pure enzyme (single band on Coomassie and silver stained SDS gel [9]) was obtained from 50 rat brains which could be maintained in an active state in elution buffer (20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 1.0 mM dithiothreitol (DTT); pH 7.5) containing 10% glycerol at $-80\,^{\circ}C$ for several months. The specific activity of the enzyme was approx. 1000 U/mg (1000 nmol P_i incorporated/mg protein per min using histone I as a substrate). Bovine serum albumin (BSA, fraction V) was obtained from Sigma (St. Louis, MO). Bovine pancreatic phospholipase A_2 (PLA_2) was purchased from Boehringer-Mannheim.

All buffers were prepared from double-distilled, deionized water that was further washed against Chelex 100 (Bio-Rad, Richmond, CA) and Amberlite MB-3 (BDH Ltd, Poole, U.K.) mixed-bed monovalent resin to reduce contaminating divalent and monovalent ions. Free Ca^{2+} for all measurements was defined according to calculations for Ca^{2+}/EGTA buffers [10]. All buffers were corrected for pH (7.5) after divalent cation addition.

The Ca^{2+} dependence of PKC activity was examined by monitoring the phosphorylation of the exogenous substrate, histone III-S. The reaction mixture routinely contained 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM DTT, histone I (1.6 mg/ml), 20 μM [γ-^{32}P]ATP (1000 cpm/pmol ATP), leupeptin (1 μg/ml), 2.5 mM $MgCl_2$, plus or minus 10 μM 'free' Ca^{2+}. Lipid vesicle mixtures used were prepared according to Lester [11]. The control inactive lipid mixture was composed of 20% bovine brain phosphatidylserine : 80% egg phosphatidylcholine (100 μM, Lipid Products). The active reaction mixture contained the above lipid mixture with 5% synthetic 1,2-diacylglycerol (w/w) (DAG, Secondary Research Products). Lipid mixtures were dried under a stream of nitrogen, followed by overnight high-vacuum aspiration. The dried mixtures were dispersed by ultrasonic irradiation in buffer minus divalent cations. Sample (50 ng) was added to the reaction mixture and incubated at $30\,^{\circ}C$ for 4 min. The reaction was stopped by the addition of 1 ml of 25% trichloroacetic acid. The samples were kept at $4\,^{\circ}C$ for 30 min and then filtered through MFS cellulose nitrate filters (0.45 μm) and washed (4×2 ml) with 5% trichloroacetic acid. The filters were placed in scintillation vials, 10 ml scintillant added and the radioactivity estimated on a Kontron β-scintillation counter. Additionally, the ability of the enzyme to undergo autophosphorylation was also examined. The same mixture as for histone III-S phosphorylation was used except the specific activity of [γ-^{32}P]ATP was different (10 μM, 5000 cpm/pmol ATP). PKC (1 μg) was used for each condition. After 5 min incubation at $30\,^{\circ}C$ concentrated SDS sample buffer was added, and the sample incubated at $100\,^{\circ}C$ for 5 min and applied to a 10% SDS polyacrylamide (SDS-PAGE) gel. The gel was run, dried and exposed to Kodak X-O-MAt X-ray film, using a Dupont Lightning Plus Intensifier.

The total amount of Ca^{2+} and Mg^{2+} in purified PKC samples containing 50–100 μg/300 μl protein was measured with a Hewlett Packard 4000 atomic absorption spectrophotometer. Ca^{2+} and Mg^{2+} concentrations were determined using a standard Ca^{2+}/EGTA solution and established conversion factors according to the instrument manual.

Fluorescence emission spectra between 300 and 400 nm were recorded on a Perkin Elmer L5 luminescence spectrophotometer (spectral pathwidth of 5 nm both for excitation and emission) with an excitation wavelength of 280 nm [12]. The measurements were carried out in a 1 cm pathlength quartz spectrophotometric cell using a pro-

tein concentration of 5 μg/ml. Samples were stirred briefly upon addition of ions. Terbium(III) chloride hexahydrate (Aldrich, Milwaukee, WI) energy-transfer fluorescence measurements were performed on a Perkin Elmer L5 spectrofluorometer at 25 °C at a wavelength of 285 nm for excitation and 540 nm for emission with 5 nm slit widths. Sample (50 or 100 μg/ml) was monitored in a 0.2 cm quartz cuvette (0.5 ml). The PKC samples were first dialyzed against buffer (pH 7.5) without EGTA or EDTA. Terbium titrations were performed by sequentially adding the fluorophore (10 mM stock solution) to a stirred protein solution, as previously described [13]. Bovine calmodulin (50 μg/ml) (Calbiochem, Lucerne, Switzerland, containing 100 μM $CaCl_2$) was used as a standard for terbium response and considered to be zero initial fluorescence for both PKC and calmodulin.

Circular dichroism (CD) spectra were measured on a Jasco 500A spectropolarimeter [12]. PKC concentrations of 100 μg/ml were used (200 μg per measurement). This absorption value enabled an optimal CD signal to be measured between 200 and 250 nm. CD spectra presented were an average of eight scans (12.5 nm/min) between 200 and 250 nm. Digitally recorded curves were fed through the Jasco J-DPY data processor for signal averaging and baseline subtraction. The estimated experimental error is less than 500 degree cm^2 $dmol^{-1}$. Concentration changes were monitored by ultraviolet absorption measurements with a Hewlett Packard diode array spectrophotometer. The samples showed an approximate ultraviolet absorption value of 0.6 at 200 nm at room temperature (25 ± 1 °C) in a 1 mm path-length cuvette. No significant protein concentration changes due to ion addition were recorded, thus, correction was not necessary. All the CD measurements were carried out in 1 mm path-length cylindrical cuvette. The pH of the sample did not significantly change (±0.1 pH unit) upon addition of the divalent cations. The molar elipticity, $[\theta]$, was calculated from the observed elipticity, θ, according to the following formula:

$$[\theta] = \frac{\theta M}{lc}$$

where M is the molecular weight, l the path-length (1 mm), and c the concentration (mg/cm^3). We used the method of Chen et al. [14] and Chang et al. [15] to estimate secondary structure of the enzyme, because of its mathematical simplicity. The spectral data were fitted by a nonlinear regression program as previously described [16].

CD spectra of BSA and bovine PLA_2 were measured under similar conditions for PKC. The protein concentration was twice that for PKC, thus, only four scans were necessary. Mole divalent cation/mole protein were corrected according to the ratios used for the divalent cation/PKC ratio assuming an M_r of 66000 for BSA and 14000 for PLA_2. Molar elipticity was calculated as for the PKC samples.

Protein was measured according to the protein dye binding procedure of Bradford [17].

3. Results

3.1. Ca^{2+} dependence of PKC activity

Analyses of the ability of PKC to transfer the γ-PO_4 of ATP to a substrate, histone III-S, were performed in a model membrane system containing the phospholipid, phosphatidylserine, and the lipid activator, DAG. It was demonstrated that Ca^{2+} was necessary in order to obtain optimal activity (fig. 1a). The Ca^{2+} concentration required to activate the enzyme was similar to that required by other laboratories using this procedure [18]. Due to the ligand dependence of the substrate, histone III-S [19], the Ca^{2+} dependence of the enzyme to undergo autophosphorylation was also analyzed. As can be seen, the autophosphorylation activity is greatly stimulated upon increasing the free Ca^{2+} concentration from 10 to 100 μM (fig. 1b). Thus, the PKC source used in the following spectroscopic studies had Ca^{2+}-dependent activation properties similar to those of previously described preparations [18,20].

3.2. Ca^{2+} and Mg^{2+} associated with purified PKC

Cytosolic PKC was extracted and purified in the presence of divalent cation chelators and ex-

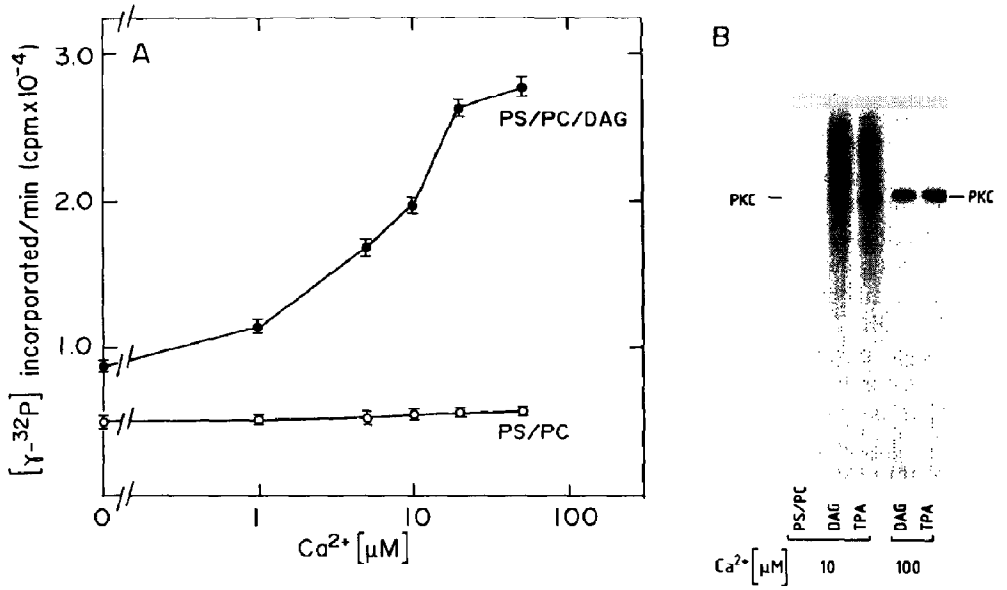

Fig. 1. Ca^{2+}-dependent activation of cytosolic protein kinase C. (a) Ca^{2+}-dependent histone III-S phosphorylating activity of PKC. Enzyme (50 ng) was activated in the presence of multilamellar vesicles (100 μM) containing PS:PC (1:4) (○) or PS:PC:DAG (1.0:3.75:0.25) (●), histone III-S (1.6 mg/ml), Mg^{2+} (5 mM), [γ-^{32}P]ATP and the designated concentration of free Ca^{2+}. (b) Autophosphorylation of cytosolic PKC. PKC (50 ng) was incubated with MLV (100 μM) containing PS:PC (1:4), PS:PC:DAG (1:3.75:0.25), or PS:PC:TPA (1:3.95:0.05), Mg^{2+} (5 mM), [γ-^{32}P]ATP and 0, 10 or 100 μM free Ca^{2+} samples were run on 10% SDS-PAGE gels, dried and exposed to X-ray films. Prestained molecular weight markers (Sigma) were used as controls.

posed to high salt concentrations (1.5 M KCl) during one of the chromatographic steps which should have removed weakly associated ions. Two atomic absorption measurements of Ca^{2+} associated with the enzyme (two different PKC preparations) resulted in readings of approx. 0.8–1 mol Ca^{2+}/mol protein PKC as determined using the Coomassie blue protein-binding assay [17]. Associated Mg^{2+} was less than 0.1 mol Mg^{2+}/mol PKC. In spite of these associated divalent cations, exogenous Ca^{2+} and Mg^{2+} were required for optimal PKC activity (see fig. 1a and b).

3.3. Divalent cation-induced changes in tryptophan fluorescence

Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} were added separately to PKC and quenching of fluorescence measured. Peak fluorescence quenching (5–7%) was at approx. 338–340 nm for all divalent cations (data not shown). The data for the curves in fig. 2 were values recorded at 340 nm emission for each of the various cations. The potency of quenching was in the following order, $Zn^{2+} > Mg^{2+} > Mn^{2+} > Ca^{2+}$. The divalent cation chelators, EGTA, CDTA and EDTA (10 mM) were incapable of reversing (less than 1%) the quenching. These results indicate that the cation-induced change in fluorescence is not specific for Ca^{2+}. The quenching is divalent cation-specific as NaCl (0.5 M) had no effect on the fluorescence spectrum of PKC.

3.4. Lack of binding of Tb^{3+}

Tb^{3+} has been used as an extrinsic fluorescence probe for demonstration of specific Ca^{2+}-binding sites on calmodulin [13,21] and the Ca^{2+}-binding proteins, such as calelectrin [22] when an energy donor (tryptophan or tyrosine) was at or near the cation-binding site. Tb^{3+} is also known to inhibit PKC [23]. Calmodulin was used as a control Ca^{2+}-binding protein to demonstrate the binding

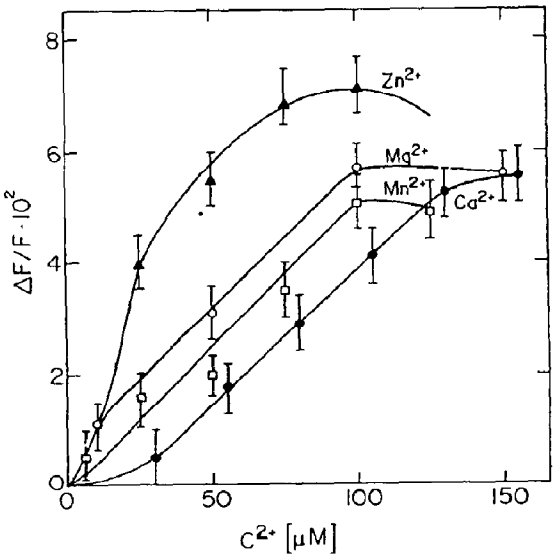

Fig. 2. Intrinsic tryptophan fluorescence quenching of protein kinase C by divalent cations. 2 ml samples (5 μg/ml) were added to 1 cm quartz cuvettes and measured for fluorescence at an excitation wavelength of 280 nm and peak emission of 340 nm. Increasing concentrations of Zn^{2+} (▲), Mg^{2+} (○), Mn^{2+} (□) or Ca^{2+} (●) were added to the stirred sample. The quenching could not be reversed by divalent cation chelators.

of Tb^{3+} (fig. 3). Optimal fluorescence energy transfer was observed at 15 μM total terbium. The relatively low fluorescence is possibly due to the endogenous Ca^{2+} (100 μM) which is present in the protein solution of the calmodulin used. In the presence of PKC (0.6 μM), no change in terbium fluorescence was observed for terbium concentrations up to 35 μM. This may be due to the lack of energy donors in potential terbium-binding sites.

3.5. CD measurements of soluble PKC

The far-ultraviolet CD spectra (200–260 nm) of PKC show a strong negative band at about 220 nm. Upon addition of 50 μM Ca^{2+}, the band becomes weaker (−17100 to 15400 degree cm^2 $dmol^{-1}$) and the peak shifts towards a longer wavelength (218 to 219 nm) (fig. 4a). The Ca^{2+} effect increased in time, inducing a relatively slow conformational change in the protein (−12800 degree cm^2 $dmol^{-1}$). When spectra were measured immediately upon addition of 50 μM Ca^{2+} (time 0, fig. 4a) the band intensity was −15400 degree cm^2 $dmol^{-1}$ as compared to −12800 degree cm^2 $dmol^{-1}$ when the protein was incubated with the same Ca^{2+} concentration for 30 min at room temperature (30 min, fig. 4a). No further change was observed at 1 h incubation (data not shown). A time course of the changes could not be made due to the relatively long time period (30 min) required to record a suitable spectrum considering the limited available protein. Additional Ca^{2+} (100 μM final) induced a larger structural change, which was no longer time dependent under the conditions employed. Ca^{2+} induced a maximal effect at 200 μM concentration (−8500 degree cm^2 $dmol^{-1}$, 222 nm). The Mg^{2+} effect on PKC structure was similar but more intense than that of Ca^{2+}. Mg^{2+} (50 μM) induced conformational changes independent of time (fig. 4b). The Mg^{2+} concentration

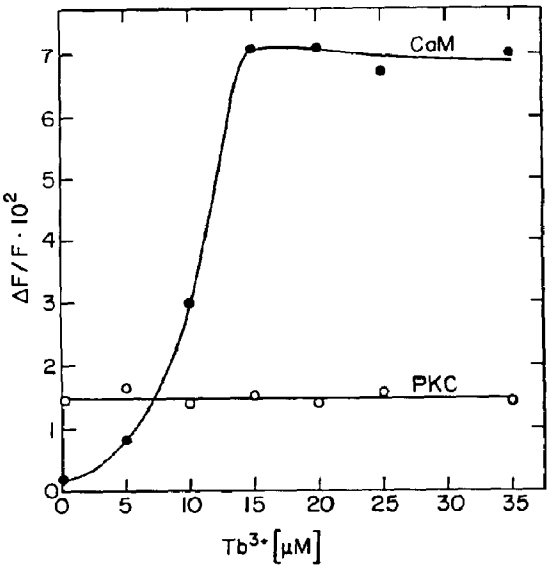

Fig. 3. Lack of Tb^{3+} binding to protein kinase C. Terbium energy transfer was measured for protein kinase C [100 μg/ml (○)] and calmodulin [50 μg/ml (●)] in a 0.2 cm quartz cuvette with excitation at 285 nm and an emission wavelength of 540 nm. Corrections were made for light scattering and dilution of sample upon addition of terbium. F_0 was the emission at 540 nm for calmodulin and was used for both proteins. Terbium did not bind to protein kinase C as demonstrated by the lack of change in fluorescence, however, there was some endogenous fluorescence at this emission wavelength hence the reading at a zero concentration of terbium.

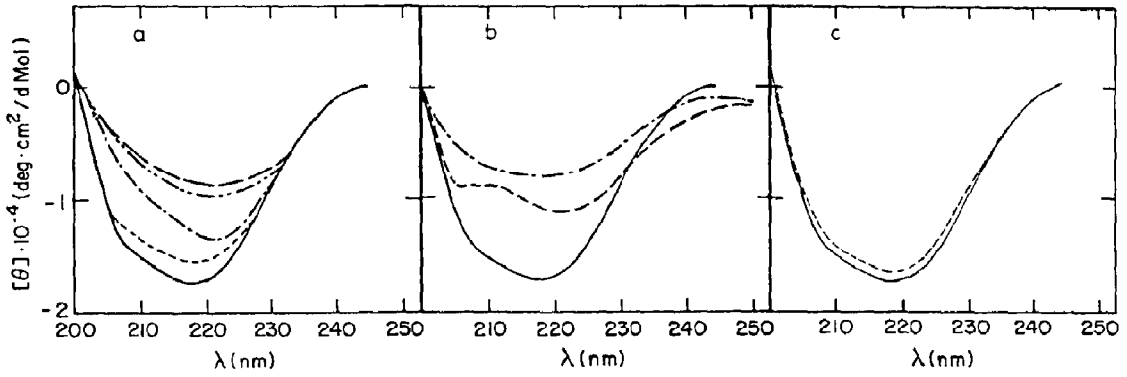

Fig. 4. Divalent-induced conformational changes in protein kinase C. Samples (200 μg) were as described in section 2. (a) Ca^{2+}-induced changes in PKC secondary structure. The various scans represent: no addition, time 0 (-); 50 μM Ca^{2+}, time 0 (- - - - -); 50 μM Ca^{2+}, time 30 (.-.-.); 100 μM Ca^{2+} (..-..-..); 200 μM Ca^{2+} (— — —). (b) Mg^{2+}-induced conformational changes in protein kinase C. Samples: time 0, no additions (-); 50 μM Mg^{2+} (- - - - -); 100 μM Mg^{2+} (.-.-.). Additional Mg^{2+} had no further effects. (c) Circular dichroism spectrum of purified protein kinase C. Scans were made at time 0 (full line) and 120 min (dashed line). NaCl (0.5 M) and EGTA (10 mM) did not induce significant changes in the CD spectrum of the untreated enzyme (full line). Similar results were observed in three separate experiments with different enzyme preparations.

required for the maximal effect was 100 μM (−7000 degree cm^2 $dmol^{-1}$, 220 nm). The gradual changes in spectra upon increasing concentrations of Ca^{2+} or Mg^{2+} were different, however, the final spectrum was similar for both divalent cations (fig. 4a and b).

High monovalent salt concentrations (0.5 M NaCl) had a negligible effect on the CD spectra (fig. 4c) showing that monovalent cations do not significantly interact with PKC. Incubation of the sample for 1 h at room temperature before the final CD measurement induced a 2% decrease in the maximal CD value. This decay may be due to the spontaneous denaturation of the enzyme. However, comparison of the molecular weight of the enzyme before and after CD measurements demonstrated no differences and protamine and histone phosphorylation activities were 90–95% of native untreated samples (data not shown). The divalent cation chelators, EDTA, EGTA and CDTA (5 mM), were incapable of reversing the induced conformational changes caused by Ca^{2+} and Mg^{2+}, even after 1 h exposure.

3.6. CD analyses of BSA and PLA_2

Two commercially available proteins were analyzed in order to determine whether the divalent cation effects were a specific characteristic of PKC. BSA was chosen as it has a molecular weight similar to that of PKC (BSA, $M_r = 66\,000$; PKC, $M_r = 82\,000$). The lipolytic enzyme, PLA_2, was analyzed as it is also predominantly in the cytosol yet active at the membrane surface [24], like PKC, and has considerable homology with PKC at the proposed lipid binding site containing portions of the sequence similar to cysteine-rich 'zinc fingers' [25]. Additionally, the activation process of bovine pancreatic PLA_2 specifically requires the binding of Ca^{2+} [24]. Neither Ca^{2+} (fig. 5a) nor Mg^{2+} (fig. 5b) had any effect on the secondary structure of either of these proteins as demonstrated in the histogram representing changes in the maximal elipticity of the three proteins, BSA, PKC and PLA_2, incubated in the presence of these divalent cations (fig. 5a and b). Upon maximal induced changes in PKC, i.e., 200 μM Ca^{2+} or 100 μM Mg^{2+}, additional Mg^{2+} (100 μM, fig. 5a) or Ca^{2+} (200 μM, fig. 5b), respectively, were added. No further changes were detected in PKC structure as for BSA and PLA_2 (fig. 5a and b).

3.7. Estimated changes in PKC secondary structure upon addition of divalent cations

Calculated secondary structures of PKC are listed in table 1. The purified enzyme in its native state has a high percentage of α-helix (42%). The

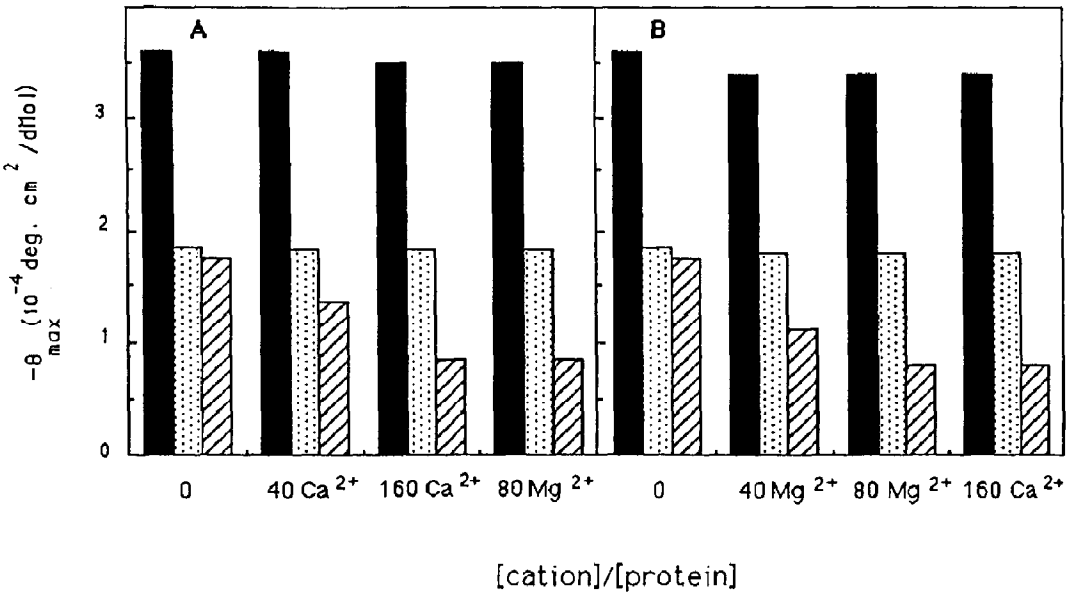

Fig. 5. The effect of divalent cations on maximal CD spectral elipticity of PKC, BSA and PLA_2. θ_{max} for PKC (hatched), BSA (filled) and PLA_2 (dotted) was at 220, 208 and 211 nm, respectively, at similar divalent cation/protein ratios (mol/mol). (a) Effect of Ca^{2+} on the θ_{max} of PKC, BSA and PLA_2. At the maximal Ca^{2+}-induced change, Mg^{2+} (80 mol Mg^{2+}/mol protein) was added and changes at θ_{max} recorded. Divalent cation concentrations are expressed as mol divalent cations/mol protein. (b) Effect of Mg^{2+} on the θ_{218} of PKC, BSA and PLA_2. Results were obtained and expressed as described in (a). At the maximal Mg^{2+}-induced change, Ca^{2+} (160 mol Mg^{2+}/mol protein) was added and changes at θ_{218} recorded.

Ca^{2+}- or Mg^{2+}-PKC structures were analyzed at divalent cation concentrations where maximal induced changes in PKC CD spectra were measured. The addition of Ca^{2+} (200 μM) or Mg^{2+} (100 μM) induced an α-helix-to-coil (undetermined structure) transition (42–24% α-helix, 31–54% coil), i.e., from a rigid to a more flexible structure. The percentage β-sheet does not significantly change (22–26%).

Table 1

Changes in protein kinase C secondary structure upon binding of divalent cations as measured by circular dichroism

Divalent cations	Secondary structure (%)		
	α-Helix	β-Sheet	Random structure
No additions	42±2.0	26±1.0	31±0.5
Mg^{2+} (100 μM) or Ca^{2+} (200 μM)	24±1.5	22±0.5	54±2.0

4. Discussion

Theoretical analyses of cDNA-derived PKC primary sequences do not reveal regions of homology to Ca^{2+}-binding proteins, such as calmodulin, calpactin, calelectrin, etc. [5,26]. One analysis showed some homology to the 'F' helix of the 'E-F hand' structure of Ca^{2+}-binding regions such as that for calmodulin [4], but this sequence is highly conserved and requires both portions for binding [27]. Thus, considering the importance of Ca^{2+} in PKC activity, it was interesting to determine if Ca^{2+} promoted a specific conformational change in cytosolic PKC. In our PKC pre-

parations a significant quantity of Ca^{2+} co-purified with the enzyme. Considering the rigid nature of soluble PKC (42% α-hellix), it may be that the Ca^{2+} molecule is trapped within this structure or that it serves a structural function. This endogenous Ca^{2+} was insufficient to maintain activity as divalent cation had to be added. By taking advantage of intrinsic spectroscopic properties of PKC, we have not been able to demonstrate a Ca^{2+}-specific binding site on the soluble enzyme. Due to the relatively large quantities of enzyme required for these analyses, cytosolic rat brain PKC was used as the enzyme source. This contains the predominant three isozyme species, α, β, and γ [3]. Genes for some other PKC isozymes (δ, ϵ and ζ) have been identified in rat brain, however, the protein products have not yet been detected [3,28]. A preliminary report of changes in tryptophan fluorescence indicates that the three predominant isozymes undergo quenching upon addition of Ca^{2+} [3]. We provide a detailed analysis of these observations using a mixture of these three isoenzymes and some other divalent cations. It should be noted that we used a similar purification procedure to obtain the isozyme mixture, however, the final hydroxyapatite chromatographic step to obtain the isozymes was omitted due to insufficient protein yield.

Due to the well-known difficulties in obtaining large quantities of this enzyme our analyses were limited. In the present report, intrinsic fluorescence analyses demonstrated that tryptophan quenching is slightly stronger by Zn^{2+}, Mg^{2+} and Mn^{2+} than by Ca^{2+}. These data were further supported by CD measurements of Ca^{2+} and Mg^{2+} effects on the secondary structure of PKC. Both cations had similar effects on structural changes but as for the intrinsic fluorescence measurements, Mg^{2+} was more potent. Maximal effects were similar at concentrations observed for the fluorescence quenching. Upon the Mg^{2+}-induced changes, addition of Ca^{2+} had no further effect on CD or tryptophan fluorescence, and the same held for the reverse treatment. The Ca^{2+}-induced changes in tryptophan and CD spectra were time-dependent, as previously demonstrated for the Ca^{2+}-induced changes in tryptophan emission of the isozymes [3]. In contrast, the other divalent cation-induced changes in tryptophan fluorescence and CD spectra were immediate which would suggest a different process of protein rearrangement by Ca^{2+}. The changes in both spectral measurements were not reversible using divalent cation chelators under the conditions we employed, in contrast to a previous report of partial reversibility of this Ca^{2+}-induced quenching [3]. However, we have shown that Ca^{2+} binding to the PKC-lipid-Mg^{2+} complex is partially reversible in both intrinsic tryptophan fluorescence and CD analyses [12]. The effect of divalent cations was at high concentrations and specific for divalent cations, as NaCl had no effect on PKC structure at very high concentrations (> 500 mM). This is in contrast to calmodulin where changes in the far-ultraviolet spectrum measured by CD were induced by both Ca^{2+} and NaCl [29]. Preincubation of PKC at Ca^{2+} and Mg^{2+} concentrations greater than required for divalent cation-induced changes in structure had no effect on subsequent activation as we have previously shown under similar conditions using the substrates, protamine and histone [8,12]. The high concentration of divalent cations required may indicate that more than one divalent cation binds per PKC molecule and, in the case of Ca^{2+}, they may do so in a sequential manner as supported by the observed time-dependent Ca^{2+}-induced structural changes. The possibility of more than one Ca^{2+}-binding site on PKC has been proposed [30].

The ion-free conformation of the purified soluble enzyme is 42% α-helix, 25–27% β-sheet, and 31% random coil (see table 1). Both Ca^{2+} and Mg^{2+} induced an α-helix-to-coil transition, the final conformation being 24% α-helix, 22% β-sheet, and 54% random. These estimates are generally accurate for percentage α-helix, while the β-sheet and random structures are relative [31]. It is interesting that such striking changes in protein structure represent a change in the order of the enzyme which cannot be related to any activation event. Upon completion of the measurements (2 h), the enzyme was analyzed and less than 10% loss of histone 1 phosphorylating activity was recorded, thus, it had not undergone denaturation (unpublished observations). These divalent cation-induced changes in PKC were specific as demon-

strated by the lack of their effect on a protein of similar molecular weight (BSA) and a protein with sequence homology in the region proposed to be involved with the ligand association (PLA_2). It has been previously shown that no change in PLA_2 conformation can be detected upon the binding of Ca^{2+} to its specific site [32]. It is interesting that the region with sequence homology for PLA_2 and PKC is proposed to be cysteine-rich zinc finger structures found in various proteins. However, this region in PKC and PLA_2 is considered to be important for penetration into lipid monolayers not for divalent cation binding [33]. Thus, there is no experimental evidence that would support the proposal that these particular regions are the divalent cation-binding domains that are responsible for the conformational changes reported here.

As to the physiological nature of these conformational changes in PKC, we consider that, due to the high Mg^{2+} concentration (10^{-3} M) in the cell cytosol [34], this Mg^{2+}-induced unfolding of the enzyme in vitro results in an enzyme conformation which most likely is the physiological structure of cytosolic localized PKC.

We have reported that penetration of PKC into lipid monolayers is stabilized to a similar degree in the presence of Ca^{2+} or Mg^{2+} [33]. However, PKC association with lipid monolayers [33] and bilayers [8] is not dependent upon the presence of divalent cations. Only after PKC associates with the lipid support is a Ca^{2+}-specific conformational change detected [12]. Interestingly, based on in vitro analyses, the binding of Mg^{2+} to the Ca^{2+}/calmodulin-dependent protein kinase was proposed to be the initial step in the process of activation of this enzyme (ref. 7; M. King, personal communication). Thus, this divalent cation-induced change in PKC conformation may be a common characteristic in other in vitro enzymatic systems.

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