Phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in MCII mast cells mediated by high-affinity Fc receptor for IgE

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In the present study we examined the activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 ($cPLA_2$) after aggregation of cell-surface high-affinity Fc receptors for IgE (FcεRI) on mast cells. MCII mast cells (a factor-dependent bone-marrow-derived murine mast cell line) produce significant amounts of leukotriene C_4 (LTC_4) (70 ng/10^6 cells) on cross-linking of FcεRI. Using enzymic and immunochemical analysis we found that $cPLA_2$ is the predominant form of this enzyme in MCII mast cells (0.2 μg/mg of total protein) and other forms (i.e. secretory PLA_2 or Ca^{2+}-independent cytosolic PLA_2) could not be detected. Therefore MCII mast cells represent an excellent cellular model for the study of the biochemical mechanism(s) responsible for FcεRI-induced activation of $cPLA_2$ and the involvement of $cPLA_2$ in FcεRI-mediated production of LTC_4. After activation of FcεRI by cross-linking, $cPLA_2$ in MCII mast cells exhibited a decreased electrophoretic mobility and its enzyme activity was increased 3-fold. Treatment with phosphatase reversed both the altered electrophoretic mobility and the enhanced enzyme activity demonstrating that they were the result of FcεRI-induced phosphorylation. On cross-linking of FcεRI, $cPLA_2$ was phosphorylated within 30 s and appeared to be an early substrate for FcεRI-activated protein kinases in MCII mast cells. Tyrosine phosphorylation may be a critical component in this process, as genistein, an inhibitor of protein tyrosine kinases, blocked the activation of $cPLA_2$. Using anti-phosphotyrosine antibodies we observed that the activating phosphorylation was not on tyrosine residues of $cPLA_2$, indicating that tyrosine kinases participate upstream in the signalling cascade that couples FcεRI to $cPLA_2$. We conclude that in MCII mast cells $cPLA_2$ is activated by kinase-dependent mechanisms and may be responsible for FcεRI-induced mobilization of arachidonic acid for the generation of LTC_4.

INTRODUCTION

Mast cells play a central role in inflammatory and immediate allergic reactions (Siraganian, 1988; Galli, 1993). A characteristic property of mast cells is the presence of the high-affinity immunoglobulin E Fc receptor (FcεRI) on their cell surface (Siraganian, 1988). On binding of IgE to FcεRI and activation by cross-linking of FcεRI-bound IgE with antigen, mast cells secrete preformed mediators, including histamine, proteoglycans and proteases (Siraganian, 1988). Activation of mast cells also results in the liberation of arachidonic acid from membrane phospholipids and production of prostaglandins, leukotrienes and platelet-activating factor (Siraganian, 1988). Oxygenated metabolites of arachidonic acid (collectively called eicosanoids) and platelet-activating factor are well-known mediators of inflammatory and allergic reactions (Larsen and Henson, 1983; Snyder, 1985). Although several phospholipid-metabolizing enzyme systems may be activated on FcεRI cross-linking in mast cells, PLA_2 was found to be largely responsible for the rapid release of arachidonic acid from membrane phospholipids (Yamada et al., 1987).

Three different PLA_2 activities have been detected and partially purified from mast cells (Murakami et al., 1992): (1) a PLA_2 recognized by antibodies raised against a cytosolic ~ 88 kDa PLA_2 purified from rabbit platelets; (2) a 14 kDa PLA_2 that reacted with antibodies prepared against the secretory PLA_2 from rat platelets; (3) a distinct phosphatidylserine-hydrolysing PLA_2 that was optimally active at acidic to neutral pH and exhibited properties characteristic of the well-known secretory PLA_2s. Recently, a PLA_2 with apparent molecular mass of ~ 90 kDa has been purified from mast cells and this enzyme exhibited the same chromatographic properties as the ~ 88 kDa PLA_2 isolated from rabbit platelets (Nakatani et al., 1994). We and others have cloned an 85 kDa cytosolic PLA_2 that is activated by submicromolar concentrations of Ca^{2+} as found in stimulated cells and exhibits great selectivity for arachidonate esterified at the *sn*-2 position of phospholipids (Clark et al., 1990, 1991; Kramer et al., 1991; Sharp et al., 1991). This novel cytosolic 85 kDa PLA_2 ($cPLA_2$) exhibits structural and functional characteristics that make it the enzyme most probably responsible for receptor-mediated release of arachidonic acid from cellular phospholipids, as shown in various cellular systems, including CHO cells that overexpress $cPLA_2$ (Lin et al., 1992a), fibroblasts (Lin et al., 1992b), monocytes (Nakamura et al., 1992), mesangial cells (Schalkwijk et al., 1992) and platelets (Kramer et al., 1993a,b).

In the present study we set out to examine whether $cPLA_2$ is a component of the FcεRI-mediated biochemical events leading to the mobilization of arachidonic acid for eicosanoid synthesis. We show that the *in vitro* enzyme activity of $cPLA_2$ is enhanced after activation of mast cells and that this increase in specific activity is due to FcεRI-mediated phosphorylation of $cPLA_2$. This phosphorylation exhibits rapid kinetics and precedes the production of leukotrienes.

Abbreviations used: FcεRI, high-affinity Fc receptor for IgE; $cPLA_2$, Ca^{2+}-sensitive cytosolic phospholipase A_2; PLA_2, phospholipase A_2; LTC_4, leukotriene C_4; MAP kinase, mitogen-activated protein kinase.

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EXPERIMENTAL

Preparation and stimulation of cells

MCII mast cells were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% D10.CAS (a mitogen-induced culture supernatant from the 'type 2' helper T-cell D10.G4 containing mast-cell growth factors, including interleukin-3, -4, -9 and -10) at 37 °C in a 5% (v/v) CO_2 atmosphere at 95% humidity. Before sensitization and activation, cells were kept in serum-free medium for at least 16 h. The cell suspension was centrifuged at 200 *g* for 5 min, resuspended at 5×10^5 cells/ml in serum-free medium and incubated at 37 °C for 60 min with or without monoclonal mouse IgE ascites (IgELb4) diluted 1:400 (Rudolph et al., 1981). The cells were then washed in Hepes-buffered saline (150 mM NaCl, 25 mM Hepes, pH 7.2) and incubated for 10 min (or as indicated) in KRH buffer (1 mM $CaCl_2$, 118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1 mM KH_2PO_4, 11.1 mM D-glucose, 1.1 mM $MgCl_2$, 5 mM Hepes, pH 7.2, and 0.1% BSA) with or without rat anti-mouse IgE monoclonal antibody ascites (EM95) diluted 1:100 (Baniyash and Eshar, 1984). Reactions were stopped by either of two methods: (1) addition of EDTA, staurosporine and genistein to a final concentration of 20 mM, 10 μM and 50 μM respectively, and then pelleting of cells for preparation of cytosolic extracts as detailed below; (2) addition of a cocktail providing final concentrations of 1% Triton X-100, 4% glycerol, 1 mM EDTA, 1 mM phenylmethanesulphonyl fluoride, 1 μg/ml leupeptin, 100 μg/ml aprotinin, 10 mM $Na_4P_2O_7$, 50 mM NaF and 1 mM Na_3VO_4. Samples were incubated on ice for 30 min before centrifugation at 10000 *g* for 20 min. The supernatants were incubated with rabbit anti-$cPLA_2$ IgG overnight and $cPLA_2$ was immunoprecipitated using Protein A–Sepharose as described previously (Kramer et al., 1993b). We found initially that treatment of MCII mast cells with IgE (for sensitization) had no effect on the enzyme activity or electrophoretic mobility of $cPLA_2$. Furthermore, MCII mast cells did not produce LTC_4 when treated with either mouse IgE (IgELb4) or rat anti-mouse IgE antibody (EM95). Therefore, as our controls we used MCII mast cells that had been processed in exactly the same way as the activated cells, but omitting the addition of IgE and anti-IgE.

Preparation of cytosols

Cells were resuspended at 10^8 per ml in Hepes-buffered saline containing protease, phosphatase and kinase inhibitors as detailed above. For the treatment with potato acid phosphatase (Sigma) cells were suspended in Bis/Tris-buffered saline, pH 6.0, containing protease and kinase inhibitors, but not phosphatase inhibitors. The cells were lysed by N_2 cavitation (6900 kPa for 30 min at 4 °C) or by sonication (four 10 s bursts at 4 °C) and subjected to centrifugation at 104000 *g* for 40 min at 4 °C. The cytosolic extracts typically contained 2–4 mg/ml of protein.

SDS/PAGE and Immunoblotting

SDS/PAGE was performed as described by Laemmli (1970) using the Novex system with 4–12% or 10% Tris–glycine gels. Electrophoresis was carried out at 30 mA for 2 h (4–12% gel) or at 25 mA for 3.5 h (10% gel). Proteins were transferred to nitrocellulose using the Sartoblot II semi-dry system (Sartorius) in transfer buffer (39 mM glycine, 1.3 mM SDS, 20% methanol, 48 mM Tris, pH 9.2). The nitrocellulose sheets were blocked in wash buffer (150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mM Tris/HCl, pH 7.4) containing 3% BSA (Sigma). The blots were incubated for 2 h at room temperature with rabbit anti-$cPLA_2$ peptide IgG (2.5 μg/ml), mouse anti-($cPLA_2$ peptide) serum (1:10000 dilution) or mouse anti-phosphotyrosine antibody (1:2000 dilution) in blotting buffer (100 mM $MgCl_2$, 0.5% Tween 20, 1% Triton X-100, 1% BSA, 100 mM Tris/HCl, pH 7.4) containing 5% fetal calf serum, then washed three times for 10 min with wash buffer and incubated for 2 h with goat anti-rabbit IgG–horseradish peroxidase conjugate (Jackson Immuno-Research, diluted 1:50000) or goat anti-mouse IgG–horseradish peroxidase conjugate (Transduction Laboratories, diluted 1:2000) in blotting buffer. The blots were washed and developed using the ECL detection system (Amersham).

Assay of PLA_2 activity

PLA_2 activity was assayed using sonicated liposomes containing 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (52 mCi/mmol; Du Pont–New England Nuclear) and *sn*-1,2-dioleoyl glycerol (Avanti Polar Lipids) at a molar ratio of 2:1 as previously described (Kramer et al., 1991) and modified as follows. The assay mixture contained 1 mM $CaCl_2$, 2 mM DTT, 150 mM NaCl, 50 mM Hepes, pH 7.5, and 1 mg/ml BSA. The substrate consisted of 2 μM radiolabelled phosphatidylcholine liposomes (50000 d.p.m. per incubation) containing 1 μM dioleoyl glycerol. Incubations were performed at 37 °C for 15 min or as indicated.

Quantification of LTC_4 production

Cells were prepared as described, suspended at 10^6 per ml in KRH buffer and incubated with or without anti-IgE. The reaction was stopped by adding EDTA to a final concentration of 20 mM. LTC_4 was assayed using the EIA kit from Cayman Chemical according to the instructions of the manufacturer.

Antibodies

Monoclonal antibodies M12 and M3-1 were raised against human $cPLA_2$ as reported previously (Kramer et al., 1991). Anti-$cPLA_2$ antibodies (against human $cPLA_2$ and the C-terminal 24 amino acids of human $cPLA_2$) were raised in rabbit and mouse as described previously (Kramer et al., 1991, 1993b). Anti-$cPLA_2$-specific and -peptide-specific IgG were purified from rabbit antiserum by affinity chromatography with $cPLA_2$ and $cPLA_2$ peptide (residues 726–749 in the $cPLA_2$ sequence) respectively coupled to Sulfolink support (Pierce). Monoclonal anti-phosphotyrosine antibody IG2 was obtained from Boehringer-Mannheim.

Other methods

Protein content was determined using the Coomassie Plus protein assay (Pierce) and BSA as standard.

RESULTS AND DISCUSSION

FcεRI-mediated production of LTC_4

Activation of MCII mast cells by cross-linking of FcεRI as described in the Experimental section results in the production of LTC_4. As demonstrated in a time-course experiment (Figure 1), LTC_4 was generated after a delay of at least 1 min in a time-dependent fashion and reached maximal levels (70 ng per 10^6 cells) after 10 min. The amount of LTC_4 formed was comparable with that produced by primary mouse mast cells of similar origin (Levi-Schaffer et al., 1987; Fonteh and Chilton, 1993). We further observed that MCII mast cells do not generate significant amounts of LTB_4 or prostaglandin D_2. Our data demonstrate

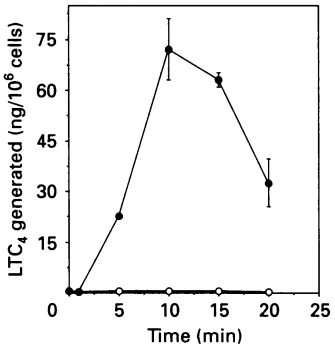

Figure 1 Time course of LTC_4 production induced by cross-linking of FcεRI in MCII mast cells

MCII mast cells were activated by cross-linking FcεRI for 0–20 min as detailed in the Experimental section. Control ('non-activated') MCII mast cells were prepared by subjecting cells to the same incubations and centrifugation steps as activated cells, but omitting both IgE and anti-IgE. LTC_4 was measured in supernatants from control (○) and activated (●) cells and is expressed as ng of LTC_4 produced per 10^6 cells. The data are representative of two experiments; values shown are means ± range of duplicate determinations.

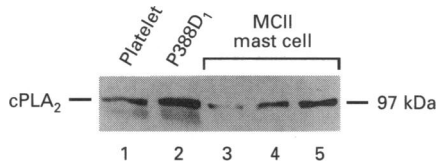

Figure 2 Immunochemical identification of $cPLA_2$ in MCII mast cells

Cytosolic extracts were prepared from MCII mast cells, human platelets and $P388D_1$ cells as described in the Experimental section and subjected to SDS/PAGE (4–12% gel) and immunoblot probing with a rabbit anti-$cPLA_2$ peptide (726–749) IgG. Lane 1, human platelets (40 μg); lane 2, $P388D_1$ cells (40 μg); lanes 3–5, MCII mast cells (5, 10 and 15 μg respectively).

that MCII mast cells possess the capacity to rapidly mobilize significant amounts of arachidonic acid for the biosynthesis of LTC_4. In addition, as discussed below, these cells contain relatively high levels of $cPLA_2$ and are deficient in other forms of the enzyme. Hence they represent an excellent cellular model for the study of the involvement of $cPLA_2$ in FcεRI-mediated production of LTC_4 and the elucidation of the biochemical mechanism(s) responsible for FcεRI-induced activation of $cPLA_2$.

PLA_2 activities in MCII mast cells

The presence of $cPLA_2$ in MCII mast cells was examined by immunoblot analysis with anti-human $cPLA_2$ peptide IgG (residues 726–749 of the encoded human protein). As shown in Figure 2, these antibodies detect $cPLA_2$ in cytosolic extracts of MCII mast cells, as well as $P388D_1$ mouse macrophages and human platelets as previously reported (Kramer et al., 1993). On incubation of MCII cytosolic extracts with radiolabelled palmitoyl-arachidonoyl-phosphatidylcholine liposomes, PLA_2 activity can be readily detected (Figure 3). We examined whether $cPLA_2$ was responsible for the PLA_2 activity detected in MCII mast-cell cytosolic extracts. As previously reported, the mouse monoclonal antibody M12 recognizes native $cPLA_2$ and is also an inhibitory antibody, whereas the antibody M3-1 binds to $cPLA_2$ but does not inhibit its enzyme activity (Kramer et al., 1991, 1993a). After pretreatment with the neutralizing antibody M12, the PLA_2 activity in MCII cytosols was inhibited 85%, but not decreased after preincubation with the control antibody M3-1 (Figure 4a). These results indicate that the hydrolysis of phosphatidylcholine liposomes observed in Figure 3 is mediated predominantly by $cPLA_2$. However, unlike in other cells that we tested (Kramer et al., 1993), there was a significant residual PLA_2 activity that was not suppressed by M12.

In order to probe for 14 kDa secretory PLA_2 known to be present in other mast cell lines (Murakami et al., 1992), we incubated MCII cytosolic extracts with [^{3}H]oleate-labelled autoclaved *Escherichia coli* which is known to be a much better substrate for secretory PLA_2 than phosphatidylcholine liposomes (Kramer and Pepinsky, 1991). In fact, the specific activity of secretory PLA_2 purified from human platelets was two to three orders of magnitude higher with *E. coli* substrate than with phosphatidylcholine liposomes (Johansen et al., 1992). Using this sensitive *E. coli* assay we were unable to detect PLA_2 activity in cytosolic extracts of MCII mast cells, indicating that they

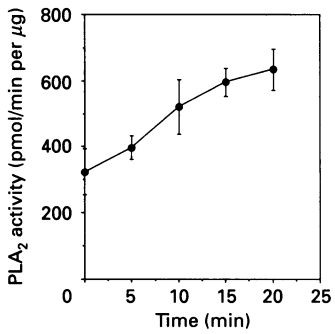

Figure 3 PLA_2 activity in MCII mast cell cytosol

MCII mast cell cytosolic extracts were incubated with phosphatidylcholine liposomes for 0–20 min and PLA_2 activity was determined as described in the Experimental section. The data shown are from a representative experiment and values are means ± S.D. from triplicate determinations.

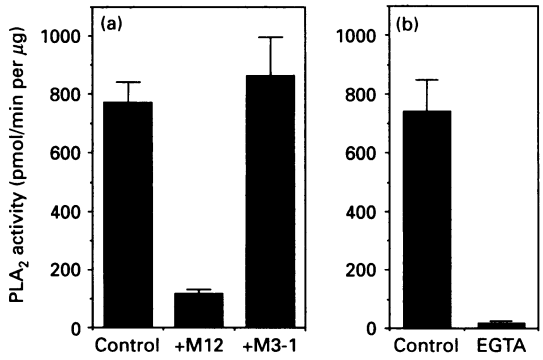

Figure 4 Effect of anti-$cPLA_2$ antibodies and EGTA on activity in MCII mast-cell cytosolic extracts

(**a**) MCII mast-cell cytosolic extracts were preincubated at 4 °C for 45 min with buffer (control) or 1 mg/ml of anti-$cPLA_2$ monoclonal antibody M12 (+M12) or M3-1 (+M3-1) and then assayed for PLA_2 activity. The data are representative of three different experiments and values shown are means ± range of duplicate determinations. (**b**) PLA_2 activity was determined in the absence (control) or presence of 5 mM EGTA (EGTA) using the standard incubation conditions described in the Experimental section.

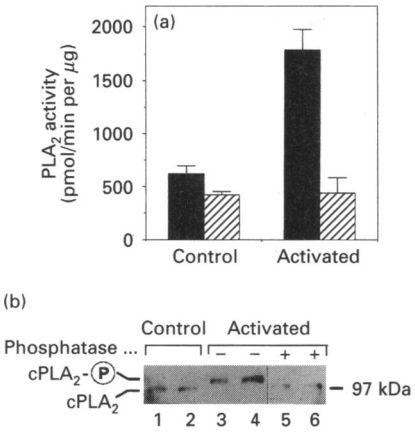

Figure 5 FcεRI-mediated activation of cPLA$_2$ in MCII mast cells

MCII mast cells were activated by cross-linking FcεRI as detailed in the Experimental section. Control ('non-activated') MCII mast cells were prepared by subjecting cells to the same incubations and centrifugation steps as activated cells, but omitting both IgE and anti-IgE. Cytosolic extracts were prepared from control and activated cells and incubated for 30 min at 25 °C in the absence and presence of 27 μg/ml potato acid phosphatase. (**a**) Determination of PLA$_2$ activity in cytosolic extracts from control and activated MCII mast cells treated with (▨) or without (■) phosphatase. The data shown are from a representative experiment and values are the means ± range from duplicate determinations. (**b**) SDS/PAGE/immunoblot analysis of cytosolic extracts (10 μg of protein) using a 10% gel and probing with rabbit anti-cPLA$_2$ peptide (726–749) IgG. Lanes 1 and 2, control cells; lanes 3 and 4, activated cells; lanes 5 and 6, phosphatase-treated activated cells. cPLA$_2$-P indicates the slowly migrating form containing the activating phosphorylation.

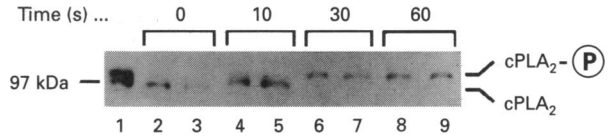

Figure 6 Time course of FcεRI-mediated phosphorylation of cPLA$_2$

MCII mast cells were activated for 0–60 s by cross-linking FcεRI as detailed in the Experimental section. Reactions were stopped by the addition of a cocktail that provided a final concentration of 20 mM EDTA, 10 μM staurosporine and 50 μM genistein. Cytosolic extracts (10 μg of protein) were analysed by SDS/PAGE (10% gel)/immunoblot probing with rabbit anti-cPLA$_2$ peptide (726–749) IgG as detailed in the Experimental section. Lane 1, cPLA$_2$ purified from a baculovirus/insect cell expression system (5 ng); lanes 2–9, cytosolic extracts from MCII mast cells activated for 0 to 60 s. The same results were obtained when a detergent cocktail (see the Experimental section) was directly added to the cell suspensions for subsequent SDS/PAGE/immunoblotting. cPLA$_2$-P indicates the slowly migrating form containing the activating phosphorylation.

contain less than 100 pg of secretory PLA$_2$ per mg of cytosolic protein. Similarly, we could not detect secretory PLA$_2$ in lysates of MCII mast cells, demonstrating that no membrane-associated secretory PLA$_2$ was present in these cells.

We further examined whether MCII mast cells contain the Ca^{2+}-independent cytosolic PLA$_2$ reported to be present in various cells and tissues (Pierik et al., 1988; Hazen et al., 1990). Since the Ca^{2+}-independent PLA$_2$ is fully active in the presence of Ca^{2+} chelators, we measured PLA$_2$ activity in MCII cytosolic extracts with assay buffer containing 5 mM EGTA. As demonstrated in Figure 4(b), PLA$_2$ activity was completely suppressed in the presence of 5 mM EGTA, demonstrating that MCII mast cells do not contain a Ca^{2+}-independent PLA$_2$. Consequently, cPLA$_2$ appears to be the predominant PLA$_2$ in MCII mast cells. Given the specific activity of purified cPLA$_2$ (5 μmol/min per mg), we calculated that MCII mast cells contain 0.2 μg of cPLA$_2$/mg of total cytosolic protein. Hence, MCII mast cells contain higher levels of cPLA$_2$ than any of the cells and tissues studied so far (Wijkander and Sundler, 1991).

FcεRI-mediated activation and phosphorylation of cPLA$_2$

MCII mast cells were activated by cross-linking FcεRI as detailed in the Experimental section. Control ('non-activated') MCII mast cells were prepared by subjecting cells to the same incubations and centrifugation steps as activated cells, but omitting both IgE and anti-IgE. In MCII mast cells with cross-linked FcεRI the activity of cPLA$_2$ increased 3-fold compared with control MCII mast cells (Figure 5a). This confirms an earlier observation by Garcia-Gil and Siraganian (1986) that PLA$_2$ activity in homogenates of FcεRI-stimulated basophilic leukaemia cells is increased compared with that in control cells. We further observed that, on FcεRI cross-linking cPLA$_2$ migrated on SDS/polyacrylamide gels with an apparently higher molecular mass (Figure 5b) than cPLA$_2$ prepared from control cells. In order to verify that the FcεRI-mediated enhancement of cPLA$_2$ activity and the electrophoretic mobility shift were due to phosphorylation, we treated cytosols from control and activated MCII mast cells with phosphatase. As demonstrated in Figure 5(a) such treatment reversed the FcεRI-mediated activation of cPLA$_2$, and the enzyme activity of cPLA$_2$ after phosphatase treatment was the same as in control cytosolic extracts. Likewise the slower migrating species of cPLA$_2$ was converted into the faster migrating form after phosphatase treatment, exhibiting the same electrophoretic mobility as cPLA$_2$ prepared from control cells (Figure 5b). These results confirm previous observations first reported by Lin and co-workers (Lin et al., 1992a,b) and confirmed in our laboratory (Kramer et al., 1993b) that the decrease in electrophoretic mobility of cPLA$_2$ is caused by phosphorylation, therefore providing a convenient measure for the receptor-mediated 'activating' phosphorylation of cPLA$_2$. The gel-shift analysis is a useful alternative method that can be used instead of assessment of radioactive phosphate incorporation after metabolic labelling of cells. In fact, the latter procedure has been found to give high background labelling of cPLA$_2$ in control cells, making it difficult to distinguish receptor-mediated from basal phosphorylation events (Qiu et al., 1993). Consequently, we have used the shift in electrophoretic mobility of cPLA$_2$ as a measure of FcεRI-mediated phosphorylation.

In order to determine the kinetics of FcεRI-mediated phosphorylation of cPLA$_2$, we performed a time-course experiment analysing the change in electrophoretic mobility of cPLA$_2$ after FcεRI cross-linking. Preliminary experiments indicated that phosphorylation was complete after 1 min. Therefore we examined earlier time points and determined phosphorylation of cPLA$_2$ after 10 s to 1 min of FcεRI cross-linking. As shown in Figure 6, FcεRI-mediated phosphorylation of cPLA$_2$ occurred within 30 s of cross-linking. The kinetics of cPLA$_2$ phosphorylation in MCII mast cells are significantly faster than those observed in other cell systems where cPLA$_2$ phosphorylation was completed only after 5 min using thrombin or ATP as agonists (Lin et al., 1992a; Kramer et al., 1993b). Furthermore, phosphorylation of cPLA$_2$ in response to cross-linking of FcεRI was more rapid than other FcεRI-mediated signalling events, including phosphorylation of phospholipase Cγ1 reaching a maximum at 3 min (Park et al., 1991) and the transient increase in the concentration of free intracellular Ca^{2+} peaking within 2–3 min (Ali et al., 1989). The delay in LTC$_4$ production (Figure 1) is probably due to the fact that both phosphorylation of

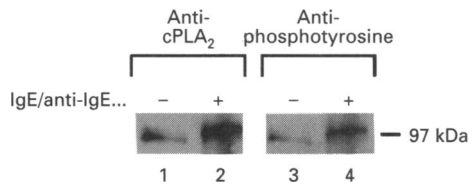

Figure 7 Tyrosine phosphorylation of $cPLA_2$ immunoprecipitated from MCII mast cells

MCII mast cells were activated for 1 min by cross-linking FcεRI as detailed in the Experimental section. Control ('non-activated') MCII mast cells were prepared by subjecting cells to the same incubations and centrifugation steps as activated cells, but omitting both IgE and anti-IgE. The $cPLA_2$ from control cells (lanes 1 and 3) and IgE/anti-IgE-treated MCII mast cells (lanes 2 and 4) was immunoprecipitated with rabbit anti-$cPLA_2$ IgG and subjected to SDS/PAGE/immunoblot probing with mouse anti-[$cPLA_2$ peptide (726–749)] serum (lanes 1 and 2) or mouse anti-phosphotyrosine monoclonal antibody (lanes 3 and 4) as described in the Experimental section.

$cPLA_2$ and increase in cytosolic free $[Ca^{2+}]$ are necessary for full activation of cellular $cPLA_2$ to liberate arachidonic acid for further conversion into eicosanoids (Lin et al., 1992b, 1993). Our data suggest that $cPLA_2$ is an early substrate for FcεRI-activated protein kinases. Amongst the earliest events in FcεRI-mediated signalling is the rapid (within seconds) phosphorylation of multiple proteins, including the β- and γ-chains of the aggregated receptor (Benhamou et al., 1990; Paolini et al., 1991; Li et al., 1992) and a 72 kDa protein tyrosine kinase (Stephan et al., 1992).

Several recent studies have indicated that tyrosine kinases play an important role in the early signal-transduction events caused by FcεRI cross-linking (Benhamou and Siraganian, 1992). As FcεRI has no intrinsic enzyme activity, cellular protein tyrosine kinases of the Src family have been proposed to constitute the link between aggregation of the receptor and subsequent phosphorylation events leading to activation of other polypeptides (Eisenman and Bolen, 1992). Notably, tyrosine kinases were found to be involved in the FcεRI-mediated activation of phospholipase Cγ1 (Park et al., 1991) and phospholipase D (Kumada et al., 1993). To determine whether tyrosine kinases are an essential component in FcεRI-mediated $cPLA_2$ activation, we treated MCII mast cells with genistein, a specific inhibitor of tyrosine kinases (Akiyama et al., 1987). We observed that pretreatment of MCII mast cells with genistein (150 μM for 30 min) inhibited FcεRI-mediated activation of $cPLA_2$ by $67 \pm 8\%$ (mean ± S.D. of three independent experiments). These findings suggest that tyrosine kinases may be critically involved in the signalling cascade that ultimately leads to the activation of $cPLA_2$. Kast et al. (1993) reported that, in HEL-30 keratinocytes, $cPLA_2$ was phosphorylated on tyrosine residues after cells were treated with transforming growth factor α, a growth factor that exerts its biological activity through binding to the epidermal-growth-factor receptor. In order to examine whether $cPLA_2$ in MCII mast cells was phosphorylated on tyrosine residues after FcεRI cross-linking, we immunoprecipitated $cPLA_2$ from control and FcεRI-activated cells using anti-$cPLA_2$ antibodies. The immunoprecipitated $cPLA_2$ was subjected to SDS/PAGE/immunoblot probing with either anti-$cPLA_2$ antibodies or anti-phosphotyrosine antibodies. As shown in Figure 7, more $cPLA_2$ protein was obtained from activated cells than control cells (lane 2 compared with lane 1) reflecting a variation in the recovery of $cPLA_2$ by immunoprecipitation. After reprobing the blot with anti-phosphotyrosine antibodies, an immunoreactive band was detected in the $cPLA_2$ immunoprecipitate from both control and FcεRI-activated cells (lanes 3 and 4), suggesting that $cPLA_2$ from control and FcεRI-activated cells contains phosphotyrosine. Although the band from activated cells was darker than that from control cells, this is due to higher amounts of $cPLA_2$ recovered from FcεRI-activated cells and cannot be attributed to increased tyrosine phosphorylation. Hence, it appears that activation of MCII mast cells does not enhance the phosphorylation of tyrosine residues on $cPLA_2$ and that the activating phosphorylation may therefore be on serine or threonine residues. In CHO cells overexpressing $cPLA_2$ it was shown convincingly that mitogen-activated protein (MAP) kinase is responsible for $cPLA_2$ phosphorylation (Lin et al., 1993). However, as noted above, in this system the kinetics of $cPLA_2$ phosphorylation were significantly slower and maximal phosphorylation was detected late (5 min) after addition of the agonist ATP (Lin et al., 1992a). Recent studies on FcεRI-mediated stimulation of MAP kinase(s) revealed that the activation peaked after 2–5 min (Tsai et al., 1993; Offermanns et al., 1994). Consequently, the maximal activation of $cPLA_2$ in MCII mast cells (after 30 s) appears to precede the maximum of MAP kinase activation. However, as noted by Beaven and Cunha-Melo (1988), the kinetics of FcεRI-mediated signalling events may depend on many different factors, including cell type and nature of the FcεRI cross-linking agent.

Conclusions

In summary, we provide support for the importance of $cPLA_2$ in the production of biologically active eicosanoids in activated mast cells. We also present the first evidence for phosphorylation and activation of $cPLA_2$ induced on cross-linking of FcεRI in mast cells. Although tyrosine kinases may be critically involved in the signalling pathway that leads to activation of $cPLA_2$, they do not appear to phosphorylate $cPLA_2$ directly on FcεRI cross-linking. The rapid kinetics of FcεRI-mediated $cPLA_2$ phosphorylation indicate that $cPLA_2$ is an early substrate for FcεRI-activated protein kinases, but the serine/threonine kinase responsible for the activating phosphorylation of $cPLA_2$ in mast cells remains to be identified.

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