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Protein kinase Cα-dependent increase in Ca^{2+}-independent phospholipase A_2 in membranes and arachidonic acid liberation in zymosan-stimulated macrophage-like $P388D_1$ cells

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Abstract

We previously reported that zymosan-stimulated, protein kinase C (PKC)-dependent arachidonic acid liberation occurs with association of Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) with the membranes of macrophage-like $P388D_1$ cells. In the present study, the possible involvement of PKC isoforms (α, β, δ, and ε) on the increase in $iPLA_2$ was examined. Stimulation of $P388D_1$ cells with zymosan induced increases in $iPLA_2$ activity and protein in the membranes and liberation of arachidonic acid. In the stimulated cells, PKCα, PKCδ, and PKCε, but not PKCβ, were increased in the membranes. The zymosan-induced increase in $iPLA_2$ activity was suppressed by pretreatment with 4β-phorbol 12-myristate 13-acetate for 10 hr, by which PKCα and PKCδ, but not PKCβ and PKCε, were depleted, and by Gö6976, a PKCα inhibitor, but not rottlerin, a PKCδ inhibitor. The zymosan-induced release of arachidonic acid was also reduced by the PKC depletion and Gö6976. However, stimulation with 4β-phorbol 12-myristate 13-acetate alone did not increase $iPLA_2$ activity in the membranes. Furthermore, the depletion of intracellular Ca^{2+} also impaired the zymosan-induced increase in $iPLA_2$ activity in the membranes. However, no increase in $iPLA_2$ activity was observed upon stimulation with Ca^{2+}-mobilizing agents (ionomycin or thapsigargin). Cytochalasin D, an inhibitor of actin polymerization, suppressed the zymosan-induced increases in $iPLA_2$ activity and protein in the membranes and the release of arachidonic acid. These results suggest that zymosan stimulates an increase in $iPLA_2$ in the membranes of $P388D_1$ cells probably through activation of PKCα in concert with cytochalasin D-sensitive events.

Keywords: Ca^{2+}-independent phospholipase A_2; Arachidonic acid; Protein kinase C; Macrophage

1. Introduction

The hydrolytic action of phospholipase A_2 (PLA_2) toward membrane glycerophospholipids mediates the liberation of arachidonic acid (AA), a rate-limiting step in the generation of eicosanoids. Among numerous types of mammalian PLA_2s, cytosolic PLA_2 ($cPLA_2$), classified as a group IVA PLA_2, and secretory PLA_2s, classified into groups IIA, V, and X, are responsible for the stimulus-induced liberation of AA and subsequent eicosanoid generation in a variety of cells [1–6]. $cPLA_2$ and secretory PLA_2 require Ca^{2+} to associate with substrates in membranes and for catalytic activity. In contrast to the two Ca^{2+}-dependent PLA_2s, intracellular $iPLA_2$, classified as a group VIA PLA_2 [7], participates in phospholipid remodeling [8] rather than stimulus-induced AA liberation [9,10]. Under certain conditions, however, $iPLA_2$ has been shown to be involved in AA liberation upon stimulation in several types of cells [2,11–13]. Furthermore, the activation of $iPLA_2$ is implicated in apoptosis [14,15], kallikrein secretion [16], superoxide anion generation [17], interleukin-1β processing [18], and fibroblast proliferation [19]. Thus, $iPLA_2$ seems to posses multiple functions, which might be dependent on cell-type (properties) and/or experimental conditions, because of the existence of multiple $iPLA_2$ splice variants [20,21].

In human monocytes, stimulation with IgG-coated glass beads has been reported to induce $iPLA_2$-mediated

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Abbreviations: AA, arachidonic acid; BEL, bromoenol lactone; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; MAFP, methyl arachidonyl fluorophosphonate; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate.

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liberation of AA [22] and an increase in $iPLA_2$ activity in the membranes [23]. In addition, the activation of PKC has been suggested to contribute to the $iPLA_2$-mediated release of AA [23,24]. Similarly, our previous study showed that $iPLA_2$ in addition to $cPLA_2$ is involved in zymosan-stimulated AA liberation in mouse macrophage-like $P388D_1$ cells, and further, that zymosan increases $iPLA_2$ in the membranes of the cells through its translocation [12]. We further demonstrated that the zymosan-induced increase in $iPLA_2$ in the membranes is suppressed under conditions where PKCα is depleted in $P388D_1$ cells [12]. It is possible that an increase in $iPLA_2$ in membranes depends on PKCα activation upon stimulation with certain particulates that induce phagocytosis. However, the contribution of other PKC isoforms, especially PKCδ and PKCε, which are also activated in $P388D_1$ cells [25], remains to be examined. In the present study, therefore, we examined contribution of PKC isoforms to the increase in $iPLA_2$ in zymosan-stimulated $P388D_1$ cells.

2. Materials and methods

2.1. Materials

Methyl arachidonyl fluorophosphonate (MAFP), bromoenol lactone (BEL), and an anti-group VI PLA_2 antibody were obtained from Cayman Chemical. Ionomycin and Gö6976 were from Calbiochem. Antibodies against PKCα, PKCβ, and PKCδ were from Transduction Laboratories. Anti-PKCε antibody was from Santa Cruz Biotechnology, Inc., 2-[1-(3-dimethylaminopropyl)-1H-indol-3yl]-3-(1H-indol-3-yl)maleimide (GF109203X) was from BIOMOL Research Laboratories, Inc., zymosan (*Saccharomyces cerevisiae*) was from Nacarai Tesque, and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) was from Dojindo Laboratories. Pyrrolidine-1 was donated by Dr. Michael H. Gelb (University of Washington, WA, USA). [^{3}H]AA (100 Ci/mmol) and 1,2-dipalmitoyl-*sn*-glycero-3-[choline-*methyl*-^{14}C]phosphocholine (159 mCi/mmol) were from Perkin–Elmer Life Sciences. Other reagents were obtained from Wako Pure Chemical Industries or Sigma.

2.2. Cell culture

$P388D_1$ cells (Lot number 032798) were obtained from the Health Science Research Resources Bank, and maintained in RPMI1640 (Nissui Pharmaceutical Co, Ltd.) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, F9423), 100 unit/mL penicillin, and 100 μg/mL streptomycin at 37° under humidified air containing 5% CO_2. Cells were plated at 1×10^6 or 8×10^6 cells in 35- or 100-mm culture dishes, respectively, and used for experiments the following day. The cells were washed twice with phosphate-buffered saline containing 0.01% BSA and then placed in 1 mL (35-mm dish) or 5 mL (100-mm dish) of RPMI1640 containing 0.01% BSA.

2.3. Detection of $iPLA_2$ activity and proteins in membranes

$P388D_1$ cells in 100-mm dishes were treated and stimulated as described in the figure legend. The membranes of the cells were prepared according to a method described previously [12] with slight modification. The cells were washed three times and sonicated in a buffer consisting of 340 mM sucrose, 2 mM EGTA, 100 μM leupeptin, 100 μM *p*-(amidinophenyl)methanesulfonyl fluoride, and 10 mM HEPES (pH 7.4). After centrifugation at 100,000 *g* for 30 min at 4°, the pellets were suspended in the same buffer containing 0.07% Triton X-100, and centrifuged at 2000 *g* for 2 min at 4° to remove incorporated zymosan particulates. The resulting supernatant, as a membrane fraction, was used for the detection of $iPLA_2$ activity and proteins. For the $iPLA_2$ assay, a substrate consisting of phosphatidylcholine was prepared in the presence of Triton X-100 according to the method of Ackermann *et al.* [26]. The $iPLA_2$ activity in the membrane fraction (50 μg protein) was determined by incubation with a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-[choline-*methyl*-^{14}C]phos-^{14}C]phosphocholine and the unlabeled compound (22.5 mCi/mmol, 25 μM) at 37° for 30 min in the presence of 5 mM EDTA, 0.04% Triton X-100 and 50 mM HEPES (pH 7.5) in a final volume of 200 μL. The reaction was terminated by adding chloroform/methanol/HCl (200:200:1, v/v). Lipids were extracted and separated by TLC on a Silica Gel G plate using chloroform/methanol/H_2O (65:35:6, v/v) as the developing system. The radioactivity of [^{14}C]lysophosphatidylcholine was determined by liquid scintillation counting and the enzyme activity was calculated. For the detection of $iPLA_2$ proteins, the membrane fraction (20 μg protein) was subjected to SDS–PAGE on a 7.5% gel. The proteins were transferred to a nitrocellulose membrane, and then antibodies against group VI PLA_2 were applied. The bound antibodies were visualized using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

2.4. AA liberation

$P388D_1$ cells were plated in 35-mm dishes at 1×10^6 cells in 1 mL of RPMI1640 containing 10% fetal bovine serum, and then incubated with [^{3}H]AA (1 μCi/mL) for 24 hr. The labeled cells were washed three times and placed in 1 mL of RPMI1640 containing 0.01% BSA. The cells were treated with various reagents in the presence of 10 μM BW755C (a cyclo-oxygenase and lipoxygenase inhibitor) [27], and then stimulated with zymosan as described in the figure legends. When cells were pretreated with BEL, they were washed three times, and then placed

in fresh RPMI1640 containing 0.01% BSA and 10 μM BW755C. The reaction was terminated by transferring the medium and cell lysate, which was prepared by adding 0.1 M NaOH, to ice-cold chloroform/methanol/HCl (100:500:1.5, v/v). Lipids in the medium and lysate were extracted and separated by TLC on a Silica Gel G plate using petroleum ether/diethyl ether/acetic acid (40:40:1, v/v) as the developing system [28]. The areas corresponding to free fatty acid and other lipids (diacylglycerol, triacylglycerol, and phospholipids) were scraped off. The radioactivity of each fraction was determined by liquid scintillation counting. The total radioactivity of the fractions recovered from a single lane of the plate was usually in the range of 2.8×10^5 to 3.2×10^5 dpm. The radioactivity of [^{3}H]AA released was corrected by adjusting the total radioactivity to 3.0×10^5 dpm.

2.5. Immunoblot analysis for PKC isoforms

$P388D_1$ cells in 100-mm dishes were stimulated with zymosan for 30 min. The cells were washed three times and sonicated as above. After the lysate had been centrifuged at 500 *g* for 5 min at 4°, the resulting supernatant was centrifuged at 100,000 *g* for 30 min to separate soluble and membrane fractions. $P388D_1$ cells in 35-mm dishes were treated with 4β-phorbol 12-myristate 13-acetate (PMA) for 10 hr and then washed three times. The cells were lysed by adding a mixture of 0.05% Triton X-100, 340 mM sucrose, 2 mMEGTA, 100 μMleupeptin, 100 μM*p*-(amidinophenyl)-methanesulfonyl fluoride, and 10 mM HEPES (pH 7.4). The membrane fraction (10 μg protein) and cell lysate (10 μg protein) obtained were subjected to SDS–PAGE on a 7.5% gel, followed by immunoblot analysis using antibodies against PKC isoforms (α, β, δ, and ε).

2.6. Statistical analysis

Values are expressed as the mean ± SEM. Data were analyzed by one-way analysis of variance followed by Bonferroni's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Zymosan-induced increase in $iPLA_2$ in membranes

As shown in Fig. 1, stimulation of $P388D_1$ cells with zymosan time- (Fig. 1A) and dose- (Fig. 1B) dependently increased $iPLA_2$ activity in the membranes with a maximal increase observed after 30 min at 0.2 mg/mL. In the zymosan (0.2 mg/mL)-stimulated cells, an increase in $iPLA_2$ protein was also observed in the membranes (Fig. 1C). Under the conditions, zymosan (0.2 mg/mL) time-dependently stimulated the liberation of AA (Fig. 2A), the maximal response being preceded by a peak of $iPLA_2$ activity in the membranes (Fig. 1A). The zymosan (0.2–1 mg/mL)-induced release of AA was partially

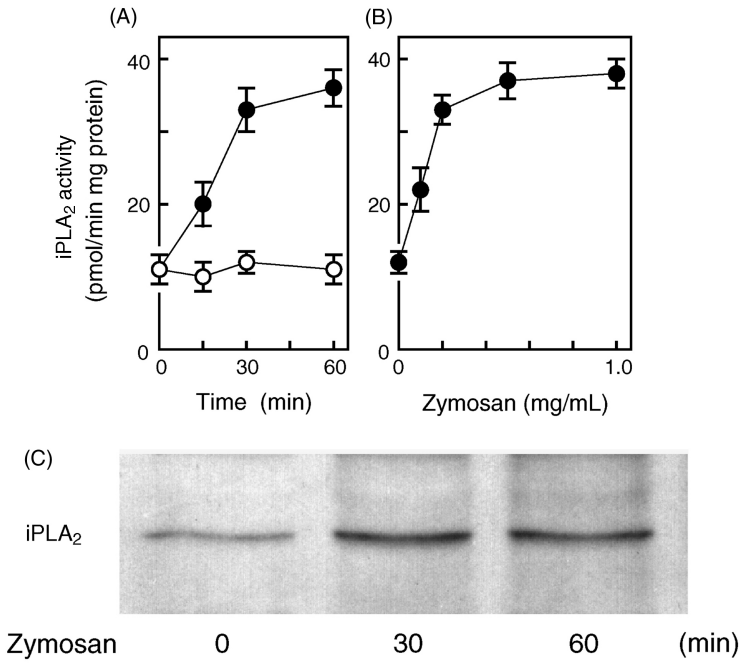

Fig. 1. Increases in $iPLA_2$ activity and protein in the membranes induced by zymosan. (A) $P388D_1$ cells were stimulated with (closed symbols) or without (open symbols) zymosan (0.2 mg/mL) for the periods indicated. (B) $P388D_1$ cells were stimulated with various concentrations of zymosan for 30 min. The $iPLA_2$ activity in the membranes prepared from the cells was measured. Each point represents the mean ± SEM of three separate experiments. (C) $P388D_1$ cells were stimulated with zymosan (0.2 mg/mL) for the periods indicated. $iPLA_2$ proteins in the membranes prepared from the cells were analyzed by immunoblotting. The results are representative of three separate experiments.

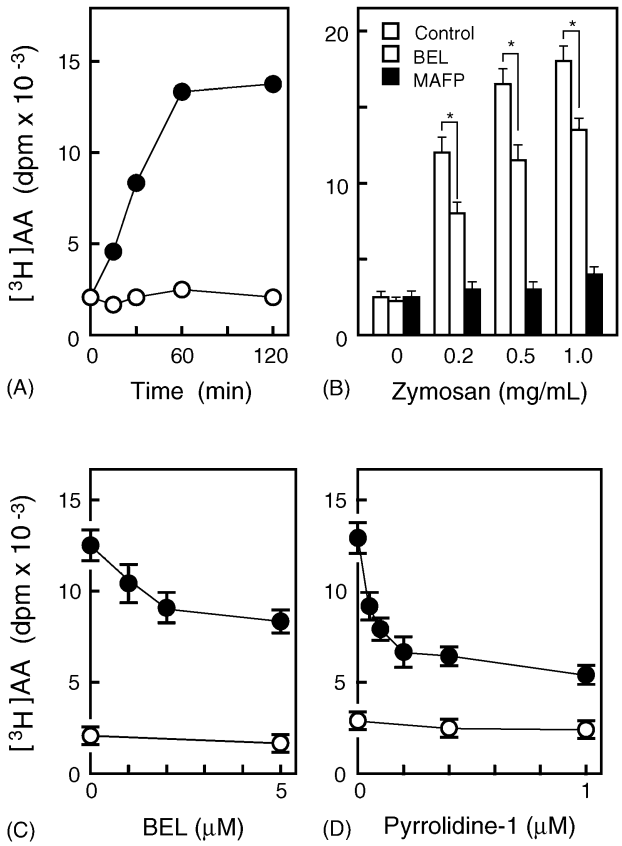

Fig. 2. Effects of PLA_2 inhibitors on the zymosan-induced AA release. (A) [^{3}H]AA-labeled P388D_1 cells were treated with BW755C (10 μM) for 30 min, and then stimulated (closed symbols) or without (open symbols) zymosan (0.2 mg/mL) for the periods indicated. Each point represents the mean of two separate experiments. (B) [^{3}H]AA-labeled P388D_1 cells were treated with or without (control) BEL (2 μM) or MAFP (30 μM) for 1 hr in the presence of BW755C (10 μM). The BEL-treated and control cells but not MAFP-treated cells were washed, and then stimulated with various concentrations of zymosan for 1 hr. (C) [^{3}H]AA-labeled P388D_1 cells were treated with various concentrations of BEL for 1 hr in the presence of BW755C (10 μM). The cells were washed, and then stimulated with (closed symbols) or without (open symbols) zymosan (0.2 mg/mL) for 1 hr. (D) [^{3}H]AA-labeled P388D_1 cells were treated with various concentrations of pyrrolidine-1 for 1 hr in the presence of BW755C (10 μM), and then stimulated with zymosan as in (C). After lipid extraction, the [^{3}H]AA liberated was determined. Values shown in (B), (C), and (D) represent the mean ± SEM of three separate experiments. (*) $P < 0.01$.

inhibited by pretreatment for 1 hr with the $iPLA_2$ inhibitor BEL (2 μM) (Fig. 2B), which was confirmed to suppress $iPLA_2$ activity in cell lysate by about 70% under the conditions (data not shown). At 2 or 5 μM, BEL reduced zymosan (0.2 mg/mL)-induced AA liberation by 30–40% (Fig. 2C). In contrast, MAFP (30 μM), a dual inhibitor of $cPLA_2$ and $iPLA_2$, almost completely inhibited the zymosan-induced liberation of AA (Fig. 2B) and activities of $cPLA_2$ and $iPLA_2$ (data not shown). Furthermore, as shown in Fig. 2D, pyrrolidine-1 (0.05–1 μM), a specific $cPLA_2$ inhibitor [29], dose-dependently suppressed zymosan (0.2 mg/mL)-induced AA liberation with a maximal inhibition (60–70%) observed at 0.2–1 μM. We confirmed that $cPLA_2$ activity (198 pmol/min mg protein, mean of two experiments), which was measured using 2-[^{3}H]arachido-[^{3}H]arachidonoyl-phosphatidylcholine as a substrate [12], was almost completely inhibited by 0.2 or 0.4 μM pyrrolidine-1 (90% inhibition at 0.2 μM; 97% inhibition at 0.4 μM).

3.2. Involvement of PKC isoforms on the increase in $iPLA_2$ in response to zymosan

Previously, we reported that the PKC activator PMA enhanced the zymosan-induced increase in $iPLA_2$ activity in the membranes of P388D_1 cells [12], suggesting the involvement of PKC on the increase in $iPLA_2$ in response to zymosan. In the present study, we further examined PKC isoforms contributing to the rise in $iPLA_2$. As shown in Fig. 3A, zymosan (0.2 mg/mL) induced increases in PKCα, PKCδ, and PKCε, but not PKCβ, in the membranes, indicating that it activates the three PKC isoforms. Furthermore, when P388D_1 cells were treated with PMA (100 nM) for 10 hr, PKCα and PKCδ, but not PKCβ and PKCε, were depleted (Fig. 3B). Under the conditions, the zymosan (0.2 mg/mL)-induced increase in $iPLA_2$ activity in the membranes and liberation of AA were significantly suppressed (Fig. 3C and D). We examined effects of inhibitors for PKCα and PKCδ on the increase in $iPLA_2$ (Fig. 4). The zymosan (0.2 mg/mL)-induced increase in $iPLA_2$ activity in the membranes was prevented by GF109203X (5 μM) or Gö6976 (0.2 or 1 μM), a PKCα inhibitor. In contrast, rottlerin (5 or 10 μM), a PKCδ inhibitor, had no effect on the increase in $iPLA_2$ activity. We confirmed that zymosan (0.2 mg/mL)-induced AA liberation (12,482 ± 1297 dpm; basal level, 2951 ± 273; mean ± SEM, N = 3) was reduced by Gö6976 (0.2 μM, 8098 ± 770 dpm; 1 μM, 6954± 451 dpm; basal level at 1 μM, 2819 ± 375 dpm). These results suggest that PKCα is required for the increase in $iPLA_2$ and release of AA induced by zymosan. However, as shown in Fig. 5, stimulation with PMA (20, 100, or 500 nM) alone for 30 min did not affect $iPLA_2$ activity in the membranes, suggesting that other factors in addition to PKCα are involved in the mechanism underlying the increase in $iPLA_2$ in the membranes of zymosan-stimulated cells.

3.3. Effects of Ca^{2+} depletion and cytochalasin D on the increase in $iPLA_2$ induced by zymosan

Fig. 5 shows that the zymosan (0.2 mg/mL)-induced increase in $iPLA_2$ activity in the membranes was attenuated when P388D_1 cells were pretreated with 100 μM BAPTA-AM and 1 mM EGTA to deplete intracellular Ca^{2+} by chelating. This suggests that Ca^{2+} is involved in the stimulatory effect of zymosan. Under similar conditions, we previously showed that Ca^{2+} depletion inhibited zymosan-induced AA liberation [12]. However, ionomycin (0.5 μM) or thapsigargin (1 μM), Ca^{2+}-mobilizing agents, did not affect $iPLA_2$ activity in the

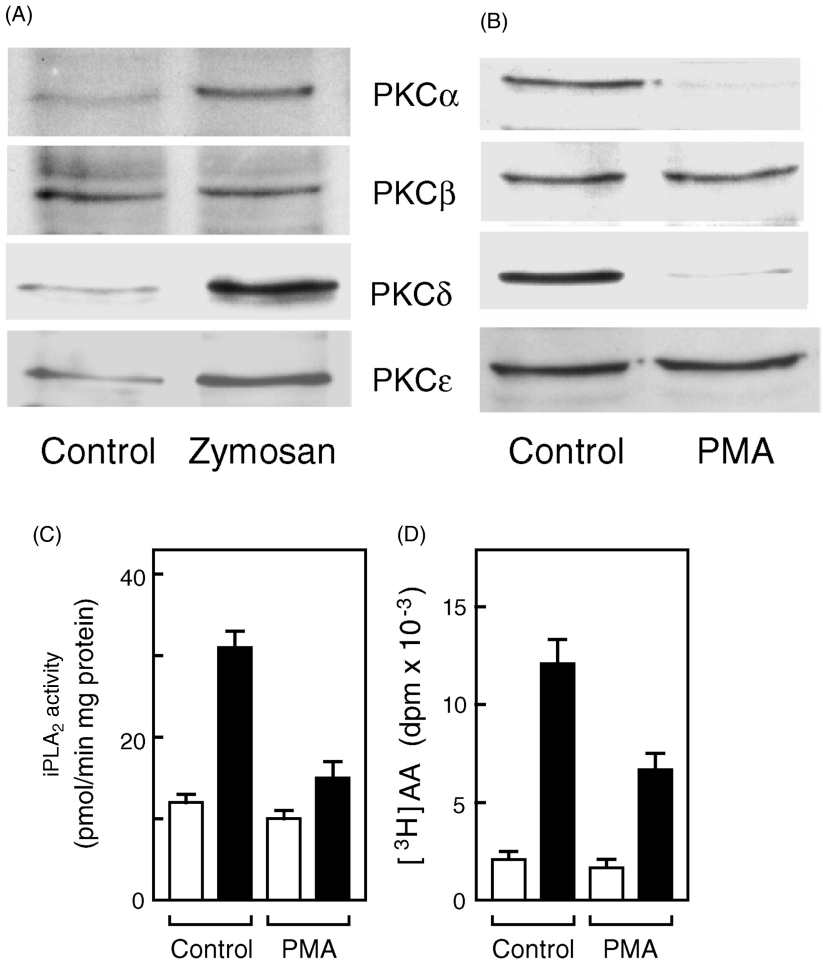

Fig. 3. Increases in PKC isoforms in the membranes induced by zymosan, and effects of PKC depletion on the zymosan-induced increase in $iPLA_2$ activity in the membranes and AA release. (A) $P388D_1$ cells were stimulated with or without (control) zymosan (0.2 mg/mL) for 30 min. PKC isoforms (α, β, δ, and ε) in the membranes prepared from the cells were analyzed by immunoblotting. (B) $P388D_1$ cells were incubated with PMA (100 nM) for 10 hr. PKC isoforms in the cells were analyzed by immunoblotting. The results shown in (A) and (B) are representative of three separate experiments. (C) $P388D_1$ cells were incubated with or without (control) PMA (100 nM) for 10 hr and washed. The cells were stimulated with (closed columns) or without (open columns) zymosan (0.2 mg/mL) for 30 min. The $iPLA_2$ activity in the membranes prepared from the cells was measured. (D) [^{3}H]AA-labeled $P388D_1$ cells were pretreated with PMA as in (C) and washed. The cells were treated with BW755C (10 μM) for 30 min, and then stimulated with (closed columns) or without (open columns) zymosan (0.2 mg/mL) for 1 hr. After lipid extraction, the [^{3}H]AA liberated was determined. Values shown in (C) and (D) represent the mean ± SEM of three separate experiments.

membranes (Fig. 5). We confirmed that stimulation with ionomycin (0.5 μM) and thapsigargin (1 μM) for 30 min induced AA liberation (7068 dpm with ionomycin, 8698 dpm with thapsigargin, 2652 dpm as in control; mean of two separate experiments), which was suppressed by 0.4 μM pyrrolidine-1 (3676 dpm with ionomycin, 3774 dpm with thapsigargin, 2754 dpm as in control) but not 5 μM BEL (6442 dpm with ionomycin, 8656 dpm with thapsigargin, 2326 dpm as in control).

It has been shown that the stimulation of human monocytes with IgG-coated glass beads induces $iPLA_2$-mediated liberation of AA [22] and an increase in $iPLA_2$ activity in the membranes [23]. Considering our results with zymosan-stimulated $P388D_1$ cells, it is possible that an increase in $iPLA_2$ in membranes occurs upon stimulation with certain particulates that induce phagocytosis. To examine this possibility, we tested the effects of cytochalasin D, an inhibitor of actin polymerization, on the increase in $iPLA_2$ in the membranes in response to zymosan, for preventing phagocytosis of zymosan. The results in Fig. 6A and B indicate that cytochalasin D (1–5 μM) dose-dependently suppressed the zymosan (0.2 mg/mL)-induced increase in $iPLA_2$ activity in the membranes and release of AA. As shown in Fig. 6C and D, we confirmed that the increase in $iPLA_2$ protein induced by zymosan (0.2 mg/mL) were inhibited at 5 μM cytochalasin D. However, 10 μM colchicine or taxol, microtubule-modifying agents, had little effect on the increase in $iPLA_2$ (zymosan alone, 34.6 pmol/min mg protein; colchicine + zymosan, 31.8 pmol/min mg protein; taxol + zymosan, 32.3 pmol/min mg protein; control, 12.5 pmol/min mg protein; mean of two separate experiments).

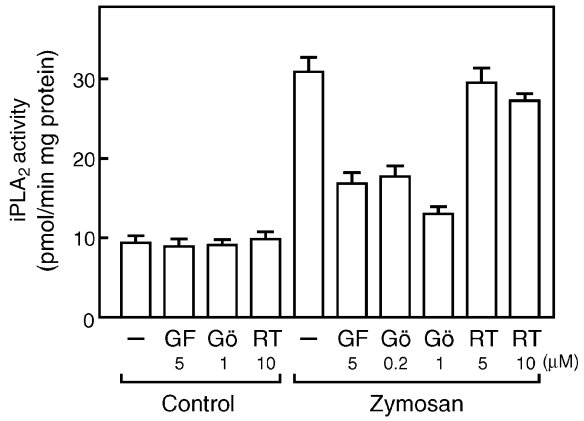

Fig. 4. Effects of PKC inhibitors on the zymosan-induced increase in $iPLA_2$ activity in the membranes. $P388D_1$ cells were treated with or without (−) GF109203X (GF, 5 μM), Gö6976 (Gö, 0.2 or 1 μM), or rottlerin (RT, 5 or 10 μM) for 1 hr, and then stimulated with or without (control) zymosan (0.2 mg/mL) for 30 min. The $iPLA_2$ activity in the membranes prepared from the cells was measured. Each value represents the mean ± SEM of three separate experiments.

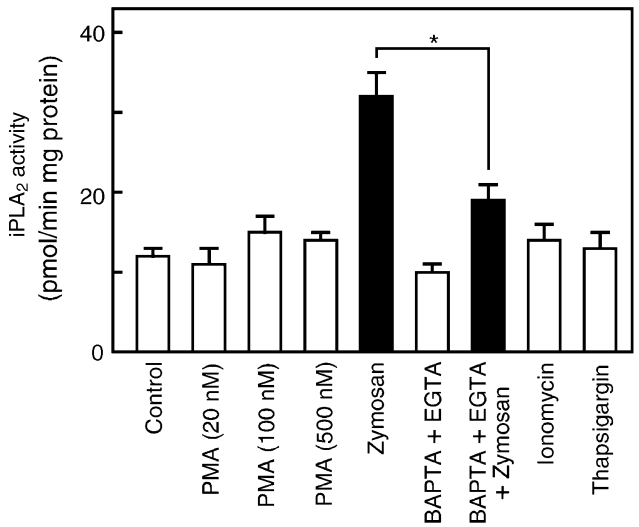

Fig. 5. Change in $iPLA_2$ activity in the membranes induced by PMA or Ca^{2+}-mobilizing agents, or by zymosan in the presence of Ca^{2+} chelators. $P388D_1$ cells were treated with or without BAPTA-AM (100 μM) and EGTA (1 mM) for 30 min. The BAPTA-loaded or -unloaded cells were washed and placed in fresh medium in the presence or absence of EGTA (1 mM), respectively. The BAPTA-unloaded cells were stimulated with or without (control) PMA (20, 100, or 500 nM), ionomycin (0.5 μM), thapsigargin (1 μM), or zymosan (0.2 mg/mL) for 30 min, while the BAPTA-loaded cells were stimulated with or without (BAPTA + EGTA) zymosan (0.2 mg/mL) for 30 min. The $iPLA_2$ activity in the membranes prepared from the cells was measured. Each value represents the mean ± SEM of three separate experiments. (*) $P < 0.05$.

4. Discussion

While zymosan-induced AA liberation has been shown to occur in parallel with PKC-dependent $cPLA_2$ activation in mouse peritoneal macrophages [30], it has been demonstrated that PKC does not participate in $cPLA_2$ regulation in $P388D_1$ macrophages [25]. We previously reported that (1) the PKC activator PMA enhanced zymosan-induced liberation of AA, whereas pretreatment with PMA to deplete PKCα inhibited the release by about 50% in $P388D_1$ cells; (2) the $iPLA_2$ inhibitor BEL suppressed zymosan- and zymosan/PMA-induced liberation of AA, but did not affect the zymosan-induced, PKC-independent liberation in the PKC-depleted cells; (3) zymosan induced an increase in $iPLA_2$ in the membranes through its translocation, which was enhanced by simultaneous stimulation with PMA and suppressed almost completely in the PKC-depleted cells [12]. Based on these findings, we have suggested that an increase in $iPLA_2$ in membranes is involved in the zymosan-stimulated, PKC-dependent release of AA in $P388D_1$ cells. In the present study, PKC isoforms contributing to an increase in $iPLA_2$ in the membranes of zymosan-stimulated $P388D_1$ cells were examined.

In this paper, we confirmed that an increase in $iPLA_2$ activity in the membranes induced by zymosan occurred in parallel with liberation of AA, which was partially suppressed by the $iPLA_2$ inhibitor BEL (Figs. 1 and 2). In the zymosan-stimulated cells, levels of PKCα, PKCδ, and PKCε, but not PKCβ, were increased in the membranes (Fig. 3A), indicating that zymosan activates the three PKC isoforms. In addition, under conditions where PKCα and PKCδ, but not PKCβ and PKCε, were depleted, the zymosan-induced increase in $iPLA_2$ activity and AA release were inhibited (Fig. 3). Moreover, Gö6976 and GF109203X, PKCα inhibitors, suppressed the zymosan-induced increase in $iPLA_2$ activity, whereas rottlerin, a PKCδ inhibitor, had no effect (Fig. 4). The zymosan-induced release of AA was also reduced by Gö6976 (present study) and GF109203X [12]. These results suggest that PKCα is involved in the zymosan-stimulated increase in $iPLA_2$, which presumably contributes in part to the liberation of AA upon the stimulation. The present study further showed that the depletion of intracellular Ca^{2+} by intra- and extracellular Ca^{2+} chelators (BAPTA-AM and EGTA) also prevented the zymosan-induced increase in $iPLA_2$ activity in the membranes (Fig. 5). This inhibitory effect may be due to an impairment of PKC activation, because intracellular Ca^{2+} has been reported to be required for the translocation of PKCα to the membranes induced by zymosan in $P388D_1$ cells [12] and by PMA in macrophage-like RAW 264.7 cells [31].

Previously, the PKC activator PMA was shown to increase $iPLA_2$ activity in the membranes of human monocytes [23]. In $P388D_1$ cells, PMA has been reported to induce translocation of PKCα, PKCδ, and PKCε, but not PKCβ and PKCζ, to the membranes [25]. In the present study, however, stimulation with PMA alone did not increase $iPLA_2$ activity in the membranes of $P388D_1$ cells (Fig. 5), although it enhanced the zymosan-induced increase in $iPLA_2$ activity in the membranes [12], suggesting that in addition to PKCα activation, zymosan-induced other intracellular events, which do not occur in response

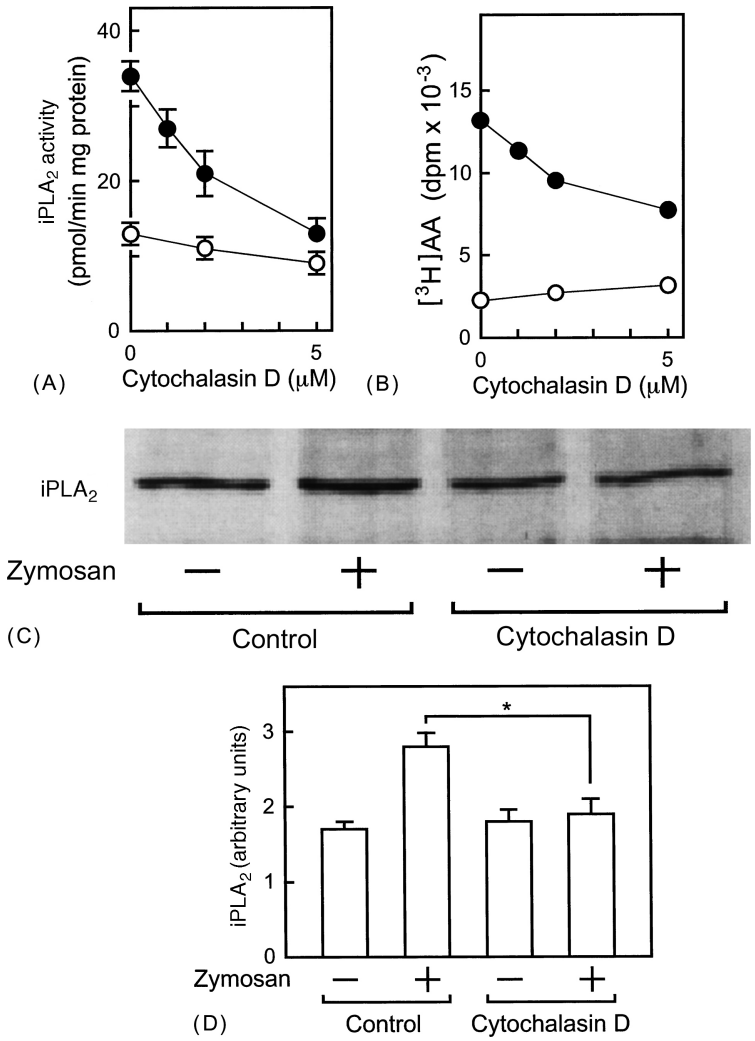

Fig. 6. Effects of cytochalasin D on the zymosan-induced increases in $iPLA_2$ activity and protein in the membranes and AA release. (A) $P388D_1$ cells were treated with various concentrations of cytochalasin D for 15 min, and then stimulated with (closed symbols) or without (open symbols) zymosan (0.2 mg/mL) for 30 min. The $iPLA_2$ activity in the membranes prepared from the cells was measured. Each point represents the mean ± SEM of three separate experiments. (B) [^{3}H]AA-labeled $P388D_1$ cells were treated with cytochalasin D as in (A) in the presence of BW755C (10 μM), and then stimulated with (closed symbols) or without (open symbols) zymosan (0.2 mg/mL) for 1 hr. After lipid extraction, the [^{3}H]AA liberated was determined. Each point represents the mean of two separate experiments performed in duplicate. (C) $P388D_1$ cells were treated with or without (control) cytochalasin D (5 μM) for 15 min, and then stimulated with (+) or without (−) zymosan (0.2 mg/mL) for 30 min. $iPLA_2$ proteins in the membranes prepared from the cells were analyzed by immunoblotting. The results are representative of three separate experiments. (D) Amounts of $iPLA_2$ proteins were estimated by measuring the density of bands of $iPLA_2$ shown in (C) and expressed as arbitrary units. Each value represents the mean ± SEM of three separate experiments. (*) $P < 0.05$.

to PMA, may be required to induce the increase in $iPLA_2$ in $P388D_1$ cells. Similarly, Ca^{2+}-mobilizing agents (ionomycin and thapsigargin) did not affect $iPLA_2$ activity in the membranes of $P388D_1$ cells (Fig. 5), while they could induce AA liberation, which was suppressed by pyrrolidine-1 but not BEL. Considering that addition of IgG-coated glass beads to human monocytes induces an increase in $iPLA_2$ activity in the membranes [23], our findings led us to assume that the increase in $iPLA_2$ in membranes depends on intracellular events, which occur upon stimulation with certain particulates that induce phagocytosis. This possibility is also suggested by the finding that cytochalasin D, known to inhibit phagocytosis by macrophages, suppressed zymosan-induced increases in $iPLA_2$ activity and protein levels in the membranes as well as the release of AA (Fig. 6). Furthermore, it has been reported that PKCα participates in the phagocytosis of IgG-opsonized erythrocytes in RAW 264.7 cells [32]. We speculate, therefore, that the rise in $iPLA_2$ in response to zymosan occurs in parallel with PKCα-dependent, cytochalasin D-sensitive events, which are elicited by zymosan but not PMA or Ca^{2+}-mobilizing agents, such as phagocytosis, in $P388D_1$ cells. However, further study is needed to clarify whether or not phagosome formation in response to zymosan is critical for the increase in $iPLA_2$ in the membranes.

It has been reported that $iPLA_2$ does not contribute to the release of AA induced by lipopolysaccharide and platelet-activating factor in $P388D_1$ cells [9], which do not respond to zymosan [33]. In the $P388D_1$ cells, used here (Fig. 2) and

in our previous study [12], which could be activated by zymosan, the release of AA upon the stimulation was suppressed almost completely by MAFP, a dual inhibitor of $cPLA_2$ and $iPLA_2$, and partially (30–40% inhibition) by the $iPLA_2$ inhibitor BEL at a certain concentration which did not affect basal levels of free AA or zymosan-induced diacylglycerol formation. Furthermore, under conditions where the $cPLA_2$ inhibitor pyrrolidine-1 (0.2 or 0.4 μM) almost completely inhibited $cPLA_2$ activity, the zymosan-induced AA liberation was prevented by 60–70%. These findings suggest that $iPLA_2$, in addition to $cPLA_2$, is involved in the zymosan-induced release of AA in $P388D_1$ cells, or that hydrolytic action of $iPLA_2$ might contribute to the $cPLA_2$-mediated AA release. In a certain clone of $P388D_1$ cells, which also respond to zymosan, however, AA liberation induced by zymosan did not change irrespective of the presence of BEL, which significantly increases basal levels of free AA, and inhibits zymosan-induced diacylglycerol formation [10]. Thus, the sensitivity of $P388D_1$ cells to zymosan (agonists) and/or BEL (inhibitors) seems to vary with the properties of the cells and/or experimental conditions employed. Furthermore, the present study showed that while zymosan (0.2–1 mg/mL) dose-dependently induced the release of AA, the degree of the inhibitory effect of BEL on the release decreased with increasing concentrations of zymosan (Fig. 2B). This is presumably explained by the result that a maximal increase in $iPLA_2$ activity in the membranes was observed at 0.2 mg/mL zymosan (Fig. 1B). These findings also suggest that the degree of AA liberation associated with $iPLA_2$ may be dependent on the stimulation.

In conclusion, we suggest that PKCα is involved in the zymosan-induced increase in $iPLA_2$ in the membranes of $P388D_1$ cells, and that the PKCα-dependent increase in $iPLA_2$ probably occurs in association with cytochalasin D-sensitive events.

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