We investigated the possible involvement of group VI Ca\(^{2+}\)-independent phospholipase A\(_{2}\) (iPLA\(_{2}\)) in arachidonic acid (AA) liberation in zymosan-stimulated macrophage-like P388D\(_{1}\) cells. Zymosan-induced AA liberation was markedly inhibited by methyl arachidonoyl fluorophosphonate, a dual inhibitor of group IV cytotoxic phospholipase A\(_{2}\) (cPLA\(_{2}\)) and iPLA\(_{2}\). We found that a relatively specific iPLA\(_{2}\) inhibitor, bromoel lactone, significantly decreased the zymosan-induced AA liberation in parallel with the decrease in iPLA\(_{2}\) activity, without an effect on diacylglycerol formation. Consistent with this, attenuation of iPLA\(_{2}\) activity by a group VI iPLA\(_{2}\) antisense oligonucleotide resulted in a decrease in zymosan-induced prostaglandin E\(_{2}\) generation. These findings suggest that zymosan-induced AA liberation may be, at least in part, mediated by iPLA\(_{2}\). A protein kinase C (PKC) inhibitor diminished zymosan-induced AA liberation, while a PKC activator, phorbol 12-myristate 13-acetate (PMA), enhanced the liberation. Bromoel lactone suppressed the PMA-enhanced AA liberation without any effect on PMA-induced PKC activation. Down-regulation of PKC\(\alpha\) on prolonged exposure to PMA also decreased zymosan-induced AA liberation. Under these conditions, the remaining AA liberation was insensitive to bromoel lactone. Furthermore, the PKC depletion suppressed increases in iPLA\(_{2}\) proteins and the activity in the membrane fraction of zymosan-stimulated cells. In contrast, the zymosan-induced increases in iPLA\(_{2}\) proteins and the activity in the fraction were facilitated by simultaneous addition of PMA. Although intracellular Ca\(^{2+}\) depletion prevented zymosan-induced AA liberation, the translocation of PKC\(\alpha\) to membranes was also inhibited. Taken together, we propose that zymosan may stimulate iPLA\(_{2}\)-mediated AA liberation, probably through a PKC-dependent mechanism.

The liberation of arachidonic acid (AA) upon stimulation is an important event leading to the generation of biologically active lipid mediators, such as prostaglandins and leukotrienes, and is mainly dependent on the hydrolysis of membrane glycerophospholipids catalyzed by phospholipase A\(_{2}\) (PLA\(_{2}\)) (1, 2). Numerous types of mammalian PLA\(_{2}\)s have been identified and classified into several groups (3). The mammalian PLA\(_{2}\)s include at least two types of intracellular PLA\(_{2}\)s, i.e. Ca\(^{2+}\)-dependent and -independent enzymes. It is widely accepted that the intracellular PLA\(_{2}\) responsible for stimulus-induced AA liberation is group IV Ca\(^{2+}\)-dependent cytosolic PLA\(_{2}\) (cPLA\(_{2}\)), which preferentially hydrolyzes glycerophospholipids with an arachidonoyl residue at the sn-2 position (4, 5). The activation of cPLA\(_{2}\) upon stimulation is mediated by Ca\(^{2+}\)-dependent translocation to membranes (6, 7), and by mitogen-activated protein kinase-catalyzed phosphorylation (8, 9). On the other hand, Ca\(^{2+}\)-independent PLA\(_{2}\) (iPLA\(_{2}\)) has been detected in a variety of cells and tissues (reviewed in Ref. 10). Among several types of iPLA\(_{2}\)s, which have been purified, sequenced, and well characterized (11–15), group VI iPLA\(_{2}\)s in mouse macrophage-like P388D\(_{1}\) cells has been proposed to participate in phospholipid remodeling rather than stimulus-induced AA liberation (16, 17).

A recent study involving P388D\(_{1}\) cells has demonstrated that platelet-activating factor (PAF)-induced AA liberation is suppressed by an inhibitor of group IIa secretory PLA\(_{2}\) or cPLA\(_{2}\), but not by a relatively specific iPLA\(_{2}\) inhibitor, bromoel lactone (BEL) (18). Furthermore, blockage of group V secretory PLA\(_{2}\) by antisense oligonucleotides partially inhibits PAF-induced prostaglandin E\(_{2}\) generation (19), while a group VI iPLA\(_{2}\) antisense oligonucleotide has no effect (20). These findings clearly indicate that PAF-induced AA liberation may be mediated by group V secretory PLA\(_{2}\) and cPLA\(_{2}\), but not by iPLA\(_{2}\). Moreover, a recent report suggested that activation of cPLA\(_{2}\) is required for the onset of secretory PLA\(_{2}\)-catalyzed hydrolysis of membrane phospholipids (21). Therefore, in PAF-stimulated P388D\(_{1}\) cells, cPLA\(_{2}\) activation may be a predominant step in the induction of AA liberation. However, while in mouse peritoneal macrophages, zymosan stimulates cPLA\(_{2}\) activity in parallel with AA liberation (22, 23), zymosan-induced AA liberation in mouse macrophage-like RAW 264.7 cells has been shown to be sensitive to BEL (24). Therefore, it is possible that iPLA\(_{2}\), in addition to cPLA\(_{2}\), might be involved in AA liberation induced by zymosan but not by PAF. Similar inhibitory effects of BEL on AA liberation have been reported in several types of cells (25–29). However, BEL is reported to inhibit phosphatidic acid phosphatase, leading to the suppression of stimulus-induced diacylglycerol formation (30, 31). This inhibitory effect may explain the inhibition of AA liberation by BEL, because diacylglycerol may contribute to AA liberation through direct and/or indirect modulation of cPLA\(_{2}\) activity (31) or through the hydrolytic action of lipases toward diacylglycerol (32). It has been shown that zymosan-induced AA liberation is decreased by a protein kinase C (PKC) inhibitor (22, 33, 34) or intracellular Ca\(^{2+}\) depletion (23, 34, 35) in mouse peritoneal macrophages. These findings may support the con-
cept that cPLA₂ contributes to the AA liberation, although it has been suggested that cPLA₂ activation in P388D₁ cells is not mediated by PKC (36). Thus, the participation of iPLA₂ in stimulus-induced AA liberation remains to be elucidated.

In the present study, to clarify the role of iPLA₂ upon stimulation, we explored the possible involvement of group VI iPLA₂ in AA liberation in zymosan-stimulated P388D₁ cells by evaluating the effects of BEL and a group VI iPLA₂ antisense oligonucleotide, which have been shown to attenuate iPLA₂ activity in P388D₁ cells (16, 20). We further examined the role of PKC in iPLA₂-mediated AA liberation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methyl arachidonyl fluorophosphonate, BEL, and a polyclonal antibody against group VI iPLA₂ were obtained from Cayman Chemical (Ann Arbor, MI). GF109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-[1H-indol-3-yl]malemide) and BRC90267 were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). 4-Phorbol 12-myristate 13-acetate (PMA) and 4-phorbol 12,13-diacetate were from Sigma. Zymosan (Saccharomyces cerevisiae) was from Nacarai Tesque (Kyoto, Japan), a monoclonal antibody against PKCα from Transduction Laboratories (Lexington, KY), BAPTA-AM (1, 2-bis(o-aminoethyl)-3-aminopropyl-N,N,N'-tetraacetoxyethylenemine) from Dade Laboratories (Kuwait City, Kuwait), and phorboline from Calbiochem (La Jolla, CA). The antisense (5'-CUC CUU CCC CCG GAA UGG GU) and sense (5'-ACC CAU UCC GGG UGA AGG AGG) oligonucleotides for murine group VI iPLA₂ (20) were obtained from Greiner Japan (Tokyo, Japan). These oligonucleotides contain phosphorothioate linkages. The prostaglandin D₂ assay kit, PKC assay kit, and enhanced chemiluminescence Western blotting detection reagents were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). [³H]Palmitic acid (50 Ci/mmol), [³H]HAA (91.8 Ci/mmol), 1,2-dipalmitoyl-sn-glycero-3-[choline-methyl]-¹⁴C]phosphocholine (159 mCi/mmol), and 1-steaoryl-2-[³H]arachidonyl-sn-glycero-3-phosphocholine (160 Ci/mmol) were from NEN Life Science Products. Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

**Cell Culture**—P388D₁ cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). Only adherent cells were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under humidified air containing 5% CO₂. Cells were plated at 1 × 10⁶ cells or 8 × 10⁵ cells per ml in 100-mm dishes, respectively, and used for experiments the following day. The cells were washed with phosphate-buffered saline containing 0.01% bovine serum albumin and then placed in 1 ml (35-mm dish) or 5 ml (100-mm dish) of RPMI 1640 containing 0.01% bovine serum albumin. The final concentrations of RPMI 1640 and 0.01% bovine serum albumin were 10 μM BEL and then plated in fresh RPMI 1640 containing 0.01% bovine serum albumin and 10 μM BEL. 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) (200:200, 1:1/v/v). Lipids in the medium and lysate were extracted and separated by thin layer chromatography on a Silica Gel G plate with the following developing systems: for the analysis of arachidonic acid and diarylglucol, petroleum ether/diethyl ether/acetonic acid (40:40:1, 1:1/v/v/v) for the first dimension and chloroform/methanol/acidic acid/H₂O (60:30:8:4, 1:1/v/v/v) for the second dimension. The area corresponding to each lipid was scraped off, and the radioactivity was determined by liquid scintillation counting.

**Measurement of Lipid Metabolism**—P388D₁ cells were plated in 35-mm dishes at 1 × 10⁶ cells or 3 ml of RPMI 1640 containing 0.01% bovine serum albumin and then placed in 1 ml of RPMI 1640 containing 0.01% bovine serum albumin. The cells were treated with BEL and then washed three times after treatment with BEL and then placed in fresh RPMI 1640 containing 0.01% bovine serum albumin and 10 μM BEL. 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 (200:200:1, 1:1/v/v). Lipids in the medium and lysate were extracted and separated by thin layer chromatography on a Silica Gel G plate with the following developing systems: for the analysis of arachidonic acid and diarylglucol, petroleum ether/diethyl ether/acetonic acid (40:40:1, 1:1/v/v/v) for the first dimension and chloroform/methanol/acidic acid/H₂O (60:30:8:4, 1:1/v/v/v) for the second dimension. The area corresponding to each lipid was scraped off, and the radioactivity was determined by liquid scintillation counting.

**Measurement of Prostaglandin D₂ Generation by Exogenous AA**—P388D₁ cells were treated with the antisense and sense oligonucleotides as above, and then placed in 1 ml of RPMI 1640 containing 0.01% bovine serum albumin. The cells were further incubated with a mixture of [³H]AA (0.5 μCi/ml) and the unlabeled compound (5 μM) at 37 °C for 15 min. Lipids in the medium and cells were separated by thin layer chromatography using an upper phase of ethyl acetate/isooctane/acetonic acid/H₂O (9:5:2:10, 1:1/v/v/v) as the development system. The area corresponding to prostaglandin D₂ was scraped off, and the radioactivity was determined by liquid scintillation counting.

**Measurement of PKC Activity**—P388D₁ cells, in 100-mm dishes were treated with BEL, washed three times, and then stimulated with PMA. The cells were scraped off, washed, and then sonicated in a buffer consisting of 340 mM sucrose, 2 mM EGTA, 100 μM leupeptin, 100 μM P-(aminophenyl)methanesulfonil fluoride, and 10 mM Heps (pH 7.4). After the lysate had been centrifuged at 100,000 × g for 30 min at 4 °C, the resulting supernatant was treated with 5 mM dithiothreitol. The activity of cPLA₂ in the supernatant (20 μg of protein) was determined by incubation with a mixture of 1-steaoryl-2-[³H]arachidonyl-sn-glycero-3-phosphocholine and the unlabeled compound (25 Ci/mmol, 10 μM) at 37 °C for 10 min in the presence of 5 mM CaCl₂ and 100 μM Tris-HCl (pH 8.5) in a final volume of 200 μl. After lipid extraction of [³H]AA liberated was determined by thin layer chromatography using petroleum ether/diethyl ether/acetonic acid (40:40:1, 1:1/v/v/v) as the development system. The radioactivity was determined, and the enzyme activity was calculated.

**Measurement of Lipid Metabolism**—P388D₁ cells were plated in 35-mm dishes at 1 × 10⁶ cells or 3 ml of RPMI 1640 containing 0.01% bovine serum albumin and then placed in 1 ml of RPMI 1640 containing 0.01% bovine serum albumin. The cells were treated with BEL and then washed three times after treatment with BEL and then placed in fresh RPMI 1640 containing 0.01% bovine serum albumin and 10 μM BEL. 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 (200:200:1, 1:1/v/v). Lipids in the medium and lysate were extracted and separated by thin layer chromatography on a Silica Gel G plate with the following developing systems: for the analysis of arachidonic acid and diarylglucol, petroleum ether/diethyl ether/acetonic acid (40:40:1, 1:1/v/v/v) for the first dimension and chloroform/methanol/acidic acid/H₂O (60:30:8:4, 1:1/v/v/v) for the second dimension. The area corresponding to each lipid was scraped off, and the radioactivity was determined by liquid scintillation counting.

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**Measurement of PKC Activity**—P388D₁ cells, in 100-mm dishes were treated with BEL, washed three times, and then stimulated with PMA. The cells were scraped off, washed, and then sonicated in a buffer consisting of 340 mM sucrose, 2 mM EGTA, 100 μM leupeptin, 100 μM P-(aminophenyl)methanesulfonil fluoride, and 10 mM Heps (pH 7.4). After the lysate had been centrifuged at 500 × g for 5 min at 4 °C, the resulting supernatant was centrifuged at 100,000 × g for 30 min to separate soluble and membrane fractions. The activity of PKC in the soluble (20 μg protein) and membrane (20 μg protein) fractions was determined using a commercial assay kit.
The iPLA2 activity in the lysate of BEL-treated P388D1 cells was inhibited in a BEL dose-dependent manner (Fig. 2A). P388D1 cells were treated with various concentrations of BEL for 15 min, and then washed. The iPLA2 activity in the cell lysate was determined as described under “Experimental Procedures.” Each point represents the mean ± S.E. of three separate experiments.

RESULTS

Effect of an iPLA2 Inhibitor on Zymosan-stimulated Lipid Metabolism—As shown in Fig. 1, stimulation of [3H]AA-labeled P388D1 cells with 1 mg/ml zymosan elicited time-dependent AA liberation, of which the time course was similar to that observed in mouse peritoneal macrophages (23). The zymosan-induced AA liberation was almost completely inhibited when the cells were treated with 30 μM methyl arachidonoyl fluorophosphonate, an inhibitor of cPLA2 and iPLA2 (40), suggesting that the inhibition was only about 20%. In addition, BEL at 2 or 5 μM did not significantly increase the zymosan-induced phosphatic acid formation in [3H]AA- or [3H]palmitic acid-labeled P388D1 cells (data not shown). Thus, under these conditions, BEL (up to 5 μM) decreased zymosan-induced AA liberation without any effect on diacylglycerol or phosphatic acid formation. Previously, we showed that antigen-induced AA liberation is mediated by diacylglycerol lipase in rat peritoneal mast cells (32). However, such a mechanism underlying the liberation is ruled out by the present work, since inhibition with methyl arachidonoyl fluorophosphonate did not affect zymosan-induced AA liberation in P388D1 cells (data not shown), in agreement with a previous result obtained with mouse peritoneal macrophages (33).

Effect of an iPLA2 Antisense Oligonucleotide—We also examined the effect of a group VI iPLA2 antisense oligonucleotide, which has been shown to attenuate iPLA2 activity in P388D1 cells (20), and obtained the results shown in Fig. 3. Under our experimental conditions, an approximately 50% decrease in iPLA2 activity was observed in P388D1 cells exposed to 1 μM antisense oligonucleotide compared with in control or sense-treated cells, as shown in Fig. 3A. Furthermore, Fig. 3B reveals that 1 μM antisense oligonucleotide was found to decrease 1 mg/ml zymosan-induced prostaglandin D2 generation by about 45%, whereas 1 μM sense oligonucleotide had no effect on the generation. However, the antisense oligonucleotide did not suppress prostaglandin D2 generation induced by ionomycin (0.5 μM, 10 min of stimulation), a Ca2+-ionophore (data not shown). We further examined the effect of the antisense oligonucleotide on the conversion of exogenous AA to prostaglandin D2, but the generation of prostaglandin D2 was not affected by the antisense oligonucleotide (37.4 pmol/dish for controls and 34.1 pmol/dish for antisense treatment, means of two experiments). These results suggest that zymosan-induced AA liberation in P388D1 cells may be, at least in part, mediated by group VI iPLA2.

Involvement of PKC in iPLA2-mediated AA Liberation—Several studies involving mouse peritoneal macrophages have demonstrated that zymosan-induced AA liberation is sensitive
to PKC inhibitors such as GF109203X (22, 33, 34). We also confirmed that GF109203X attenuated 1 mg/ml zymosan-induced AA liberation dose-dependently in P88D1 cells (Fig. 4A). Furthermore, as shown in Fig. 4B, when the cells were stimulated with 1 mg/ml zymosan and 5–20 nM PMA, the synergistic enhancement of zymosan-induced AA liberation by PMA was observed. Stimulation with 20 nM PMA caused about 2-fold enhancement of the AA liberation induced by zymosan alone, although PMA did not cause AA liberation by itself. However, 20 nM 4α-phorbol 12,13-didecanoate, an inactive phorbol ester, had no effect (data not shown). In addition, the PMA-enhanced AA liberation was completely inhibited by pretreatment with 5 μM GF109203X (Fig. 4B). Under these conditions, 20 nM PMA did not exhibit a significant effect on zymosan-induced diacylglycerol or phosphatidic acid formation (data not shown). These findings indicate that PKC may be involved in zymosan-induced AA liberation in P88D1 cells.

In order to evaluate the involvement of PKC in iPLA2-mediated AA liberation, we examined the effect of BEL on the PMA-enhanced AA liberation. As shown in Fig. 5, BEL at 2 or 5 μM markedly suppressed the enhancement by 20 nM PMA of 1 mg/ml zymosan-induced AA liberation. At 5 μM BEL, the PMA-enhanced AA liberation was reduced to the level of BEL-insensitive AA liberation in response to zymosan alone, suggesting that iPLA2 may contribute to the PKC-dependent AA liberation in response to zymosan. Furthermore, we examined whether or not BEL affects PMA-induced PKC activation, the results being shown in Fig. 6. Stimulation of P88D1 cells with 20 nM PMA resulted in an increase in PKC activity in the membrane fraction, with a concomitant decrease in the activity in the cytosol fraction. However, pretreatment of the cells with 20 μM BEL did not affect the exchange of PKC activity between the fractions. In addition, even on treatment of the membrane fraction of PMA-treated cells with 20 μM BEL, the PKC activity in the fraction was not affected (data not shown).

In mouse peritoneal macrophages, zymosan-induced AA liberation has been suggested to be mediated by PKCα (33). To study the involvement of PKCα in AA liberation in P88D1 cells, we examined whether or not down-regulation of PKCα affects zymosan-induced AA liberation (Fig. 7). The exposure of the cells to PMA (100 or 200 nM) for 10 h, which led to a decrease in the amount of PKCα (Fig. 7A), caused the suppression of 1 mg/ml zymosan-induced AA liberation by about 50% (Fig. 7B). Under these conditions, 5 μM BEL did not further decrease the remaining AA liberation in PMA-pretreated cells, although it attenuated zymosan-induced liberation in control cells. Furthermore, as shown in Fig. 7C, we confirmed that 1 mg/ml zymosan caused translocation of PKCa to the membrane fraction from the cytosol, and that 5 μM BEL did not exert any effect on the translocation. However, translocation of PKCaβ to the membranes was not observed in zymosan-stimulated P88D1 cells (data not shown), as already reported elsewhere (36). These results suggest that iPLA2-mediated AA liberation may occur in parallel with PKCa activation.

To examine the mechanism underlying PKC-dependent regulation of iPLA2-mediated AA liberation, we determined iPLA2 activity in PKC-depleted P88D1 cells upon stimulation with zymosan. As shown in Fig. 8A, stimulation with 1 mg/ml zymosan resulted in an increase in iPLA2 activity in the membrane fraction with a concomitant decrease in the activity in
P388D1 cells were treated with (closed columns) or without (open columns) 20 μM BEL for 15 min, and then washed. The cells were stimulated with or without 20 nM PMA for 30 min. The PKC activity in the cytosol fraction. Pretreatment with 100 nM PMA for 10 h significantly suppressed the zymosan-induced increase in iPLA2 activity (Fig. 8B). Under these conditions, zymosan-induced iPLA2 activity (Fig. 8C) or without (open columns) 1 mg/ml zymosan for 30 min. The PKC activity in the cytosol fraction. Pretreatment with 100 nM PMA for 10 h, and then washed. The cells were stimulated with or without 5 μM BEL for 15 min. The cells were washed, and then stimulated with or without 1 mg/ml zymosan for 1 h. [3H]AA liberated was determined as described under “Experimental Procedures.” Each value represents the mean ± S.E. of three separate experiments.

The results in Fig. 10 suggest that the inhibition of AA liberation by intracellular Ca²⁺ depletion may be ascribed to, at least in part, suppression of PKC activation.

**DISCUSSION**

In the present study, we explored the possible involvement of iPLA2 in stimulus-induced AA liberation using zymosan-stimulated mouse macrophage-like P388D1 cells, which possess group VI iPLA2, one of the purified and sequenced iPLA2s (11, 13). The group VI iPLA2 in P388D1 cells has been shown to be inhibited by...
BEL, a relatively selective iPLA₂ inhibitor (42). Based on the inhibitory effect of BEL on the incorporation of arachidonic acid into phospholipids (16), it has been recognized that group VI iPLA₂ participates in phospholipid remodeling. Furthermore, in PAF-stimulated P388D₁ cells, AA liberation is partially suppressed by a secretory PLA₂ inhibitor or methyl arachidonyl fluorophosphonate, a dual inhibitor of cPLA₂ and iPLA₂, but not by BEL (18), suggesting that PAF-induced AA liberation is mediated by cPLA₂ and secretory PLA₂, but not by group VI iPLA₂. However, as shown in this study, we found that BEL significantly decreased the zymosan-induced AA liberation under the conditions where BEL actually suppressed iPLA₂ activity but not cPLA₂ activity in P388D₁ cells (Fig. 2), suggesting the involvement of group VI iPLA₂ in the response to zymosan.

A recent report demonstrated that BEL inhibits phosphatidic acid phosphatase activity, resulting in suppression of the conversion of phosphatidic acid to diacylglycerol in P388D₁ cells (30). Furthermore, in human amnionic WISH cells, the inhibition by BEL of stimulus-induced AA liberation has been suggested to be due to the impairment of diacylglycerol-mediated cPLA₂ regulation through the suppression of diacylglycerol formation (31). Although a number of studies have also demonstrated that stimulus-induced AA liberation is inhibited by BEL in a variety of cells including zymosan-stimulated macrophage-like RAW 264.7 cells (24–29), it is unclear whether or not BEL affects diacylglycerol liberation in these cells. However, the present study showed that 2–5 μM BEL suppressed zymosan-induced AA liberation by about 45%, without a significant change in diacylglycerol formation (Fig. 2) or phosphatidic acid formation (data not shown). Therefore, we suggest that the attenuation by BEL of zymosan-induced AA liberation may be due to inhibition of group VI iPLA₂ rather than phosphatidic acid phosphatase.

We further found that a group VI iPLA₂ antisense oligonucleotide decreased iPLA₂ activity and zymosan-induced prostaglandin D₂ generation, while a sense oligonucleotide had no effect (Fig. 3). Furthermore, the antisense oligonucleotide did not affect Ca²⁺ ionophore-induced prostaglandin D₂ generation (data not shown) or the conversion of exogenous AA to prostaglandin D₂. These results appear to indicate that attenuation by the antisense oligonucleotide of zymosan-induced prostaglandin D₂ generation is due to the inhibition of iPLA₂ activity but not cPLA₂ or cyclooxygenase activity. In contrast to zymosan, a recent report has shown that the group VI iPLA₂ antisense oligonucleotide does not affect PAF-induced AA liberation despite the attenuation of iPLA₂ activity in P388D₁ cells (20). Although it is unclear whether or not the different effects of the antisense oligonucleotide on zymosan- and PAF-induced AA liberation are due to differences in the signaling pathways responsible for zymosan and PAF, we propose that group VI iPLA₂ may be, at least in part, involved in zymosan-induced AA liberation in P388D₁ cells.

In mouse peritoneal macrophages, zymosan-induced AA liberation has been shown to occur in parallel with cPLA₂ activation (22, 23). Furthermore, a PKC inhibitor has been shown to suppress the AA liberation with concomitant decreases in mitogen-activated protein kinase and cPLA₂ activities (22). These findings suggest that cPLA₂-mediated AA liberation may be regulated by PKC-dependent activation of mitogen-activated protein kinase in mouse peritoneal macrophages. We showed here that GF109203X, a PKC inhibitor, decreased zymosan-induced AA liberation, while PMA, a PKC activator, potentiated the AA liberation in P388D₁ cells (Fig. 4), suggesting that PKC may be involved in zymosan-induced AA liberation. However, it is possible that the PKC-dependent AA liberation is not mediated by cPLA₂, since it has been demonstrated that PKC does not participate in cPLA₂ regulation in P388D₁ cells (36). In this study, BEL partially inhibited zymosan-induced AA liberation (Fig. 2), while methyl arachidonyl fluorophosphonate did so almost com-

![Fig. 9. Effect of PMA on zymosan-induced iPLA₂ translocation to the membrane fraction. A, P388D₁ cells were stimulated with 1 mg/ml zymosan, 100 nM PMA, zymosan plus PMA, or the vehicle for 30 min. The iPLA₂ activity in the membrane fraction was determined. Each value represents the mean ± S.E. of three separate experiments. B, P388D₁ cells were stimulated as in A. The membrane fraction (20 μg) of the cells was subjected to the detection of iPLA₂ as described under “Experimental Procedures.” The results are representative of three experiments.](http://www.jbc.org/)
demonstrated that zymosan increased iPLA2 activity in the presence of PKCα on prolonged exposure to PMA reduced zymosan-induced AA liberation, and further that the treatment of PKC-depleted cells with PMA did not inhibit the remaining, PKC-independent AA liberation (Fig. 7). Thus, BEL seems to affect only PKC-dependent AA liberation in response to zymosan. In addition, we confirmed that BEL had no effect on zymosan-induced PKCα translocation (Fig. 7). Therefore, it is conceivable that iPLA2-mediated AA liberation may occur downstream of PKC activation in P388D1 cells. We further demonstrated that zymosan increased iPLA2 activity in the membrane fraction with a decrease in the activity in the cytosolic fraction (Fig. 8). Of interest was the finding that the depletion of PKCα inhibited zymosan-induced increases in iPLA2 activity and the enzyme proteins in the membrane fraction (Fig. 8). Moreover, the increases in iPLA2 proteins and the activity in response to zymosan were potentiated by simultaneous stimulation with PMA (Fig. 9), this being consistent with the result that PMA enhanced BEL-sensitive AA liberation, and exposure to PMA reduced zymosan-induced AA liberation, and further that the treatment of PKC-depleted cells with PMA did not inhibit the remaining, PKC-independent AA liberation (Fig. 7). The present study demonstrated that BEL suppressed PMA-enhanced AA liberation in response to zymosan without any effect on PMA-induced PKC activation (Figs. 5 and 6), suggesting the possible involvement of iPLA2 in PKC-dependent AA liberation. We also showed that the depletion of PKCα on prolonged exposure to PMA reduced zymosan-induced AA liberation, and further that the treatment of PKC-depleted cells with PMA did not inhibit the remaining, PKC-independent AA liberation (Fig. 7). Thus, BEL seems to affect only PKC-dependent AA liberation in response to zymosan.

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Involvement of Group VI Ca$^{2+}$-independent Phospholipase A$_2$ in Protein Kinase C-dependent Arachidonic Acid Liberation in Zymosan-stimulated Macrophage-like P388D$_1$ Cells

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