Inhibition of Ca^{2+}-independent phospholipase A_2 by bromoenol lactone attenuates prostaglandin generation induced by interleukin-1β and dibutyryl cAMP in rat mesangial cells

Satoshi Akiba*, Misako Hayama, Takashi Sato

Department of Pathological Biochemistry, Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan

Received 10 August 1998; received in revised form 14 September 1998

Abstract Cytokine-induced prostaglandin generation in rat mesangial cells has been suggested to be dependent on the expression of secretory phospholipase A_2 ($sPLA_2$). In the present study, we investigated the possible involvement of Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) in the generation. The results showed that bromoenol lactone, a relatively selective $iPLA_2$ inhibitor, significantly attenuated prostaglandin E_2 generation induced by interleukin-1β and dibutyryl cAMP in parallel with the inhibition of $iPLA_2$ activity. However, the inhibitor did not affect $sPLA_2$ release upon stimulation, activities of $sPLA_2$ or cytosolic phospholipase A_2, or Ca^{2+} ionophore-induced arachidonic acid liberation. These results suggest that prostaglandin E_2 generation upon stimulation may be partially mediated by $iPLA_2$ in addition to $sPLA_2$.

© 1998 Federation of European Biochemical Societies.

Key words: Phospholipase A_2; Prostaglandin; Bromoenol lactone; Rat mesangial cell

1. Introduction

Phospholipase A_2 (PLA_2) plays a central role in providing arachidonic acid, a precursor for prostaglandins and leukotrienes, through the hydrolysis of membrane glycerophospholipids [1]. Among numerous types of mammalian PLA_2s, stimulus-induced arachidonic acid liberation is thought to be mediated by group IIA or V secretory PLA_2 ($sPLA_2$), or by group IV cytosolic PLA_2 ($cPLA_2$) [2–4]. The $sPLA_2$ is released from the intracellular store upon stimulation or through the expression of the $sPLA_2$ protein, and subsequently hydrolyzes membrane phospholipids [2]. On the other hand, the hydrolytic action of $cPLA_2$ is regulated by Ca^{2+}-dependent translocation to membranes and by mitogen-activated protein kinase-catalyzed phosphorylation with an increase in $cPLA_2$ activity [4]. In contrast to these PLA_2s, group VI Ca^{2+}-independent PLA_2 ($iPLA_2$) has been suggested to contribute to phospholipid remodeling [5]. However, the role of $iPLA_2$ in stimulus-induced arachidonic acid liberation remains to be elucidated.

Arachidonic acid liberation induced by Ca^{2+}-mobilizing agonists like platelet-activating factor rapidly plateaus and occurs in parallel with transient intracellular Ca^{2+} elevation and $cPLA_2$ translocation [6,7]. In contrast, arachidonic acid liberation induced by proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor, continues for a relatively long time and occurs in parallel with the expression of $sPLA_2$ [8,9]. The possible contribution of $sPLA_2$ to the continual arachidonic acid liberation has been well investigated with rat mesangial cells upon stimulation with cytokines and/or cAMP-elevating reagents [10–13]. Stimulation of the cells with interleukin-1β has been shown to drastically increase prostaglandin E_2 (PGE_2) and $sPLA_2$ 16–24 h after stimulation [10]. An inhibitor of or an antibody against $sPLA_2$ suppresses the PGE_2 generation by 50–70% [11]. The remaining PGE_2 generation might be dependent on the hydrolytic action of $cPLA_2$, because an increase in $cPLA_2$ activity has also been observed after the stimulation period [12]. Furthermore, a recent report has shown that addition of $sPLA_2$ to the cells induces the phosphorylation of $cPLA_2$, probably through protein kinase C activation [13]. However, it is unclear whether or not interleukin-1β sustains the intracellular Ca^{2+} elevation needed for $cPLA_2$ activation during such a long period. Therefore, we assumed that $iPLA_2$ may also be involved in the cytokine-induced PGE_2 generation. In the present study, to examine this possibility, we studied the effect of bromoenol lactone (BEL), a relatively specific $iPLA_2$ inhibitor [14,15], on PGE_2 generation in rat mesangial cells stimulated with interleukin-1β and dibutyryl cAMP.

2. Materials and methods

2.1. Materials

Interleukin-1β was obtained from Collaborative Biomedical Products (Bedford, MA, USA). Dibutyryl cAMP was from Sigma (St. Louis, MO, USA), BEL from Cayman Chemical (Ann Arbor, MI, USA), and ionomycin from Calbiochem (La Jolla, CA, USA). [^{3}H]Arachidonic acid (100 Ci/mmol), 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine (55 mCi/mmol), 1,2-dipalmitoyl-*sn*-glycero-3-[choline-*methyl*-^{14}C]phosphocholine (159 mCi/mmol), and 1-stearoyl-2-[^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine (160 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA).

2.2. Cell culture

Glomerular mesangial cells were obtained from a culture of glomeruli isolated from Sprague-Dawley rats, and grown in RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite [16]. Confluent mesangial cells (passages 3–8) were made quiescent by incubating them in RPMI 1640 containing 0.1 mg/ml bovine serum albumin for 24 h, and then used for the following experiments.

2.3. Measurement of PGE_2 generation and arachidonic acid liberation

The quiescent cells in 35 mm dishes were incubated with [^{3}H]arachidonic acid (0.5 μCi/ml) for 24 h. The labeled cells were washed and incubated with BEL at 37°C for 1 h in 1 ml of RPMI 1640 containing 0.1 mg/ml bovine serum albumin. After the medium had been removed, the cells were stimulated in fresh medium. Lipids

*Corresponding author. Fax: (81) (75) 595-4759.
E-mail: akiba@mb.kyoto-phu.ac.jp

Abbreviations: BEL, bromoenol lactone; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; $sPLA_2$, secretory PLA_2; PGE_2, prostaglandin E_2

0014-5793/98/$19.00 © 1998 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(98)01236-8

in the medium and the cells were extracted [16], and separated by thin-layer chromatography on a silica gel G plate with the following development systems: for the analysis of arachidonic acid, petroleum ether/diethyl ether/acetic acid (40:40:1, v/v/v); and for the analysis of PGE_2, an upper phase of ethyl acetate/isooctane/acetic acid/H_2O (9:5:2:10, v/v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting.

2.4. Assay for $sPLA_2$ activity

The quiescent cells in 35 mm dishes were treated with BEL, and then the medium was removed. The cells were stimulated with interleukin-1β and dibutyryl cAMP in 1 ml of fresh medium. After the medium had been centrifuged, the supernatant (50 μl), as an enzyme source, was incubated with 2 μM 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine at 37°C for 1 h in the presence of 5 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.5). [^{14}C]Linoleic acid liberated was determined as described previously [17].

2.5. Assays for $iPLA_2$ and $cPLA_2$ activity

The quiescent cells in 100 mm dishes were treated with BEL at 37°C for 1 h. The cells were scraped off and washed with 340 mM sucrose, 2 mM EGTA, 100 μM leupeptin, 100 μM *p*-(amidinophenyl)methanesulfonyl fluoride, and 10 mM HEPES (pH 7.4). After lysis of the cells by sonication, the lysate was centrifuged at 100 000×*g* for 30 min. The supernatant (10 μg protein) obtained was treated with 5 mM dithiothreitol at 37°C for 10 min to inhibit $sPLA_2$ activity. For the $iPLA_2$ assay, the supernatant was incubated with a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-[choline-*methyl*-^{14}C]phosphocholine and the unlabeled compound (2.5 mCi/mmol, 100 μM) at 37°C for 1 h in the presence of 5 mM EDTA, 800 μM Triton X-100 and 50 mM HEPES (pH 7.5). For the $cPLA_2$ assay, the supernatant was incubated with a mixture of 1-stearoyl-2-[^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine and the unlabeled compound (0.5 Ci/mol, 2 μM) at 37°C for 1 h in the presence of 5 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.5). After lipid extraction, [^{14}C]lysophosphatidylcholine generated or [^{3}H]arachidonic acid liberated was determined by thin-layer chromatography using chloroform/methanol/H_2O (65:35:6, v/v/v) or petroleum ether/diethyl ether/acetic acid (40:40:1, v/v/v) as the development system, respectively. The radioactivity was determined and the enzyme activity was calculated.

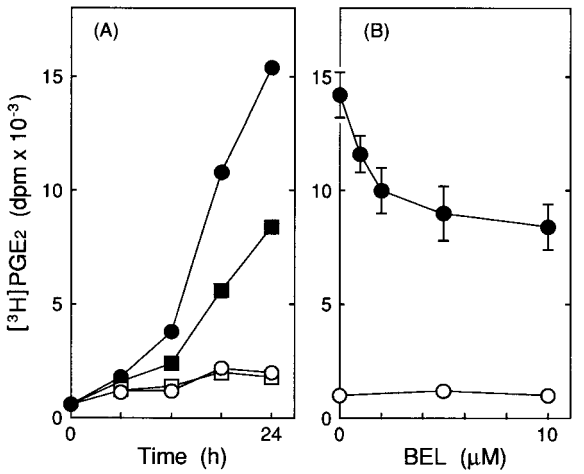

Fig. 1. Effect of BEL on PGE_2 generation induced by interleukin-1β and dibutyryl cAMP. A: [^{3}H]Arachidonic acid-labeled mesangial cells were treated with (squares) or without (circles) 5 μM BEL for 1 h. The cells in fresh medium were stimulated with (closed symbols) or without (open symbols) 1 nM interleukin-1β and 1 mM dibutyryl cAMP for the indicated periods. B: [^{3}H]Arachidonic acid-labeled mesangial cells were treated with various concentrations of BEL for 1 h. The cells in fresh medium were stimulated with (closed symbols) or without (open symbols) 1 nM interleukin-1β and 1 mM dibutyryl cAMP for 24 h. [^{3}H]PGE_2 generated was determined as described in Section 2.3. Data represent the means (±S.E.M.) for two or three separate experiments performed in duplicate.

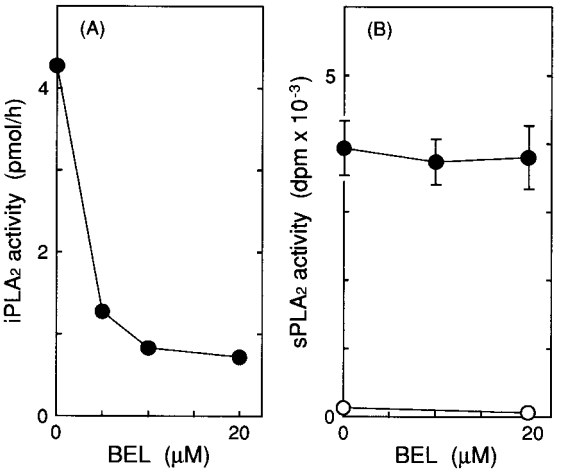

Fig. 2. Effect of BEL on $iPLA_2$ activity (A) and $sPLA_2$ release (B). A: Mesangial cells were treated with various concentrations of BEL for 1 h. The activity of $iPLA_2$ in the supernatant derived from the cells was determined as described in Section 2.5. B: Mesangial cells were treated with BEL and then stimulated as in Fig. 1B. The activity of $sPLA_2$ in the extracellular medium was determined as described in Section 2.4. Data represent the means (±S.E.M.) for two or three separate experiments performed in duplicate.

3. Results

As shown in Fig. 1A, stimulation of rat mesangial cells with 1 nM interleukin-1β and 1 mM dibutyryl cAMP induced PGE_2 generation time-dependently, which had progressively increased by 24 h after the stimulation. The increase in PGE_2 was found to be significantly attenuated by pretreatment with 5 μM BEL, a relatively selective $iPLA_2$ inhibitor. The inhibitory effect of BEL occurred in a dose-dependent manner, and 5 μM BEL suppressed the PGE_2 generation by about 40% (Fig. 1B). To determine whether or not this inhibitory effect of BEL is due to suppression of cyclooxygenase activity, we examined the effect of BEL on the conversion of exogenous arachidonic acid to PGE_2. The addition of [^{3}H]arachidonic acid to mesangial cells resulted in an increase in [^{3}H]PGE_2. However, 10 μM BEL did not affect the increase in PGE_2 caused by exogenous arachidonic acid in stimulated or intact cells (data not shown).

Fig. 2A illustrates the inhibitory effect of BEL on $iPLA_2$ activity in rat mesangial cells. When the cells were treated with BEL (5–20 μM), $iPLA_2$ activity in the supernatant derived from the cell lysate was markedly inhibited. Under our experimental conditions, the addition of $CaCl_2$ to the assay system for $iPLA_2$ activity did not facilitate the increase in lysophosphatidylcholine, a product generated by PLA_2, indicating that the increase in the product in our assay system reflects $iPLA_2$ activity. Thus, we confirmed that BEL actually suppressed $iPLA_2$ activity. In rat mesangial cells, PGE_2 generation induced by cytokines has been suggested to be dependent on the expression of $sPLA_2$ [10,11]. Therefore, we determined the effect of BEL on $sPLA_2$ release upon stimulation. As shown in Fig. 2B, however, treatment of the cells with BEL (10, 20 μM) did not affect an increase in $sPLA_2$ activity in the extracellular medium in response to 1 nM interleukin-1β and 1 mM dibutyryl cAMP. Furthermore, even though the medium of the stimulated cells was treated with 20 μM BEL for 30 min, $sPLA_2$ activity in the medium was not affected

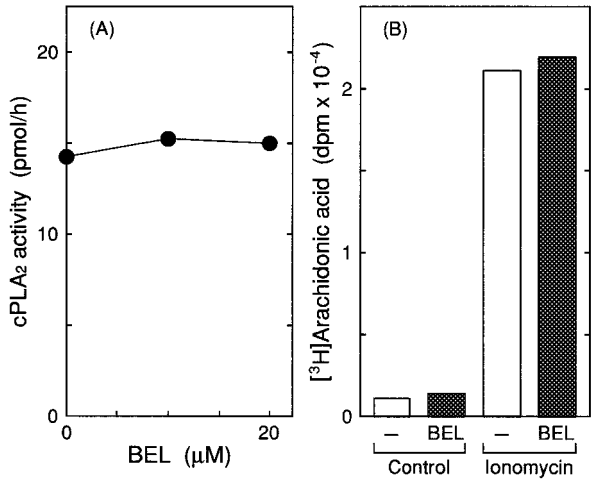

Fig. 3. Effect of BEL on $cPLA_2$ activity (A) and ionomycin-induced arachidonic acid liberation (B). A: Mesangial cells were treated with BEL for 1 h. The activity of $cPLA_2$ in the supernatant derived from the cells was determined as described in Section 2.5. B: [^{3}H]Arachidonic acid-labeled mesangial cells were treated with or without 10 μM BEL for 1 h, and then stimulated with or without 2 μM ionomycin for 15 min. [^{3}H]Arachidonic acid liberated was determined as described in Section 2.3. Data represent the means for two separate experiments performed in duplicate.

(4327 ± 173 dpm and 4453 ± 254 dpm for the activities in the untreated and BEL-treated medium, respectively, mean ± S.E.M., $n = 3$).

Recently, exogenous $sPLA_2$ was shown to induce the phosphorylation of $cPLA_2$ [13], suggesting the involvement of $cPLA_2$ in $sPLA_2$-dependent PGE_2 generation. Therefore, the effect of BEL on $cPLA_2$ activity was determined. However, treatment with BEL (10, 20 μM) had no effect on $cPLA_2$ activity in rat mesangial cells (Fig. 3A). Furthermore, we showed that 10 μM BEL did not affect ionomycin-induced arachidonic acid liberation, which is presumably catalyzed by $cPLA_2$ (Fig. 3B).

4. Discussion

In order to investigate the possible involvement of $iPLA_2$ in stimulus-induced arachidonic acid liberation, we examined whether or not BEL, a relatively selective $iPLA_2$ inhibitor, affects PGE_2 generation in rat mesangial cells stimulated with interleukin-1β and dibutyryl cAMP. In the present study, we found that BEL decreased PGE_2 generation upon the stimulation by about 40% (Fig. 1) in parallel with inhibition of $iPLA_2$ activity (Fig. 2A). This suggests that BEL-sensitive $iPLA_2$ may be partially implicated in the PGE_2 generation. The stimulation of rat mesangial cells with interleukin-1β and/or cAMP-elevating reagents has been shown to induce PGE_2 generation in parallel with the expression of $sPLA_2$ [10]. Furthermore, an inhibitor of or an antibody against $sPLA_2$ has been reported to attenuate the PGE_2 generation by 50–70% [11]. These findings clearly suggest the contribution of $sPLA_2$ to the generation. We further demonstrated that BEL did not affect $sPLA_2$ release (Fig. 2B) or its activity, indicating that the inhibition of PGE_2 generation by BEL does not result from impairment of intracellular signaling leading to the $sPLA_2$ expression or the hydrolytic action of $sPLA_2$ released. Therefore, it is possible that PGE_2 generation induced by interleukin-1β and dibutyryl cAMP is mediated by $iPLA_2$ in addition to $sPLA_2$.

Previously, it was shown that interleukin-1β induces an increase in $cPLA_2$ activity at the time when $sPLA_2$ expression is sufficiently observed [12]. A recent report has shown that exogenous $sPLA_2$ induces the phosphorylation of $cPLA_2$ through protein kinase C activation [13]. In that report, therefore, it was proposed that interleukin-1β-induced PGE_2 generation may be mediated by $cPLA_2$, which is activated by $sPLA_2$ released. Although it is unclear whether or not $sPLA_2$-activated $cPLA_2$ actually participates in PGE_2 generation induced by interleukin-1β and dibutyryl cAMP, the present study demonstrated that BEL had no effect on $cPLA_2$ activity or ionomycin-induced arachidonic acid liberation (Fig. 3). Therefore, based on the present results, we suggest that BEL attenuates PGE_2 generation induced by interleukin-1β and dibutyryl cAMP probably through inhibition of $iPLA_2$ activity without suppression of the actions of $sPLA_2$ and $cPLA_2$.

Several types of $iPLA_2$s have been detected in a variety of cells and tissues [18]. Among the $iPLA_2$s, 80 kDa group VI $iPLA_2$ has been purified, sequenced, and well characterized [19,20]. It has been proposed that group VI $iPLA_2$ does not participate in stimulus-induced arachidonic acid liberation, because BEL or an antisense oligonucleotide for the $iPLA_2$ does not affect platelet-activating factor-induced arachidonic acid liberation [21,22]. In contrast, the results we obtained here suggest the involvement of $iPLA_2$ in arachidonic acid liberation upon stimulation, although it is unclear whether or not $iPLA_2$ detected in rat mesangial cells is identical to group VI $iPLA_2$. This discrepancy may be due to differences in the types of agonists used. Platelet-activating factor is one of the Ca^{2+}-mobilizing agonists, while interleukin-1β and dibutyryl cAMP are not. Recent studies with $iPLA_2$ inhibitors including BEL have also suggested the contribution of $iPLA_2$ to stimulus-induced arachidonic acid liberation [23,24]. In Fas-stimulated human leukemic U937 cells, however, $iPLA_2$ activity did not change upon the stimulation [24]. We also observed no change in $iPLA_2$ activity in stimulated rat mesangial cells (data not shown). Thus, the mechanism underlying acceleration of the $iPLA_2$ action has not been elucidated at present. Further studies will focus on the regulation of the hydrolytic action of $iPLA_2$ upon stimulation.

In conclusion, based on the present results, we propose that BEL-sensitive $iPLA_2$ may be partially involved in PGE_2 generation induced by interleukin-1β and dibutyryl cAMP in rat mesangial cells.

References

[1] Waite, M. (1985) J. Lipid Res. 26, 1379–1388.
[2] Kudo, I., Murakami, M., Hara, S. and Inoue, K. (1993) Biochim. Biophys. Acta 1170, 217–231.
[3] Dennis, E.A. (1997) Trends Biochem. Sci. 22, 1–2.
[4] Leslie, C.C. (1997) J. Biol. Chem. 272, 16709–16712.
[5] Balsinde, J. and Dennis, E.A. (1997) J. Biol. Chem. 272, 16069–16072.
[6] Kramer, R.M., Roberts, E.F., Hyslop, P.A., Utterback, B.G., Hui, K.Y. and Jakubowski, J.A. (1995) J. Biol. Chem. 270, 14816–14823.
[7] Qiu, Z.-H., Gijón, M.A., de Carvalho, M.S., Spencer, D.M. and Leslie, C.C. (1998) J. Biol. Chem. 273, 8203–8211.
[8] Bingham III, C.O., Murakami, M., Fujishima, H., Hunt, J.E.,

Austen, K.F. and Arm, J.P. (1996) J. Biol. Chem. 271, 25936–25944.
[9] Kuwata, H., Nakatani, Y., Murakami, M. and Kudo, I. (1998) J. Biol. Chem. 273, 1733–1740.
[10] Pfeilschifter, J., Pignat, W., Vosbeck, K. and Märki, F. (1989) Biochem. Biophys. Res. Commun. 159, 385–394.
[11] Pfeilschifter, J., Schalkwijk, C., Briner, V.A. and van den Bosch, H. (1993) J. Clin. Invest. 92, 2516–2523.
[12] Schalkwijk, C.G., de Vet, E., Pfeilschifter, J. and van den Bosch, H. (1992) Eur. J. Biochem. 210, 169–176.
[13] Huwiler, A., Staudt, G., Kramer, R.M. and Pfeilschifter, J. (1997) Biochim. Biophys. Acta 1348, 257–272.
[14] Ackermann, E.J., Conde-Frieboes, K. and Dennis, E.A. (1995) J. Biol. Chem. 270, 445–450.
[15] Balsinde, J., Bianco, I.D., Ackermann, E.J., Conde-Frieboes, K. and Dennis, E.A. (1995) Proc. Natl. Acad. Sci. USA 92, 8527–8531.
[16] Hayama, M., Akiba, S., Fukuzumi, M. and Sato, T. (1997) J. Biochem. 122, 1196–1201.
[17] Akiba, S., Nagatomo, R., Hayama, M. and Sato, T. (1997) J. Biochem. 122, 859–864.
[18] Ackermann, E.J. and Dennis, E.A. (1995) Biochim. Biophys. Acta 1259, 125–136.
[19] Ackermann, E.J., Kempner, E.S. and Dennis, E.A. (1994) J. Biol. Chem. 269, 9227–9233.
[20] Tang, J., Kriz, R.W., Wolfman, N., Shaffer, M., Seehra, J. and Jones, S.S. (1997) J. Biol. Chem. 272, 8567–8575.
[21] Balsinde, J. and Dennis, E.A. (1996) J. Biol. Chem. 271, 6758–6765.
[22] Balsinde, J., Balboa, M.A. and Dennis, E.A. (1997) J. Biol. Chem. 272, 29317–29321.
[23] Gross, R.W., Rudolph, A.E., Wang, J., Sommers, C.D. and Wolf, M.J. (1995) J. Biol. Chem. 270, 14855–14858.
[24] Atsumi, G., Tajima, M., Hadano, A., Nakatani, Y., Murakami, M. and Kudo, I. (1998) J. Biol. Chem. 273, 13870–13877.