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Inhibitory Effect of Prostaglandin E_2, Forskolin, and Dibutyryl cAMP on Arachidonic Acid Release and Inositol Phospholipid Metabolism in Guinea Pig Neutrophils*

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The effect of prostaglandin E_2 (PGE_2), forskolin, and dibutyryl cAMP on arachidonic acid release, inositol phospholipid metabolism, and Ca^{2+} mobilization was investigated. The chemotactic tripeptide (formylmethionyl-leucyl-phenylalanine (fMLP))-induced arachidonic acid release in neutrophils was significantly inhibited by PGE_2, forskolin, and dibutyryl cAMP. Among them, PGE_2 was found to be the most potent inhibitor. However, when neutrophils were stimulated by Ca^{2+} ionophore A23187, such inhibitory effect by these agents was less marked. PGE_2 also suppressed the enhanced incorporation of [^{32}P]P_i into phosphatidic acid (PA) and phosphatidylinositol in a dose-dependent manner in fMLP-stimulated neutrophils. Also in this case, Ca^{2+} ionophore-induced alterations were hardly inhibited by PGE_2. As well, PGE_2 inhibited the fMLP-induced decrease of [^{3}H]arachidonic acid in phosphatidylcholine and phosphatidylinositol and the increase in PA very significantly. But the inhibitory effect by PGE_2 was found to be weak in Ca^{2+} ionophore-stimulated neutrophils. These results suggest that a certain step from receptor activation to Ca^{2+} influx is mainly inhibited by PGE_2. Concerning polyphosphoinositide breakdown, PGE_2 did not affect the fMLP-induced decrease of [^{32}P]phosphatidylinositol 4,5-bisphosphate which occurred within 10 s but inhibited the subsequent loss of [^{32}P]phosphatidylinositol 4-phosphate and [^{32}P]phosphatidylinositol, suggesting that the compensatory resynthesis of phosphatidylinositol 4,5-bisphosphate was inhibited. On the other hand, fMLP-induced diacylglycerol formation was suppressed for the early period until 1 min, but with further incubation, diacylglycerol formation was rather accelerated by PGE_2. Moreover, the inhibition of PA formation by PGE_2 became evident after a 30-s time lag, suggesting that the conversion of diacylglycerol to PA is inhibited by PGE_2. The formation of water-soluble products of inositol phospholipid degradation by phospholipase C, such as inositol phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate, was also suppressed by PGE_2 treatment. However, the inhibition was not so marked as that of arachidonic acid release and PA formation. Thus, PGE_2 appeared to inhibit not only initial events such as polyphosphoinositide breakdown but also turnover of inositol phospholipids. PGE_2, forskolin, and dibutyryl cAMP did not block the rapid elevation of intracellular Ca^{2+} which was observed within 10 s in fMLP-stimulated neutrophils. However, subsequent increase in intracellular Ca^{2+} which was caused from 10 s to 3 min after stimulation was inhibited by PGE_2, forskolin, and dibutyryl cAMP. These data may demonstrate that PGE_2 inhibits Ca^{2+} influx rather than Ca^{2+} mobilization from intracellular stores. Considering that arachidonic acid release is completely dependent on extracellular Ca^{2+}, PGE_2 may inhibit Ca^{2+} influx through the suppression of inositol phospholipid metabolism and then inhibit arachidonic acid release.

Neutrophils have been considered as playing a major role in host defenses against microorganisms and have been implicated in contributing to inflammatory events. The release of lysosomal enzymes and the generation of superoxide anion in activated neutrophils have been suggested as possible mechanisms by which some of these events are mediated (1–3).

We (4) and other investigators (5–9) have demonstrated that phosphatidylinositol response and arachidonic acid release are closely associated with neutrophil activation. Recently, the degradation of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2)[1] by phospholipase C rather than phosphatidylinositol (PI) has been proposed to be a primary event in stimulus-response coupling in a variety of tissues (for review see Refs. 10 and 11). Also in chemotactic peptide-stimulated neutrophils, PI-4,5-P_2 breakdown to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) has been found to occur very rapidly compared to that of other inositol lipids such as phosphatidylinositol 4-phosphate (PI-4-P) and PI (8, 9, 12). Thus, two biologically important factors, DG and IP_3, are produced in response to receptor activation. DG, one of the products of PI-4,5-P_2, is known to act as an activator for protein kinase C (for review see Refs. 13 and 14). Therefore, the signal-induced DG accumulation leads to the activation of protein kinase C. Concerning another product, since Streb *et al.* (15) suggested that IP_3 had roles in the mobilization of intracellular free Ca^{2+}, much evidence has been presented that IP_3 is able to release Ca^{2+} from intracellular store in various

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[1] The abbreviations used are: PI-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; fMLP, formylmethionyl-leucyl-phenylalanine; PI, phosphatidylinositol; PI-4-P, phosphatidylinositol 4-phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PG, prostaglandin; DG, diacylglycerol; IP, inositol phosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EMEM, Eagle's minimal essential medium; NDGA, nordihydroguaiaretic acid; BPB, bromphenacyl bromide.

tissues and cells (for review see Refs. 16 and 17). Thus, it now appears to be the most promising intermediate of hormone-stimulated inositol phospholipid metabolism capable of acting as a messenger for the mobilization of Ca^{2+} from intracellular storage.

It is well known that in activated neutrophils arachidonic acid is liberated from phospholipids and converted to prostaglandins and leukotrienes that have very strong biological activities. In this case, the liberation of arachidonic acid is thought to be the rate-limiting step. However, it is not clear how arachidonic acid release is regulated. Recently, numerous reports have shown that an increase of cAMP in leukocytes generally leads to subsequent inhibition of physiological functions (18–20). Substances that are able to elevate cAMP have therefore been considered as factors that contribute to the modulation of leukocyte activities. Particularly, prostaglandins E have attracted attention, since they seem to regulate leukocyte under physiological conditions (20). Among the E series of prostaglandins, prostaglandin E_2 (PGE_2) is thought to be one of the most important regulators because PGE_2 is one of the major prostaglandins produced from arachidonic acid in activated neutrophils (21, 22). However, so far it is not known whether PGE_2 is able to affect inositol phospholipid metabolism, arachidonic acid release, and Ca^{2+} mobilization, although PGE_2 can inhibit the subsequent responses such as lysosomal enzyme secretion and superoxide anion production (18–20) in neutrophils.

In this report, the effect of PGE_2 on the metabolism of inositol phospholipids, arachidonic acid release, and Ca^{2+} mobilization is studied.

MATERIALS AND METHODS

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (95.4 Ci/mmol), myo[2-^{3}H]inositol (12.5 Ci/mmol), and [^{32}P]orthophosphoric acid (carrier-free) were obtained from New England Nuclear. Ca^{2+} ionophore A23187 was from Calbiochem-Behring. Nordihydroguaiaretic acid, bromophenacyl bromide, forskolin, formylmethionyl-leucyl-phenylalanine (fMLP), *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide, and dibutyryl cAMP were from Sigma. Prostaglandin E_2 was from Funakoshi Chemical Co., Osaka. Eagle's minimum essential medium (EMEM) and Hanks' balanced salt solution were from Nissui Co., Tokyo. 2-[(Amino-5-methylphenoxy)methyl]-6-methoxy-8-aminoquinoline-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (Quin 2AM), was from Dojin Chemical Laboratories, Kumamoto. BW775C was a gift from Mitsui Pharmaceutical Co., Tokyo.

Neutrophils—Female Hartley guinea pigs (300–400 g) were injected with 10 ml of 12% polypepton. The peritoneal exudate cells were collected after 12 h in Hanks' balanced salt solution. The cells were washed three times with the buffer and were suspended in EMEM containing 10% guinea pig serum and 20 mM HEPES/NaOH buffer (pH 7.4).

Incorporation of [^{32}P]P_i into Phospholipids and the Alterations of Distribution of ^{32}P-Phospholipids—Neutrophils ($2–5 \times 10^8$ cells/20 ml) were incubated with 1 mCi of [^{32}P]P_i in phosphate-free EMEM containing 10% guinea pig serum and 20 mM HEPES/NaOH buffer pH 7.4) for 1 h at 37 °C. The cells were washed twice with the buffer. Aliquots of the suspended cells ($2–4 \times 10^6$ cells/0.5 ml) were transferred to a tube and then stimulated with fMLP or Ca^{2+} ionophore A23187 for the indicated time.

Redistribution of [^{3}H]Arachidonic Acid-labeled Lipid—Neutrophils ($2–5 \times 10^8$ cells) were incubated with 25 μCi of [^{3}H]arachidonic acid in EMEM containing 10% guinea pig serum and HEPES/NaOH buffer (pH 7.4) for 1 h at 37 °C. The cells were then sedimented by centrifugation and washed twice with the buffer. The cells were suspended at $2–5 \times 10^6$ cells/0.5 ml in EMEM and then stimulated by fMLP or Ca^{2+} ionophore at 37 °C for the indicated time.

Inositol Phosphate Formation—After neutrophils (1×10^9 cells/20 ml) were incubated with 25 μCi of myo[2-^{3}H]inositol in Hanks' balanced salt solution containing 10% fetal calf serum for 2 h, the cells were washed twice with the buffer and then treated with 10 mM LiCl for 10 min at 37 °C. The cells suspended at 3.5×10^7 cells/ml were stimulated with 1 μM fMLP at 37 °C for the indicated time. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. After the resultant precipitate was removed by centrifugation, the supernatant was diluted with 4 volumes of water and neutralized to pH 7.0. Separation of various inositol phosphates was carried out by a similar method as described by Downes and Michell (23).

Measurement of Cytosolic Free Ca^{2+} Using Quin 2—Neutrophils (1×10^8 cells/ml) were loaded with 50 μM Quin 2AM in a manner similar to that described by Tsien *et al.* (24) for 20 min at 37 °C and then diluted to 10-fold and allowed to incubate for 40 min. At the end of the incubation period, the cells were washed and suspended in Hanks' balanced salt solution containing calcium (1.6 mM) or 2 mM EGTA (1×10^7 cells/ml). Fluorescence measurements were performed using a Hitachi fluorescence spectrometer Type 650-40 according to the method of Tsien *et al.* (24).

Measurement of cAMP Content—After neutrophils ($1–2 \times 10^6$ cells/0.5 ml) were incubated with fMLP or Ca^{2+} ionophore A23187 in the presence or absence of PGE_2 or forskolin for the indicated time at 37 °C, the reaction was stopped in a boiling water bath. The cells were centrifuged at $2000 \times g$ for 20 min, and the supernatants were retained for the measurement of cAMP contents. Assay of cAMP was carried out using a radioimmunoassay kit (Yamasa Co., Chiba, Japan).

Extraction and Separation of Lipids—Various lipids except PI-4,5-P_2 and PI-4-P were extracted and separated by the methods as described previously (4). After PI-4,5-P_2 and PI-4-P were precipitated with 10% trichloroacetic acid, the residues were extracted by the same method as described before (4).

Lysosomal enzyme (*N*-acetyl-β-D-glucosaminidase) secretion and superoxide anion production were measured as described previously (12, 25).

RESULTS

Effect of Inhibitors for Arachidonic Acid Cascade on Superoxide Anion Production and Lysosomal Enzyme Secretion—The chemotactic tripeptide, fMLP, has been shown to stimulate superoxide anion production and lysosomal enzyme secretion very significantly (3, 26–30). This enhanced production of superoxide anion was further stimulated by the addition of indomethacin, a cyclooxygenase inhibitor (Fig. 1*A*). On the contrary, a lipoxygenase inhibitor such as nordihydroguaiaretic acid (NDGA) and BW755C were found to be strong

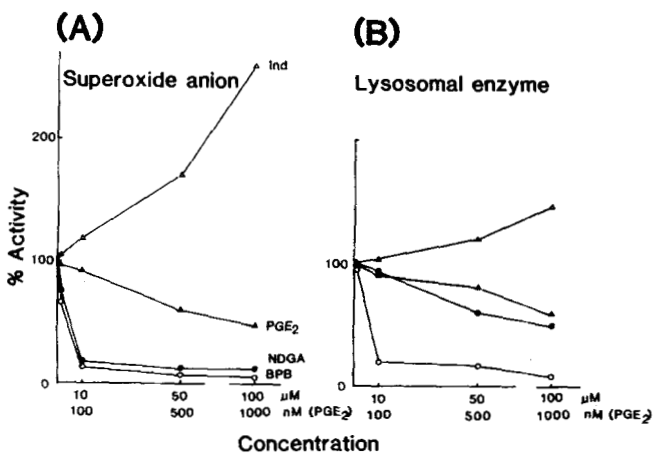

FIG. 1. **Effect of inhibitors for arachidonic acid cascade on superoxide anion (*A*) and lysosomal enzyme secretion (*B*).** *A*, neutrophils (2×10^6 cells/0.5 ml) were stimulated by 1 μM fMLP in the presence of various concentrations of drugs with 80 μM cytochrome *c* for 10 min at 37 °C, and then cytochrome *c* reduction was measured at 550 nm with a reference of 540. Before the treatment by fMLP, neutrophils were preincubated with various drugs or vehicle for 3 min. *B*, after neutrophils were treated with 5 μg/ml cytochalasin B for 10 min at 37 °C, reaction was started by the addition of 1 μM fMLP in the presence of various concentrations of drugs and continued for 5 min. Aliquots of the supernatant were used to measure secreted *N*-acetyl-β-D-glucosaminidase. Concentrations of agents are indicated in nanomolar for PGE_2 or micromolar for indomethacin (*Ind*), NDGA, and BPB.

inhibitors for superoxide anion production. Bromophenacyl bromide (BPB), a phospholipase A_2 inhibitor, also suppressed the production. PGE_2 suppressed the production of superoxide anion. For lysosomal enzyme secretion, similar results were obtained, namely, NDGA, BW755C, BPB, and PGE_2 inhibited the secretion of *N*-acetyl-β-D-glucosaminidase and indomethacin activated the secretion (Fig. 1*B*). These results suggest that cyclooxygenase products inhibit the neutrophil functions such as superoxide anion production and lysosomal enzyme secretion, but lipoxygenase products activate those functions. PGE_2 is one of the major products of arachidonic acid metabolites when neutrophils were stimulated by chemotactic peptide (21, 22). Therefore, there is a possibility that PGE_2 causes a negative feedback effect on neutrophils.

Inhibitory Effect of PGE_2, Forskolin, and Dibutyryl cAMP on Arachidonic Acid Release—It has become clear that arachidonic acid metabolites make important roles on neutrophil activation (6, 31, 33). Therefore, the effect of PGE_2, forskolin, and dibutyryl cAMP on arachidonic acid release was examined. As shown in Fig. 2, not only PGE_2 but also forskolin and dibutyryl cAMP inhibited arachidonic acid release in a dose-dependent manner which is induced by fMLP or Ca^{2+} ionophore stimulation. Among them, PGE_2 was found to be the most potent inhibitor and 1 μM PGE_2 almost completely inhibited arachidonic acid release in chemotactic peptide-stimulated neutrophils. The inhibitory effect was also obtained by the treatment of forskolin which is known as a potent cAMP-increasing agent, although dibutyryl cAMP caused a slight inhibition. Therefore, some parts of the inhibitory effect of PGE_2, if not all, may be explained by cAMP elevation. This inhibition was more significant in fMLP-stimulated neutrophils than that in Ca^{2+} ionophore-activated neutrophils. Since Ca^{2+} ionophore can activate phospholipase A_2 through intracellular Ca^{2+} elevation, these data may suggest that PGE_2 mainly inhibits a certain step in pathways from receptor activation to arachidonic acid release rather than inhibit directly the arachidonic acid-cleaving enzyme phospholipase A_2.

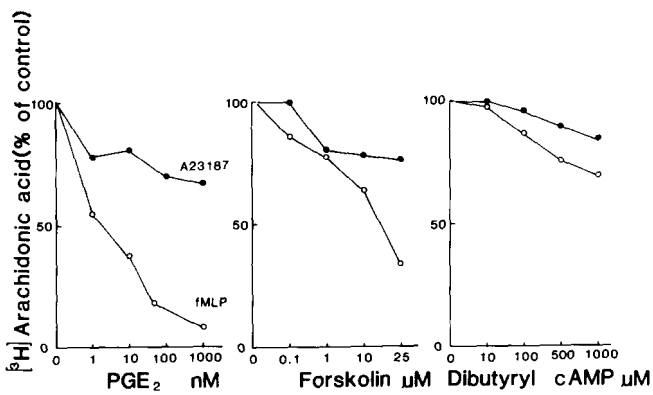

FIG. 2. **Effect of PGE_2, forskolin, and dibutyryl cAMP on the release of arachidonic acid.** Neutrophils were incubated with [^{3}H]arachidonic acid for 1 h at 37 °C. The cells were washed and stimulated with 1 μM fMLP (○) or 1 μM Ca^{2+} ionophore A23187 (●). Before stimulation, neutrophils were pretreated by PGE_2 for 3 min, forskolin for 7 min, or dibutyryl cAMP for 10 min.

Effect of PGE_2 on [^{32}P]P_i Incorporation into Phospholipids—When neutrophils were stimulated by fMLP, a marked increase of ^{32}P incorporation into phosphatidic acid (PA) and PI and the formation of lysophosphatidylcholine were observed (Fig. 3). These increases were significantly antagonized by the addition of PGE_2 in a dose-dependent manner. Metabolisms of other phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine, and phosphatidylserine were not affected by fMLP. As observed on the effect of arachidonic acid release, this inhibitory effect was more significant in chemotactic peptide-activated neutrophils than that in Ca^{2+} ionophore-activated neutrophils.

Effect of PGE_2 on the Distribution of [^{3}H]Arachidonic Acid—After neutrophils were prelabeled with [^{3}H]arachidonic acid, the effect of PGE_2 on the redistribution of [^{3}H]arachidonic acid was examined (Fig. 4). When neutrophils were stimulated with fMLP, the decrease of [^{3}H]arachidonic acid in PC and PI and the increase in PA and free arachidonic acid were significant. The decrease in PC and PI was blocked

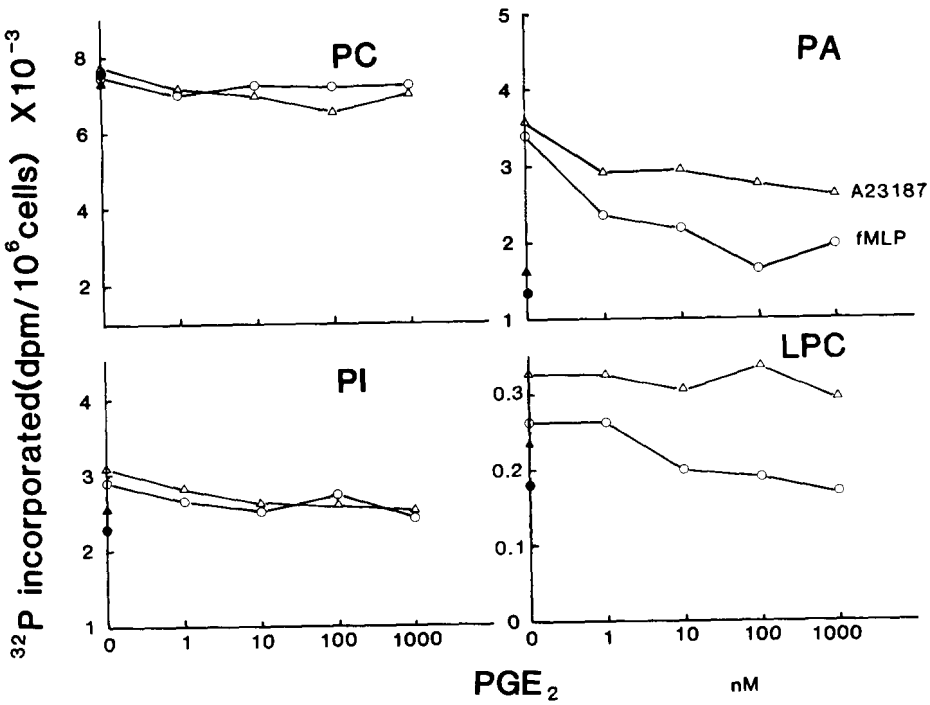

FIG. 3. **Effect of PGE_2 on the labeling of various ^{32}P-phospholipids.** Neutrophils were incubated with 1 mCi of [^{32}P]P_i in phosphate-free EMEM containing 10% guinea pig serum and 10 mM HEPES/NaOH buffer (pH 7.4) for 1 h at 37 °C. After the cells were washed and resuspended in fresh medium, the cells were pretreated with PGE_2 or vehicle for 3 min before the stimulation. The reaction was started by the addition of 1 μM fMLP (○) or 1 μM A23187 (△) and continued for 3 min. PGE_2 alone did not affect the basal labeling of ^{32}P-phospholipids. *Open symbols* indicate the values in stimulated neutrophils by fMLP (○) or A23187 (△). *Closed symbols* (●, control for fMLP treatment; ▲, control for A23187 treatment) indicate the values in normal neutrophils without stimulation. *LPC*, lysophosphatidylcholine.

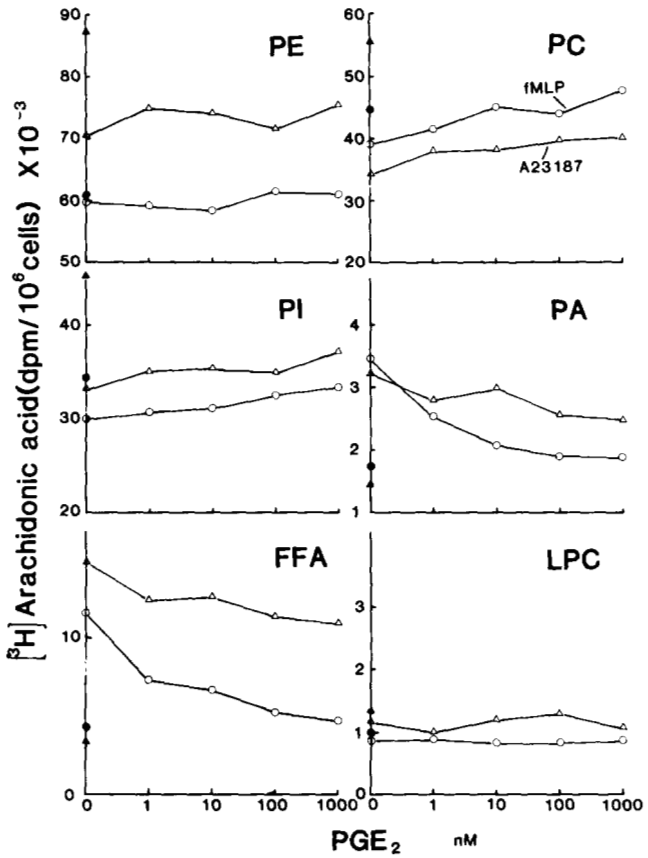

FIG. 4. **Effect of PGE_2 on the redistribution of [^{3}H]arachidonic acid moiety in [^{3}H]arachidonyl lipids.** Neutrophils were incubated with [^{3}H]arachidonic acid for 1 h at 37 °C. The cells were washed to remove free [^{3}H]arachidonic acid and stimulated with 1 μM fMLP or 1 μM A23187 for 3 min at 37 °C. Other details were the same as described under "Materials and Methods." Under these conditions, PGE_2 did not affect the basal distribution of [^{3}H]arachidonic acid in nonstimulated neutrophils. ●, control for fMLP; ○, treatment with fMLP; ▲, control for A23187; △, treatment with A23187. *FFA*, free fatty acid; *LPC*, lysophosphatidylcholine; *PE*, phosphatidylethanolamine.

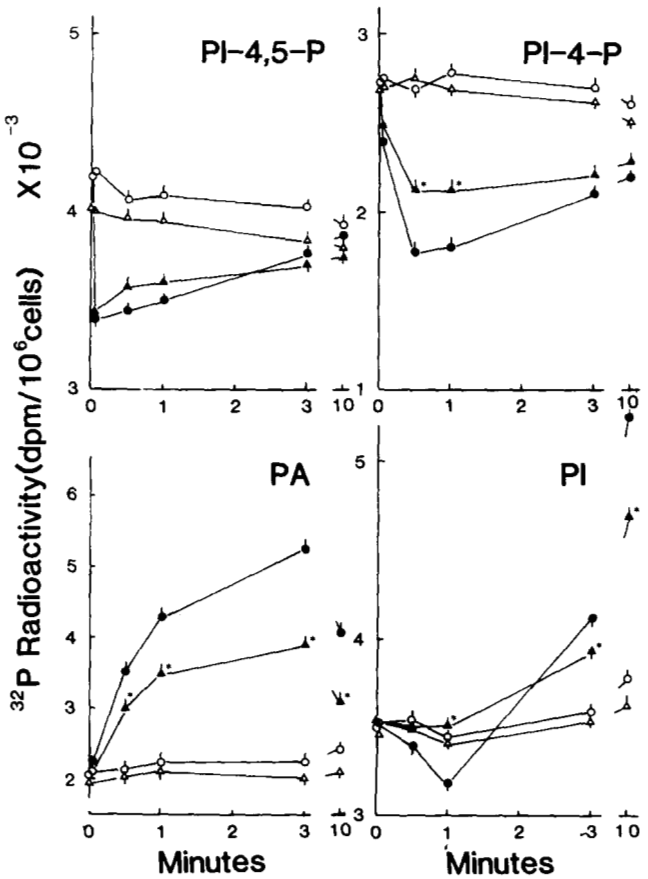

FIG. 5. **Effect of PGE_2 on the labeling of [^{32}P]PI-4,5-P_2, PI-4-P, PI, and PA.** Neutrophils were incubated with [^{32}P]P_i for 1 h at 37 °C. The cells were washed and stimulated with 1 μM fMLP in the presence or absence of 1 μM PGE_2 for 3 min at 37 °C. Before stimulation by fMLP, neutrophils were pretreated with 1 μM PGE_2 for 3 min. Each *point* represents the mean ± S.E. of triplicate determinations. This experiment is representative of four others that gave similar results. *, significantly different from fMLP-treated control ($p < 0.01$). ○, control; ●, fMLP; △, PGE_2; ▲, PGE_2 + fMLP.

by the addition of PGE_2 in a dose-dependent manner. Moreover, the increase in PA and free arachidonic acid was almost completely inhibited by the treatment of more than 100 nM PGE_2. Ca^{2+} ionophore also enhanced the loss of [^{3}H]arachidonic acid in PC, phosphatidylethanolamine, and PI. However, this decrease was not inhibited so strongly by PGE_2 as that observed in fMLP-stimulated neutrophils. As well, the increase of [^{3}H]arachidonic acid in PA and free fatty acid which was caused by Ca^{2+} ionophore was also weakly inhibited.

Effect of PGE_2 on ^{32}P-Labeled PI-4,5-P_2, PI-4-P, PI, and PA—It has been reported that polyphosphoinositide breakdown is closely related to Ca^{2+} mobilization and arachidonic acid release (for review see Refs. 17 and 34). As shown in Fig. 5, chemotactic peptide caused very rapid decreases in [^{32}P]PI-4,5-P_2 and [^{32}P]PI-4-P, followed by the compensatory resynthesis of PI-4,5-P_2 and PI-4-P. On the other hand, PA labeling by ^{32}P increased with time without transient decrease by chemotactic peptide. PGE_2 did not inhibit the decrease in [^{32}P]PI-4,5-P_2 significantly but suppressed the subsequent decrease in [^{32}P]PI-4-P. This experiment was repeated four times. PGE_2 always inhibited the decrease of PI-4-P more markedly than that of PI-4,5-P_2, although the inhibitory effect was varied in each experiment. Concerning [^{32}P]PI, both the initial decrease which occurred within 1 min and the subsequent resynthesis were inhibited by PGE_2. These results may show that recovery synthesis of PI-4,5-P_2 from PI is inhibited by PGE_2. Under these conditions, ^{32}P incorporation into PA was markedly inhibited. However, this inhibition became evident in 30 s after stimulation, suggesting that the conversion of DG to PA is blocked by PGE_2. Ca^{2+} ionophore did not cause the significant decrease of PI-4-P and PI-4,5-P_2 (data not shown).

Effect of PGE_2 on Inositol Phosphates Formation—It seemed likely that PGE_2 did not inhibit the initial reactions such as PI-4,5-P_2 breakdown in the ^{32}P experiment, although PI-4-P decrease was inhibited. Thus, the effect of PGE_2 on the formation of inositol phosphate which should be formed by the result of inositol phospholipids breakdown was studied. As shown in Fig. 6, fMLP enhanced the formation of various inositol phosphates such as IP_3, IP_2, and IP. In this case, formation of IP_3 occurred rapidly and reached a maximum at 10 s after stimulation. The formation preceded that of IP_2 or IP. PGE_2 could inhibit not only IP and IP_2 formation but also IP_3 formation. But the inhibition seemed to be slight compared to that of arachidonic acid release and PA formation. These results may suggest that there are some steps to be inhibited by PGE_2 except PI-4,5-P_2 breakdown to DG and IP_3. Under these conditions, arachidonic acid release and PA formation were strongly blocked.

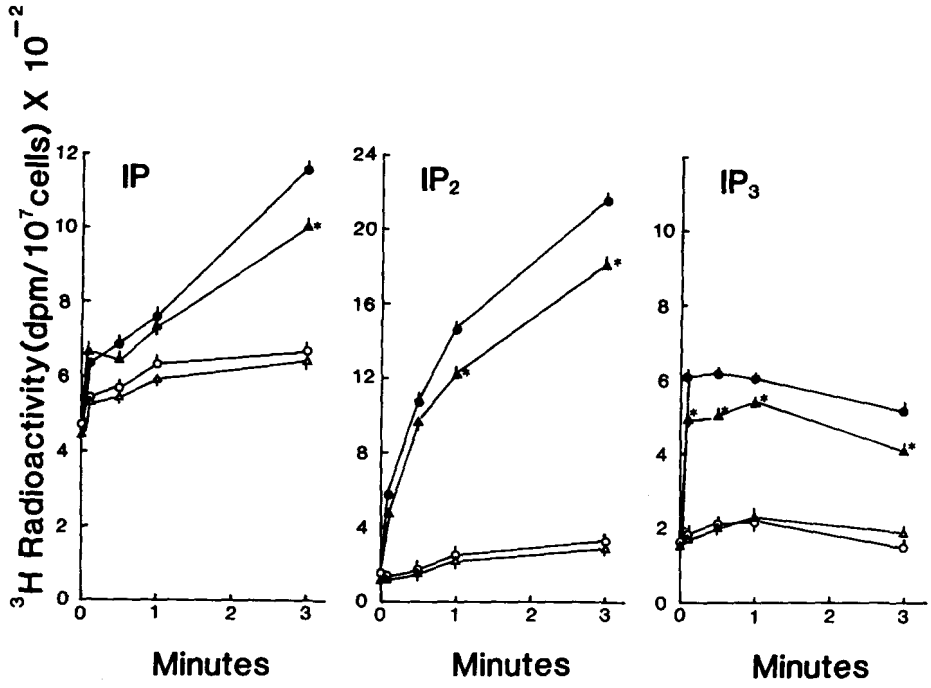

FIG. 6. **Effect of PGE_2 on fMLP-induced formation of inositol phosphates.** Neutrophils labeled with myo[2-^{3}H]inositol were incubated with 10 mM LiCl for 10 min and then treated with 1 μM PGE_2 or vehicle for 3 min. The cells were then stimulated with 1 μM fMLP. The treatment by PGE_2 without fMLP did not show the significant effect on the formation of inositol phosphates. Each *point* represents the mean ± S.E. of triplicate determinations. *, significantly different from fMLP-treated control ($p < 0.01$). ○, control; ●, fMLP; △, PGE_2; ▲, PGE_2 + fMLP.

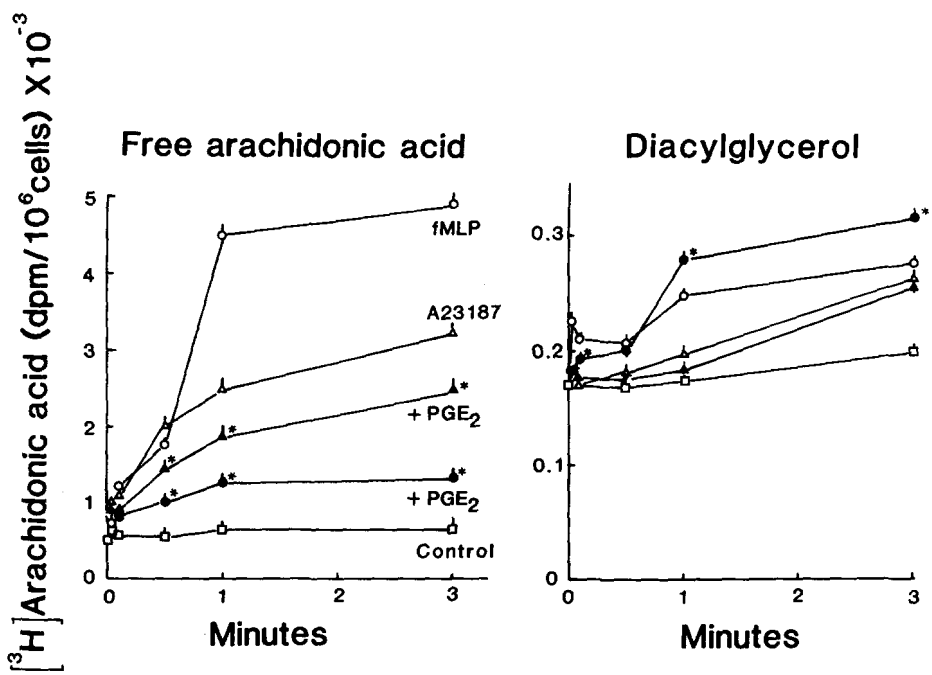

FIG. 7. **Time course of fMLP- or A23187-induced accumulation of [^{3}H]arachidonic acid and [^{3}H]arachidonyl-DG.** [^{3}H]Arachidonic acid-labeled neutrophils were preincubated with 1 μM PGE_2 or vehicle for 3 min at 37 °C. The cells were stimulated by 1 μM fMLP or 1 μM A23187 for the indicated time at 37 °C. PGE_2 alone did not affect the basal [^{3}H]arachidonic acid or [^{3}H] arachidonyl-DG. Each *point* represents the mean ± S.E. of triplicate determinations. *, significantly different from fMLP- or Ca^{2+} ionophore-treated control. □, control; ○, fMLP; △, A23187; ●, fMLP + PGE_2; ▲, A23187 + PGE_2.

Time Course of the Formation of [^{3}H]Arachidonyl-DG and Free [^{3}H]Arachidonic Acid—DG formation was increased by fMLP during the incubation time up to 3 min (Fig. 7). This increase seemed to be inhibited for the early period until 1 min, but with further incubation the increase was, on the contrary, potentiated a little with PGE_2. On the other hand, increased formation of DG by Ca^{2+} ionophore was not affected significantly by PGE_2. fMLP-induced liberation of arachidonic acid was inhibited very strongly. However, Ca^{2+} ionophore-induced liberation was partially blocked by the addition of PGE_2. Considering the results that DG accumulation after 1 min was rather stimulated by PGE_2, the conversion of DG to PA may be inhibited. This may be the reason why PA formation was markedly inhibited.

Effect of PGE_2 and Forskolin on fMLP- or Ca^{2+} Ionophore A23187-induced cAMP Elevation—As shown in Table I, PGE_2 alone caused approximately a 2-fold elevation of cAMP levels relative to control values, but stimulated a 5–6-fold rise in the presence of 1 μM fMLP or Ca^{2+} ionophore A23187. On the other hand, forskolin alone also elicited a 1.4-fold rise in cAMP levels. However, the combination of forskolin and fMLP or forskolin and Ca^{2+} ionophore A23187 caused only additive increases in cAMP levels. These results strongly suggest that the inhibitory effect of PGE_2 on inositol phospholipids metabolism is produced mainly through cAMP elevation.

Effect of PGE_2 on Intracellular Ca^{2+} Level—It was already demonstrated that arachidonic acid release was completely dependent on extracellular Ca^{2+} (4). Therefore, the effect of PGE_2 on free intracellular Ca^{2+} levels was monitored by Quin

2 in the presence or absence of extracellular Ca^{2+}. When neutrophils were activated by fMLP in Ca^{2+} containing medium, the intracellular Ca^{2+} level increased from 170 to 370 nM very rapidly within 10 s and then gradually increased up to 900 nM until 3 min (Fig. 8A). In this case, PGE_2 did not inhibit the rapid increase in Ca^{2+} which occurred within 10 s, but it suppressed the following increase in Ca^{2+} which occurred between 10 s and 3 min in a dose-dependent manner. Forskolin and dibutyryl cAMP also gave the same effect on Ca^{2+} mobilization, namely, they did not inhibit the initial increase in Ca^{2+} but inhibited the following increase (data not shown). Similar experiments were carried out in the presence of 2 mM EGTA (Fig. 8B). Even under this condition, the initial increase by fMLP stimulation still occurred, although the amount of increased Ca^{2+} was less significant; but the secondary increase in Ca^{2+} was not observed. In this condition, PGE_2 did not block Ca^{2+} increase which was induced by fMLP. These data suggest that PGE_2 inhibits the secondary Ca^{2+} increase which may be due to Ca^{2+} influx; but PGE_2 does not inhibit the initial burst increase, which may be due to Ca^{2+} release from the intracellular store.

DISCUSSION

PGE_2 inhibited the functions of leukocytes such as lysosomal enzyme secretion, superoxide anion production, and aggregation. This effect was thought to be caused through intracellular cAMP elevation (18, 19). But it is not certain how cAMP inhibited those events. On the other hand, when neutrophils are activated with fMLP, rapid alterations of inositol phospholipid metabolism are caused. Such enhanced inositol phospholipid metabolism is believed to have played an important role in neutrophil functions through Ca^{2+} mobilization and protein kinase C activation. In this case, breakdown of PI-4,5-P_2 to DG and IP_3 rather than that of PI to DG and IP has been proposed as an initial event (12). If this is the case, inhibition of these steps must lead to suppression of neutrophil functions. However, this initial breakdown of PI-4,5-P_2 appeared not to be inhibited markedly by the addition of PGE_2, although fMLP-induced decrease of PI and PI-4-P was blocked. If the decrease of PI and PI-4-P mainly caused by the conversion to PI-4,5-P_2 through the pathway, PI to PI-4-P, and then to PI-4,5-P_2 as suggested by Berridge (35), inhibition of loss of ^{32}P-labeled PI and PI-4-P may indicate the suppressive conversion from PI to PI-4,5-P_2. In this experiment, although fMLP-induced decrease of PI-4,5-P_2 was not significantly altered by PGE_2, IP_3 formation was found to be inhibited. This discrepancy may be explained by the reason why inhibition of [^{32}P]PI-4,5-P_2 breakdown to DG and IP_3 is apparently masked, because basal labeling of [^{32}P] PI-4,5-P_2 is slightly lower in PGE_2-treated neutrophils. A second possibility is that PGE_2 stimulates the phosphomonoesterase that hydrolyzes PI-4,5-P_2 to PI-4-P (36) in addition to the inhibition of PI-4,5-P_2 breakdown to DG and IP_3. A third possibility is that the inhibition of decrease in [^{32}P]PI-4,5-P_2 is hardly detected since the decrease is rapidly recovered with the resynthesis. On the contrary, formed IP_3 may be detected cumulatively, although IP_3 gradually degraded to IP_2 and then to IP with the further incubation. Therefore, inhibition of IP_3 formation may be easily measured. On the other hand, synthesis of PA and PI was strongly inhibited to PGE_2. These results suggest that PGE_2 inhibits not only an initial event such as PI-4,5-P_2 breakdown weakly but also turnover of inositol phospholipids strongly. Forskolin and dibutyryl cAMP seemed to mimic PGE_2 effect on arachidonic acid release and phospholipid metabolism. If the inhib-

TABLE I

Effect of PGE_2 and forskolin on fMLP- or Ca^{2+} ionophore A23187-induced cAMP elevation

After neutrophils were preincubated with 1 μM PGE_2 for 3 min or 50 μM forskolin for 5 min at 37 °C, the cells were stimulated with 1 μM fMLP or Ca^{2+} ionophore A23187 for 3 min. The reaction was stopped by placing the tube in a boiling bath for 2 min. Measurement of cAMP was carried out by the same methods as described under "Materials and Methods." The data represent the mean ± S.E. of triplicate determinations.

Treatment	Control	fMLP	Ca^{2+} ionophore
		pmol/10^7 cells	
None	7.06 ± 0.20	7.55 ± 1.28	8.11 ± 1.05
PGE_2	14.71 ± 1.34	38.24 ± 1.00	36.28 ± 1.97
Forskolin	10.39 ± 0.39	15.00 ± 1.27	15.69 ± 2.23

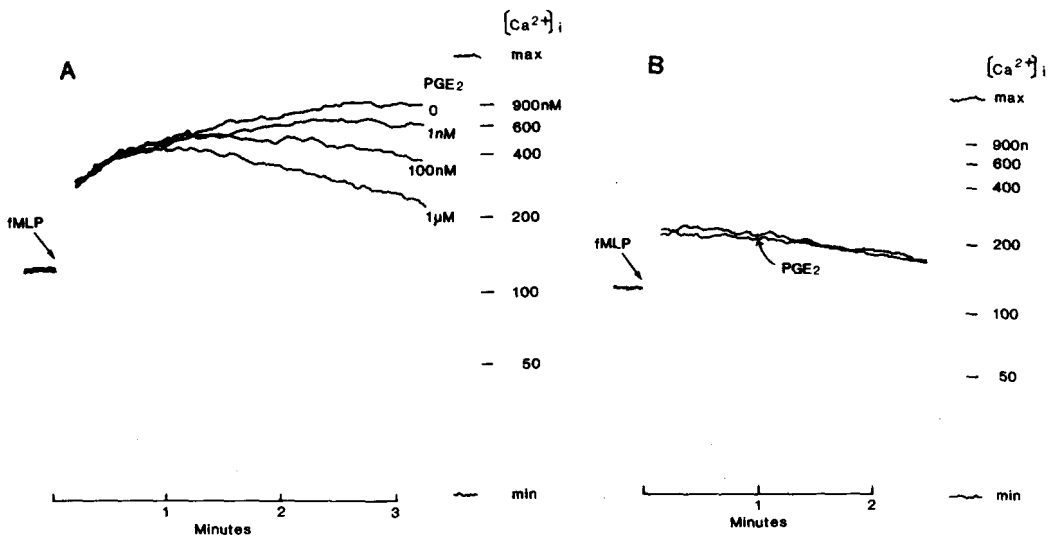

FIG. 8. **Time course of changes in fluorescence of Quin 2-loaded neutrophils.** *A*, effect of PGE_2 on fluorescence response of Quin 2-loaded, fMLP-activated neutrophils in Ca^{2+}-containing medium; *B*, effect of PGE_2 on fluorescence response of Quin 2-loaded, fMLP-activated neutrophils in 2 mM EGTA-containing medium. The concentration of PGE_2 used in this experiment was 1 μM. Other details were the same as described under "Materials and Methods."

itory effect is derived from the increase in cAMP, the potency must be dependent on the ability for raising cAMP. Indeed, the inhibitory effect was correlated with cAMP elevation. Interestingly, the combined treatment of PGE_2 and fMLP caused the dramatic increase of cAMP (Table I). This result also supports the idea that the potent effect of PGE_2 may be derived mainly from cAMP elevation. When neutrophils were activated with Ca^{2+} ionophore, the inhibitory effect of PGE_2 on arachidonic acid release and phospholipid metabolism was not so effective as that in fMLP-activated neutrophils. This fact may indicate that events leading to intracellular Ca^{2+} increase such as enhanced metabolism of inositol phospholipids are mainly suppressed by PGE_2. But, it is still unclear whether the inhibitory effect of PGE_2 on lysosomal enzyme secretion and superoxide anion production is caused by the inhibition of enhanced inositol phospholipid turnover.

It is well known that Ca^{2+} plays an important role in lysosomal enzyme secretion and superoxide anion production, although extracellular Ca^{2+} is not an absolute requirement for the activation of these functions (37, 38). In this experiment, we showed that PGE_2 could inhibit Ca^{2+} influx but not Ca^{2+} mobilization from intracellular stores. Recently, much evidence has been presented that newly formed IP_3, which is a resultant product of PI-4,5-P_2 breakdown by phospholipase C, can mobilize Ca^{2+} from the intracellular store (for review see Refs. 15 and 34) and another product of PI-4,5-P_2 breakdown, DG, activates protein kinase C (for review see Refs. 13 and 14). However, in this experiment, IP_3 and DG formation induced by fMLP could not be inhibited effectively by PGE_2. If IP_3 could release Ca^{2+} from intracellular store, this may be the reason why the initial Ca^{2+} increase within 10 s was hardly blocked by PGE_2. Recently, Togni *et al.* (39) have also reported that the inhibitory effect of cAMP on neutrophil responses is not due to the suppression of the initial burst elevation of Ca^{2+}. Since arachidonic acid release is completely dependent on extracellular Ca^{2+} (4), inhibition of Ca^{2+} influx perhaps causes the suppression of arachidonic acid release in fMLP-stimulated neutrophils. However, PGE_2 also could inhibit the release of arachidonic acid and inositol phospholipid metabolism that was induced by Ca^{2+} ionophore, although its effect was not so marked. These results may indicate that PGE_2 also inhibits a step in the pathways from Ca^{2+} influx to arachidonic acid release, such as phospholipid metabolism, phospholipase A_2 activation, and DG lipase-catalyzed arachidonic acid liberation (40). Considering the fact that PA can act as Ca^{2+} ionophore in membranes (41) and PA synthesis is tightly coupled to lysosomal enzyme secretion (42), enhanced metabolism of inositol phospholipids may play an important role in not only Ca^{2+} mobilization from intracellular store but also Ca^{2+} influx. In this case, PGE_2 seemed to affect Ca^{2+} influx through inhibiting the enhanced metabolism of inositol phospholipids rather than inhibiting directly phospholipase C activity which catalyzes the degradation of PI-4,5-P_2 to IP_3 and DG. In fact, phospholipase C, which was isolated from neutrophils treated with PGE_2 or dibutyryl cAMP, was found to have a similar activity to that of nontreated neutrophils.[2]

[2] T. Takenawa, J. Ishitoya, and Y. Nagai, unpublished data.

However, considering the fact that fMLP-induced IP_3 formation was also suppressed by PGE_2, phospholipase C activation may be inhibited indirectly through blocking the signal transduction between receptor and phospholipase C activation. Recently, Watson *et al.* (32) have reported that the inhibition of thrombin-induced production of inositol phosphates by prostacyclin in platelets may be due to the direct or indirect inhibition of phospholipase C. This problem remains to be solved in the future.

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