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PROSTACYCLIN AND BERAPROST SODIUM AS SUPPRESSORS OF ACTIVATED RAT POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—Beraprost sodium (beraprost) is a stable analogue of prostaglandin I_2 (PGI_2), which can be administrated orally. In the present study, the effect of beraprost on the activation process of polymorphonuclear leukocytes (PMNs) was examined *in vitro*. Beraprost effectively inhibited chemotaxis of PMNs induced by formyl-methionyl-leucyl-phenylalanine (FMLP). Like prostaglandin E_2 (PGE_2), beraprost elevated intracellular cAMP level and inhibited the influx of extracellular Ca^{2+} in PMNs. The concentration–response curves showed that the inhibitory effect of beraprost on chemotaxis was correlated with the increment of intracellular cAMP level of the PMNs and inhibition of influx of extracellular Ca^{2+}. Beraprost also inhibited inositol phospholipid metabolic turnover and superoxide anion production of PMNs induced by FMLP at relatively high concentration. These results suggest that the inhibitory effect of beraprost on the PMN function especially chemotaxis is mediated through the elevation of the intracellular cAMP level, which interferes with the signal transduction process probably through the inhibition of Ca^{2+} mobilization in PMNs. The above-mentioned effects of beraprost were also the case with PGI_2. The potency of beraprost was comparable to PGI_2 in the present study. Considering its stability, these results thus raise a possibility that beraprost might exert anti-inflammatory effect *in vivo*.

It is well known that polymorphonuclear leukocytes (PMNs†) play important roles in the host defense mechanisms especially in acute inflammation. On activation, they exert a number of functions such as chemotaxis to the inflammatory site, phagocytosis, superoxide anion production and release of lysosomal enzyme [1, 2]. Prostaglandins (PGs) and leukotrienes, the metabolites of arachidonic acid cascade, are also produced concomitantly on activation of PMNs [3]. These metabolites of arachidonic acid are also involved in the inflammation as either mediators or inhibitors of the inflammatory reactions [4]. PGE_1 and PGE_2, for example, have been reported to inhibit PMN chemotaxis, superoxide anion production and release of lysosomal enzyme [5–8], while they could increase the permeability of the blood vessels to potentiate inflammatory reaction. These activities are considered to be mediated by the modulation of intracellular cAMP level.

On the other hand, PGI_2 is well established to have a potent inhibitory effect on platelet aggregation [9] and vasodilation [10] by elevating cAMP level in the platelets and endothelial cells [9, 10]. However, the effect of PGI_2 on the PMN functions still remains controversial [6, 11–13]. This seems to be primarily due to its chemical instability. PGI_2, which possibly increases cAMP level on PMNs, might be expected to inhibit PMN functions as PGE_2, but the direct experimental approach of the effect using PGI_2 *per se* has been hampered by its instability [14].

In the present study we examined the effect of a stable and orally active agent with a PGI_2-like structure, beraprost sodium (sodium (±)-(1*R**, 2*R**, 3*aS**, 8*bS**)-2,3,3*a*,8*b*-tetrahydro-2-hydroxyl-1-[(*E*)-(3*S**)-3-hydroxy-4-methyl-1-octen-6-ynyl]-1H-cyclopenta[*b*]benzofuran-5-butyrate; beraprost) [15], on PMN functions in comparison with other PGs.

MATERIALS AND METHODS

Reagent. Formyl-methionyl-leucyl-phenylalanine (FMLP), forskolin, isobutylmethylxanthine (IBMX) and cytochrome *c* (Type III horse heart) were purchased from the Sigma Chemical Co. (St Louis, MO). Fura-2/AM was from Dojin Chemical Lab. (Kumamoto, Japan), and superoxide dismutase (SOD) from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Beraprost, PGI_2, PGE_2 and $PGF_{2\alpha}$ were synthesized in our chemical laboratory. Beraprost and PGI_2 used were sodium salts.

Animals. Male Wistar SPF rats were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). In all the experiments, rats aged 25–30 weeks and weighing about 300 g were used.

Preparation of PMNs. Rat peritoneal PMNs were

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† Abbreviations used: PMN, polymorphonuclear leukocyte; FMLP, formyl-methionyl-leucyl-phenylalanine; PG, prostaglandin; PGI_2, prostaglandin I_2; PGE_2, prostaglandin E_2; PGE_1, prostaglandin E_1; cAMP, cyclic 3′5′-adenosine monophosphate; EGTA, ethylene glycol bistetraacetic acid; IBMX, isobutylmethylxanthine; HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; SOD, superoxide dismutase; TPI, phosphatidyl inositol-4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; DG, diacylglycerol.

purified according to the method of Inoue and Sendo [16]. In brief, 15 mL of 3% proteose pepton (Difco Lab., Detroit, MI) was injected into the peritoneal cavity of rat, twice at the interval of 12–14 hr. Two hours after the last proteose pepton injection, peritoneal exuded cells were collected and suspended in RPMI1640 medium supplemented with 1% fetal calf serum (RPMI-1% FCS medium). Cells were then placed on the Ficoll-Isopaque gradient (Pharmacia, Piscataway, NJ) and centrifuged. The supernatant was discarded, and the cell pellet was treated with hypotonic buffer to lyse erythrocytes and washed twice with RPMI-1% FCS medium. The purity of the PMNs thus obtained was over 95% as estimated by May–Giemsa staining.

Chemotaxis assay. Chemotaxis assay was performed by the modified Boyden method [17] using a millipore filter with 3 μm pore size. FMLP was added to the RPMI-1% FCS medium in the lower compartment as an attractant of PMNs. PMNs were placed in the upper compartment at 3×10^6 cells/mL in RPMI-1% FCS medium. The chambers were incubated for 90 min at 37° to allow PMN migration. The filters were then removed and the cells were stained with Giemsa. The number of PMNs which migrated to the lower compartment was counted by optical microscope. The chemotactic activity was expressed as the average number (PMN/high power field; HPF) of PMNs in five random fields per filter at a magnification of 400×. Control experiment was performed in the absence of FMLP.

Measurement of superoxide anion production. Superoxide anion production was measured by the reduction rate of ferricytochrome *c* to ferrocytochrome *c* at 550 nm [18]. PMNs were suspended at a final concentration of 2×10^6 cells/mL in HEPES buffer (NaCl, 150 mM; KCl, 5 mM; $MgCl_2$, 1.29 mM; $CaCl_2$, 1 mM; *d*-glucose, 5.55 mM; HEPES, 10 mM; pH 7.4). For estimation of blank value, SOD (60 μg/mL) was added to incubation mixture. After incubation for 5 min at 37°, cells were stimulated by FMLP at a final concentration of 10^{-7} M for 10 min at 37°. The reaction was terminated by placing in an ice bath for 5 min and centrifuged at 1000 rpm for 10 min. Absorbance of the supernatant at 550 nm was determined using a spectrophotometer (Hitachi, U-2000). The amount of superoxide anion production was calculated from molar extinction at 20,000 M^{-1} cm^{-1}.

Measurement of cAMP level. PMNs were suspended at 1×10^7 cells/mL in HEPES buffer and treated with 10^{-5} M IBMX at 37° for 1 min. They were then stimulated by 10^{-7} M FMLP at 37° for 1 min. The reaction was stopped by addition of boiled 0.004 N glacial acetic acid. The cells were boiled for another 5 min and centrifuged at 12,000 rpm for 15 min. Supernatant was collected for the cAMP assay with RIA kit (New England Nuclear, Boston, MA).

Inositol phospholipid metabolic turnover assay. The assay of total [^{3}H]inositol phosphates was performed according to the method of Imboden and Stobo [19] with modifications. Briefly, myo-[^{3}H]inositol was incorporated into the phospholipids of PMNs ($7–9 \times 10^7$ cells/mL) in the 199 medium containing 10 mM HEPES, 0.2% bovine serum albumin and 40 μCi/mL of myo-[^{3}H]inositol (Amersham, Bucks, U.K.) by incubating for 3 hr at 37° [20]. The cells were washed twice with HEPES buffer (Ca^{2+}-free) and resuspended in HEPES buffer at

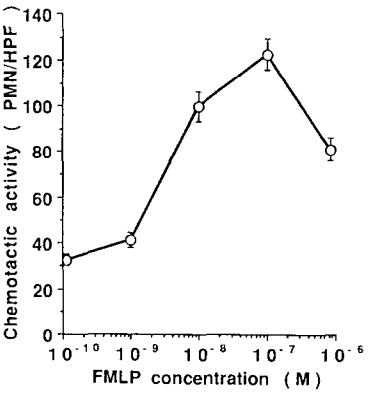


Fig. 1. Concentration–response curve of FMLP-induced chemotactic activity in rat PMNs. PMNs were incubated with FMLP at various concentrations shown in the figure. Chemotactic activity was assayed as described in the Materials and Methods. All assays were performed in triplicate. Each point and vertical bar represent mean ± SE for four separate experiments.

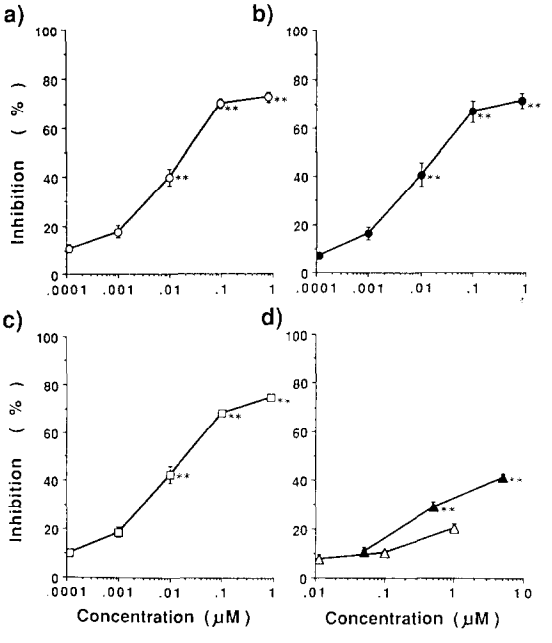


Fig. 2. Inhibitory effect of beraprost, PGI_2, PGE_2, $PGF_{2\alpha}$ and forskolin on FMLP-induced chemotaxis in rat PMNs. PMNs were preincubated with beraprost (a); PGI_2 (b); PGE_2 (c); $PGE_{2\alpha}$ (d: △) or forskolin (d: ▲) at various concentrations shown in the figure. After treatment with drugs, 10^{-7} M FMLP-induced chemotaxis of PMNs during the incubation for 90 min at 37° was determined. All assays were performed in triplicate. Effect of drugs is expressed as % inhibition to non-treated control. Each point and vertical bar represent mean ± SE of four separate experiments. *$P < 0.05$; **$P < 0.01$.

2×10^7 cells/mL. After incubation of the labeled PMNs at 37° for 5 min, 10 mM LiCl was supplemented to the solution and the cells were incubated for additional 10 min at 37°. They were then treated with IBMX for 1 min at 37° and stimulated by FMLP at 10^{-7} M. The reaction was terminated by addition of chloroform–methanol–HCl (20:40:1 v/v). The upper phase of the solution, containing water soluble inositol phosphates, was purified according to Bligh–Dyer method [21], and eluted on AG-1-X8 anion exchange columns (Bio-Rad, Richmond, CA). Radioactivity in each fraction of column chromatography was determined by a liquid scintillation counter.

Measurement of intracellular free calcium concentration. Ten μM fura-2/AM was added to the PMN suspension in RPMI-1% FCS medium at a concentration of 1×10^8 cells/mL and the cells were incubated at 37° for 5 min [22]. Cells were diluted to 1×10^7 cells/mL and further incubated at 37° for 20 min. They were centrifuged at 1000 rpm for 8 min and resuspended in HEPES buffer at 5×10^6 cells/mL. Fluorescence of fura-2 was measured using calcium analyser (Japan Spectroscopic, CAF-100) with excitation at 340 and 380 nm, and with emission at 505 nm. Concentration of intracellular free calcium was calculated as described by Grynkiewicz *et al.* [23].

Statistics. Statistical analysis was done by Anova-test and Dunnett-test.

RESULTS

Inhibitory effect of beraprost on FMLP-induced chemotaxis of PMNs. The effect of beraprost on the FMLP-induced chemotaxis of PMNs was examined. As shown in Fig. 1, FMLP induced chemotaxis of rat PMNs at the range from 10^{-9} M to 10^{-6} M, and the maximum response was obtained at 10^{-7} M. Therefore, 10^{-7} M FMLP was employed for the following experiments. PMNs were pretreated for 1 min with the various drugs at the concentrations shown in Fig. 2. Beraprost inhibited the chemotaxis induced by 10^{-7} M FMLP in a concentration-dependent fashion. IC_{50} value for the inhibition of chemotaxis by beraprost was about 30 nM (Fig. 2a). PGI_2 and PGE_2 also inhibited the FMLP-induced chemotaxis and the concentration–response curves were essentially identical to that of beraprost (Fig. 2b and c). In contrast, the inhibitory effect of $PGF_{2\alpha}$ was minimal, if any (Fig. 2d). Higher concentration of $PGF_{2\alpha}$ (1 μM) resulted in only 20% inhibition of the chemotaxis. Forskolin also inhibited chemotaxis significantly ($P < 0.01$) at the concentration of more than 0.5 μM (Fig. 2d). None of these agents showed any cytotoxic effect on PMNs as determined by the dye-exclusion (data not shown).

These results indicate that beraprost might inhibit PMN activation in the early step of inflammation like other PGs. Inhibitory effect of forskolin, known to increase cAMP level, on chemotaxis also supports that cAMP is possibly involved in the inhibitory effect by beraprost.

Effect on superoxide anion production. PMNs play a major role in the host defense mechanism by migrating to the inflammatory sites and phagocyting of foreign materials. One of the reactions induced

Table 1. Effect of beraprost, PGs and forskolin on FMLP-induced superoxide anion production in rat PMNs

Treatment (μM)	O_2^- production	(nmol/10^6 cells/10 min)	Inhibition (%)
Control[a]		1.19 ± 0.066	0
Beraprost	0.01	1.11 ± 0.077	6.5
	0.1	1.00 ± 0.058	14.9
	1	0.85 ± 0.074*	30.1
	10	0.66 ± 0.085**	44.9
PGI_2	0.01	1.15 ± 0.065	3.9
	0.1	1.00 ± 0.089	17.3
	1	0.84 ± 0.047**	30.1
	10	0.71 ± 0.038**	40.0
PGE_2	0.01	1.11 ± 0.038	5.1
	0.1	1.01 ± 0.080	14.2
	1	0.76 ± 0.047**	36.4
	10	0.59 ± 0.066**	49.8
$PGF_{2\alpha}$	10	1.04 ± 0.066	12.9
	100	0.88 ± 0.075*	25.5
Forskolin	0.5	1.11 ± 0.063	4.9
	5	0.99 ± 0.103	18.4
	50	0.76 ± 0.072**	34.0

PMNs (1×10^6 cells/mL) were preincubated in the presence or absence of beraprost and other drugs at 37° for 10 min, and further incubated with 10^{-7} M FMLP for 10 min at 37°. The amount of superoxide anion (O_2^-) production was determined as described in the Materials and Method. All assays were performed in triplicate. Effect of drugs is expressed in % inhibition to non-treated control. Each value represents mean ± SE of four separate experiments.

[a] Stimulated by FMLP alone.

* $P < 0.05$; ** $P < 0.01$.

Table 2. Effect of beraprost, PGI_2 and PGE_2 on cAMP level of rat PMNs stimulated by FMLP

Concentration (μM)	Beraprost		PGI_2		PGE_2	
	cAMP (pmol/10^7 cells)	Increase (% control)	cAMP (pmol/10^7 cells)	Increase (% control)	cAMP (pmol/10^7 cells)	Increase (% control)
Control[a]	4.84 ± 0.34	0	4.99 ± 0.40	0	4.81 ± 0.24	0
0.01	5.40 ± 0.35	11.6	6.31 ± 0.38	26.5	5.45 ± 0.33	13.3
0.1	6.79 ± 0.44**	40.3	7.63 ± 0.41**	52.9	6.51 ± 0.38*	35.3
1	8.22 ± 0.67**	69.8	9.18 ± 0.73**	84.0	7.64 ± 0.72**	58.8

PMNs were preincubated at 37° for 1 min in the presence or absence of beraprost, PGI_2 or PGE_2 with IBMX, and further stimulated by 10^{-7} M FMLP for 1 min. cAMP level was determined by radioimmunoassay. All assays were performed in duplicate. Effect of drugs is expressed as % increase to non-treated control. Each value represents mean ± SE of at least four separate experiments.

[a] Stimulated by FMLP alone.

* $P < 0.05$; ** $P < 0.01$.

during this process is the production of superoxide anion [1, 2], which in excess may lead to the tissue injury as well [24]. As shown in Table 1, pretreatment of PMNs with beraprost inhibited the superoxide anion production by FMLP in a concentration-dependent fashion. However, the inhibitory action of beraprost on the superoxide anion production was less effective as compared to the inhibition of chemotaxis. The inhibition was observed significantly at 1 and 10 μM of beraprost and the inhibitory rates were only 30 and 45%, respectively. Either PGI_2 or PGE_2 showed essentially identical effect to beraprost on superoxide anion production, whereas $PGF_{2\alpha}$ little affected it even at 10 μM. Forskolin inhibited superoxide anion production significantly ($P < 0.01$) at 50 μM. The inhibitory effect of forskolin on superoxide anion production as well as chemotaxis suggests that cAMP modulates PMN functions.

Effect on cAMP level. As summarized in Table 2, beraprost did elevate the cAMP level of PMNs in a concentration-dependent fashion. Beraprost increased cAMP level at above 10 nM, although the effect was significant above 100 nM. Similar effect was observed in the cases of PGI_2 and PGE_2. The result clearly indicates that beraprost indeed elevates the intracellular cAMP level at the concentration range which affects the biological activity of PMNs such as chemotaxis.

Effect on inositol phospholipid metabolic turnover. It is well known that PMN activation induced by

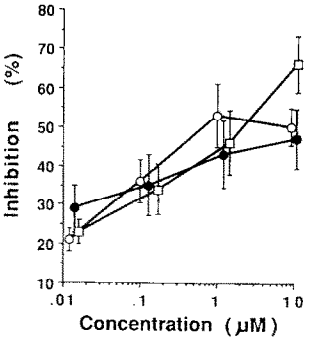
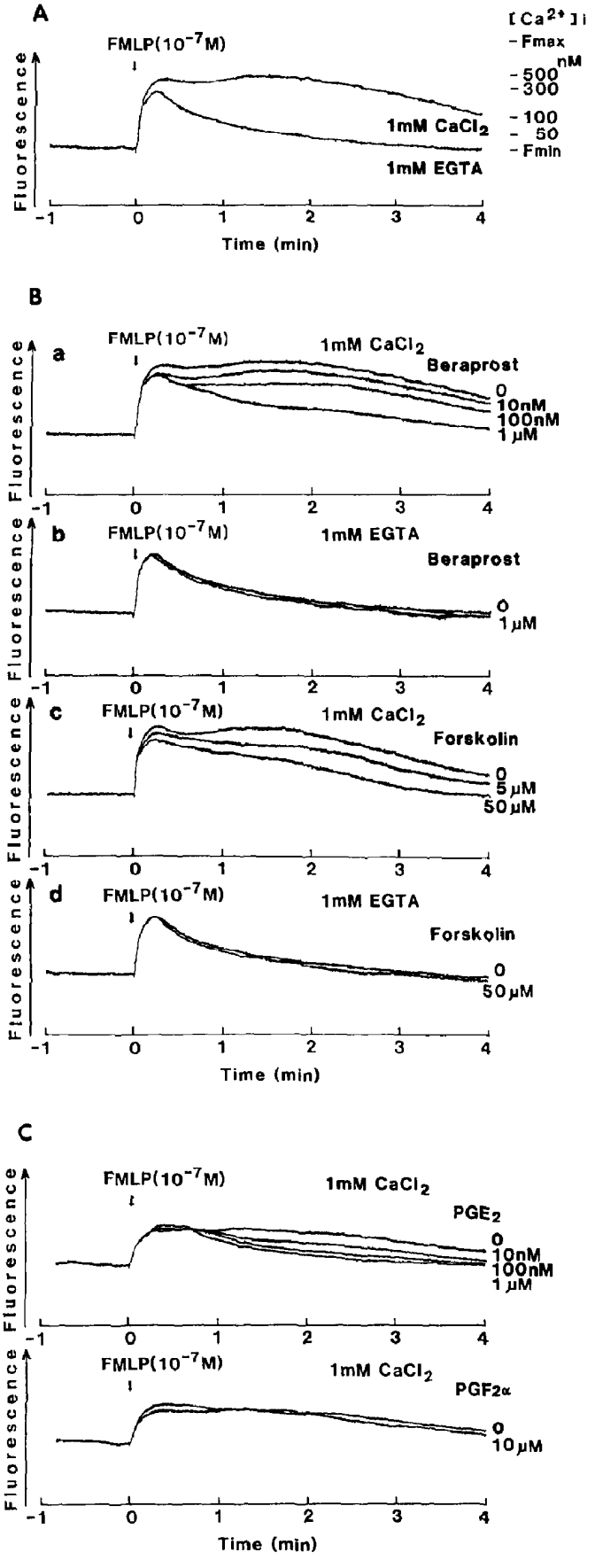


Fig. 3. Inhibitory effect on FMLP-induced inositol phospholipid metabolic turnover by beraprost, PGI_2 and PGE_2 in rat PMNs. Labelling of PMNs was made by the treatment with myo-[^{3}H]inositol and then cells were further incubated with 10 mM LiCl for 10 min. After pretreatment by beraprost (○–○), PGI_2 (●–●) or PGE_2 (□–□) of various concentration for 1 min at 37°, PMNs (2×10^7 cells/mL) were stimulated by 10^{-7} M FMLP for 1 min. All assays were performed in triplicate. Effect of drugs is expressed as % inhibition of [^{3}H]inositol phosphate production to non-treated control. Each point and vertical bar represent mean ± SE of four separate experiments.

FMLP is mediated through inositol phospholipid metabolic turnover [25–27]. We then examined the effect of beraprost on this pathway.

Treatment of PMNs with 10^{-7} M FMLP for 1 min

Fig. 4. (A) Time course of fura-2 fluorescence of PMNs stimulated by FMLP in the presence or absence of 1 mM $CaCl_2$. All samples were taken from the same bath of fura-2 loaded cells. Each experiment was done in 1 mM $CaCl_2$ containing buffer or 1 mM EDTA containing buffer. PMNs (5×10^6 cells/mL) were stimulated by 10^{-7} M FMLP. Fura-2 fluorescence of PMNs (excitation, 340 nm; emission, 505 nm) is indicated in each trace. $[Ca^{2+}]_i$ represents the concentration of intracellular free Ca^{2+}. (B) Effect of beraprost and forskolin on fura-2 fluorescence change of PMNS stimulated by FMLP. After pretreatment with beraprost (a, b) or forskolin (c, d) for 1 or 5 min at 37°, fura-2 loaded PMNs were stimulated by 10^{-7} M FMLP at time 0 in the presence of 1 mM $CaCl_2$ (a, c) and in the presence of 1 mM EGTA (b, d). Each tracing shows a representative result from three separate experiments (excitation, 340 nm; emission, 505 nm). (C) Effect of PGE_2 and $PGF_{2\alpha}$ on fura-2 fluorescence changes of PMNs stimulated by FMLP. After pretreatment with PGE_2 and $PGE_{2\alpha}$ for 1 min at 37°, fura-2 loaded PMNs were stimulated by 10^{-7} M FMLP. Each tracing shows a representative result from three separate experiments (excitation, 340 nm; emission, 505 nm).


A
FMLP(10⁻⁷M)
[Ca²⁺]i
- Fmax
nM
- 500
- 300
- 100
- 50
- Fmin
1mM CaCl₂
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Fluorescence
-1
0
1
2
3
4
Time (min)
B
a
FMLP(10⁻⁷M)
1mM CaCl₂
Beraprost
0
10nM
100nM
1 μM
b
FMLP(10⁻⁷M)
1mM EGTA
Beraprost
0
1 μM
c
FMLP(10⁻⁷M)
1mM CaCl₂
Forskolin
0
5 μM
50 μM
d
FMLP(10⁻⁷M)
1mM EGTA
Forskolin
0
50 μM
Fluorescence
Time (min)
C
FMLP(10⁻⁷M)
1mM CaCl₂
PGE₂
0
10nM
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1 μM
FMLP(10⁻⁷M)
1mM CaCl₂
PGF2α
0
10 μM
Fluorescence
Time (min)

accelerated inositol phospholipid metabolic turnover by two and a half times of the control level (data not shown). As shwn in Fig. 3, preincubation of PMNs with beraprost inhibited FMLP-induced inositol phospholipid metabolic turnover in a concentration-dependent fashion. However, the only partial inhibition was observed at higher concentration (10 μM) of beraprost and IC_{50} value was estimated to be about 1 μM. PGI_2 and PGE_2 similarly inhibited FMLP-induced inositol phospholid metabolic turnover.

Effect on intracellular free calcium level. Finally, we examined the effect of beraprost on the intracellular Ca^{2+} mobilization of PMNs on activation.

As shown in Fig. 4A, concentration of intracellular free Ca^{2+} can be measured by fluorescence of fura-2, although, we must beware that the relation between them is non-linear. Stimulation of PMNs by FMLP in the presence of 1 mM $CaCl_2$ induced the elevation of intracellular free Ca^{2+} level, which apparently consisted of two phases, that is, a rapid transient elevation immediately after the stimulation (first phase) and subsequent much gradual elevation (second phase). When 1 mM EGTA instead of $CaCl_2$ was added to the system, the second phase of Ca^{2+} elevation was inhibited drastically, while the first transient elevation was little affected. The result indicates that the second phase of the response is fully dependent on the extracellular Ca^{2+}, whereas the former is not. Therefore, it is possible that the second phase of the response is due to the increase of influx of extracellular Ca^{2+}. The pretreatment of PMNs with beraprost before FMLP stimulation at more than 10 nM selectively suppressed the second phase elevation of intracellular free Ca^{2+} level in presence of 1 mM $CaCl_2$ in a concentration-dependent fashion and 1 μM of beraprost almost completely inhibited it [Fig. 4B (a)]. Meanwhile, in the presence of 1 mM EGTA, however, beraprost (1 μM) hardly affected the first phase of Ca^{2+} elevation by FMLP [Fig. 4B (b)]. The approximate IC_{50} value for the inhibition of the second phase elevation of intracellular free Ca^{2+} level lies roughly between 10–100 nM. Forskolin exhibited essentially identical effect to beraprost on the FMLP-induced Ca^{2+} elevation of PMNs [Fig. 4B (c, d)]. PGE_2 in the presence of extracellular Ca^{2+} inhibited elevation of intracellular free Ca^{2+} at more than 10 nM, whereas $PGF_{2\alpha}$ little affected even at 10 μM (Fig. 4C). These data suggest that beraprost inhibits the influx of extracellular Ca^{2+}, that is, the secondary gradual Ca^{2+}elevation without affecting the initial rapid Ca^{2+} release from the intracellular Ca^{2+} storage sites.

DISCUSSION

In the present study, using beraprost, a stable and orally active agent with a PGI_2-like structure [15], we have confirmed that this compound, as well as PGE_1 and PGE_2, could inhibit the chemotaxis and superoxide anion production of PMNs in a concentration-dependent fashion. The inhibitory effect of PGE series on PMN functions was reported to be based on cAMP elevating effect [5–8]. Beraprost was also observed to elevate the cellular cAMP level of PMNs. The concentration–response curve of the inhibition of PMN function such as chemotaxis by beraprost was similar to that of the increment of intracellular cAMP level, indicating that beraprost might also inhibit PMN function through intracellular cAMP elevating effect. This speculation is also supported by the finding that forskolin inhibited chemotaxis of PMNs in a relatively low concentration. Meanwhile, the inhibition of superoxide anion production by beraprost was less effective than chemotaxis in PMN, that is to say, the inhibition of superoxide production was observed at relatively high concentrations of beraprost. Therefore, it seems that inhibitory mechanisns of beraprost for superoxide anion production and chemotaxis are not due to the identical pathway.

PMN activation by FMLP was reported to be mediated by the signal transduction systems in which phospholipase C is initially activated [25, 27]. It is believed that activation of phospholipase C accelerates inositol phospholipid metabolic turnover, that is to say, promotes the breakdown of phosphatidyl inositol-4,5-bisphosphate (TPI) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) [26], and that this IP_3 induces the mobilization of free Ca^{2+} from intracellular storage sites. Subsequently, IP_3-induced elevation of intracellular Ca^{2+} level probably causes alteration in various Ca^{2+}-dependent steps. Such mechanism might also be true in the present study. In fact, treatment of PMNs with FMLP accelerated inositol phospholipid metabolic turnover and caused early increase (first phase) in intracellular Ca^{2+} level probably through either the activation of phospholipase C or following increase in IP_3 production. Subsequently, the continuous increase of intracellular Ca^{2+} i.e. second phase increase, was observed due to augmentation of the influx of extracellular Ca^{2+}.

Beraprost failed to inhibit Ca^{2+} mobilization from intracellular storage sites immediately after the stimulation by FMLP. Therefore, it seems that beraprost does not affect directly the process of degradation of TPI to IP_3 and DG. On the other hand, beraprost effectively inhibited the sustained increase of intracellular Ca^{2+} level i.e. the influx of extracellular Ca^{2+}, and the inhibition by beraprost occurred in accordance with the intracellular cAMP elevation. Furthermore, forskolin also inhibited the influx of extracellular Ca^{2+}. These findings imply that there may be a tightly coupled linkage between cAMP elevation by beraprost and modulation of the influx of extracellular Ca^{2+} in PMNs. However, the detailed mechanisms in which cAMP inhibits the influx of extracellular Ca^{2+} remain to be proved.

The present results also showed that inositol phospholipid metabolic turnover was also inhibited by beraprost, although the inhibition was not complete at even high concentration of beraprost. In addition, as mentioned above, beraprost does not affect directly the process of initial IP_3 production after FMLP stimulation. Therefore, these findings suggest that beraprost modifies the inositol phospholipid metabolic turnover not directly but indirectly. The precise mechanism of such inhibitory action of beraprost on inositol phospholipid metabolic turnover remains to be clarified. A possible explanation is that an increase of intracellular Ca^{2+} level accelerates the inositol phospholipid metabolic turnover through

phospholipase C activation in a direct or indirect fashion, and that the suppressive effect of beraprost on the increase of intracellular Ca^{2+} level by the inhibition of Ca^{2+} influx decreases inositol phospholipid metabolic turnover rate. In the present results effect of beraprost on superoxide anion production was inhibitory. However, the incomplete inhibition of beraprost was observed even at higher concentrations similarly to the inhibition of inositol phospholipid metabolic turnover. Beraprost possibly inhibits superoxide anion production through the indirect inhibition of inositol phospholipid metabolic turnover, because the decreased DG production causes the suppression of protein kinase C/$NADPH^+$ oxidase system. However, we cannot exclude other mechanisms of inhibition of superoxide anion production by beraprost.

In addition, in the present study, the inhibition of FMLP-induced chemotaxis by beraprost is relatively well correlated with the inhibitory effect on the influx of extracellular Ca^{2+} and the cAMP elevating effect of the drug. That is to say, it is suggested that elevation of intracellular cAMP level induced by beraprost inhibits PMN function such as chemotaxis mainly through the effect on intracellular Ca^{2+} level especially inhibition of the influx of extracellular Ca^{2+}.

PGs have been considered to be the mediator to modify the various inflammatory processes [4]. PGE_1 and PGE_2, for instance, promote inflammation by augmenting vascular permeability and enhancing chemotaxis of PMNs. In addition, there are substantial evidences that they also exhibit inhibitory effects on inflammation [5–8]. These results indicate that PGE_1 and PGE_2 have equivocal actions for inflammation depending on the stages and the types of inflammation. Meanwhile, our present results clearly indicate that beraprost has a potent inhibitory effect on PMN function especially FMLP-induced chemotaxis. These effects were similar to the effect of PGI_2. This supports the notion that both beraprost and PGI_2 inhibit the inflammatory process at the stages of migration of PMNs to the inflammatory sites and the subsequent activation including superoxide anion production. However, in the present study, the inhibition of superoxide anion production was only effective at relatively high concentration of beraprost. Therefore, inhibition of chemotaxis by beraprost seems to be a much more important mode of action than that of superoxide anion production in PMNs. The stability of beraprost allows *in vivo* application and may be useful for the elucidation of the role of PGI_2 in the inflammatory disease.

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