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Stimulation of protein kinase C redistribution and inhibition of leukotriene B_4-induced inositol 1,4,5-trisphosphate generation in human neutrophils by lipoxin A_4

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1 To test the hypothesis that protein kinase C (PKC) is involved in the inhibitory actions of lipoxin A_4 (LXA_4) on second messenger generation, we studied the effects of LXA_4 on PKC in human neutrophils and on leukotriene B_4 (LTB_4)-stimulated inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) generation.

2 LXA_4, 1 μM, caused a fall in cytosolic PKC-dependent histone phosphorylating activity to 23.5% of basal levels.

3 LXA_4, caused an increase in particulate PKC-dependent histone phosphorylating activity with a bell-shaped dose-response fashion; maximal stimulation was observed at 10 nM LXA_4.

4 Western blot analysis with affinity-purified antibodies to α- and β-PKC showed that only the β-PKC isotype was translocated by LXA_4.

5 LXA_4 inhibited LTB_4-stimulated $Ins(1,4,5)P_3$ generation in a bell-shaped fashion with maximal inhibition at 1 nM LXA_4. The observed inhibition was dose-dependently removed by pre-incubation with a PKC inhibitor (Ro-31-8220).

6 These results show that LXA_4 activates PKC in whole cells and supports a role for PKC activation in the inhibitory action of LXA_4 on LTB_4-induced $Ins(1,4,5)P_3$ generation.

7 LXA_4 (1-1000 nM) pre-incubation did not affect specific binding of [^{3}H]-LTB_4 to neutrophils. Thus, the inhibitory effect of LXA_4 on LTB_4-stimulated $Ins(1,4,5)P_3$ generation could not be attributed to an effect on LTB_4 receptors.

Keywords: Lipoxin A_4; neutrophil; protein kinase C; inositol 1,4,5-trisphosphate; Ro-31-8220; leukotriene B_4

Introduction

Lipoxin A_4 (LXA_4) is a metabolite of arachidonic acid which is biosynthesized either via interactions between 5- and 15-lipoxygenases in cells possessing both enzymes, such as neutrophils, or by transcellular metabolism involving 5- and 12-lipoxygenases such as that which occurs with mixed suspensions of leukocytes and platelets (Serhan, 1991). LXA_4 exhibits a unique profile of biological actions distinct from those of other eicosanoids. It contracts smooth muscle and promotes neutrophil degranulation, superoxide anion generation and chemotaxis, without causing aggregation (Serhan *et al.*, 1984; Dahlen *et al.*, 1987; Lee *et al.*, 1989). In addition to these proinflammatory properties, LXA_4 suppresses leukotriene D_4-induced vasoconstriction, it antagonizes leukotriene B_4 (LTB_4)-induced leukocyte migration and plasma leakage in the hamster cheek pouch model and it inhibits LTB_4 and N-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe)-induced chemotaxis of human neutrophils (Badr *et al.*, 1989; Lee *et al.*, 1989; Hedqvist *et al.*, 1989). These inhibitory actions suggest that LXA_4 may be a potential anti-inflammatory compound. LXA_4 also inhibits signal transduction events, including LTB_4 and fMet-Leu-Phe-induced generation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and subsequent Ca^{2+} release in neutrophils (Grandordy *et al.*, 1990; Moores & Merritt, 1991). The ability of LXA_4 to inhibit these signal transduction events may explain the inhibitory actions of LXA_4 at a functional level; however, the mechanism for inhibition of signal transduction by LXA_4 is not known. Since stimulation of PKC by phorbol esters is known to inhibit agonist-induced $Ins(1,4,5)P_3$ generation and Ca^{2+} mobilization in cells (MacIntyre *et al.*, 1985; Rickard & Sheterline, 1985; Zavoico *et al.*, 1985; Nigam *et al.*, 1992) and since LXA_4 is also known to inhibit agonist-induced $Ins(1,4,5)P_3$ generation (Grandordy *et al.*, 1990) we have tested the hypothesis that PKC stimulation is involved in the inhibitory action of LXA_4 on second messenger generation.

PKC exists as a family of isozymes, of which α- and β-PKC are the predominant isoforms found in neutrophils (Majumdar *et al.*, 1991; Smallwood & Malawista, 1992). LXA_4 is known to stimulate isolated PKC (Hansson *et al.*, 1981), but it is not known whether LXA_4 can stimulate PKC in whole cells. In order to test the hypothesis that PKC is involved in the inhibitory actions of LXA_4 we have studied the effects of LXA_4 on the subcellular distribution of PKC levels in intact neutrophils by measurement of histone phosphorylating activity and by Western blotting with antibodies to α- and β-PKC, and assessed the effects of the PKC inhibitor Ro-31-8220 on LXA_4 inhibition of LTB_4-stimulated $Ins(1,4,5)P_3$ accumulation. The effect of LXA_4 on [^{3}H]-LTB_4 binding was also studied to investigate whether the inhibitory activity of LXA_4 on LTB_4-stimulated cells could be attributed to LTB_4 receptor down-regulation.

Methods

Neutrophil isolation

Peripheral blood from healthy volunteers was collected in heparinized syringes and the neutrophils were isolated by dextran sedimentation and lymphoprep isolation as previously described (Lee *et al.*, 1989). These suspensions contained >98% neutrophils as determined by light microscopy and the viability was >99% as determined by trypan blue dye exclusion.

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Stimulation of neutrophils and preparation of subcellular fractions

Cells were resuspended in HBSS/HEPES (10 μM) buffer (pH 7.4) at a concentration of 10^7 cells ml^{-1}. Following pre-incubation at 37°C for 5 min, LXA_4 (1–1000 nM) or HBSS/HEPES buffer (pH 7.8) was added and the incubation continued for a further 8 min. In preliminary experiments (results not shown) optimal stimulation occurs at 8 min with LXA_4 (0.1 μM). The reaction was stopped by the addition of a 5 fold excess of ice-cold HBSS/HEPES buffer (pH 7.4) and the cells were collected by centrifugation at 600 *g*, 5 min, 4°C. The cells were resuspended in 1 ml of extraction buffer consisting of 20 μM Tris (pH 7.5), 10 μM β-mercaptoethanol, 5 mM EDTA, 1 mM phenylmethyl-sulphonylfluoride and 1 mM leupeptin and disrupted by sonication. The homogenate was made up to 5.5 ml with extraction buffer and centrifuged at 100,000 *g* for 30 min at 4°C. The supernatant (cytosolic fraction) was collected and the pellet was treated with 1 ml of 0.2% (v/v) Triton X-100 for 30 min to extract PKC (particulate fraction). The fractions were assayed for PKC activity within 24 h.

PKC assay

PKC was determined in triplicate according to a modification of the method of Gay & Stitt (1988). The reaction mixture (200 μl) consisted of 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 200 μg ml^{-1} histone and 50 μl of enzyme fraction (cytosolic fractions diluted 1:5 with extraction buffer, particulate fractions undiluted) in the presence or absence of 0.6 mM $CaCl_2$, 50 μg ml phosphatidyl-L-serine and 1 μg ml^{-1} 1,2- diolein. The reaction was initiated by the addition of ATP (50 μM, containing 0.5 μCi [^{32}P]-ATP) and the incubation was carried out for 15 min at 30°C. At the end of the incubation, 100 μl aliquots were transferred onto 2.5 cm squares of phosphocellulose binding paper (Whatman P81), and the binding paper squares were washed twice for 10 min with 1% (v/v) orthophosphoric acid. The bound radioactivity was measured by liquid scintillation counting. PKC activity was defined as the amount of ^{32}P incorporated into histone per min per 10^7 cells minus that obtained in the absence of calcium and lipids.

Western blotting

Cytosol and particulate fractions obtained from neutrophils treated with LXA_4 (10 nM, 1 μM) were separated by SDS–PAGE (10%) at 10 μg/lane for cytosolic fractions and 20 μg/lane for particulate fractions. Proteins were electroblotted onto nitrocellulose membranes at 14 V overnight as described by Towbin *et al.* (1979). Molecular weight markers were identified by staining with Ponceau S in 1% (v/v) acetic acid. The nitrocellulose membranes were blocked with 5% (w/v) fat-free dry milk in 25 mM Tris-Cl (pH 7.5), 0.15 M NaCl, 0.2% (v/v) Tween-20 (TBST) for 4 h at room temperature, washed with TBST and immunostained overnight with 1:2500 dilution of anti-peptide affinity purified antibodies to α- and β-PKC. Following 3 washes of 20 min each with TBST, the membranes were incubated with a 1:4000 dilution of peroxidase-labelled anti-rabbit IgG for 2 h at room temperature and the protein bands were visualised with enhanced-chemiluminescent reagents according to the manufacturer's instructions.

Stimulation of neutrophils and extraction of Ins(1,4,5)P_3

Neutrophils (10^7 cells ml^{-1}, 0.25 ml final volume) were pre-incubated with LiCl (5 mM) for 5 min at 37°C. LXA_4 or HBSS/HEPES buffer (pH 7.4) was added and the incubation continued for a further 8 min. An optimal concentration of LTB_4 (1 μM) for Ins(1,4,5)P_3 stimulation, which has previously been defined, was added. The reaction was stopped after 10 s by adding 0.3 ml of ice-cold trichloroacetic acid (1 M). Tubes were left on ice for 20 min, after which they were centrifuged at 12,000 *g* for 5 min. EDTA (10 mM, 0.125 ml.) and 0.5 ml of 1,1,2-trichlorofluoroethane/tri-n-octylamine (1:1 v/v) mixture were added and samples were vortex-mixed and centrifuged at 12,000 *g* for 4 min. The aqueous phase was removed and neutralised with 125 μl of $NaHCO_3$ (160 mM). Aliquots were assayed for Ins(1,4,5)P_3 content as described below. In inhibition experiments, cells were incubated with the PKC inhibitor, Ro-31-8220, at 37°C before an optimal concentration of LXA_4 (1 nM) was added, and the cells were stimulated with LTB_3 (1 μM) and Ins(1,4,5)P_3 extracted as before.

Measurement of Ins(1,4,5)P_3

Ins(1,4,5)P_3 binding protein was isolated from bovine adrenal glands as described (Challis *et al.*, 1990) and the protein content was determined by the method of Bradford (1976) and adjusted to 20 mg ml^{-1}. Aliquots were frozen at −20°C until used. On the day of the assay, 30 μl of the binding protein was added to tubes containing 30 μl of [^{3}H]-Ins(1,4,5)P_3 (15–30 Ci $mmol^{-1}$, diluted to 8000 c.p.m. 30 μl^{-1}) and 30 μl of Tris/EDTA buffer (pH 7.8) plus either 30 μl of standard Ins(1.4.5)P_3 (0.3–300 μM/tube, prepared by serial dilution) or 30 μl of sample. The tubes were vortex-mixed and incubated at 4°C for 30 min, after which they were centrifuged at 2200 *g* for 15 min at 4°C and the supernatant removed by decantation. Deionised water (60 μl) was added to the pellet and after vortex-mixing, the radioactivity was determined by liquid scintillation counting. Total binding was determined in the absence of cold Ins(1,4,5)P_3 and non-specific binding was determined in the presence of 40 mM cold Ins(1,4,5)P_3.

Binding of [^{3}H]-LTB_4 to neutrophils

Cells were resuspended in HBSS/HEPES (30 mM) buffer (pH 7.4) containing 0.1% (w/v) BSA, at a concentration of 15×10^6 cells ml^{-1}. LXA_4 (1 nM–1 μM), or buffer, was added and the cells were incubated at 37°C for 8 min. At the end of the incubation, 100 μl of the cell suspension was added to tubes containing 50 μl of [^{3}H]-LTB_4 (0.1 nM final concentration) and 50 μl of increasing concentrations of cold LTB_4 (0.1–1000 nM) or 50 μl of buffer to determine total binding. The cells were incubated at 4°C for 40 min. The bound and free radioactivity were separated by vacuum filtration using GF-C filters and rapid washing with ice-cold BSA-free HBSS/HEPES buffer (pH 7.4). The filters were left to soak in 80% ethanol overnight and the radioactivity was measured by liquid scintillation.

Materials

Hanks balanced salt solution (HBSS), with and without calcium and magnesium, and affinity-purified antipeptide antibodies to α-PKC (AGNKVISPSQNRRG, residues 313–326 of α-PKC) and β-PKC (GPKTPQQKTANTISKFD, residues 313–329 of β-PKC) were obtained from Gibco Life Technologies Ltd. Lymphoprep was purchased from Nycomed. L-Phosphatidyl-L-serine, 1,2-diolein, phorbol 12-myristate 13-acetate (PMA),1-oleoyl-2-acetylglycerol(OAG),leupeptin, phenylmethylsulphonylfluoride, histone (Type IIIS), 2-mercaptoethanol, BSA, ATP, Ins(1,4,5)P_3, EDTA, HEPES, dextran, ovalbumin, dithiothreitol, Tween-20 and nitrocellulose membranes were purchased from Sigma Chemical Company Ltd. P81-ion exchange paper and GF-C filters were obtained from Whatman Ltd. [γ^{32}P]-ATP (3000 Ci $mmol^{-1}$), [^{3}H]-Ins(1,4,5)P_3 (15–30 Ci $mmol^{-1}$), and [^{3}H]-LTB_4 (170–230 Ci $mmol^{-1}$) were obtained from New England Nuclear. Horseradish peroxidase-labelled anti-rabbit IgG and enhanced chemiluminescent detection reagents were purchased from Amersham PLC. 1,1,2-Trichlorofluoroethane and tri-n-octylamine were obtained from Aldrich Chemical Company. Ro-31-8220 was a gift from Roche Company Ltd. All other reagents were analytical grade purchase from BDH Ltd. LTB_4 was synthesised according to the method previously described

(Nicolaou *et al.*, 1984). The methyl ester form of LXA_4 was synthesized as described by Lee *et al.* (1991), and prior to use an aliquot was subjected to alkaline hydrolysis to generate the free acid form of LXA_4, as described by Brezinski & Serhan (1991).

Statistical analysis

Results are expressed as means ± s.e.mean. To allow comparison of values within individual experiments, the 2-tailed paired *t* test was used to compare groups of experiments using PMN from different donors.

Results

Effect of LXA_4 on PKC

In unstimulated cells, 97.6% of the total PKC activity (Ca^{2+}, phospholipid-dependent histone phosphorylating activity) was found in the cytosol and 2.4% was found in the particulate fraction. LXA_4-treated cells showed a fall in cytosolic PKC activity to 23.5% of basal levels at 1 μM LXA_4 (Figure 1). LXA_4 had a biphasic effect on particulate PKC activity with maximal activity observed at 10 nM LXA_4 which was 3.6 fold higher than basal levels (Figure 1). At higher concentrations of LXA_4 there was a progressive decline from the peak level such that at 1 μM LXA_4, the particulate activity was similar to that observed with unstimulated cells.

To compare with the results of LXA_4 on PKC activity, the effects of PMA and OAG were assessed. PMA caused a dose-dependent loss of cytosolic PKC activity to 9.7% of basal levels at 160 nM PMA (Figure 2). The particulate PKC activity increased progressively to a maximum of 2.5 fold higher than basal levels at 160 nM PMA (Figure 2). Like PMA, OAG caused a dose-dependent loss of cytosolic PKC activity and a dose-dependent increase in particulate PKC activity (Figure 3).

To confirm the histone phosphorylation data showing a stimulatory effect of LXA_4 on PKC and to determine the effects on the major PKC isotypes found in neutrophils, the experiments with LXA_4 were repeated at 10 nM and 1 μM LXA_4 and α- and β-PKC was measured in cytosol and particulate fractions by Western blotting using affinity-purified isozyme-specific antibodies to PKC. The results are shown in Figure 4a, b. Antibody to α-PKC detected a 80 kDa immunoreactive band in cytosol fractions from unstimulated cells (Figure 4a). LXA_4 appeared to increase the intensity of this immunoreactive band but this band was not observed in particulate fractions from either LXA_4-stimulated or unstimulated cells, suggesting that there was no stimulation of α-PKC. A lower molecular weight band was also detected in the cytosol fraction, which appeared to be increased in intensity with LXA_4 treatment. However, as this band was not translocated with LXA_4 stimulation, which would have indicated possible proteolysis of PKC, the difference in intensity observed was most likely an artefact, due to slight inequalities of protein loading between the lanes in the gel. This would be supported by the finding that the 80 kDa band was also more intense in the same lane in this gel.

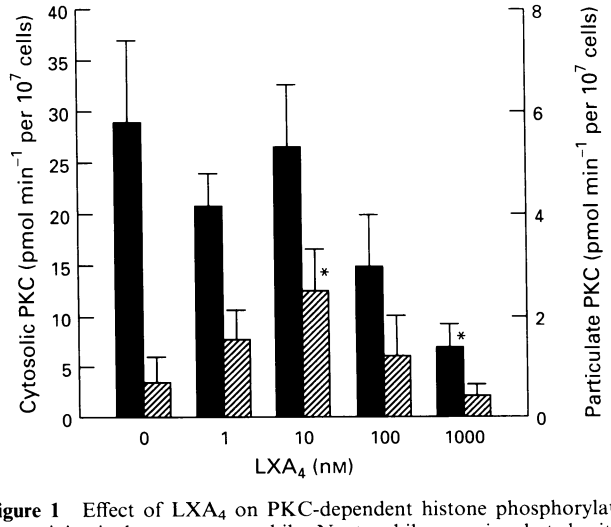

Figure 1 Effect of LXA_4 on PKC-dependent histone phosphorylating activity in human neutrophils. Neutrophils were incubated with LXA_4 for 8 min at the indicated concentrations and the amount of PKC in cytosolic and particulate fractions determined by enzyme assay. The results represent the mean ± s.e.mean of four experiments and are shown as solid columns for cytosolic PKC activity and hatched columns for particulate activity. *$P<0.05$ compared to 0 control.

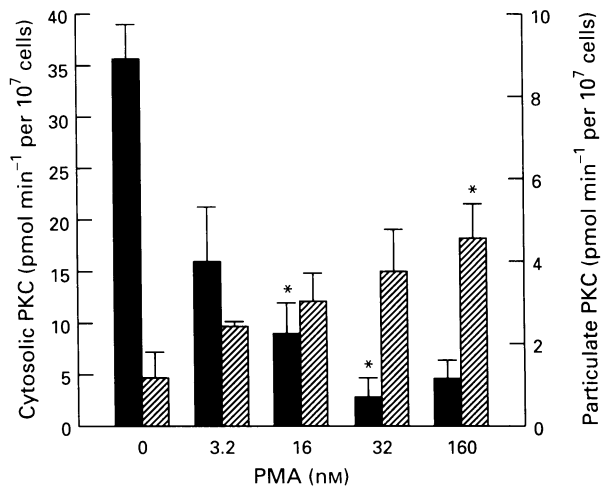

Figure 2 PMA-induced redistribution of PKC-dependent histone phosphorylating activity in neutrophils. Neutrophils were incubated with various concentrations of PMA for 8 min and the amount of PKC determined in subcellular fractions. Cytosolic and particulate PKC activities are represented by solid columns, and hatched columns, respectively. Results are mean ± s.e.mean of three experiments.

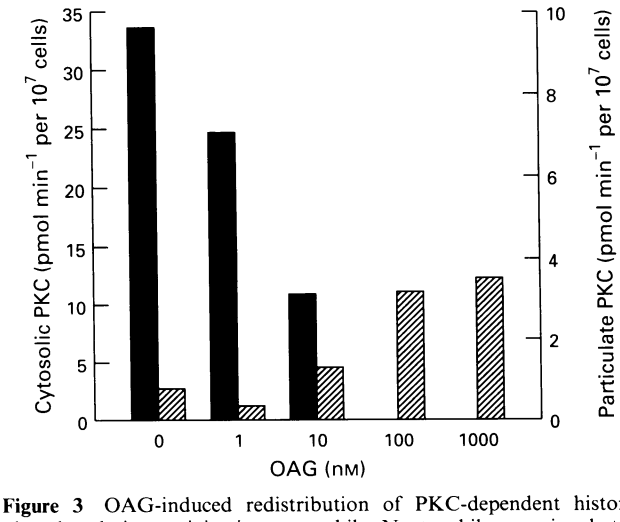

Figure 3 OAG-induced redistribution of PKC-dependent histone phosphorylating activity in neutrophils. Neutrophils were incubated with various concentrations of OAG for 8 min and the amount of PKC determined in subcellular fractions. Cytosolic and particulate PKC activities are represented by solid columns, and hatched columns, respectively. Results are mean of two experiments.

Immunoblots with antibody to β-PKC showed a prominent 80 kDa immunoreactive band present in cytosol fraction of unstimulated cells (Figure 4b). The intensity of this band did not change appreciably in cells treated with 10 nM LXA_4. However, at 1 μM LXA_4 the intensity of this band decreased markedly. A faint 80 kDA immunoreactive band was detected in particulate fractions from unstimulated cells which became more intense on treatment with LXA_4. The effect was more marked at 10 nM LXA_4 compared to 1 μM LXA_4 as observed with PKC-dependent histone phosphorylating activity.

Effect of LXA_4 pretreatment on LTB_4-induced Ins(1,4,5)P_3 generation

In the absence of LXA_4, LTB_4 (1 μM) induced an increase in Ins(1,4,5)P_3 accumulation from a basal level of 3.6±1.7 pmol per 10^7 cells to 35.7±11.9 pmol per 10^7 cells representing an 8.9 fold increase over basal levels. Preincubation of neutrophils with increasing concentrations of LXA_4 from 0.01–1 nM for 8 min dose-dependently inhibited the subsequent generation of Ins(1,4,5)P_3 elicited by LTB_4 (1 μM) to a maximum 70% inhibition at 1 nM LXA_4 (Figure 5). At higher concentrations of LXA_4 the inhibition was progressively reduced to levels which were similar to those observed in the absence of LXA_4 treatment. LXA_4 had no significant effect on Ins(1,4,5)P_3 generation in unstimulated neutrophils (Table 1).

Effect of the PKC inhibitor (Ro-31-8220) on the suppression of LTB_4-induced Ins(1,4,5)P_3 generation by LXA_4

In order to examine the possible role of PKC in mediating the inhibitory action of LXA_4 on LTB_4-induced Ins(1,4,5)P_3 generation, neutrophils were preincubated with the PKC inhibitor, Ro-31-8220, before incubation with an optimal dose of LXA_4 (1 nM) and subsequent stimulation of the cells with LTB_4 (1 μM). Following preincubation of neutrophils for 15 min with increasing doses of Ro-31-8220 there was a dose-dependent attenuation of the inhibitory effects of LXA_4 on LTB_4-induced Ins(1,4,5)P_3 generation (Figure 6). Preincubation of cells for 5 min with 0.01–1000 nM Ro-31-8220 had no effect on LXA_4-induced inhibition of LTB_4-stimulated Ins(1,4,5)P_3 generation (data not shown).

To test whether the PKC inhibitor affected LTB_4-stimulated Ins(1,4,5)P_3 generation, neutrophils were incubated with 100 nM and 1 μM Ro-31-8220 for 15 min and LTB_4-induced Ins(1,4,5)P_3 levels were measured. Mean values (±s.e.mean) obtained from 4 experiments were 22.8±5.8 pmol per 10^7 cells at 100 nM Ro-31-8220 and 22.7±5.9 pmol per 10^7 cells at 1 μM Ro-31-8220 which were similar to the control value of 21.9±6.1 pmol per 10^7 cells obtained with buffer alone. Therefore, the ability of Ro-31-8220 to attenuate the inhibitory effects of LXA_4 on LTB_4-induced Ins(1,4,5)P_3 generation could not be attributed to a direct effect of Ro-31-8220 on LTB_4-induced Ins(1,4,5)P_3 generation.

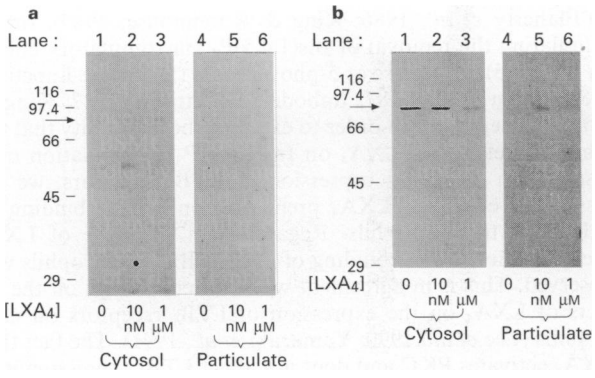

Figure 4 Effect of LXA_4 on the subcellular distribution of α- and β-PKC isotypes in human neutrophils. Neutrophils were treated with 10 nM and 1 μM LXA_4 and cytosol and membrane fractions were electrophoresed on 10% SDS–PAGE gels and immunoblotted. (a) Immunoblot using anti-peptide antibody to α-PKC. (b) Immunoblot using anti-peptide antibody to β-PKC. The positions of the PKC isoforms are indicated by arrows.

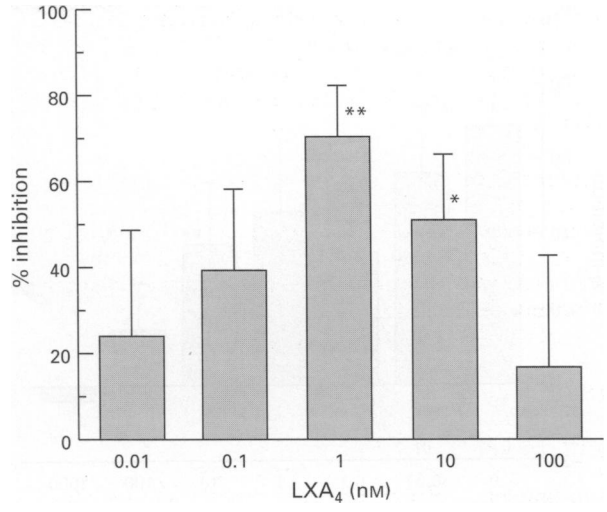

Figure 5 Inhibitory effect of LXA_4 on LTB_4-induced Ins(1,4,5)P_3 generation. Neutrophils were incubated with increasing concentrations of LXA_4 before challenging with LTB_4 (1 μM) and the amount of Ins(1,4,5)P_3 released was measured by radioassay. The values represent the mean±s.e.mean of seven experiments and are expressed as percentage inhibition defined as 100% of control. The mean control value (vehicle-treated control) = 35.7±11.9 pmol per 10^7 cells. *$P<0.05$ compared to control. **$P<0.01$ compared to control.

Table 1 Neutrophil Ins(1,4,5)P_3 levels in response to LXA_4 stimulation

Conc. of LXA_4 (nM)	*Ins(1,4,5)P_3* (pmol per 10^7 cells)
0	5.0±0.8
0.01	4.6±0.4
0.1	4.6±1.1
1.0	3.4±0.3
10.0	3.1±0.4
100.0	4.0±0.7

Values are mean±s.e.mean, $n=3$.

The effect of LXA_4 on LTB_4 receptor expression on neutrophils

The possibility that the observed inhibitory effects of LXA_4 on LTB_4-induced Ins(1,4,5)P_3 accumulation was due to down-regulation of LTB_4 receptors was examined by preincubating cells with 1 nM, 10 nM and 1 μM LXA_4 for 8 min at 37°C and then assessing the specific binding of [^{3}H]-LTB_4. The data for all concentrations of LXA_4 were similar. Neutrophils which had been preincubated with LXA_4 demonstrated identical binding of [^{3}H]-LTB_4 as compared to neutrophils which had been preincubated with buffer alone (data not shown). Thus, LXA_4 did not change LTB_4 receptor expression on human neutrophils.

Discussion

LXA_4 is formed in nanomolar and sub-nanomolar amounts in whole blood and in mixed suspensions of platelets exposed to stimuli (Brezinski & Serhan, 1991; Levy *et al.*, 1993). Our

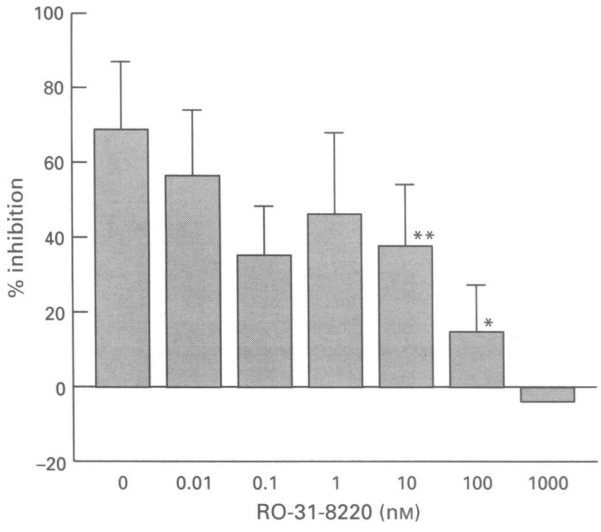

Figure 6 Effect of protein kinase C inhibitor (Ro-31-8220) on the LXA_4 (1 nM) elicited suppression of $InsP_3$ generation induced by LTB_4 (1 μM). Cells were incubated with varying concentrations of Ro-31-8220, or HBSS/HEPES (pH 7.4) buffer as control, for 15 min before challenging with an optimal concentration of LXA_4 and the amount of Ins(1,4,5)P_3 generated in response to LTB_4 was measured by radioassay. Values represent the mean ± s.e.mean of three experiments except at 1000 nM Ro-31-8220 where only 2 experimental values were obtained. $^*P<0.05$ compared to 0 control. $^{**}P<0.01$ compared to 0 control.

findings show that PKC stimulation occurs with LXA_4 treatment at physiologically relevant concentrations of LXA_4. PKC is largely found as a cytosolic enzyme in resting cells (Nishizuka, 1984). Upon stimulation, the enzyme becomes translocated to the cell membrane (Melloni *et al.*, 1986). Both LXA_4 and PMA caused a fall in cytosolic PKC-dependent histone phosphorylating activity. However, the pattern of redistribution of PKC to the membrane fraction was unlike that achieved with the classical PKC activator, PMA, where a progressive increase in particulate PKC occurs with increasing doses of PMA. With LXA_4 there was a bell-shaped dose-response in the particulate PKC activity. This suggests that LXA_4 may exert other effects which are not exhibited by PMA.

PMA stimulates PKC without a net increase in intracellular Ca^{2+} (Pontremoli *et al.*, 1986). However, LXA_4 is known to cause detectable rises in intracellular Ca^{2+} when present at concentrations of 0.1 μM or greater (Moores & Merritt, 1991). This effect on Ca^{2+} produced by LXA_4 may explain the high dose inhibition of particulate PKC observed at concentrations of 0.1 – 1.0 μM LXA_4 since Ca^{2+} is known to stimulate calpain and the neutral serine protease found in neutrophils which catalyse the proteolytic conversion of PKC to a Ca^{2+}, phospholipid-independent form (Melloni *et al.*, 1985; Pontremoli *et al.*, 1990a, b).

PMA activates PKC irreversibly whereas PKC association with membranes by stimulation with its endogenous activator, diacylglycerol, is readily reversible (Thomas *et al.*, 1987). The effects of PMA on PKC are also known to be different from those of diacylglycerol with respect to longevity and permeability (Thomas *et al* 1987). Therefore the effects of OAG, a synthetic diacylglycerol, on PKC were also studied to compare with the effects of LXA_4. Our results showed that the effects of LXA_4 on particulate PKC were also different from that of OAG. Therefore potential differences in longevity of action, permeability or reversibility of PKC translocation are unlikely to explain the LXA_4 effect on particulate PKC.

The maximal amount of PKC activity recovered in the particulate fraction did not equal the maximal amount of cytosolic PKC activity lost on LXA_4 treatment. This is a common finding with PKC stimulation and may reflect the presence of endogenous inhibitors in the membrane fraction (Balazovich *et al.*, 1986) or changes in substrate specificity of particulate PKC (Cochet *et al.*, 1986).

In neutrophils, α- and β-PKC are the predominant isotypes found (Majumdar *et al.*, 1991; Smallwood & Malawista, 1992). Our study showed that of these two PKC isotypes β-PKC was the isotype affected by LXA_4 treatment, and is the first study to demonstrate a selective effect of LXA_4 on a particular PKC isotype. The pattern of redistribution of β-PKC immunoreactivity in response to LXA_4 correlated with the pattern seen by histone phosphorylation. The anti-α-PKC antibody detected a lower molecular weight band found in cytosolic fractions. Since this band was not found to translocate to the particulate fraction it is unlikely to be a proteolytic fragment of PKC. Similar low molecular weight bands have been observed in other studies using antipeptide antibodies (Majumdar *et al.*, 1991; Smallwood & Malawista, 1992). The presence of this band suggests that the oligopeptide sequence which the antibody recognises is present in other neutrophil proteins.

The finding that LXA_4 inhibited LTB_4-induced Ins(1,4,5)P_3 generation cannot be explained by removal of a stimulatory gradient, since prior exposure of cells to LTB_4 at concentrations which stimulate IP_3 generation do not affect the subsequent Ins(1,4,5)P_3 response to other agonists, such as leukotriene D_4 (Winkler *et al.*, 1988). Moreover, our study showed that LXA_4 did not stimulate Ins(1,4,5)P_3 generation at the doses used in our experiments. Instead, our findings that LXA_4 stimulates PKC redistribution and that the PKC inhibitor Ro-31-8220 reversed the effect of LXA_4 on LTB_4-induced Ins(1,4,5)P_3 generation, suggests that LXA_4 exerts its inhibitory effects on Ins(1,4,5)P_3 generation via PKC stimulation. The finding that maximal inhibition of IP_3 generation occurred at 1 nM LXA_4 whereas maximal increase in particulate PKC occurred at 10 nM LXA_4 may indicate the involvement of an additional PKC isoform, possibly nPKC, a novel phospholipid-dependent, but Ca^{2+}-independent family of isozymes which differs in substrate specificity to PKC (Majumdar *et al.*, 1991). Our PKC assay measures total Ca^{2+}, phospholipid-dependent histone phosphorylating activity and therefore may not reflect nPKC isozyme activity profile. Another possibility is that LXA_4 at nonomolar concentrations may exert other at present unknown biochemical signals which influence agonist-induced Ins(1,4,5)P_3 generation.

LXA_4, acting via PKC, may exert its inhibitory effect on LTB_4-induced Ins(1,4,5)P_3 accumulation either by inhibiting the activity of phospholipase C, which is known to occur in platelets stimulated with PKC activators (Rittenhouse & Sasson, 1985), by down-regulation of the LTB_4 receptors as has been demonstrated in neutrophils stimulated with PMA (O'Flaherty *et al.*, 1986; King & Rittenhouse, 1989), or by stimulating the removal of Ins(1,4,5)P_3 via stimulatory effects on Ins(1,4,5)P_3 3-kinase or 5-phosphatase (Molina & Lapetina, 1986; Biden *et al.*, 1987; Imboden & Pattison, 1987: King & Rittenhouse, 1989). In order to examine the possibility that the inhibitory effects of LXA_4 on Ins(1,4,5)P_3 accumulation may result from decreased expression of LTB_4 receptors, we assessed the effects of LXA_4 preincubation on the binding of [^{3}H]-LTB_4 to neutrophils. Regardless of the dose of LXA_4 used, no effect on the binding of [^{3}H]-LTB_4 to neutrophils was observed. This is in agreement with earlier findings on the effects of LXA_4 on the expression of LTB_4 receptors on neutrophils (Lee *et al.*, 1990; Yamazaki *et al.*, 1991). The fact that LXA_4 activates PKC and does not affect LTB_4 expression is in contrast to the inhibitory effects observed with PMA on LTB_4 receptor binding (O'Flaherty *et al.*, 1986; Yamazaki *et al.*, 1991).

LXA_4 is a labile arachidonic acid metabolite and it must be considered whether the effects observed with LXA_4 under our conditions are a result of an active metabolite. However, previous studies with neutrophils and radiolabelled LXA_4 have

shown that LXA_4 is not metabolized when neutrophils are incubated for 30 min at 37°C (Brezinski & Serhan, 1991). LXA_4 may act intracellularly at its site of synthesis as LXA_4 is known to activate isolated PKC (Hansson, 1989), or it may act extracellularly after release from cells (Serhan, 1994). The mechanism by which LXA_4 acts on whole cells to stimulate PKC is not known, but may occur after active transport into the cytoplasm. A specific uptake carrier that is distinguishable from the LXA_4 receptor has been described in neutrophils (Serhan, 1994).

In conclusion, we have found that LXA_4 stimulates PKC redistribution in intact neutrophils with β-PKC the major isotype affected. Our results support a role for PKC activation in the mechanism of LXA_4 inhibition of LTB_4-induced $Ins(1,4,5)P_3$ generation.

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