Early signalling events implicated in leukotriene B_4-induced activation of the NADPH oxidase in eosinophils: role of Ca^{2+}, protein kinase C and phospholipases C and D

Rosie S. PERKINS, Mark A. LINDSAY, Peter J. BARNES and Mark A. GIEMBYCZ*

Department of Thoracic Medicine, Royal Brompton National Heart and Lung Institute, London SW3 6LY, U.K.

The early signalling events that may ultimately contribute to the assembly and subsequent activation of the NADPH oxidase in guinea-pig peritoneal eosinophils were investigated in response to leukotriene B_4 (LTB_4). LTB_4 promoted a rapid, transient and receptor-mediated increase in the rate of H_2O_2 generation that was potentiated by R 59 022, a diradylglycerol (DRG) kinase inhibitor, implicating protein kinase C (PKC) in the genesis of this response. This conclusion was supported by the finding that the PKC inhibitor, Ro 31-8220, attenuated (by about 30%) the peak rate of LTB_4-induced H_2O_2 generation under conditions where the same response evoked by 4β-phorbol 12,13-dibutyrate (PDBu) was inhibited by more than 90%. Paradoxically, Ro 31-8220 doubled the amount of H_2O_2 produced by LTB_4 which may relate to the ability of PKC to inhibit cell signalling through phospholipase C (PLC). Indeed, Ro 31-8220 significantly enhanced LTB_4-induced Ins(1,4,5)P_3 accumulation and the duration of the Ca^{2+} transient in eosinophils. Experiments designed to assess the relative importance of DRG-mobilizing phospholipases in LTB_4-induced oxidase activation indicated that phospholipase D (PLD) did not play a major role. Thus, although H_2O_2 generation was abolished by butan-1-ol, this was apparently unrelated to the inhibition of PLD, as LTB_4 failed to stimulate the formation of Ptd[^{3}H]BuOH in [^{3}H]butan-1-ol-treated eosinophils. Rather, the inhibition was probably due to the ability of butan-1-ol to increase the eosinophil cyclic AMP content. In contrast, Ca^{2+}- and PLC-driven mechanisms were implicated in H_2O_2 generation, as LTB_4 elevated the Ins(1,4,5)P_3 content and intracellular free Ca^{2+} concentration in intact cells, and co-chelation of extracellular and intracellular Ca^{2+} significantly attenuated LTB_4-induced H_2O_2 generation. Pretreatment of eosinophils with wortmannin did not affect LTB_4-induced H_2O_2 production at concentrations at which it abolished the respiratory burst evoked by formylmethionyl-leucylphenylalanine in human neutrophils. Collectively, these data suggest that LTB_4 activates the NADPH oxidase in eosinophils by PLD- and PtdIns 3-kinase-independent mechanisms that involve Ca^{2+}, PLC and PKC. Furthermore, the activation of additional pathways that do not require Ca^{2+} is also suggested by the finding that LTB_4 evoked a significant respiratory burst in Ca^{2+}-depleted cells.

INTRODUCTION

Eosinophil leucocytes were originally believed to act as tissue-preserving cells; their ability to release neutralizing enzymes such as histaminase, phospholipase D (PLD) and arylsulphatase were proposed to counter the potentially damaging effects of mast cell degranulation products [1]. Furthermore, the elevated levels of circulating eosinophils noted in diseases involving tissue-invading parasites together with their status as the major cytotoxic cell for parasitic larvae [2] suggested that, like neutrophils, they play an important role in host defence. However, it is now appreciated that eosinophils, when activated, are cytotoxic for many mammalian cells, particularly those of the gut, skin and lung, a finding that has implicated them in the pathogenesis of a number of non-parasitic inflammatory disorders, notably Crohn's disease, atopic dermatitis and allergic asthma [3].

A major property of eosinophils that has the potential to promote and/or exacerbate allergic inflammation in the airways is their ability to undergo a massive increase in O_2 consumption in response to soluble and particulate stimuli [4,5] culminating in the generation of highly reactive O_2-derived free radicals [6–8]. Indeed, the capacity of eosinophils to produce these toxic O_2 intermediates is significantly greater than that of neutrophils [6–8]. As in other phagocytic cells, the increase in O_2 utilization is due to the activation of a membrane-associated enzyme, NADPH–O_2 oxidoreductase [9–11], which catalyses the single-electron reduction of molecular O_2 to $O_2^{\cdot-}$, a powerful oxidizing and reducing agent [12]. In the presence of superoxide dismutase (SOD), which has been localized to the cytosol of eosinophils with an activity comparable with neutrophils [13], $O_2^{\cdot-}$ undergoes reduction (dismutation) to H_2O_2 the other principal product of the oxidative burst from these cells. Under the influence of eosinophil peroxidase (which may be co-released from activated eosinophils with $O_2^{\cdot-}$), halogens (normally bromide [14,15]) are oxidized by H_2O_2 to their respective hypohalous acid which may exacerbate asthmatic inflammation by promoting the cytolysis of pneumocytes and epithelial cells [16,17] and by degranulating mast cells [18].

In human neutrophils, activation of the respiratory-burst oxidase depends minimally on the translocation of a small GTP-

Abbreviations used: PL, phospholipase; PKC, protein kinase C; $O_2^{\cdot-}$, superoxide anion; LTB_4, leukotriene B_4; PDBu, phorbol 12,13-dibutyrate; fMLP, *N*-formylmethionyl-leucylphenylalanine; OZ, serum-opsonized zymosan; SOD, superoxide dismutase; DRG, diradylglycerol; PtdBuOH, phosphatidylbutan-1-ol; HRPO, horseradish peroxidase; HBSS, Hanks balanced-salt solution; Percoll, polyvinylpyrrolidine-coated silica gel; BAPTA/AM, 1,2-bis-(*O*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular free calcium concentration; U-75,302, 6-[6-(3-hydroxy-1*E*,5*Z*-undecadien-1-yl)-2-pyridinyl]-1,5-hexanediol; U-73,122, 1-(6{-[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione; U-73,343, 1-(6{-[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrolidine-2,5-dione.

* To whom correspondence should be addressed.

binding protein, p21^{rac2}, and two other proteins, p47*phox* and p67*phox*, from the cytosol to the plasmalemma where $O_2^{\cdot-}$ ions are generated in NADPH-dependent manner by the flavoprotein, cytochrome *b*-558 [9–11]. Evidence for an apparently identical mechanism of oxidase activation in eosinophils is also available [19,20]. Bolscher and colleagues [19] have identified by Western blotting two cytosolic proteins of molecular mass 47 and 67 kDa which, in a reconstituted cell-free system, activate both the eosinophil and neutrophil oxidase. Furthermore, immunoblot and absorption spectroscopy analyses indicate that eosinophil membranes contain large amounts of cytochrome *b*-558 which is absent from eosinophils obtained from patients with X-chromosome-linked chronic granulomatous disease [19,20].

The intracellular sequence of events that precede, and ultimately promote, the assembly of an active oxidase complex is incompletely understood but appears to depend on the nature of the activating stimulus [11,21]. In neutrophils, persuasive evidence is available that soluble agonists utilize a pathway(s) that involves the participation of phospholipase A_2 (PLA_2), phospholipase C (PLC), PLD, protein kinase C (PKC), PtdIns 3-kinase and one or more tyrosine kinases that are not necessarily mutually exclusive [22–32]. Mechanistically, perhaps the best studied stimulant of $O_2^{\cdot-}$ generation in the neutrophil is formylmethionylleucylphenylalanine (fMLP). Recent studies suggest that the interaction of fMLP with its cognate receptor activates the respiratory burst, at least in part, by enhancing PKC activity [24]. The generation of diradylglycerol (DRG) that is prerequisite for this biochemical response arises predominantly, although not exclusively, from phosphatidylcholine under the influence of PLD [22,23,29] via a catalysis that apparently requires the activation of a tyrosine kinase(s) [25]. Furthermore, there is increasing evidence that arachidonic acid and PtdIns(3,4,5)P_3 play a pivotal role in receptor-mediated oxidase activation [27,31] implicating PLA_2 [26] and PtdIns 3-kinase [32] in the genesis of this response.

In eosinophils, little is known of the cell signalling pathway(s) that governs the activation of the respiratory-burst oxidase, despite the importance of this response in host defence and inflammatory diseases. Experiments were therefore designed to gain fundamental information about the early signalling events that may be involved in the production of O_2-derived free radicals in eosinophils. Particular emphasis was placed on establishing whether the intracellular pathways utilized by neutrophils are shared by eosinophils. To this end, the possible role that Ca^{2+}, PKC, PLC, PLD and PtdIns 3-kinase may play in the generation of H_2O_2 evoked by leukotriene B_4 (LTB_4), a potent pro-inflammatory mediator that is a consistent and effective stimulant of the respiratory-burst oxidase [4,5], was assessed.

EXPERIMENTAL

Drugs and analytical reagents

The following drugs and analytical reagents were used: 4α- and 4β-PDBu (Scientific Marketing Associates, Barnet, Kent, U.K.); Percoll and Ficoll-Paque (Pharmacia, Uppsala, Sweden); Hanks balanced-salt solution (HBSS; Flow Laboratories, Rickmansworth, Herts., U.K.); R59 022 (Janssen Life Sciences, Beerse, Belgium); fura 2/AM, wortmannin and BAPTA/AM (Calbiochem, Nottingham, U.K.), D-*myo*-Ins(1,4,5)P_3 (Semat Technical Ltd., St. Albans, Herts., U.K.); [^{3}H]butan-1-ol (25.8 Ci/mmol) and [^{14}C]PtdBuOH (11 Ci/mmol) (Amersham International, Bucks., U.K.); D-[*inositol*-1-^{3}H(n)]Ins(1,4,5)P_3 (21 Ci/mmol) and adenosine 3′,5′-cyclic monophospho-2-*O*-succinyl-3-[^{125}I]iodotyrosyl methyl ester (2000 Ci/mmol) (NEN/DuPont, Stevenage, Herts., U.K.); Ro 31-8220 (Roche Products Ltd., Welwyn, Herts., U.K.); LTB_4 (Bayer, Stoke Poges, Slough, Bucks., U.K.); U-75,302, U-73,122 and U-73,343 (Upjohn, Kalamazoo, MI, U.S.A.). Whatman GF/B glass-fibre filters and silica-gel TLC plates were from Whatman (Maidstone, Kent, U.K.) and organic solvents and buffer reagents (AnalaR grade) were obtained from BDH (Poole, Dorset, U.K.). All other drugs and reagents were purchased from Sigma (Poole, Dorset, U.K.).

Stock solutions of all drugs were made up in DMSO, except for U-75,302 and dibutyryl cyclic AMP, which were dissolved in ethanol and distilled water respectively. Zymosan A (from *Saccharomyces cerevisiae*) was opsonized by incubation with 20% autologous human serum at 37 °C for 30 min, washed and resuspended in HBSS.

Induction, harvesting and purification of eosinophils

Eosinophils were elicited into the peritoneum of male Dunkin–Hartley guinea pigs (800–1400 g) by weekly intraperitoneal injection of human serum (1 ml per animal), obtained as a by-product of human granulocyte isolations. This procedure led to the production of eosinophil/macrophage-rich peritoneal exudates, substantially or entirely devoid of neutrophils and platelets, within 2–6 weeks.

Some 3–6 days after plasma injection guinea pigs were anaesthetized with ketamine (25 mg/kg body weight) and xylazine (5 mg/kg) and the peritoneal cavity of each animal lavaged with 50 ml of sterile glucose (5%, w/v) injected via a 17G cannula. The lavage fluid was aspirated into conical polypropylene centrifuge tubes and centrifuged at 240 ***g*** for 10 min at 4 °C to pellet cells. These were then washed in HBSS, pooled and finally resuspended in Percoll (1.070 g/ml)-containing buffer A (25 mM Pipes, pH 7.2, 110 mM NaCl, 5 mM KCl, 40 mM NaOH and 5.4 mM glucose) supplemented with foetal calf serum (20%, v/v).

Eosinophils were separated from other cell types by centrifugation of the pooled cell preparation at 1600 ***g*** for 20 min at 18 °C over discontinuous Percoll density gradients (1.080, 1.085, 1.090 and 1.100 g/ml) in buffer A as described by Gartner [33]. Eosinophils were recovered by this procedure from the 1.085/1.090 and 1.090/1.100 g/ml Percoll interfaces and were more than 97% pure and more than 95% viable as assessed by Trypan Blue exclusion. Cells were pooled, washed twice in HBSS and resuspended in the appropriate assay buffer (see below).

Harvesting and purification of human neutrophils

Blood was collected from normal healthy volunteers by antecubital venepuncture into acid/citrate/dextrose (120 mM citric acid, 50 mM trisodium citrate, 110 mM glucose, pH 7.4) and mixed with dextran (6%, w/v) to sediment erythrocytes. The leucocyte-rich plasma was removed and layered on to 10 ml cushions of Ficoll-Paque followed by centrifugation at 400 ***g*** for 40 min at 18 °C. The granulocyte-containing pellet was resuspended in 9 ml of ice-cold sterile distilled water, and osmolarity was restored by the addition of 1 ml of 10 × concentrated HBSS. Granulocytes were pelleted by centrifugation at 1200 ***g*** for 10 min at 4 °C and washed twice in HBSS before resuspension in the appropriate assay buffer. Cell suspensions containing more than 95% neutrophils were used for subsequent experiments.

Measurement of respiratory burst

The ability of eosinophils to generate H_2O_2 was used as an index of respiratory-burst activity and was measured as the horseradish

peroxidase (HRPO)-catalysed oxidation of scopoletin using a modification of that described by Root et al. [34]. Eosinophils (10^6 cells) were resuspended at 10^8 cells/ml in buffer B (10 mM Hepes, pH 7.4, 138 mM NaCl, 6 mM KCl, 1 mM NaH_2PO_4, 5 mM $NaHCO_3$, 5.5 mM glucose) and 10 μl was added to 990 μl of buffer B supplemented with $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), SOD (30 units), HRPO (1 unit) and scopoletin (4 μM) in polystyrene cuvettes and incubated for 5 min at 37 °C. When inhibitors were used, the cell suspension was incubated for a further 5 min before the addition of LTB_4 or 4β-PDBu. Peroxide generation was measured fluorimetrically ($\lambda_{excitation}$ = 350 nm; $\lambda_{emission}$ = 460 mm; slit width = 5 nm) using a thermostatically controlled recording spectrophotofluorimeter fitted with a Peltier stirrer. Changes in fluorescence were monitored continuously for 5–20 min and negative first-derivative plots of the reduction in fluorescence were constructed to obtain the peak rates of scopoletin absorbance. These values were converted into rates of H_2O_2 generation and quantified by interpolation from a standard curve constructed to known concentrations of H_2O_2. This method allows both the total amount and peak rate of H_2O_2 to be measured. Unless stated otherwise, all results are expressed as peak rates of H_2O_2 production which preliminary experimentation identified as being the most reproducible measure of oxidase activity.

Measurement of intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ and chelation with BAPTA/AM

Eosinophils (10^7/ml) were suspended at 37 °C in buffer B supplemented with 0.1% BSA (w/v), and incubated for 30 min with fura 2/AM (1 μM). The cell suspension was then divided equally and incubated for an additional 30 min with or without BAPTA/AM (2 μM) to chelate intracellular Ca^{2+}. After washing of the eosinophils three times in buffer B [+0.1% (w/v) BSA], the ability of LTB_4 to generate H_2O_2 was assessed in the absence (+100 μM EGTA) and presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}. Changes in $[Ca^{2+}]_i$ were monitored in parallel experiments using well-established spectrofluorimetric methods ($\lambda_{excitation}$ = 340/380 nm; $\lambda_{emission}$ = 510 nm; slit width = 4 nm).

Measurement of Ins(1,4,5)P_3 mass

Eosinophils were suspended at 230×10^6/ml in buffer C (10 mM Hepes, pH 7.4, 124 mM NaCl, 4 mM KCl, 0.64 mM NaH_2PO_4, 0.66 mM K_2HPO_4, 5.2 mM $NaHCO_3$, 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose) and stored on ice until required. Assays, performed in duplicate, were conducted at 37 °C in a total volume of 300 μl and were initiated by the addition of 30 μl of eosinophil suspension (7×10^6 cells) to 240 μl of prewarmed buffer C. The cell suspensions were incubated for 5 min, after which LTB_4 (30 μl) was added for the time intervals indicated below. Reactions were terminated by the addition of 300 μl of trichloroacetic acid (1 M), and Ins(1,4,5)P_3 mass was subsequently extracted [35] and measured using a competitive protein-binding assay [36]. The detection limit and sensitivity (IC_{50}) of this assay is 0.4 and 1 pmol of Ins(1,4,5)P_3 respectively.

Measurement of PLD activity

PLD was measured using the method of Randall et al., [37]. Briefly, eosinophils (10^7) and neutrophils (10^7) were resuspended at 37 °C in 500 μl (final volume) of buffer C containing [^{3}H]butan-1-ol (20 μCi) and, where appropriate, the pharmacological agents under investigation. After 5 min preincubation, LTB_4, PDBu, fMLP or serum-opsonized zymosan (OZ) was added (see the Results section for details) and the reaction terminated after 30 min by the addition of 2 ml of ice-cold buffer C. The cells were pelleted by centrifugation, the supernatant was discarded and the cell pellet extracted with 1.5 ml of chloroform/methanol (1:2, v/v). The chloroform was then evaporated under N_2 and the dry lipid extract washed three times with 2 ml of the upper phase of chloroform/methanol/1 M NaOH/distilled water (2:2:1:1, by vol.). Radiolabelled products were separated by TLC (LK6DF silica-gel 60A plates) in the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/distilled water (5:11:2:10, by vol). When the solvent front had reached the top of the plate (running time about 90 min), the chromatogram was dried and the amount of Ptd[^{3}H]BuOH formed quantified by liquid-scintillation counting by scraping each lane of the TLC plate in 1 cm strips and counting them in 2 ml of Filtron X. Authentic Ptd[^{14}C]BuOH (about 2000 d.p.m.) was used as standard and had an R_F value of 0.36.

Measurement of cyclic AMP

Eosinophils were resuspended at 40×10^6/ml in buffer C and stored on ice until required. Assays, performed in duplicate, were conducted at 37 °C in a total volume of 300 μl and were initiated by the addition of 30 μl (1.5×10^6 cells) of eosinophil suspension to 240 μl prewarmed buffer C. The cell suspensions were incubated for 10 min after which 30 μl of butan-1-ol was added. Reactions were terminated after 5 min by the addition of 300 μl of trichloroacetic acid (1 M) and supernatants neutralized as described by Downes et al. [35]. Aliquots (500 μl) of the neutralized extracts were acetylated, by the consecutive addition of triethylamine (20 μl) and acetic anhydride (10 μl), and cyclic AMP mass was measured immediately by radioimmunoassay. Briefly, to 200 μl of acetylated sample, was added 50 μl of adenosine 3′,5′-cyclic monophospho-2-*O*-succinyl-3-[^{125}I]-iodotyrosine methyl ester (2000–3000 d.p.m.) in 0.2% BSA and 100 μl of anti-(cyclic AMP) antibody in 0.2% BSA. After vortex-mixing, samples were incubated overnight at 4 °C and free and antibody-bound cyclic AMP was separated by charcoal precipitation with ice-cold potassium phosphate buffer (100 mM, pH 7.4) in 0.2% BSA and quantified by γ-counting. The detection limit and sensitivity (IC_{50}) of this assay is 10 and 145 fmol of cyclic AMP respectively.

Statistical analysis

Data in the text and Figure legends are means ± S.E.M. of n independent determinations taken from different cell preparations. Where appropriate, Student's t test (two-tailed) or one-way analysis of variance/Newman–Keuls test was used to assess significance between control and treatment groups. The null hypothesis was rejected when $P < 0.05$.

RESULTS

Effect of LTB_4 and 4β-PDBu on H_2O_2 generation

The spontaneous rate of H_2O_2 production from guinea-pig eosinophils was very low (0.30 ± 0.10 nmol/min per 10^6 cells, n = 10). LTB_4 stimulated the production of H_2O_2 in a concentration-dependent manner with an EC_{50} of 28.0 ± 4.7 nM and a maximal rate of production of 5.27 ± 0.14 nmol/min per 10^6 cells (Figure 1a). The kinetics of oxidant production were both rapid and transient. When LTB_4 was used in a high concentration (100 nM; about EC_{90}), the maximal rate of H_2O_2 production was achieved within 45 s of exposing the cells to the stimulus which returned quickly to baseline levels within 2 min (results not shown). Preincubation (5 min) of eosinophils with U-75,302, a

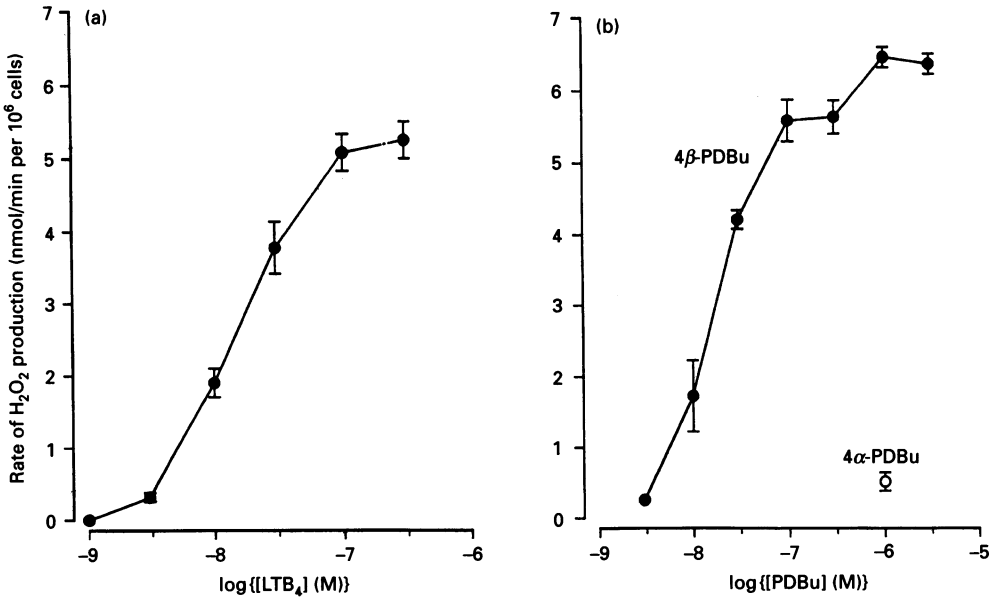

Figure 1 Effect of LTB_4 and PDBu on the rate of H_2O_2 generation

Highly purified (more than 97%) eosinophils (10^6) were added to buffer B at 37 °C in polystyrene fluorimeter cuvettes supplemented with Ca^{2+} (1 mM), Mg^{2+} (1 mM), SOD (30 units), HRPO (1 unit) and the fluorophore, scopoletin (4 μM). Eosinophils were allowed to equilibrate (5 min) and were then challenged with stimulus. The generation of H_2O_2 by the cells was measured spectrophotofluorimetrically by monitoring the reduction in fluorescence of scopoletin from which peak rates of H_2O_2 generation were calculated. (**a**) and (**b**) Concentration–response relationship of LTB_4- and PDBu-induced H_2O_2 generation respectively. Data points represent means ± S.E.M. of eight and four independent experiments respectively performed with different cell preparations. See the Experimental section for further details.

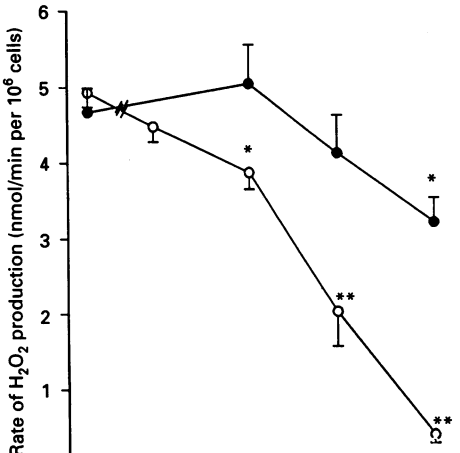

Figure 2 Effect of Ro 31-8820 on LTB_4- and 4β-PDBu-induced H_2O_2 generation

Eosinophils, equilibrated in buffer B at 37 °C, were pretreated for 5 min with Ro 31-8220 (300 nM–10 μM) and then challenged with equi-effective concentrations of LTB_4 (100 nM; ●) or 4β-PDBu (1 μM; ○). Changes in fluorescence were monitored and converted into rates of H_2O_2 generation. Data points represent means ± S.E.M. of four independent experiments performed with different cell preparations. See the legend to Figure 1 and the Experimental section for further details. *$P < 0.05$, **$P < 0.01$; significant inhibition of LTB_4- and 4β-PDBu-induced H_2O_2 generation by Ro 31-8220.

selective LTB_4-receptor antagonist [38], inhibited LTB_4 (100 nM)-induced H_2O_2 generation in a concentration-dependent manner with an IC_{50} of 386 ± 69 nM.

Exposure of eosinophils to the phorbol diester, 4β-PDBu, increased H_2O_2 production in a concentration-dependent manner with an EC_{50} of 21.6 ± 2.1 nM and a maximal rate of production (6.37 ± 0.14 nmol/min per 10^6 cells) not significantly different from that elicited by a maximally effective concentration of LTB_4 (Figure 1b). Kinetically, however, the response promoted by the phorbol diester was different from that induced by LTB_4. Thus 4β-PDBu caused a delayed and prolonged generation of H_2O_2 where the lag period before the onset of the response decreased with increasing concentration of stimulus. 4α-PDBu (1 μM) did not stimulate a respiratory burst in eosinophils, indicating that the effect of the phorbol diester was enantiomerically specific (Figure 1b). Pretreatment of eosinophils with a concentration of U-75,302 (3 μM) that abolished LTB_4 (100 nM)-induced H_2O_2 production did not antagonize the effect of 4β-PDBu.

Role of PKC in LTB_4- and 4β-PDBu-induced H_2O_2 generation: effect of Ro 31-8220

Pretreatment (5 min) of eosinophils with the PKC inhibitor Ro 31-8220 (0.3–10 μM) did not affect the spontaneous generation of H_2O_2. Similarly, Ro 31-8220 failed to inhibit the response elicited by LTB_4 (100 nM) except at the highest concentration examined where the rate of H_2O_2 generation was significantly suppressed (by 30.8 ± 6.8% at 10 μM; $P < 0.05$; Figure 2).

The effect of Ro 31-8220 (10 μM) on the concentration–response relationship of LTB_4-induced H_2O_2 generation is shown in Figure 3(a). Ro 31-8220 reduced the rate of oxidant production in an apparently non-competitive manner. There was a reduction (about 30%) in the maximum response attained by LTB_4 but no significant decrease in its potency for H_2O_2 generation (EC_{50} values: control = 17.1 ± 3.2 nM; + Ro 31-8220 = 27.5 ± 5.2 nM; $P > 0.05$). Curiously, when a submaximal concentration (30 nM; approx. EC_{50}) of LTB_4 was used, Ro 31-8220 (10 μM) para-

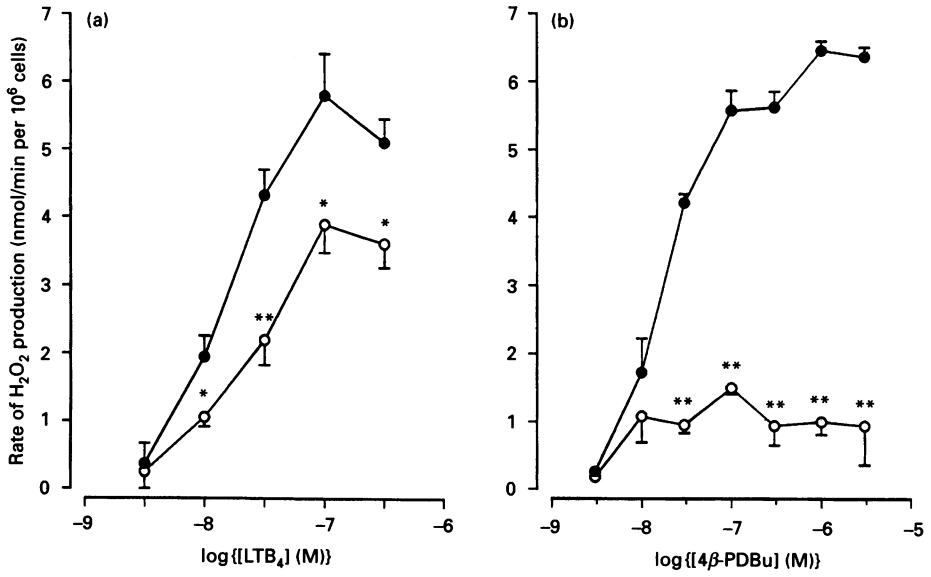

Figure 3 Effect of Ro 31-8220 on the concentration–response relationship of the rate of LTB_4- and 4β-PDBu-induced H_2O_2 generation

Eosinophils, equilibrated in buffer B at 37 °C, were pretreated for 5 min with 10 μM Ro 31-8220 (○) or its vehicle (●) and then challenged with LTB_4 (3 nM–3 μM) (**a**) or 4β-PDBu (3 nM–3 μM) (**b**). Changes in fluorescence were monitored continuously and peak rates of H_2O_2 generation calculated. Data points represent means ± S.E.M. of four independent experiments performed with different cell preparations. See the legend to Figure 1 and the Experimental section for further details. $^*P < 0.05$, $^{**}P < 0.01$; significant inhibition of LTB_4- and 4β-PDBu-induced H_2O_2 generation by Ro 31-8220

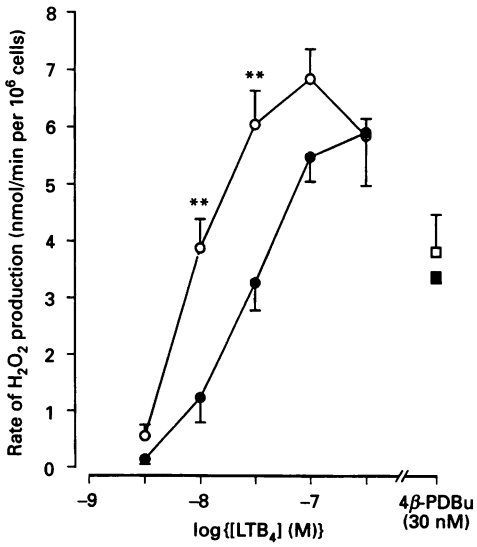

Figure 4 Potentiation of LTB_4-induced H_2O_2 generation by R 59 022

Eosinophils, equilibrated in buffer B at 37 °C, were pretreated for 5 min with 10 μM R 59 022 (○, □) or its vehicle (●, ■) and then challenged with LTB_4 (○, ●) or 4β-PDBu (□, ■). Changes in fluorescence were monitored continuously and peak rates of H_2O_2 generation calculated. Data points represent means ± S.E.M. of five independent experiments performed with different cell preparations. See the legend to Figure 1 and the Experimental section for further details. $^{**}P < 0.01$; significant enhancement of LTB_4-induced H_2O_2 generation by R 59 022.

doxically doubled the absolute amount of H_2O_2 generated by eosinophils (from 2.62 ± 0.17 to 5.28 ± 0.57 nmol/10^6 cells; $n = 4$; $P < 0.01$) but, in agreement with the data reported above, markedly inhibited the peak rate of generation (from 4.32 ± 0.38 to 2.21 ± 0.37 nmol/min per 10^6 cells; $n = 4$; $P < 0.01$). The effect of Ro 31-8220 on LTB_4-induced respiratory-burst activity thus depended on which parameter (rate or amount) of oxidative metabolism was measured.

In contrast with its effect on the LTB_4-elicited response, Ro 31-8220 almost completely suppressed (by 91.8 ± 2.4% at 10 μM) the generation of H_2O induced by 4β-PDBu (Figure 3b). This effect was concentration-dependent (IC_{50} = 2.72 ± 0.51 μM) but was non-competitive in nature (Figure 3b). Thus there was a reduction in the maximum response attained without a shift to the right in the 4β-PDBu concentration–response curve.

Role of PKC in LTB_4 and 4β-PDBu-induced H_2O_2 generation: effect of R 59 022

The involvement of PKC in the activation of the respiratory-burst oxidase by LTB_4 was investigated further by examining the effect of the purported diradylglycerol kinase inhibitor R 59 022. Pretreatment (5 min) of eosinophils with R 59 022 (10 μM) had no effect on the basal production of H_2O_2, but caused a parallel 2.65-fold leftwards shift of the LTB_4 concentration–response curve (EC_{50} values: control = 28.0 ± 4.7 nM; + R 59 022 = 10.5 ± 1.6 nM; Figure 4) without significantly changing the maximum rate of H_2O_2 production (7.08 ± 1.15 nmol/min per 10^6 cells). In contrast, R 59 022 (10 μM) did not affect the response elicited by 30 nM 4β-PDBu, a submaximal concentration (about EC_{55}) that would allow stimulatory and inhibitory effects of R 59 022 to be seen (Figure 4).

Role of PLD in LTB_4- and 4β-PDBu-induced H_2O_2 generation: effect of butan-1-ol

Pretreatment of eosinophils with butan-1-ol [10–100 mM; 0.091–0.91% (v/v) respectively], a primary alcohol that selectively inhibits PLD [39], caused a concentration-dependent inhibition

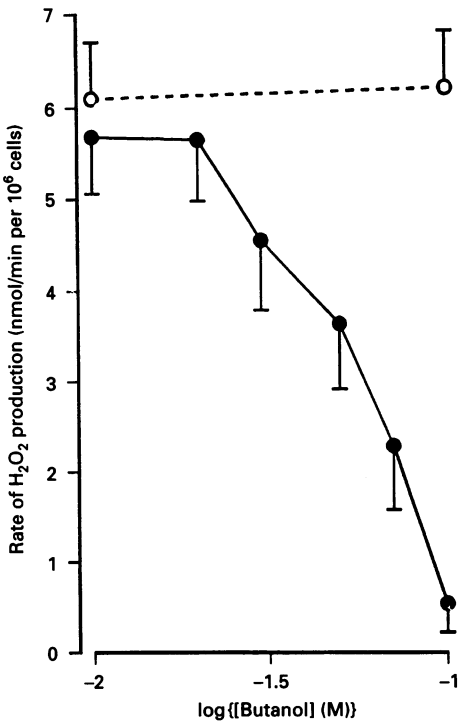

Figure 5 Inhibition of LTB_4-induced H_2O_2 generation by butan-1-ol

Eosinophils, equilibrated in buffer B at 37 °C, were pretreated for 5 min with butan-1-ol (10–100 mM) and then challenged with 100 nM LTB_4 (●) or 1 μM 4β-PDBu (○). Changes in fluorescence were monitored continuously and peak rates of H_2O_2 generation calculated. Data points represent means ± S.E.M. of four independent experiments performed with different cell preparations. See the legend to Figure 1 and the Experimental section for further details.

of LTB_4-stimulated H_2O_2 production (IC_{50} = 66.1 ± 6.4 mM; approx 0.6%, v/v). In contrast, butan-1-ol did not inhibit the generation of H_2O_2 induced by 4β-PDBu (1 μM) at any concentration examined (Figure 5).

Effect of LTB_4 and 4β-PDBu on PLD activity

The ability of butan-1-ol to inhibit LTB_4-induced H_2O_2 generation provided evidence that PLD-dependent mechanisms may be implicated in the genesis of this response. Further studies were therefore conducted to assess directly and unequivocally whether LTB_4 stimulates PLD in intact eosinophils. This was performed by exploiting the fact that PLD catalyses a transphosphatidylation reaction in which a primary alcohol is converted into the corresponding phosphatidylalcohol at the expense of phosphatidic acid [39]. Eosinophils were therefore incubated with [^{3}H]butan-1-ol of high specific radioactivity and the formation of Ptd[^{3}H]BuOH was monitored.

Under control conditions, there was a low but detectable generation of Ptd[^{3}H]BuOH by eosinophils indicating a basal turnover of PLD in resting cells (Figure 6a). Treatment of cells with a concentration of LTB_4 (1 μM) that supramaximally stimulated H_2O_2 generation did not measurably augment PLD activity whereas, under identical experimental conditions, 4β-PDBu (1 μM) enhanced the formation of Ptd[^{3}H]BuOH 5.2-fold. This effect was concentration-dependent (EC_{50} = 206 ± 49 nM) and maximal at 1.5 μM 4β-PDBu.

To confirm that the method used to measure PLD activity [37] was sufficiently sensitive to detect receptor-mediated Ptd[^{3}H]BuOH formation, the ability of fMLP and OZ to activate PLD in human neutrophils was assessed. These stimuli have been shown previously to be effective activators of PLD in this cell type [22,23,25]. At rest, the basal amount of Ptd[^{3}H]BuOH in neutrophils was approximately 3-fold greater than in eosinophils (Figure 6b). After treatment of cells with fMLP (1 μM in the presence of 5 μM cytochalasin B) and OZ (2 mg/ml), the Ptd[^{3}H]BuOH content of neutrophils was increased 4- and 3-fold respectively (Figure 6b).

Figure 7(a) shows the results of experiments designed to determine whether activation of PLD by 4β-PDBu was mediated via the activation of PKC. Pretreatment (5 min) of eosinophils with Ro 31-8220 (10 μM) elicited a biphasic effect on 4β-PDBu-induced Ptd[^{3}H]BuOH biosynthesis: Ro 31-8220 significantly augmented the basal formation of Ptd[^{3}H]BuOH and the generation induced by concentrations of 4β-PDBu less than 150 nM. However, when 4β-PDBu was used above 500 nM, Ro 31-8220 significantly suppressed Ptd[^{3}H]BuOH generation (Figure 7a). When 4β-PDBu was used at a concentration of 100 nM (approximately EC_{40}), Ro 31-8220 enhanced PLD activity in a concentration-dependent manner (Figure 7b).

Effect of butan-1-ol on cyclic AMP accumulation

The observation that LTB_4 did not significantly enhance Ptd[^{3}H]BuOH formation in eosinophils suggests that the inhibitory effect of butan-1-ol on H_2O_2 generation may be unrelated to the inhibition of PLD. As previous reports have documented that primary alcohols can significantly increase cyclic AMP formation in a variety of cells at concentrations required to inhibit PLD activity [40,41], experiments were performed to determine whether butan-1-ol stimulates cyclic AMP accumulation in eosinophils. Treating cells with 30 mM (0.27%, v/v) and 100 mM (0.91%, v/v) butan-1-ol, concentrations that inhibited LTB_4-induced H_2O_2 generation by 32 and 95% respectively (Figure 3), significantly ($P < 0.05$) increased the cyclic AMP content in guinea-pig eosinophils from 0.27 ± 0.09 to 0.42 ± 0.11 and 1.30 ± 0.44 pmol/10^6 cells respectively (n = 4).

Effect of dibutyryl cyclic AMP on LTB_4- and 4β-PDBu-induced H_2O_2 generation

Pretreatment of eosinophils for 10 min with dibutyryl cyclic AMP (100 μM–10 mM) inhibited LTB_4 (100 nM)-induced H_2O_2 generation in a concentration-dependent manner with an IC_{50} of 276.4 ± 34.2 μM (n = 4). In contrast, dibutyryl cyclic AMP did not significantly suppress oxidase activation evoked by 4β-PDBu (1 μM) at any concentration examined.

Role of PLC in LTB_4-induced H_2O_2 generation: effect of U-73,122

Pretreatment (5 min) of eosinophils with U-73,122, an inhibitor of PLC-dependent processes [42], had no effect on basal oxidase activity at any concentration examined, but inhibited the rate of LTB_4 (100 nM)-induced H_2O_2 generation in a concentration-dependent manner (IC_{50} = 187 ± 24 nM; Figure 8). In contrast, the respiratory burst evoked by 4β-PDBu (1 μM) was completely unaffected by U-73,122 at a concentration (500 nM) that abolished the production of H_2O_2 evoked by 100 nM LTB_4 (results not shown). U-73,343 (1 μM), a structural analogue of U-73,122 that does not inhibit signalling through PLC [42], failed to inhibit LTB_4-induced H_2O_2 generation.

Effect of LTB_4 on Ins(1,4,5)P_3 levels

Under resting conditions, the mass of Ins(1,4,5)P_3 was 0.50 ± 0.38 pmol/10^6 cells. After the addition of LTB_4 (1 μM),

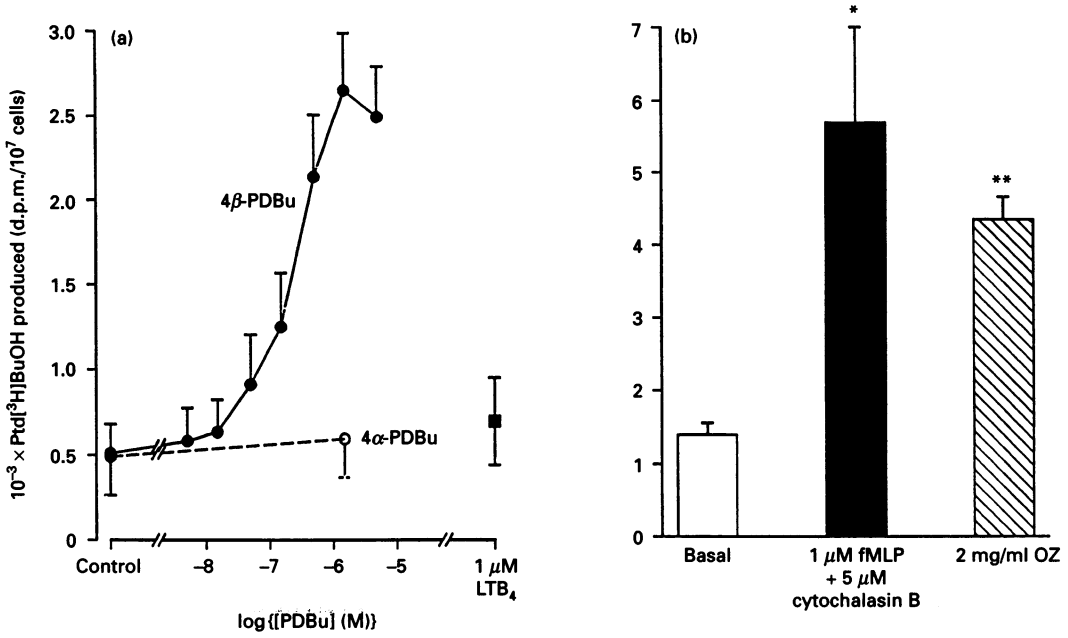

Figure 6 Effect of soluble and particulate stimuli on Ptd[^{3}H]BuOH formation in guinea-pig eosinophils and human neutrophils

Cells (10^7) were incubated in buffer C containing [^{3}H]butan-1-ol (20 μCi) and after 5 min preincubation were challenged with the soluble or particulate stimulus indicated. Reactions were terminated 30 min later and radiolabelled lipids extracted and subsequently separated by TLC as detailed in the Experimental section. (**a**) Effect of the enantiomers of PDBu (○, ●), and 1 μM LTB_4 (■) on the Ptd[^{3}H]BuOH content of intact eosinophils. (**b**) Effect of fMLP (1 μM + 5 μM cytochalasin B) and OZ (2 mg/ml) on Ptd[^{3}H]BuOH formation in intact neutrophils. Data points and bar charts represent means ± S.E.M. of five and three independent experiments respectively performed with different cell preparations. $^*P < 0.05$, $^{**}P < 0.01$; significant enhancement of fMLP- and OZ-induced Ptd[^{3}H]BuOH formation.

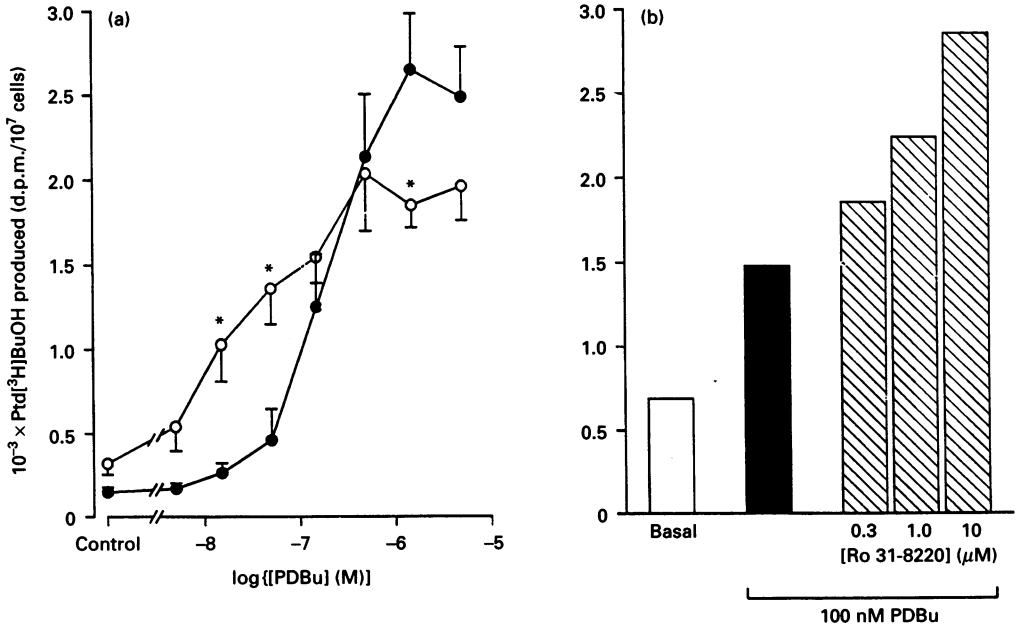

Figure 7 Effect of Ro 31-8220 on 4β-PDBu-induced Ptd[^{3}H]BuOH formation

(**a**) Cells (10^7) were incubated for 5 min in buffer C containing [^{3}H]butan-1-ol (20 μCi) in the absence (●) and presence (○) of Ro 31-8220 (10 μM) and then challenged with 4β-PDBu (30 nM–5 μM). (**b**) Eosinophils were pretreated with Ro 31-8220 (0.3, 1 and 10 μM) or vehicle for 5 min before the addition of 4β-PDBu (100 nM). Reactions were terminated 30 min later and Ptd[^{3}H]BuOH extracted and subsequently measured by TLC. Data points and bar charts represent means ± S.E.M. of four and two independent experiments respectively performed with different cell preparations. $^*P < 0.05$; significant change in 4β-PDBu-induced Ptd[^{3}H]BuOH formation induced by Ro 31 8220.

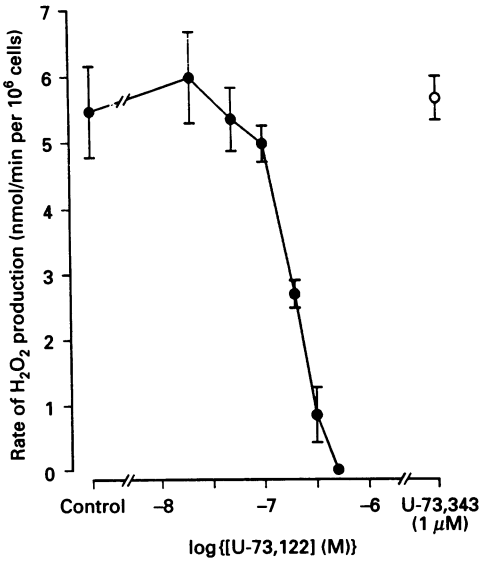

Figure 8 Inhibition of LTB_4-induced H_2O_2 generation by U-73,122

Eosinophils, equilibrated in buffer B at 37 °C, were pretreated for 5 min with U-73,122 (●) or U-73,343 (○; 1 μM) and then challenged with LTB_4 (100 nM). Changes in fluorescence were monitored continuously and peak rates of H_2O_2 generation calculated. Data points represent means ± S.E.M. of four independent experiments performed with different cell preparations. See the legend to Figure 1 and the Experimental section for further details.

there was an immediate increase in the level of Ins(1,4,5)P_3 which peaked at 5 s (to 2.87 ± 0.43 pmol/10^6 cells; 5.7-fold increase) and then rapidly decayed to baseline levels after 10 s stimulation despite the continued presence of LTB_4 (Figure 9a). The increase in Ins(1,4,5)P_3 mass measured at the 5 s time-point was concentration-dependent (EC_{50} = 200.3 ± 50 nM), maximal between 1 and 10 μM LTB_4 (Figure 9b) and was suppressed by the LTB_4 antagonist, U-75,302 (IC_{50} = 455 ± 128 nM; Figure 9c). Curiously, pretreatment of eosinophils with U-73,122, at a concentration (500 nM) that abolished LTB_4-stimulated H_2O_2 generation (see Figure 8), failed to inhibit the concomitant accumulation of Ins(1,4,5)P_3 under identical experimental conditions (control: 2.91 ± 0.45 pmol/10^6 cells; +Ro 31-8220: 3.12 ± 0.37 pmol/10^6 cells; n = 3).

Effect of wortmannin on LTB_4-induced H_2O_2 generation

Wortmannin, a potent inhibitor of PtdIns 3-kinase [32], inhibited LTB_4 (100 nM)-induced H_2O_2 generation in a concentration-dependent manner (IC_{50}: 2.5 ± 0.8 μM; n = 4) but, in contrast with the same response evoked by fMLP (100 nM) in human neutrophils (IC_{50} = 61.1 ± 16.1 nM; n = 3), was about 40-fold less potent under identical experimental conditions. In neutrophils and eosinophils the concentration–response curve that described the inhibition of H_2O_2 generation was very steep (slope factor > 3), and the fMLP-evoked response was abolished at a concentration of wortmannin that did not affect the LTB_4-induced respiratory burst (results not shown).

Role of Ca^{2+} and Mg^{2+} in LTB_4-induced H_2O_2 generation

The resting $[Ca^{2+}]_i$ in guinea-pig eosinophils was approx. 80 nM (Figure 10a; Table 1). Exposure of these cells to LTB_4 (1 μM) produced a typical Ca^{2+} transient characteristic of many cell types. Thus there was a rapid 6-fold increase in the $[Ca^{2+}]_i$ to about 500 nM which peaked after about 5 s and which, in some

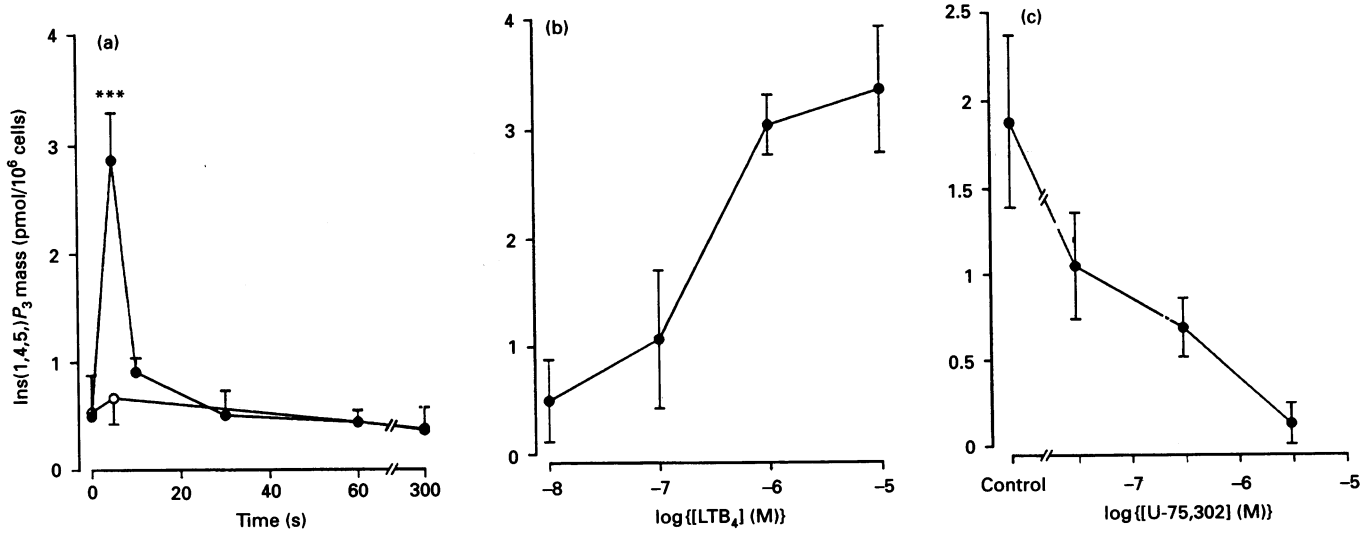

Figure 9 Effect of LTB_4 on eosinophil Ins(1,4,5)P_3 content

Eosinophils (7 × 10^6 per tube) were incubated for 5 min in buffer C at 37 °C, challenged with LTB_4 and the reaction was subsequently quenched with an equal volume of trichloroacetic acid (1 M). Ins(1,4,5)P_3 was subsequently extracted and measured using a competitive protein-binding assay. (**a**) and (**b**) Time course (measured at 1 μM LTB_4) and concentration–response relationship (determined at 5 s) of LTB_4-induced (●) and basal (○) Ins(1,4,5)P_3 accumulation respectively. (**c**) Antagonism of the response evoked by 100 nM LTB_4 at 5 s by U-75,302. Data points represent means ± S.E.M. of three independent experiments performed with different cell preparations. See the Experimental section for further details. $^{**}P < 0.01$; significant increase in LTB_4-induced Ins(1,4,5)P_3 mass over control level.

Table 1 Effect of EGTA and BAPTA/AM on LTB_4-induced H_2O_2 generation and Ca^{2+} mobilization

Eosinophils (10^7/ml) were loaded with fura 2/AM (1 μM) with or without BAPTA/AM (2 μM). After a wash, the ability of LTB_4 (1 μM) to generate H_2O_2 and to increase $[Ca^{2+}]_i$ was assessed in the absence (+100 μM EGTA) and presence of extracellular Ca^{2+} (1 mM) and Mg^{2+} (1 mM). Changes in fluorescence were monitored continuously and peak rates of H_2O_2 generation and absolute changes in $[Ca^{2+}]_i$ were calculated. See the Experimental section for further details. Data represent means ± S.E.M. of five independent experiments performed with different cell preparations. *$P < 0.05$; significant inhibition of LTB_4-induced H_2O_2 generation and/or Ca^{2+} mobilization. **$P < 0.05$; resting $[Ca^{2+}]_i$ significantly lower than in control (untreated) eosinophils.

Stimulus conditions	Rate of H_2O_2 generation (nmol/min per 10^6 cells)	$[Ca^{2+}]_i$ (nM)	
		Resting	Peak
LTB_4 (1 μM)	8.07 ± 1.40	81 ± 17	494 ± 411
LTB_4 + EGTA (100 μM)	7.37 ± 0.99	58 ± 24	83 ± 26*
LTB_4 + BAPTA/AM (2 μM)	6.43 ± 0.94	91 ± 14	177 ± 26*
LTB_4 + EGTA + BAPTA/AM	4.53 ± 0.91*	22 ± 3**	22 ± 4*

preparations, quickly returned to a new steady-state about 50 nM above resting (non-stimulated) level (compare Figures 10a and 11; Table 1).

To determine the sources of Ca^{2+} mobilized by LTB_4 and the role of intracellularly and extracelluarly derived bivalent cations in the activation of the respiratory-burst oxidase, fura 2-loaded eosinophils were either treated with the intracellular Ca^{2+} chelator, BAPTA/AM (2 μM), or preincubated in Ca^{2+}/Mg^{2+}-free buffer B containing EGTA (100 μM) to chelate extracellular Ca^{2+}. Under both experimental conditions the magnitude of the LTB_4 (1 μM)-induced Ca^{2+} transient was markedly suppressed (by 79 and 94% respectively; Table 1), whereas the oxidative burst was not significantly altered with respect to both the rate of production and the absolute amount of product formed (Figure 10b; Table 1). Quantitatively identical results were obtained when the experiment was performed in nominally Ca^{2+}-free buffer B in the absence of EGTA (results not shown). In contrast, when BAPTA/AM-loaded cells were incubated in Ca^{2+}/Mg^{2+}-free buffer B, the resting $[Ca^{2+}]_i$ was reduced to about 20 nM and LTB_4-induced Ca^{2+} mobilization was abolished (Figure 10a); however, under these conditions the rate of H_2O_2 generation and the absolute amount formed was reduced by only 44 and 57% respectively (Figure 10b; Table 1).

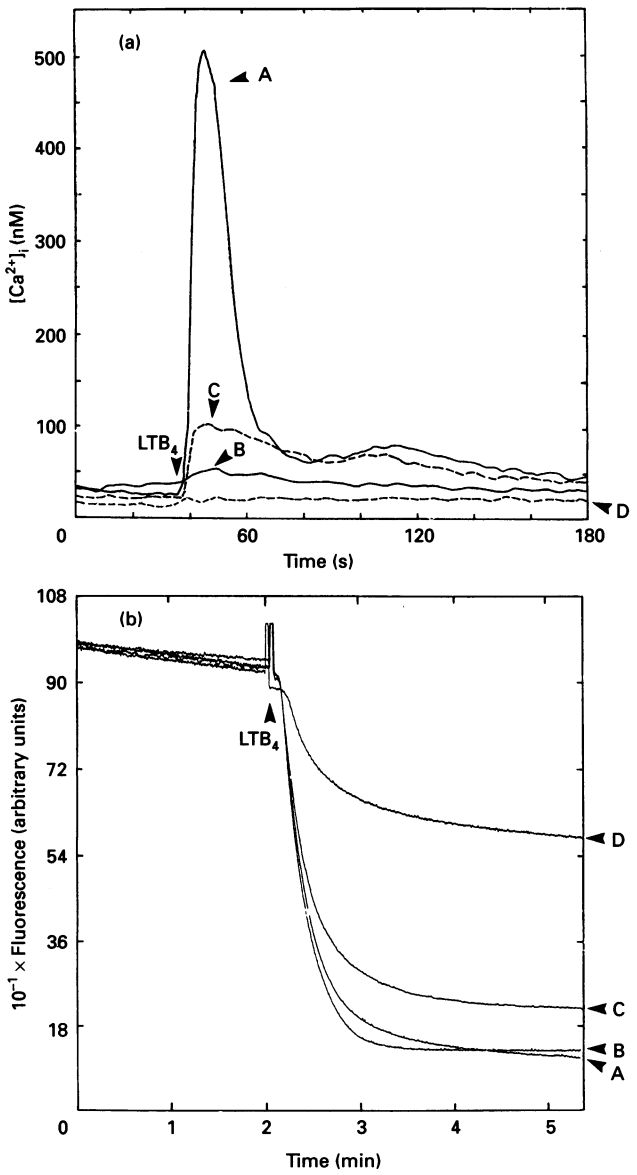

Figure 10 Representative trace of the effect of EGTA and of BAPTA/AM on LTB_4-induced Ca^{2+} mobilization and H_2O_2 generation

Eosinophils (10^7/ml) were loaded with fura 2/AM (1 μM) with or without BAPTA/AM (2 μM). After a wash, the ability of LTB_4 (1 μM) to increase $[Ca^{2+}]_i$ (**a**) and to generate H_2O_2 (**b**) was assessed in the absence (+100 μM EGTA) and presence of extracellular Ca^{2+} (1 mM) and Mg^{2+} (1 mM). Changes in fluorescence were monitored continuously and peak rates of H_2O_2 generation and absolute changes in $[Ca^{2+}]_i$ were calculated. See the Experimental section for further details. Data represent means ± S.E.M. of five independent experiments performed with different cell preparations. A, LTB_4; B, LTB_4 + EGTA; C, LTB_4 + BAPTA/AM; D, LTB_4 + EGTA + BATPA/AM.

Effect of Ro 31-8220 on LTB_4-induced Ins(1,4,5)P_3 accumulation and Ca^{2+} mobilization

The finding that Ro 31-8220 enhanced the amount of H_2O_2 produced in response to a submaximal concentration of LTB_4 (see above) implies that activation of PKC may exert an inhibitory influence at one or more stages in the sequence of events leading to oxidase activation. To determine whether PLC may be regulated by PKC in eosinophils, the effect of Ro 31-8220 on LTB_4-induced Ins(1,4,5)P_3 accumulation and Ca^{2+} mobilization was examined. Pretreatment of eosinophils for 5 min with Ro 31-8220 (10 μM) did not affect the basal level of Ins(1,4,5)P_3 (control: 0.41 ± 0.07; +Ro 31-8220: 0.48 ± 0.09 pmol/10^6 cells; $n = 3$) but significantly enhanced (1.7-fold) the amount of Ins(1,4,5)P_3 produced by LTB_4 (300 nM) at 5 s (from 2.63 ± 0.33 to 4.31 ± 0.67 pmol/10^6 cells; $n = 4$; $P < 0.05$). Similar data were obtained when changes in intracellular Ca^{2+} were measured. Thus Ro 31-8220 did not affect the resting $[Ca^{2+}]_i$ or the amplitude of the Ca^{2+} transient evoked by LTB_4 (1 μM) but markedly enhanced the duration of the Ca^{2+} signal (Figure 11). Indeed, after the addition of LTB_4 (300 nM) the time required for the increase in $[Ca^{2+}]_i$ to decay to 50% of its peak value was increased from 11 ± 3 to 131 ± 34 s ($P < 0.001$; $n = 3$).

LTB_4 did not detectably increase the $[Ca^{2+}]_i$ in Ro 31-8220-treated eosinophils in Ca^{2+}/Mg^{2+}-free buffer B containing 100 μM EGTA.

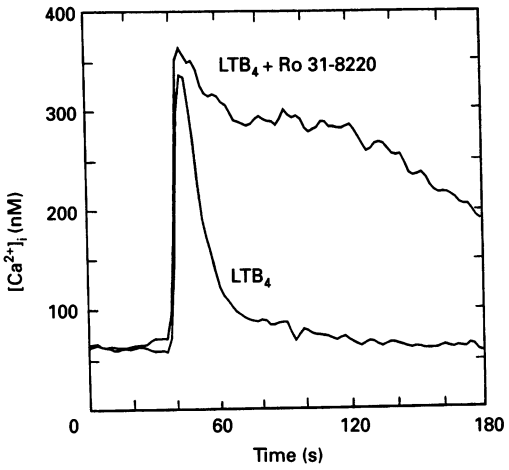

Figure 11 Representative trace of the effect of Ro 31-8220 on LTB_4-induced Ca^{2+} mobilization

Eosinophils (10^7/ml) were loaded with fura 2/AM (1 μM) as described in the Experimental section. Cells were then treated with Ro 31-8220 (10 μM) or its vehicle for 5 min before being challenged with LTB_4 (1 μM). Changes in fluorescence were monitored continuously and absolute changes in $[Ca^{2+}]_i$ were calculated. See the text for further details. Data represent means $\pm$ S.E.M. of four independent experiments performed with different cell preparations.

Effect of U-73,122 on LTB_4-induced Ca^{2+} mobilization

Pretreatment of eosinophils with 500 nM U-73,122, a concentration that abolished H_2O_2 generation (Figure 8), did not inhibit LTB_4-induced Ca^{2+} mobilization. These data are thus consistent with the inability of U-73,122 to suppress LTB_4-induced Ins(1,4,5)P_3 accumulation.

DISCUSSION

The eosinophil undergoes a massive respiratory burst when exposed to appropriate stimuli but, unlike the neutrophil, little is known of the intracellular signalling pathways that regulate this response. The documented participation of eosinophils in the pathogenesis of a variety of inflammatory disorders [3] prompted us to investigate the signal-transduction pathway(s) that may ultimately govern the assembly and subsequent activation of the NADPH oxidase in these cells. LTB_4 was studied, as it is a potent proinflammatory mediator and an effective stimulant of oxidative metabolism in eosinophils (see ref. [5]).

Previous studies have unequivocally identified binding sites on guinea-pig eosinophils for [^{3}H]LTB_4 which have the characteristics of *bona fide* receptors [43–45]. Moreover, two populations of this receptor apparently exist which are proposed to mediate different functional responses: the receptor for which LTB_4 has high affinity subserving chemotaxis and chemokinesis, and the receptor for which LTB_4 has low affinity mediating respiratory burst and prostanoid generation. The finding that LTB_4-stimulated H_2O_2 generation from guinea-pig peritoneal eosinophils was inhibited by the LTB_4 antagonist, U-75,302 [38], confirms that oxidant production is evoked by a receptor-mediated mechanism in these cells. Furthermore, the potency of LTB_4 (EC_{50} = 23 nM) is in good agreement with data reported previously for LTB_4-induced $O_2^{\cdot-}$ generation by guinea-pig alveolar eosinophils (EC_{50} = 50 nM) and is consistent with the proposal that oxidative metabolism in these cells is mediated via a population of low-affinity LTB_4 receptors [43,44].

Evidence that PKC plays a role in LTB_4-induced H_2O_2 generation was provided by the finding that the PKC inhibitor, Ro 31-8220 (10 μM), reduced the rate of oxidant production by 30% under conditions in which 4β-PDBu-evoked responses were suppressed by more than 90%. Furthermore, the DRG kinase inhibitor, R 59 022, which is purported to prevent the conversion of diacylglycerols into phosphatidic acid, potentiated LTB_4-induced H_2O_2 generation in eosinophils.

If PKC is intimately involved in oxidase activation, then LTB_4 must promote the formation of DRG via the stimulation of PLD and/or PLC. PLD was initially implicated in this response because butan-1-ol abolished H_2O_2 generation, a finding entirely consistent with what has been reported in agonist-stimulated neutrophils [e.g. 22,46–48] where stimulation of PLD by LTB_4 is regarded as an important early signalling event in the pathway(s) leading to oxidase activation [25,49–51]. Intriguingly, however, LTB_4 failed to stimulate the formation of PtdBuOH (an unambiguous index of PLD activity) at concentrations up to, and in excess of, those required to activate the oxidase maximally, casting some doubt over the inhibitory mechanism of action of butan-1-ol.

At least two explanations could account for these irreconcilable data. The first is that the method [37] used to measure the transphosphatidylation reaction was not sufficiently sensitive to detect changes in PLD activity. Although this cannot be excluded unequivocally, it is unlikely as 4β-PDBu increased Ptd[^{3}H]BuOH mass more than 5-fold in eosinophils under identical experimental conditions, and human neutrophils generated significant amounts of Ptd[^{3}H]BuOH in response to a number of receptor-mediated stimuli that are established activators of PLD [22,23,25]. An alternative possibility is that butan-1-ol attenuated H_2O_2 generation by a mechanism divorced from the inhibition of PLD. As butan-1-ol elevated the cyclic AMP content in eosinophils over the same concentration range at which H_2O_2 generation was inhibited, activation of the cyclic AMP/cyclic AMP-dependent protein kinase cascade may explain these findings. Indeed, there is compelling evidence that cyclic AMP-elevating drugs generally inhibit receptor-mediated eosinophil activation (see ref. [5]), a finding confirmed in the present study where dibutyryl cyclic AMP suppressed H_2O_2 generation. Although the mechanism(s) by which butan-1-ol increased cyclic AMP was not formally investigated, primary alcohols at concentrations similar to those required inhibit PLD can increase cyclic AMP through their ability to activate the stimulatory G-protein, G_s [40], and/or inhibit the inhibitory G-protein, G_i [40]. It is clear therefore that caution should be exercised when using primary alcohols as inhibitors of PLD.

It is noteworthy that neither dibutyryl cyclic AMP nor butan-1-ol inhibit 4β-PDBu-induced H_2O_2 generation in eosinophils, which is concordant with the inability of cyclic AMP-elevating drugs to suppress eosinophil activation by stimuli that by-pass the receptor (see ref. [5]).

Although LTB_4 failed to stimulate PLD in guinea-pig eosinophils, Minnicozzi and colleagues [52] reported that the complement fragment, C5a, was an effective stimulant of PLD in human eosinophils. The reason(s) for this disparity is obscure, but the nature of the activation stimulus (C5a compared with LTB_4) or a fundamental difference in the cell signalling mechanism(s) that governs oxidase activation between human and guinea-pig eosinophils could be responsible.

In common with its effect in many other cells, LTB_4 evoked a rapid increase in the Ins(1,4,5)P_3 content of guinea-pig eosinophils, indicating that PLC was stimulated. By definition, a stoicheiometrically equivalent amount of DRG will also be generated from PtdIns(4,5)P_2 which could be of sufficient magnitude to activate the PKC-driven component of the respiratory

burst described above. Curiously, 10-fold higher concentrations of LTB_4 were required to elevate the Ins(1,4,5)P_3 content (ED_{50} about 200 nM) than to promote the generation of H_2O_2 (EC_{50} about 22 nM). One explanation of these data is that there is a large receptor reserve for Ins(1,4,5)P_3 production in eosinophils such that a modest activation of PLC is sufficient to activate the oxidase maximally. Alternatively, PLC-derived signal molecules may not participate in LTB_4-Stimulated H_2O_2 production. Although this latter interpretation is inconsistent with the idea that PLC-derived DRG may be necessary for complete activation of the NADPH oxidase (see above), U-73,122, a purported inhibitor of PLC-dependent processes [42,53], failed to attenuate LTB_4-induced Ins(1,4,5)P_3 production at a concentration that abolished the respiratory burst. Bleasdale and colleagues [42,53] documented a similar effect of U-73,122 in human neutrophils but concluded that U-73,122 was acting at the level of PLC to inhibit the NADPH oxidase. That contention was based on the knowledge that Ins(1,4,5)P_3 binds to receptors on the endoplasmic reticulum in a non-co-operative manner leading to the highly co-operative opening of Ca^{2+} channels [54,55]. Consequently, a small increment in Ins(1,4,5)$_3$ mass evokes a large increase in $[Ca^{2+}]_i$. Bleasdale et al. [42,53] logically extended this paradigm by proposing that a small decrease in Ins(1,4,5)P_3 mass imposed by U-73,122 should effect a much larger reduction in PLC-dependent Ca^{2+} mobilization [42,53]. Although evidence for such a mechanism is available [42,53], U-73,122 did not detectably suppress LTB_4-induced Ca^{2+} mobilization in guinea-pig eosinophils. Taken together therefore these data suggest that U-73,122 is not a selective inhibitor of PLC-dependent processes and that the suppression of LTB_4-induced H_2O_2 generation is mediated by an, as yet, ill-defined mechanism(s).

In human neutrophils, the generation of oxygen-derived free radicals by agonists such as LTB_4 and fMLP involves PtdIns(3,4,5)P_3 which is formed enzymically from PtdIns(4,5)P_2 by a PtdIns 3-kinase ([32] and references therein). Surprisingly, experiments designed to assess whether a similar pathway is implicated in activation of the NADPH oxidase in eosinophils yielded results inconsistent with this postulate. Thus wortmannin, a selective inhibitor of PtdIns 3-kinase [32], failed to suppress LTB_4-induced H_2O_2 generation at concentrations where the same response evoked in neutrophils was abolished. These findings thus identify another difference in the signal-transduction pathways that control the activity of the respiratory-burst oxidase between eosinophils and neutrophils.

Another aspect of cell signalling in eosinophils that merits discussion concerns the sources of Ca^{2+} mobilized by LTB_4 and the Ca^{2+}-dependence of H_2O_2 generation. Initial Ca^{2+}-chelation studies suggested that LTB_4 mobilized Ca^{2+} principally from intracellular stores. Thus eosinophils, loaded with a low concentration (2 μM) of the intracellular Ca^{2+} chelator (BAPTA/AM) that does not affect intracellular pH, responded with a Ca^{2+} transient that was reduced in amplitude by approximately 80%. Curiously, however, LTB_4-induced Ca^{2+} mobilization was abolished in eosinophils that were incubated in EGTA (100 μM)-containing buffer, implying that Ca^{2+} entry was the sole determinant of this response. Why EGTA should exert such a dramatic effect on the Ca^{2+} transient is unclear but it is unlikely to be due to the chelation of Ca^{2+} released intracellularly by LTB_4 as identical results were obtained when the experiment was performed in nominally Ca^{2+}-free media. This raises the possibility that BAPTA/AM may be acting non-specifically to attenuate the fura 2 signal.

In BAPTA/AM-loaded cells incubated in an EGTA-containing buffer, LTB_4 generated an appreciable amount of H_2O_2 in the absence of any detectable increase in $[Ca^{2+}]_i$. These data thus indicate that this response is mediated, at least in part, by a Ca^{2+}-independent mechanism(s). Intriguingly, this conclusion disagrees fundamentally with a large number of other investigations performed with neutrophils where an increase in $[Ca^{2+}]_i$ is considered essential for agonist-induced activation of the respiratory-burst oxidase (see ref. [56] and references therein). These disparate results may be attributable to the low concentration of Ca^{2+} chelators used in the present study which was just sufficient to suppress completely LTB_4-induced Ca^{2+} transients. Indeed, our data are in complete agreement with those of O'Flaherty and colleagues [56] who reported that fMLP, platelet-activating factor and LTB_4 promoted a substantial respiratory burst in Ca^{2+}-depleted human neutrophils loaded with low concentrations (0.1–1 μM) of fura 2/AM. The relatively high concentrations of Ca^{2+} indicators used by other investigators may have produced effects in cells unrelated to Ca^{2+} chelation.

A perplexing finding that emerged from these studies was that Ro 31-8220 inhibited the rate of LTB_4-induced H_2O_2 generation but markedly enhanced the total amount of product formed. To account for this apparent paradox it is conceivable that these two indices of oxidase activation are regulated, at least in part, independently (see ref. [10]). The increase in the amount of H_2O_2 formed in response to LTB_4 may be explained if PKC, when activated, exerts a negative feedback influence on one or more effectors of the NADPH oxidase which is relieved by Ro 31-8220. The finding that the duration of the Ca^{2+} transient and amount of Ins(1,4,5)P_3 formed in response to LTB_4 was augmented by Ro 31-8220 indicates that PLC and/or Ins(1,4,5)P_3 5-phosphatase may be such effectors. Indeed, phosphorylation of the PLC-β isoenzyme family is now regarded as a ubiquitous mechanism of PLC regulation [57] which results in a decrease in phosphoinositide hydrolysis, and coincident desensitization of PLC-coupled receptors. In addition, there is good evidence that PKC can phosphorylate (and thereby inactivate) Ins(1,4,5)P_3 5-phosphatase [58,59]. Thus, in its simplest form, we suggest that, as the mobilization of Ca^{2+} is necessary for the complete activation of the respiratory-burst oxidase (see above), Ro 31-8200, by preventing the phosphorylation of PLC and/or Ins(1,4,5)P_3 5-phosphatase, would increase the amount of Ins(1,4,5)P_3 produced and hence extend the duration of the Ca^{2+} transient; the amount of H_2O_2 formed via the Ca^{2+}-driven component would thereby be augmented. At the same time the reduction in the rate of H_2O_2 generation would reflect an inhibitory effect of Ro 31-8220 on the PKC-dependent pathway(s) of NADPH oxidase activation.

A final discussion point relates to the actions of Ro 31-8220 on 4β-PDBu-induced Ptd[^{3}H]BuOH formation. In contrast with its effect in most cells and tissues [e.g. 25], Ro 31-8220 paradoxically potentiated rather than suppressed the ability of 4β-PDBu to activate PLD in eosinophils; only when relatively high concentrations (> 150 nM) of the phorbol diester were employed was a modest (approx. 20%) inhibition observed. These results are difficult to interpret but it is possible that 4β-PDBu activates two or more PKC isoforms that may theoretically exert opposite effects on PLD activity and for which Ro 31-8220 exhibits a different inhibitory potency.

In conclusion, the results of this study indicate that the early signalling events leading to oxidase activation in eosinophils apparently differ, at least in part, from those established in neutrophils. Thus, although Ca^{2+}, PLC and PKC play a significant role in the genesis of this response, no compelling evidence was obtained implicating either PLD or PtdIns 3-kinase. Furthermore, LTB_4 elicited an appreciable respiratory burst in EGTA/BAPTA-treated eosinophils indicating that Ca^{2+}-independent mechanisms are also involved.

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