Protein kinase C regulates leukotriene B_4 receptors in human neutrophils

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Three protein kinase C (PKC) activators, viz. phorbol myristate acetate, mezerein, and *rac*-1-*O*-myristoyl-2-acetylglycerol, inhibited human neutrophil binding of [^{3}H] leukotriene B_4 (LTB_4) by reducing the number of high-affinity receptors available to the arachidonic acid metabolite. The inhibitory effect occurred in whole cells and cytoplasts but not in isolated membranes; it appeared to involve the activation of PKC rather than direct competition for binding sites. PKC may govern cellular responsiveness by regulating the receptor-linked bioactions of endogenous mediators like LTB_4.

(Polymorphonuclear leukocyte) Protein kinase C Leukotriene B_4 receptor

1. INTRODUCTION

PKC is rapidly activated during cell stimulation. The enzyme then initiates biological responses by phosphorylating critical effector proteins [1]. PKC also transmits negative signals [2] which may dampen cellular function through modification of Ca^{2+} pumps [3], ion channels [4], contractile elements [5], GTP-binding proteins [6], nucleotide cyclases [7], or receptors for exogenous stimuli [2]. We show here that PKC regulates receptors for an endogenous mediator, LTB_4, in human PMN.

2. MATERIALS AND METHODS

2.1. *Reagents and buffers*

[^{3}H]LTB_4 (186 Ci/mmol), unlabelled LTB_4, MAG, HEG, PMA, MEZ, GF/C glass microfiber filters, 0.25 cm silica gel TLC plates (preheated at 180°C for 3 h before use) and BSA were obtained as described [8]. SOD (Diagnostic Data, Mountain View, CA), catalase (Worthington, Freehold, NJ) and sodium azide (Fisher, Fair Lawn, NJ) were purchased. Our buffer was a modified Hanks' balanced salt solution (1.4 mM $CaCl_2$) [8].

Abbreviations: PMN, polymorphonuclear neutrophils; PKC, protein kinase C; SOD, superoxide dismutase; LTB_4, leukotriene B_4; PMA, phorbol myristate acetate; MEZ, mezerein; MAG, *rac*-1-*O*-myristoyl-2-acetylglycerol; HEG, 1-*O*-hexadecyl-2-ethylglycerol; ETYA, eicosatetraynoic acid; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide

2.2. *Binding assays*

PMN were prepared [8] and suspended in buffer at 37°C for 20 min. Cells were challenged with an indicated reagent or its vehicle at 37°C, centrifuged (16000 × *g* for 5 s), and washed with 4°C buffer before distributing 5×10^6 cells into a final volume of 1 ml buffer (4°C). The buffer contained [^{3}H]LTB_4, unlabelled LTB_4, and 125 μg BSA. Incubation was continued on ice for 60 min and then suspensions were filtered. Details of the assay and counting of radioactivity adherent to filters have been described [8]. Membrane fractions, isolated from Percoll gradients of nitrogen-cavitated PMN and twice washed by centrifugation (140000 × *g* for 2 h, 4°C) [8], were suspended in a final volume of 1 ml Hanks' buffer a 4 or 37°C. Membranes (125 μg protein) were incubated with [^{3}H]LTB_4, unlabelled LTB_4, and a PKC activator for 60 min

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before filtration. Cytoplasts, prepared as in [9], were assayed for binding sites exactly like intact cells. Cytoplast/PMN ratios were: 0.04 for granule-bound enzymes (lysozyme and β-glucuronidase); 0.23 for plasmalemma (alkaline phosphatase); 0.32 for cytosol (lactic acid dehydrogenase); and 0.35 for volume (based on mean corpuscular volumes determined with a Coulter Counter particle size analyser). Particle size analysis and microscopic examination indicated <1 PMN per 1000 cytoplasts.

2.3. *Metabolic assays*

5×10^6 PMN were challenged with 50 nM PMA for 10 min at 37°C, washed (4°C), resuspended (4°C) with 100 pM [^{3}H]LTB$_4$ for 60 min, and extracted with acidic (final pH ~3–4) chloroform:methanol. Extracts were analyzed on TLC developed with diethyl ether:acetic acid:water (100:1:0.5, v/v) which resolves LTB$_4$ (R_f = 0.68) from its 20-hydroxy (R_f = 0.18) and 20-carboxy (R_f = 0.24) metabolites [8].

3. RESULTS AND DISCUSSION

PMN and their isolated membranes bound [^{3}H]LTB$_4$ at 4°C. Binding progressed to equilibrium over 40 min; it was saturable, reversible, and specific for LTB$_4$ [8]. At 37°C but not 4°C, PMN quantitatively metabolized [^{3}H]LTB$_4$ [8]. Accordingly, we activated PMN at 37°C but assayed binding only after the cells were chilled, twice washed, suspended at their original concentration (4°C) and incubated with ligand for 60 min (4°C). This procedure completely avoided [^{3}H]LTB$_4$ metabolism: in cells pretreated with DMSO or 50 nM PMA, ~92% of initially added radioactivity (0.1 nM [^{3}H]LTB$_4$) comigrated with authentic LTB$_4$ on TLC. However, PMN pretreated with increasing concentrations of PMA progressively lost their capacity to bind [^{3}H]LTB$_4$ (fig.1, upper, solid line). The phorbol diester reduced principally the specific component of binding; non-specific binding (i.e. binding observed in the presence of 316 nM unlabelled LTB$_4$) was minimally changed by PMA challenge (fig.2, upper).

PMA did not appear to inhibit binding by causing release of oxygen radicals or granule-bound enzymes. The compound stimulates secretion of

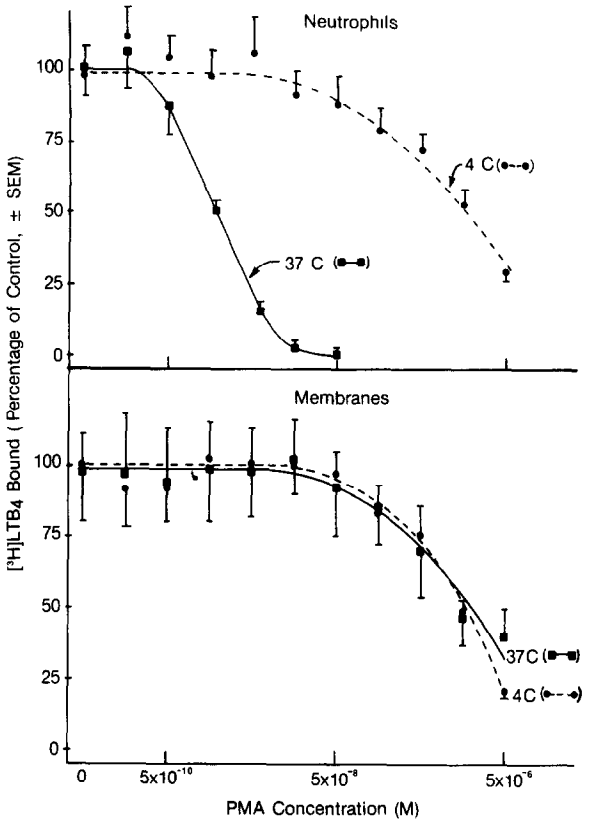

Fig.1. Effect of PMA on [^{3}H]LTB$_4$ binding to PMN or their membranes. PMN were incubated at 37 or 4°C for 10 min with the indicated concentration of PMA or DMSO (0.4%, final concentration). Cells were rapidly chilled, washed, and resuspended (4°C) with 100 pM [^{3}H]LTB$_4$, ± 316 nM unlabelled LTB$_4$, for 60 min. Membranes were incubated at 37 or 4°C for 60 min with the indicated concentration of PMA or DMSO, 100 pM [^{3}H]LTB$_4$, and ± 316 nM unlabelled LTB$_4$. Suspensions were filtered and radiolabel adherent to filters measured. Specific binding values (i.e. fraction of initially added radiolabel bound by samples incubated with 100 pM [^{3}H]LTB$_4$ minus that bound by samples incubated with 100 pM [^{3}H]LTB$_4$ plus 316 nM LTB$_4$) were derived and presented as the mean percentage of specific binding observed in DMSO-treated samples (0.195 ± 0.6), ± SE, for at least 6 experiments.

superoxide anion or lysozyme only after a substantial (>1 min) lag; it acted to reduce binding rapidly ($t_{1/2}$ <1 min, fig.2, upper, inset). Furthermore, three anti-oxidants did not influence results (table 1); PMA stimulated degranulation only at concentrations (>15.8 nM) well above those that affected

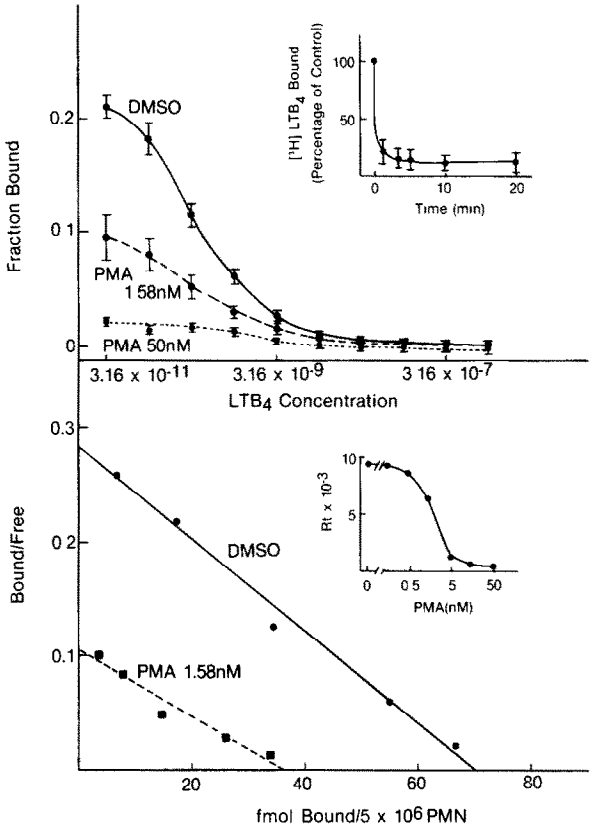

Fig.2. Effect of PMA on PMN receptor-mediated binding of [^{3}H]LTB$_4$. Cells were incubated with DMSO or PMA at 37°C for 10 min, rapidly chilled, washed, and resuspended with 31.6 pM–1 μM ligand ([^{3}H]LTB$_4$ plus unlabelled LTB$_4$) for 60 min at 4°C. (Upper) Fraction of added label bound by PMN treated with DMSO, 5 nM PMA, or 50 nM PMA; (inset) label specifically bound (as percentage of DMSO-treated control suspensions) by cells treated with 5 nM PMA at 37°C for the indicated time period. (Lower) Scatchard plots of high-affinity receptor binding data taken from the upper panel; (inset) extrapolated receptor numbers (R_t) for the high-affinity receptors, as determined on PMN pretreated with the indicated concentration of PMA. All data points are the mean of at least 6 experiments.

binding; and inhibition occurred in cytoplasts. PMN cytoplasts release oxygen radicals when stimulated but are depleted of granule-bound enzymes [9]. When 1 × 10^7 cytoplasts were treated with DMSO for 10 min at 37°C, chilled, washed (4°C), and incubated (4°C for 60 min) with

Table 1

Effects of inhibitors on the PMA-induced blockage of [^{3}H]LTB$_4$ binding

Inhibitor	Stimulus	
	DMSO	PMA
None	19.6 ± 0.8[a]	0.8 ± 0.2
SOD + catalase	23.4 ± 0.7	0.9 ± 0.2
SOD + catalase + azide	18.2 ± 0.9	1.1 ± 0.3
ETYA	12.4 ± 0.2	0.7 ± 0.2

[a] Percentage of initially added radioactivity bound by cells incubated with 0.1 nM [^{3}H]LTB$_4$ minus the percentage bound by cells incubated with 0.1 nM [^{3}H]LTB$_4$ plus 316 nM unlabelled LTB$_4$. Values are means ± SE for at least 4 experiments

PMN (1 × 10^7 in 1 ml) were incubated at 37°C with 100 μg SOD, 1000 U catalase, 100 μM sodium azide, and 30 μM ETYA, as indicated, for 4 min. Cells were then challenged with 5 nM PMA or DMSO for 10 min, chilled, washed, and assayed for [^{3}H]LTB$_4$ binding as described in section 2. In studies with SOD, catalase, and azide, cells were washed and resuspended in buffer containing the same inhibitors

100 pM [^{3}H]LTB$_4$ (± 316 nM unlabelled LTB$_4$), they specifically bound 3.0 fmol radiolabel. Following treatment with 0.5, 5 or 50 nM PMA, specific binding fell to 1.5, 0.6 and 0.1 fmol, respectively. It also appeared unlikely that inhibition resulted from receptor blockade by endogenously formed LTB$_4$: unlike other stimuli, PMA does not trigger arachidonic acid metabolism in PMN [10] and an arachidonate antimetabolite, ETYA [11], did not reduce the action of PMA (table 1). Finally, direct competition for binding sites did not explain results. PMA was 1000-fold weaker in reducing binding when preincubated with PMN at 4°C or when incubated with PMN membranes at 37°C as well as 4°C (fig.1). Several findings implicated PKC in reduced binding. First, corresponding with the effects of PMA, PKC is temperature-sensitive, virtually absent from our washed membrane preparations [12], and present as an activatable enzyme in cytoplasts [9]. Second, two other PKC activators, MEZ and a diacylglycerol, MAG, also inhibit binding by a temperature- and whole cell-dependent mechanism (table 2). Third, a dialkylglycerol, HEG, neither

Table 2

Potencies of selected compounds in inhibiting [^{3}H]LTB$_4$ binding

Stimulus	Whole cells		Membranes	
	4°C	37°C	4°C	37°C
PMA	2 μM[a]	3 nM	2 μM	2 μM
MEZ	4 μM	5 nM	2 μM	4 μM
MAG	>100 μM	50 μM	>100 μM	>100 μM
HEG	nd	>100 μM	nd	nd

[a] Concentration of stimulus producing a 50% reduction in [^{3}H]LTB$_4$ specific binding, as extrapolated from concentration-response curves (e.g. see fig.1) of at least 4 experiments. HEG did not reduce binding at 10–100 μM. nd, not done

Binding of [^{3}H]LTB$_4$ was conducted as described in fig.1

activates PKC [12] nor influences binding (table 2). Lastly, PMA, MEZ, and MAG inhibited binding at concentrations virtually identical to those activating PKC [12].

PMN possess high- and low-affinity LTB$_4$ receptors [8]. PMA acted principally on the high-affinity receptors. Thus, Scatchard analyses revealed that 1.58 nM of the phorbol diester reduced the number of high-affinity receptors from 10000 to 4000/cell while changing their K_d from 0.3 to 0.4 nM (fig.2, lower). For low-affinity receptors, these values changed from 200000 to 400000/cell and from 0.3 to 0.7 μM, respectively. The number of high-affinity receptors decreased progressively with increasing concentrations of PMA (fig.2, lower, inset). Similar results were observed with cytoplasts: following treatment with 0, 0.5, 5 or 50 nM PMA, each cytoplast had 1600, 1000, 300 and <100 high-affinity receptors, respectively, for [^{3}H]LTB$_4$. All K_d values were ~0.5 nM.

Stimulus-response coupling is mediated by several pathways including those activating PKC and metabolizing arachidonate [1]. The negative interaction between these two pathways demonstrated here provides a novel, possibly fundamental explanation for the unresponsiveness of PMA-pretreated PMN not only to LTB$_4$ [13] but also to stimuli dependent upon arachidonate metabolism, such as chemotactic peptides [14]. Indeed, down-regulation of receptors for endogenous mediators by PKC may be a general mechanism that operates to limit functional responses and produce a state of desensitization in a wide variety of cell types.

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