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Inhibitors of arachidonic acid lipoxygenase impair the stimulation of inositol phospholipid hydrolysis by the T lymphocyte mitogen phytohaemagglutinin

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Piriprost and nordihydroguiaretic acid (NDGA), specific inhibitors of arachidonate lipoxygenase, inhibited phytohaemagglutinin (PHA)-stimulated breakdown of inositol lipids in human T lymphocytes. The dual inhibitors eicosatetraynoic acid (ETYA) and BW 755C, which inhibit both lipoxygenase and cyclooxygenase, also had similar actions, whereas indomethacin and acetylsalicyclic acid, which inhibit cyclooxygenase alone, did not. The effects of lipoxygenase inhibitors and dual inhibitors were reversible. These agents did not inhibit phosphatidylinositol-4,5-bisphosphate-specific phospholipase C (PIP_2-PLC) in vitro. Bromophenacyl bromide, and irreversible inhibitor of phospholipase A_2, also abolished PHA-stimulated inositol lipid breakdown without affecting PIP_2-PLC in vitro. The results are consistent with a role for the PHA-stimulated generation of arachidonic acid and its conversion to lipoxygenase metabolites (e.g. leukotrienes and/or hydroxyeicosatetraenoic acids) as intermediate steps in the signal transduction pathway between cell-surface mitogen receptors and the stimulation of PIP_2-PLC in lymphocytes.

Lymphocyte transformation; Second messenger; Phosphatidylinositol; Phospholipase C; Lipoxygenase

1. INTRODUCTION

The cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) by a specific phospholipase C (PLC) and the subsequent generation of the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) is thought to play a crucial role in the transduction of mitogenic signals following the binding of mitogens such as phytohaemagglutinin (PHA) or specific monoclonal antibodies to cell-surface structures on T lymphocytes [1,2]. DAG stimulates protein kinase C whereas IP_3 mediates elevation of cellular Ca^{2+} concentrations [3]. Cascades of biochemical processes consequent to these early events eventually synergize in securing commitment of the lymphocyte to mitosis [4].

The mechanisms which link the binding of mitogens to cell-surface structures and the stimulation of PIP_2-PLC are unclear. Considerable evidence supports a role for guanine nucleotide-binding proteins (G proteins) in coupling receptor stimulation to the activation of polyphosphoinositide breakdown in several biological systems [5] including lymphocytes [6–8]. However, reconstitution experiments using purified G proteins and PIP_2-PLC have not been carried out. Therefore, it is unclear whether a G protein is the only mediator linking receptors to PIP_2-PLC. Since there is some evidence that G proteins may activate phospholipase A_2 [9], the enzyme which generates free arachidonic acid from phospholipids, we asked whether metabolites of arachidonic acid may in turn stimulate PIP_2-PLC following PHA stimulation of T lymphocytes. The results here show that two inhibitors of arachidonic acid lipoxygenase and two drugs which inhibit both lipoxygenase and cyclooxygenase enzymes impair the stimulation of T lymphocyte PIP_2-PLC by PHA. By contrast, two agents which inhibit cyclooxygenase (but not lipoxygenase) did not impair this process. The data suggest that the generation of arachidonic acid and its metabolism to leukotrienes or hydroxyeicosatetraenoic acids (HETEs) [10] may be an obligatory step in the coupling of T lymphocyte mitogen receptors to PIP_2 breakdown.

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Abbreviations: PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; PHA, phytohaemagglutinin; HETE, hydroxyeicosatetraenoic acid; ETYA, eicosatetraynoic acid; NDGA, nordihydroguiaretic acid; PLA_2, phospholipase A_2

2. MATERIALS AND METHODS

2.1. *Materials*

Piriprost was a gift from Upjohn Co., Kalamazoo, MI, and BW 755C from Wellcome Research Laboratories, Beckenham, Kent. Eicosatetraynoic acid (ETYA) was purchased from Cayman Chemicals and nordihydroguiaretic acid (NDGA), indomethacin and acetylsalicylic acid were from Sigma Chemical Co., Poole, Dorset.

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[2-^{3}H]Myo-inositol and [^{3}H]PIP_2 were supplied by Amersham International, England.

2.2. *Cells*

Lymphocytes were prepared from freshly drawn peripheral blood from healthy volunteers by sedimentation on Lymphoprep (Nyegaard, Oslo) gradients and essentially freed of monocytes as described [11]. They were cultured in inositol-free RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum [12].

2.3. *Assay for PHA-stimulated inositol lipid breakdown*

Lymphocytes were labelled overnight with 5 $\mu Ci \cdot ml^{-1}$ [2-^{3}H]myo-inositol. The cells were washed twice in Hanks' salts, and resuspended (at $1-5 \times 10^6$ ml^{-1}) in RPMI 1640 medium supplemented with 10% fetal calf serum. LiCl was added to 10 mM and the cells incubated at 37°C for 20 min. PHA (Wellcome) was added to 10 $\mu l \cdot ml^{-1}$ and the incubation continued for a further 30 min. Water-soluble compounds were extracted as described [13] and radiolabel in inositol phosphates was quantitated by chromatography on Dowex A1-X8 columns [14].

2.4. *Assay for PIP_2-PLC*

Lymphocytes were lysed by hypotonic shock and resolved into particulate and cytosolic fractions following the removal of nuclei [15]. PIP_2-PLC was assayed by incubating 10 μg of protein with 50 pmol [^{3}H]PIP_2 in 40 μl reactions containing 20 mM Tris-HCl, pH 7.5 and either 400 nM (particulate fractions) or 150 nM (cytosolic fractions) free Ca^{2+} (buffered with EGTA). The release of radiolabelled IP_3 was quantitated after 5 min incubation at 37°C as described [16].

3. RESULTS AND DISCUSSION

Piriprost and NDGA, two inhibitors of lipoxygenase [17,18], both abolished the PHA-stimulated generation of labelled inositol phosphates in lymphocytes whose inositol lipids were prelabelled with [^{3}H]inositol (fig.1). The response to each drug was clearly dose-dependent. ETYA and BW 755C, dual inhibitors which inhibit both lipoxygenase and cyclooxygenase [17] also inhibited PHA-stimulated inositol lipid breakdown in a dose-responsive manner (fig.1). However, indomethacin, which inhibits cyclooxygenase but not lipoxygenase [17] did not affect PHA-stimulated generation of inositol phosphates (fig.2). Similar results were obtained using another cyclooxygenase inhibitor, acetylsalicylic acid (not shown). We therefore conclude that the abolition of inositol lipid breakdown by the dual inhibitors ETYA and BW 755C cannot be attributed to their effects on cyclooxygenase, and is more likely the result of their actions on the lipoxygenase pathway. Additionally, the results obtained with the dual inhibitors ETYA and BW 755C show that the effect of inhibiting the lipoxygenase pathway is not attributable to the increased metabolism of arachidonate via the cyclooxygenase pathway, with the consequent generation of prostaglandins, which may in turn inhibit inositol lipid breakdown via generation of cyclic AMP and activation of cyclic AMP-dependent protein kinase [19,20].

The effects of the lipoxygenase inhibitors on inositol lipid breakdown were not attributable to irreversible damage to cellular structure. The effect of 100 μM piriprost was immediately reversed by washing and resuspension of the cells (fig.3). However, the effect of higher concentrations of the drug were only partially reversed. Similar results were obtained using ETYA (not shown).

We then considered the possibility that the lipoxygenase inhibitors may directly affect PIP_2-PLC. Table 1 shows that lymphocytes contain both membrane-bound and cytosolic forms of PIP_2-PLC which could be assayed in vitro using [^{3}H]PIP_2 as a substrate in the presence of Ca^{2+} ion buffered in the nanomolar range. Even at the highest levels of NDGA, ETYA or piriprost used in the intact cell experiments (fig.1) only a marginal inhibition of PIP_2-PLC was observed in vitro (table 1). Similar results were obtained using BW 755C in a separate experiment (not shown). These results also suggest that the effects of the drugs on inositol lipid breakdown in intact cells could not be attributed to the perturbation of Ca^{2+} levels, since the in vitro PIP_2-PLC assay is critically dependent on nanomolar levels of Ca^{2+}. As a control, 100 μM neomycin sulfate, a known inhibitor of PIP_2-PLC, was shown to inhibit the in vitro assay almost completely (table 1).

Taken together, the data suggest that piriprost, NDGA, ETYA and BW 755C reversibly inhibit a regulatory process which links the binding of PHA to cell-surface structures on lymphocytes to the stimulation of PIP_2-PLC. We propose that the observed effects may be explained by a requirement for the generation of lipoxygenase metabolites as an intermediate step in the coupling process. While it remains theoretically possible that each of these 4 drugs affects a target other than lipoxygenase, which is critical in the coupling process, this is unlikely given their widely differing chemical structures. Piriprost is a derivative of prostaglandin I (6,9-(deepoxy)-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1), whereas NDGA (4,4′-(2,3-dimethyl-1,4-butanediyl)-bis-(1,2-benzenediol) contains catechol residues. The dual inhibitors BW 755C (3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline HCl) and the arachidonic acid analogue ETYA (a competitive inhibitor of lipoxygenase and cyclooxygenase) also have widely differing structures. We also wish to stress that although the IC_{50} values observed for each drug (fig.1) appear to be higher than those found for inhibition of lipoxygenase in phosphate-buffered saline [17], we and others [18] have observed that these drugs bind tightly to serum proteins and that their effective free concentrations may be as much as 10-fold lower than the concentrations added. Since the experiments described here were carried out in serum-containing medium (necessary for maintaining cell viability during [^{3}H]inositol labelling and subsequent manipulations), the effective IC_{50} of the inhibitors are probably of the order of 10-fold less than is apparent from fig.1.

In many systems the rate-determining step in the pro-

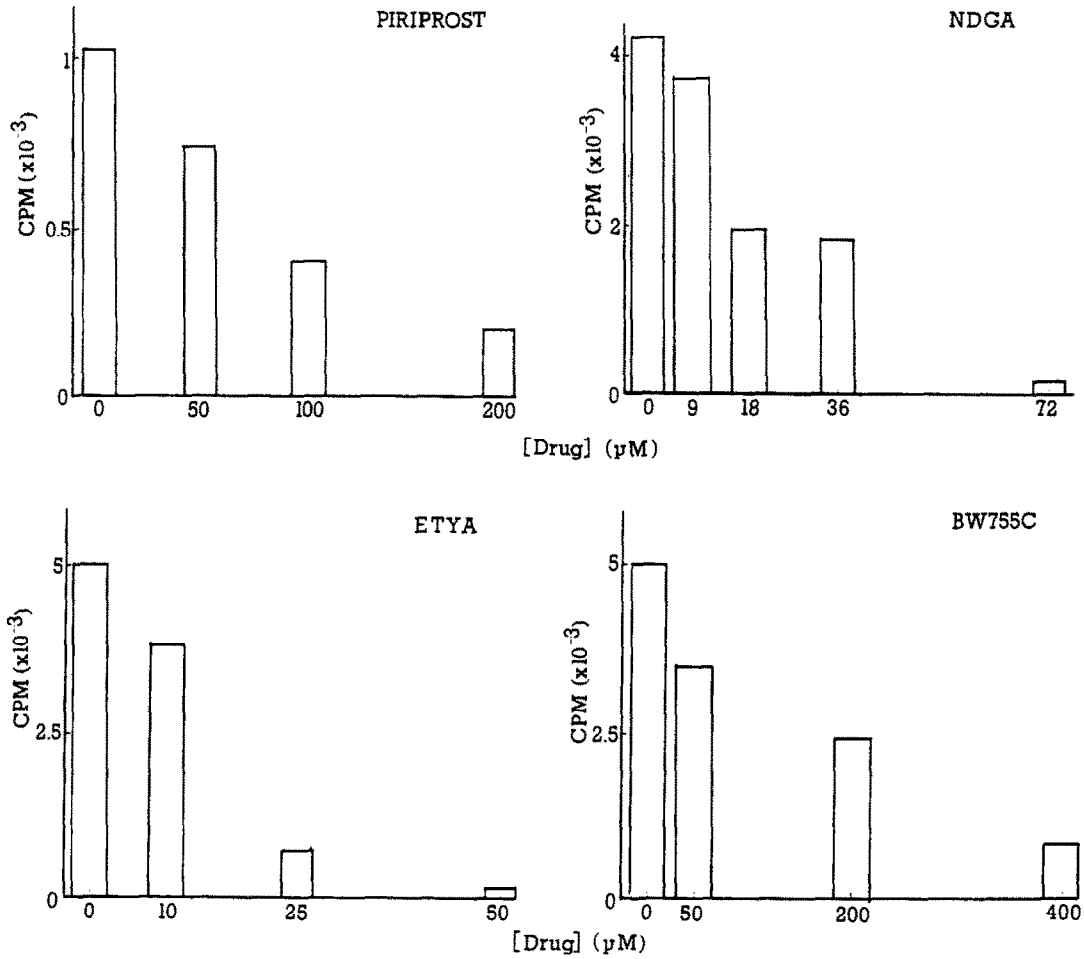

Fig.1. PHA-stimulated generation of [^{3}H]inositol phosphates (vertical axis) by [^{3}H]inositol-labelled lymphocytes treated for 1 h with the indicated concentrations (horizontal axis) of 4 different lipoxygenase inhibitors. In each experiment the PHA-stimulated increase in [^{3}H]inositol phosphates in the absence of drugs was 4–6-fold over basal levels. The results in each panel are typical of at least 5 separate experiments. 1.5×10^6 cells per determination were used in the experiment using piriprost, and 5×10^6 cells for each of the other titrations.

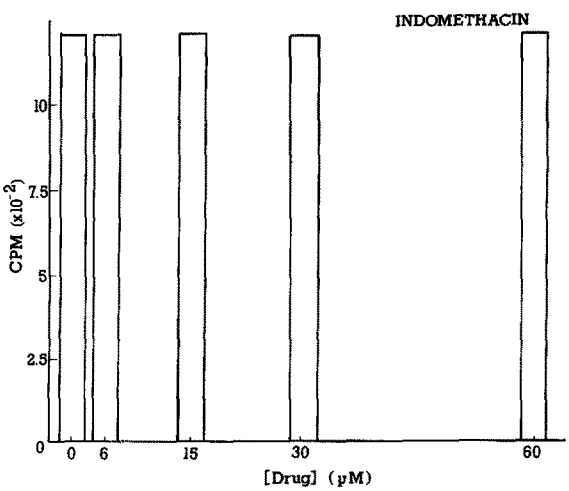

Fig.2. PHA-stimulated generation of [^{3}H]inositol phosphates (vertical axis) in lymphocytes treated for 1 h with the indicated concentrations (horizontal axis) of indomethacin. The results are typical of 3 experiments.

duction of lipoxygenase metabolites is the generation of arachidonic acid by the cleavage of phospholipids by phospholipase A_2 (PLA_2). We therefore tested the effects of bromophenacyl bromide, an irreversible inhibitor of PLA_2 [21] on the PHA-stimulated generation of inositol phosphates. Fig.4 shows that as little as 1 μM of this inhibitor reduced mitogen-stimulated inositol lipid breakdown by 50%. However, the precise specificities of bromophenacyl bromide are unknown. Although this inhibitor also did not affect PIP_2-PLC in vitro (table 1), the conclusion that the action described here depends on its inhibition of PLA_2 must remain tentative until more specific inhibitors become available. (We were unable to use steroids or mepacrine as PLA_2 inhibitors because the former are toxic to lymphocytes whereas mepacrine inhibits PIP_2-PLC in vitro.)

The data presented here support the hypothesis that lymphocyte mitogens such as PHA trigger the generation of arachidonic acid which is then converted to a

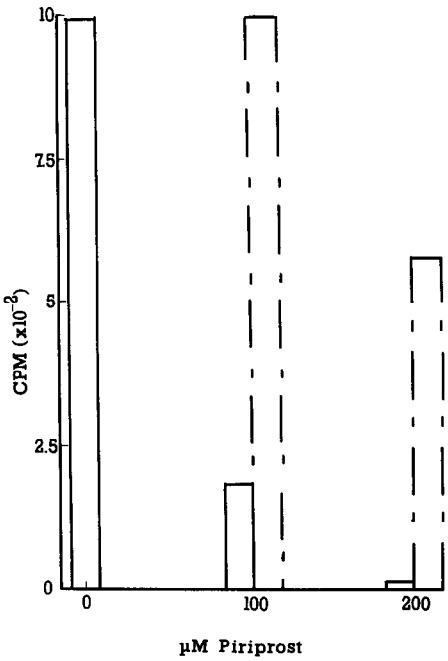

Fig.3. [^{3}H]Inositol pre-labelled lymphocytes were incubated for 1 h with the indicated concentrations (horizontal axis) of piriprost, and the PHA-stimulated generation of [^{3}H]inositol phosphates (vertical axis) was measured (solid lines). One-half of each culture was washed free of the drug immediately before the addition of PHA (broken lines).

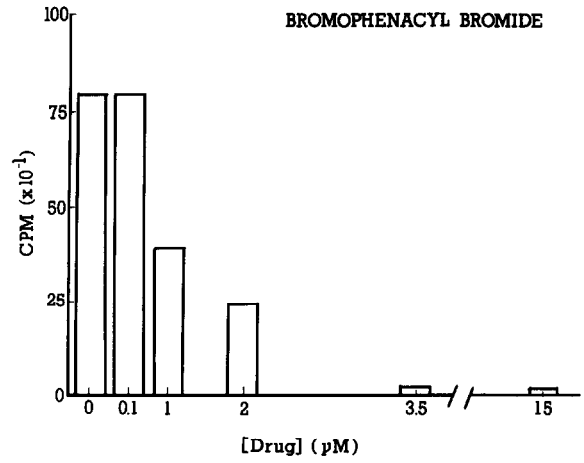

Fig.4. PHA-stimulated generation of [^{3}H]inositol phosphates by lymphocytes treated with the indicated concentrations of bromophenacyl bromide for 1 h.

lipoxygenase product(s), and that this product stimulates PIP_2-PLC in turn. Stable products of lipoxygenase include the leukotrienes and the hydroxyeicosatetraenoic acids (HETEs) [10]. Several actions of exogenous leukotrienes on lymphocytes have been documented [22] and lipoxygenase inhibitors have been shown to suppress the synthesis of the lymphokine interleukin-2 by T leukaemia cells [23]. Mitogenic effects of leukotrienes have also been described in other systems [24], and leukotriene D_4 has been shown to stimulate inositol lipid hydrolysis in rat basophilic leukaemia cells [25]. The generation of lipoxygenase products following mitogen-stimulation of T lymphocytes is a controversial issue (e.g. [26,27]). However, by pre-labelling of highly purified T lymphocytes with [^{3}H]arachidonic acid we have repeatedly observed that PHA stimulates the dose-dependent generation of a metabolite, tentatively identified as 5-HETE (Ayub Khan, M., Jeremy, J.Y., A.V.H. and R.G.W., in preparation). This metabolite is not liberated from the cells and may therefore have escaped detection in previous studies, many of which employed radioimmunoassay procedures. Furthermore, the PHA-stimulated generation of this metabolite was inhibited by NDGA with a dose-response similar to the effect of this drug on inositol lipid breakdown. These observations extend our earlier tentative conclusions that PHA treatment of lymphocytes stimulated PLA_2 activity as judged by the accumulation of glycerophosphoinositol [7].

Table 1

The effect of lipoxygenase inhibitors and bromophenacyl bromide on lymphocyte PIP_2-PLC activity in vitro

Additions to assay	PIP_2-PLC activity[a] (fmol IP_3 released/5 min/µg protein)	
	Cytosolic PLC	Membrane-bound PLC
None	830	868
70 µM NDGA	761	800
50 µM ETYA	867	806
200 µM piriprost	909	735
15 µM bromophenacyl bromide	869	804
100 µM neomycin sulphate	< 10	22

[a] PIP_2-PLC was assayed as described in section 2

It would be of interest to determine whether the putative role of lipoxygenase metabolites proposed here is restricted to lymphocytes or is a general feature of other growth factor signalling pathways as well. However, whereas many growth factors trigger very rapid generation of IP_3 (i.e. within seconds) [28], production of this second messenger in mitogen stimulated lymphocytes is delayed by about 10 min, suggesting that (an)other event(s) precede the breakdown of inositol lipids in this system [29], in accord with the observations reported here.

We are currently attempting to elucidate the mechanism by which a putative arachidonate metabolite may stimulate PIP_2-PLC during the mitogenic response of T lymphocytes. It will be of particular interest to localize the site(s) of action of the G proteins involved in stimulation of lymphocyte PIP_2-PLC [6–8] with respect to the mechanism proposed here.

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