Biochem. J. (1986) 238, 425–436 (Printed in Great Britain)

The role of polyphosphoinositides and their breakdown products in A23187-induced release of arachidonic acid from rabbit polymorphonuclear leucocytes

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Stimulation of rabbit polymorphonuclear leucocytes with A23187 causes phospholipase C mediated breakdown of polyphosphoinositides, as evidenced by accumulation of [^{3}H]inositol-labelled inositol bisphosphate and inositol trisphosphate. At the same time the polyphosphoinositides and the products of their breakdown, diacylglycerol and phosphatidic acid, label rapidly with radioactive arachidonic acid. Enhancement of polyphosphoinositide labelling is not as great as enhancement of diacylglycerol or phosphatidic acid labelling, suggesting additional early activation of a second independent synthetic pathway to the last named lipids. Experiments using double (^{3}H/^{14}C) labelling, to distinguish pools with different rates of turnover, suggest the major pool of arachidonic acid used for synthesis of lipoxygenase metabolites turns over more slowly than arachidonic acid in diacylglycerol, but at about the same rate as arachidonic acid esterified in phosphatidylcholine or phosphatidylinositol. Further, when cells are prelabelled with [^{14}C]arachidonic acid, then stimulated for 5 min, it is only from phosphatidylcholine, and to a lesser extent phosphatidylinositol, that radiolabel is lost. Release of arachidonic acid is probably via phospholipase A_2, since it is blocked by the phospholipase A_2 inhibitor manoalide. The absence of accumulated lysophosphatides can be explained by reacylation and, in the case of lysophosphatidylinositol, deacylation. The importance of phospholipase A_2 in phosphatidylinositol breakdown contrasts with the major role of phospholipase C in polyphosphoinositide hydrolysis. Measurements of absolute free fatty acid levels, as well as studies showing a correlation between production of radiolabelled hydroxyeicosatetraenoic acids and release of radiolabel from the phospholipid pool, both suggest that hydrolysis of arachidonic acid esterified into phospholipids is the limiting factor regulating formation of lipoxygenase metabolites. By contrast with A23187, fMet-Leu-Phe (a widely used polymorphonuclear leucocyte activator) is a poor stimulant for arachidonic acid release unless a 'second signal' (e.g. cytochalasin B, or a product of A23187-stimulated cells) is also present. In the presence of cytochalasin B, fMet-Leu-Phe, like A23187, stimulates release of radiolabelled arachidonic acid principally from phosphatidylcholine.

INTRODUCTION

Polymorphonuclear leucocytes congregate at inflammatory sites, especially during the early stages of inflammation, and are probably an important source of arachidonic acid derived inflammatory mediators. In the resting condition these cells have only low levels of free arachidonic acid, so that production of HETE or prostaglandin metabolites must either require an exogenous source of substrate, or be from arachidonic acid derived from esterified stores (reviewed by Irvine, 1982). Despite considerable investigation, the mechanism of release of arachidonic acid from esterified stores within the polymorphonuclear leucocyte is still unclear. Although some workers have provided evidence for involvement of phospholipase A_2 mediated hydrolysis of phosphatidylcholine and phosphatidylinositol, this hypothesis alone does not explain all observations. For example, it does not explain why, in A23187-stimulated polymorphonuclear leucocytes, arachidonic acid is acylated into and deacylated from phosphatidic acid much more rapidly than it is acylated into and deacylated from phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol or triacylglycerols (Lapetina *et al.*, 1980). Studies in other cell systems have suggested that a major source of phosphatidate following cell stimulation is breakdown of polyphosphoinositides (Michell *et al.*, 1981). No previous studies have investigated the contribution of polyphosphoinositides to arachidonic acid release in stimulated polymorphonuclear leucocytes. The object of this work was to determine what significance, if any, polyphosphoinositide breakdown had in the direct provision of arachidonic acid for conversion to lipoxygenase metabolites.

EXPERIMENTAL

Preparation of cells

Cells were obtained from young male New Zealand White rabbits by peritoneal lavage 16 h after intraperitoneal injection of 150 ml of 0.2% rabbit glycogen in saline. To increase the purity of the cell population to > 99% polymorphonuclear leucocytes, the original population (> 90% pure) was mixed with 65% Percoll in saline (Pharmacia) and centrifuged for 40 min at 32000 *g*. The cell population with density > 1.075 was removed and washed twice with Dulbecco's saline. Dulbecco's saline had the composition (per litre): 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 0.13 g of $CaCl_2,2H_2O$ and 0.1 g of $MgCl_2,6H_2O$.

Abbreviations used: DMSO, dimethyl sulphoxide; fMLP, fMet-Leu-Phe; (di)HETE, (di)hydroxyeicosatetraenoic acid.

Labelling and stimulation of cells

Cells were labelled at 1×10^7/ml in Dulbecco's saline kept (with shaking) at 37 °C. Two types of labelling experiments were performed. In the first type, unlabelled cells were stimulated in the presence of isotope. The second type were pulse–chase experiments where the cells were preincubated with isotope followed by non-radioactive material, then stimulated.

Prelabelling with [^{3}H]inositol. Pulse–chase labelling with [^{3}H]inositol was as follows. Cells were incubated for 4 h with 15 μCi of [2-^{3}H]inositol/ml (Amersham; item TRK 317, sp. radioactivity 14 Ci/mmol), then washed twice in Dulbecco's saline supplemented with 10 mg of unlabelled inositol/l, incubated at 37 °C for 15 min in the inositol-containing medium, washed and resuspended in 1 ml portions at 1×10^7 cells/ml in medium of composition (per litre): 8.47 g of NaCl, 0.31 g of KCl, 0.13 g of $CaCl_2$, $2H_2O$, 0.1 g of $MgCl_2$,$6H_2O$ and 0.238 g of Hepes.

Prelabelling with radioactive arachidonic acid. Prelabelling with arachidonic acid involved incubation with 0.2 μCi of [1-^{14}C]arachidonic acid/ml (Amersham; item CFA 504, sp. radioactivity 55 mCi/mmol). The arachidonic acid was first dissolved at 50 μCi/ml in ethanol and the appropriate volume of this was added to the cells. After 1 h incubation, the cells were washed twice and dispensed at 1×10^7 cells/ml in 1 ml portions in Dulbecco's saline. In a few experiments, the isotope employed was a mixture of 0.2 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid/ml (Amersham; item TRK 508, sp. radioactivity 135 Ci/mmol) and 0.4 μCi of [1-^{14}C]stearic acid/ml (Amersham; item CFA 24, sp. radioactivity 55.7 mCi/mmol). In other experiments (as described in Table 3) ^{3}H-labelled arachidonic acid (5 μCi/ml) was added to incubations 5 min before the end of the 1 h period of incubation with [1-^{14}C]arachidonic acid.

Stimulation of prelabelled cells. Whatever the radiolabelled compound(s) used for prelabelling, the washed, labelled cells were equilibrated at 37 °C prior to addition (with mixing) of either 0.5 μg of ionophore A23187 (Lilly) in 20 μl of DMSO, or 20 μl of DMSO (control). (A dose of 0.5 μg of A23187 was selected because, in preliminary experiments, this amount of ionophore was found to be sufficient to cause maximal loss of [^{14}C]arachidonic acid from the phospholipid pool without significantly affecting viability, as assessed by release of lactate dehydrogenase.) In the [^{14}C,^{3}H]-arachidonic acid double label experiments the cells were equilibrated for 5 min before addition of ionophore. In all other experiments, 10 min pre-equilibration was allowed. If inhibitors were used, these were added 5 min before addition of A23187. DMSO alone (20 μl) had no effect on either arachidonic acid release or phosphoinositide breakdown. In a few experiments, fMLP, from Sigma, was included as a stimulus. This was added to the cells dissolved in 10 μl of DMSO in place of ionophore. If cytochalasin B (Sigma) was also included, this was provided in 10 μl of DMSO, 5 min before addition of fMLP. The final concentration of cytochalasin B was always 5 μg/ml.

Experiments in which radiolabel and cell-stimulating agent were added simultaneously. For the experiments in which ^{14}C-labelled arachidonic acid was added to unlabelled cells at the same time as the A23187 stimulus, [1-^{14}C]arachidonic acid was added to each culture dissolved in ethanol (final concentration 0.2%). Equal (0.5 ml) volumes of cells (2×10^7/ml) and arachidonic acid (1.67 μCi/ml), plus or minus A23187, were mixed to initiate stimulation. A similar mixing technique was used to study metabolism of ^{14}C-labelled lysophosphatidylcholine and ^{14}C-labelled lysophosphatidylinositol. No ethanol was necessary in these cultures. [1(3)-^{14}C]Glycerol (Amersham; item CFA 47, sp. radioactivity 30 mCi/mmol) was used at 2.5 μCi/ml. L-Lysophosphatidylcholine (1-[1-^{14}C]palmitoyl) (Amersham; item CFA 633, sp. radioactivity 57 mCi/mmol) was present at 0.1 μCi/ml. L-3-Lysophosphatidyl[U-^{14}C]inositol, prepared by phospholipase A_2 digestion of L-3-phosphatidyl[U-^{14}C][inositol (Amersham; item CFA 641, sp. radioactivity 282 mCi/mmol) was present in incubations at approx. 0.1 μCi/ml.

Lipid extraction

After stimulation, cells were cultured at 37 °C for varying periods between 1 and 600 s. Incubations were terminated by addition of HCl. Three methods of lipid extraction were employed, the particular extraction technique selected depending on the metabolite for which optimum extraction efficiency was required. Most samples were acidified to pH 3 and extracted with 2 vol. of butan-1-ol as described by Bjerve *et al.* (1974). This technique gave a high efficiency of extraction of lysophospholipids. Cooling the extractions overnight to −20 °C improved the efficiency of separation of water and butanol. Lysophospholipid was added where appropriate as a carrier. In the experiments in which arachidonic acid incorporation into polyphosphoinositides was measured, incubations were terminated by the successive addition of 0.20 ml of 0.22 M-HCl and 4.5 ml of chloroform/methanol (1:2, v/v), and 10 μg of phosphatidylinositol 4,5-bisphosphate, 10 μg of phosphatidylinositol phosphate and 50 μg of phosphatidic acid were added as carriers. Phases were then separated by successive addition of 1.5 ml of chloroform and 1.5 ml of water. In a few experiments, in which neither lysophospholipids nor polyphosphoinositides were being quantified, Folch extraction was considered sufficient. Each sample was brought to pH 3 by addition of HCl and shaken with 5 ml of chloroform/methanol (2:1, v/v). The aqueous layer was removed and the lower (chloroform) phase washed twice with 3 ml of 0.5M-$MgCl_2$.

Analytical methods

Analysis of phospholipids and acylglycerols. Phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, total HETEs, free arachidonic acid, diacylglycerol and triacylglycerol were satisfactorily separated by the three-stage t.l.c. technique described by Irvine *et al.* (1984). Phosphatidic acid was assayed separately by t.l.c. on oxalate-impregnated silica gel G plates using the solvent system chloroform/methanol/10 M-HCl (87:13:0.5, by vol.) (Cohen & Derksen, 1969). In most experiments, radioactivity was quantified by scanning with a Berthold LB2722-2 t.l.c. scanner linked to a Commodore Pet 4000 computer. Where phosphatidic acid was measured, or where ^{3}H or double (^{3}H/^{14}C) labelling was employed, the scanner was only used to locate radioactivity peaks. Silica gel from the positions of the peaks was scraped off, digested in 1 ml of Soluene

(Packard) and counted in 8 ml of Dimilume scintillant (Packard). As a check on the phospholipid quantifications made by t.l.c. a small number of samples were also separated by h.p.l.c. (Hax & Guerts van Kessel, 1977). Polyphosphoinositides were separated by chromatography on immobilized neomycin (Schacht, 1978) or, when separation of lysophosphoinositides was also required, by chromatography on silica gel H (Analtech) using the solvent system chloroform/methanol/4M-NH_4OH (9:7:2, by vol.) (Gonzalez-Sastre & Folch-Pi, 1968).

Analysis of water-soluble inositol derivatives. Water-soluble inositol metabolites were separated by ion-exchange chromatography on Dowex 1-X8 in a modification of the technique of Ellis *et al.* (1963) as described by Bijsterbosch *et al.* (1985). In this technique, successively greater concentrations of formate ion were used to elute, in turn, glycerophosphoinositol, inositol cyclic 1,2-phosphate, inositol monophosphate, inositol bisphosphate and inositol trisphosphate. No attempt was made to resolve isomers of inositol trisphosphate and the radioactivity measurements for inositol trisphosphate may include trace amounts of inositol derivatives with more than three phosphate groups. Glycerophosphoinositol and inositol cyclic 1,2-phosphate were also incompletely resolved so that results for these two metabolites are combined or, where results for these inositol derivatives are quoted individually, t.l.c. was used to resolve further the two compounds (Koch-Kallnbach & Diringer, 1977). Identity of the peaks obtained by ion-exchange chromatography was also checked by paper chromatography (Grado & Ballou, 1961) or high voltage electrophoresis (Dawson & Clarke, 1972).

Analysis of arachidonic acid and its metabolites. Radiolabelled HETEs and other metabolites of arachidonic acid were separated at 4 °C by t.l.c. on silica gel G using the solvent system toluene/dioxan/acetic acid (65:34:1.5, by vol.) (Harvey & Osborne, 1983). Non-labelled lipoxygenase metabolites of arachidonic acid were quantified by h.p.l.c. as described by Osborne *et al.* (1983). Free and total arachidonic acid content of cell extracts was determined by packed column g.l.c. of samples methylated before (free) and after (total) alkaline hydrolysis. The column used was a Silar 10C, temperature programmed from 160°C to 240°C at 4°C/min.

Inhibitor studies

The phospholipase inhibitor manoalide was supplied by Professor J. Faulkner, Scripps Institution of Oceanography, and was added to cells dissolved in DMSO (final DMSO concentration 0.1%). Mepacrine hydrochloride (Sigma) was water soluble. To ensure that these compounds were not acting through an effect on cell viability, in some experiments released lactate dehydrogenase was measured as described by Wacker *et al.* (1956). The specificity of these inhibitors was checked on crude enzyme preparations made by disrupting rabbit polymorphonuclear leucocytes. Cells, suspended at 1×10^7/ml in saline, were disrupted with a Wright–Edwards pump (Stansted Fluid Power, Stansted, Essex, U.K.). The pump was used with a bias air pressure of 280 kPa corresponding to a back pressure of 5660 kPa. Disrupted cells were centrifuged successively for 15 min at 300 ***g*** (to remove nuclei), 15 min at 4000 ***g*** (to remove mitochondria), then 30 min at 20000 ***g***. The washed microsomal pellet from the final spin was used as a source of phospholipase A_2 and the 20-fold concentrated supernatant was used as a source of phospholipase C. Phospholipase A_2 was assayed by hydrolysis of L-3-phosphatidylcholine (1-stearoyl-2-[1-^{14}C]arachidonyl) (Amersham; item CFA 655, sp. radioactivity 60 mCi/mmol) at pH 8 in 100 mM-Tris/HCl/1 mM-$CaCl_2$ (Cosentino & LeGrande, 1981). Phospholipase C was assayed by hydrolysis of phosphatidyl[U-^{14}C]inositol liposomes as substrate in 1 mM $CaCl_2$/50 mM-Tris/maleate buffer, pH 7.0. Butanol extraction (Bjerve *et al.*, 1974) was used to separate radiolabelled inositol phosphates from phosphatidylinositol and (if present) lysophosphatidylinositol. Of the radioactivity released into the aqueous phase by our polymorphonuclear leucocyte cytosol preparation, > 90% was inositol 1-phosphate or inositol cyclic 1,2-phosphate.

RESULTS AND DISCUSSION

Phosphoinositide breakdown following stimulation with A23187

Within 10 s of addition of A23187 to [^{3}H]inositol-labelled polymorphonuclear leucocytes there was significant release of labelled inositol phosphates, notably

Table 1. [^{3}H]Inositol phosphates produced 10 s after exposure of cells to A23187

Polymorphonuclear leucocytes (> 99% pure) were labelled with [^{3}H]inositol, followed by a 'cold' inositol chase, as described in the Experimental section. They were then exposed for 10 s to A23187 (0.5 μg/ml) or an equivalent volume of DMSO vehicle, and labelled inositol phosphates were separated from the aqueous phase after butanol extraction. Results shown are quadruplicate determinations on two different cell populations. Values in parentheses are the 95% confidence limits of each stimulation index, calculated by using Fieller's theorem ($n = 4$). Statistically significant stimulation: *0.05 > P, **0.01 > P.

	Ratio $\left(\frac{^3\text{H in extracts from A23187-treated cells}}{^3\text{H in extracts from control cells}}\right)$		
Inositol metabolite	Cell population 1	Cell population 2	Mean ratio
Inositol monophosphate	1.05 (0.88–1.25)	1.27 (1.11–1.46)*	1.16
Inositol bisphosphate	1.22 (1.05–1.42)*	1.42 (1.24–1.62)**	1.32
Inositol trisphosphate	1.27 (1.05–1.56)*	1.24 (1.04–1.48)*	1.26

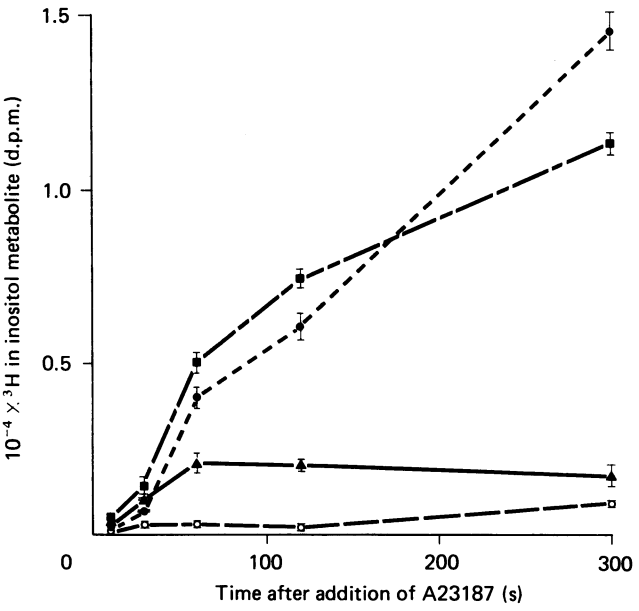

Fig. 1. Production of [^{3}H]inositol phosphates at various times after exposure of [^{3}H]inositol-labelled cells to A23187 (0.5 μg/ml)

Each point is the mean (±S.D.) of four determinations on a single cell preparation. A repeat experiment on a different cell preparation gave similar results. Radioactivity at zero time has been deducted. ▲——▲, Inositol trisphosphate (radioactivity at zero time, 646±107 d.p.m.); ■—·—■, inositol bisphosphate (radioactivity at zero time, 952±100 d.p.m.); ●---●, inositol monophosphate (radioactivity at zero time, 1153±271 d.p.m.); ○———○, inositol cyclic 1,2-phosphate /glycerophosphoinositol (radioactivity at zero time, 412±95 d.p.m.).

inositol bisphosphate and inositol trisphosphate (Table 1). Release of inositol monophosphate at early time periods was variable from experiment to experiment, and was always appreciably less than the overall release of inositol polyphosphates. This predominance of inositol polyphosphates is remarkable in view of the small proportion of labelled phosphatidylinositol phosphate and phosphatidylinositol bisphosphate as a fraction of total [^{3}H]inositol-labelled lipids. Phosphatidylinositol phosphate comprised 1.4% of [^{3}H]inositol-labelled lipids (mean value, range 0.9–2.0%, $n = 6$). Phosphatidylinositol bisphosphate comprised 0.5% of [^{3}H]inositol-labelled lipids (mean value, range 0.3–0.9%, $n = 6$). Almost all the remaining [^{3}H]inositol label was present as phosphatidyl[^{3}H]inositol. These results suggest that, in the whole cell, phospholipase C preferentially breaks down polyphosphoinositides. Such preference does not necessarily require a specific phospholipase C enzyme; it could be a result of the way phosphoinositides and polyphosphoinositides are organized in the cell membrane. With more prolonged exposure of leucocytes to A23187, the proportion of inositol phosphates accounted for by polyphosphates declined and that accounted for by inositol monophosphate increased (Fig. 1). This pattern of kinetics suggests that most, if not all, labelled inositol monophosphate was formed by breakdown of polyphosphates by phosphatases. It is possible that inositol bisphosphate was also produced as a result of phosphatase action upon inositol trisphosphate. However, the very significant production of inositol bisphosphate, even at only 10 s after A23187 stimulation, makes it likely that at least some of the inositol bisphosphate released was formed by direct hydrolysis of phosphatidylinositol phosphate. Although most work on stimulation of polyphosphoinositide breakdown has emphasized the importance of inositol trisphosphate production (Michell *et al.*, 1981; Michell, 1982; Berridge & Irvine, 1984), production of inositol bisphosphate predominates during A23187 stimulation of macrophages (Emilsson & Sundler, 1984), and both inositol bisphosphate and inositol trisphosphate are formed when rabbit polymorphonuclear leucocytes are stimulated with fMLP (Bradford & Rubin, 1985).

Arachidonic acid turnover following A23187 stimulation

The earliest changes in arachidonic acid turnover following A23187 treatment were marked increases in incorporation of labelled arachidonic acid into phosphatidylinositol phosphate, phosphatidylinositol bisphosphate, diacylglycerol and phosphatidic acid, but not other

Table 2. Lipid labelling with [1-^{14}C]arachidonic acid 10 s after exposure of cells to A23187

Polymorphonuclear leucocytes (> 99% pure) were incubated for 10 s with A23187 (0.5 μg/ml) in the presence of 1.67 μCi of arachidonic acid/ml in 0.2% ethanol. Controls received DMSO vehicle in place of A23187. Lipids were extracted with chloroform/methanol (1:2, v/v) and separated as described in the Experimental section. Pooled results from two cell preparations are shown (four determinations per cell preparation; total eight samples in each control and A23187-treated group). Values in parentheses are the 95% confidence limits of each stimulation index, calculated by using Fieller's theorem. Statistically significant stimulation: *0.05 > P, **0.01 > P.

Lipid	Ratio $\left(\frac{^{14}C \text{ in extracts from A23187-treated cells}}{^{14}C \text{ in extracts from control cells}}\right)$
Phosphatidylinositol	1.13 (0.92–1.39)
Phosphatidylinositol phosphate	2.50 (2.15–2.95)**
Phosphatidylinositol bisphosphate	2.61 (2.29–3.20)**
Diacylglycerol	4.55 (3.08–8.64)**
Phosphatidic acid	5.94 (4.26–9.67)**
Triacylglycerol	0.36 (0.16–0.61)
Phosphatidylcholine	1.12 (0.95–1.34)
Phosphatidylethanolamine	1.01 (0.87–1.16)

lipids (Table 2). The four lipids showing markedly increased levels of radiolabelled arachidonic acid can be linked in a metabolic sequence, i.e. phosphatidylinositol bisphosphate can be formed by phosphorylation of phosphatidylinositol phosphate, diacylglycerol by phospholipase C mediated breakdown of phosphatidylinositol bisphosphate and phosphatidic acid by phosphorylation of diacylglycerol (Berridge, 1984). It is to be expected that increased incorporation of radiolabel into the first lipid of a metabolic sequence will lead to increased incorporation into subsequent members of the chain. Thus some, but not necessarily all, of the increased labelling of diacylglycerol and phosphatidic acid can be explained if they are derived from polyphosphoinositides. Phosphatidylinositol itself does not label as rapidly as the polyphosphoinositides. Hence it is necessary, if the rapid labelling of the polyphosphoinositides is to be explained, to postulate either an acylation step preceding phosphatidylinositol phosphate (as, for example, if this lipid was formed by acylation and phosphorylation of glycerophosphoinositol), or a small pool of phosphatidylinositol with very different kinetics of [^{14}C]arachidonic acid labelling from the bulk phosphatidylinositol pool [as described by Monaco (1982), using WRK-1 cells].

As Table 2 shows, A23187 stimulation of [^{14}C]arachidonic acid incorporation into diacylglycerol and phosphatidic acid was significantly greater than stimulation of [^{14}C]arachidonic acid incorporation into polyphosphoinositides. It is for this reason that we propose acylation of monoacylglycerol as an additional pathway to the rapidly labelled diacylglycerol pool. Labelled phosphatidic acid might also be formed by acylation of glycerol 1-phosphate. When cells were stimulated for 1 min with A23187 in the presence of [^{14}C]glycerol, both ^{14}C-labelled monoacylglycerol and ^{14}C-labelled glycerol phosphate could be detected. Thus the components for an acylation pathway to diacylglycerol and phosphatidic acid are both present in polymorphonuclear leucocytes, and activated by A23187. Early activation of a pathway to diacylglycerol other than phosphoinositide breakdown has previously been described during hormonal activation of a number of tissues, such as adrenal cortex by adrenocorticotropic hormone (Farese, 1985). Its significance is unclear.

A23187 has previously been shown to stimulate incorporation of radiolabelled arachidonic acid into phosphatidic acid of horse neutrophils (Lapetina *et al.*, 1980) or guinea pig neutrophils (Takenawa *et al.*, 1983). Our results provide an explanation for this rapid labelling alternative to that proposed earlier (Lapetina *et al.*, 1980). In earlier work, the rapid labelling of phosphatidic acid was explained in terms of a phosphatidic acid specific phospholipase A_2. Our results suggest that it is unnecessary to postulate such an enzyme. Instead the rapid labelling of phosphatidic acid can be explained largely in terms of derivation of this phosphatidic acid from a rapidly labelling pool of diacylglycerol, which in turn could be derived either by acylation of monoacylglycerol or by breakdown of phosphatidylinositol phosphate and bisphosphate pools which themselves label rapidly with [^{14}C]arachidonic acid. The studies with [^{3}H]inositol-labelled cells showed that breakdown of phosphatidylinositol phosphate and bisphosphate occurred sufficiently rapidly to account for the observed rate of diacylglycerol and phosphatidic acid labelling.

At 30 s or more after exposure to A23187, [^{14}C]arachidonic acid incorporation into phosphatidylinositol was also significantly enhanced (Fig. 2). This agrees with observations made by Rubin *et al.* (1981). The slower kinetics of increased radiolabelled arachidonic acid incorporation into phosphatidylinositol in comparison with diacylglycerol and polyphosphoinositides suggests that [^{14}C]arachidonic acid-labelled diacylglycerol was derived from monoacylglycerol or polyphosphoinositides rather than phosphoinositides, and that labelling of phosphatidylinositol was probably an indirect consequence of recycling labelled diacylglycerol.

Significance of difference lipids as sources of arachidonic acid for HETEs production

The rapid labelling of diacylglycerol and phosphatidic acid provides no information about the absolute amounts of arachidonic acid released from these two lipids. In other cell types, evidence has been presented for direct release of arachidonic acid from diacylglycerol via a diacylglycerol lipase (Rittenhouse-Simmons, 1979) and from phosphatidic acid via a specific phospholipase A_2 (Lapetina & Billah, 1981). However, the absolute amounts of diacylglycerol and phosphatidic acid in rabbit polymorphonuclear leucocytes are small relative to triacylglycerol and phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, which can, respectively, be hydrolysed by triacylglycerol lipase (Elsbach & Farrow, 1969) or phospholipase A_2 (Bormann *et al.*, 1984). What are the most important mechanisms for release of arachidonic acid in the A23187-stimulated rabbit polymorphonuclear leucocyte?

A major fate of released arachidonic acid is conversion to metabolites, in the A23187-stimulated rabbit polymorphonuclear leucocyte predominantly 5-HETE and 5,12-diHETE (Borgeat & Samuelsson, 1979). We found that resting polymorphonuclear leucocytes contained 3.00 ± 0.20 nmol of arachidonic acid/10^7 cells, of which 0.06 ± 0.03 nmol/10^7 cells were present as free acid and the rest was esterified. This meant that arachidonic acid accounted for 4% of total and 5% of free fatty acid (all values are the mean $\pm$ S.D. for three determinations). Levels of lipoxygenase metabolites in resting cells were too low to measure (i.e. below 0.01 nmol/10^7 cells). Pulse–chase experiments showed that not only was arachidonic acid rapidly incorporated into diacylglycerol, it was also rapidly lost, even in resting cells. Thus reacylation and deacylation of diacylglycerol and phosphatidic acid could occur in the absence of appreciable release of arachidonic acid. When cells were stimulated with A23187, increased labelling of diacylglycerol and phosphatidic acid occurred within seconds, yet release of arachidonic acid occurred much more slowly. Exposure to A23187 caused very little change in measured free arachidonic acid, as virtually all released arachidonic acid was converted to lipoxygenase metabolites. At 10 s after stimulation, when labelling of diacylglycerol was increased 4.6-fold and phosphatidic acid 5.9-fold (Table 2), release of 5-HETE and 5,12-diHETE was still below 0.01 nmol/10^7 cells. At 5 min after exposure to A23187, when labelling of diacylglycerol and phosphatidic acid was in decline (Fig. 2), release of arachidonic acid metabolites totalled 0.34 ± 0.02 nmol/10^7 cells, comprising 5-HETE (0.16 ± 0.01 nmol/10^7 cells) and 5,12-diHETE isomers

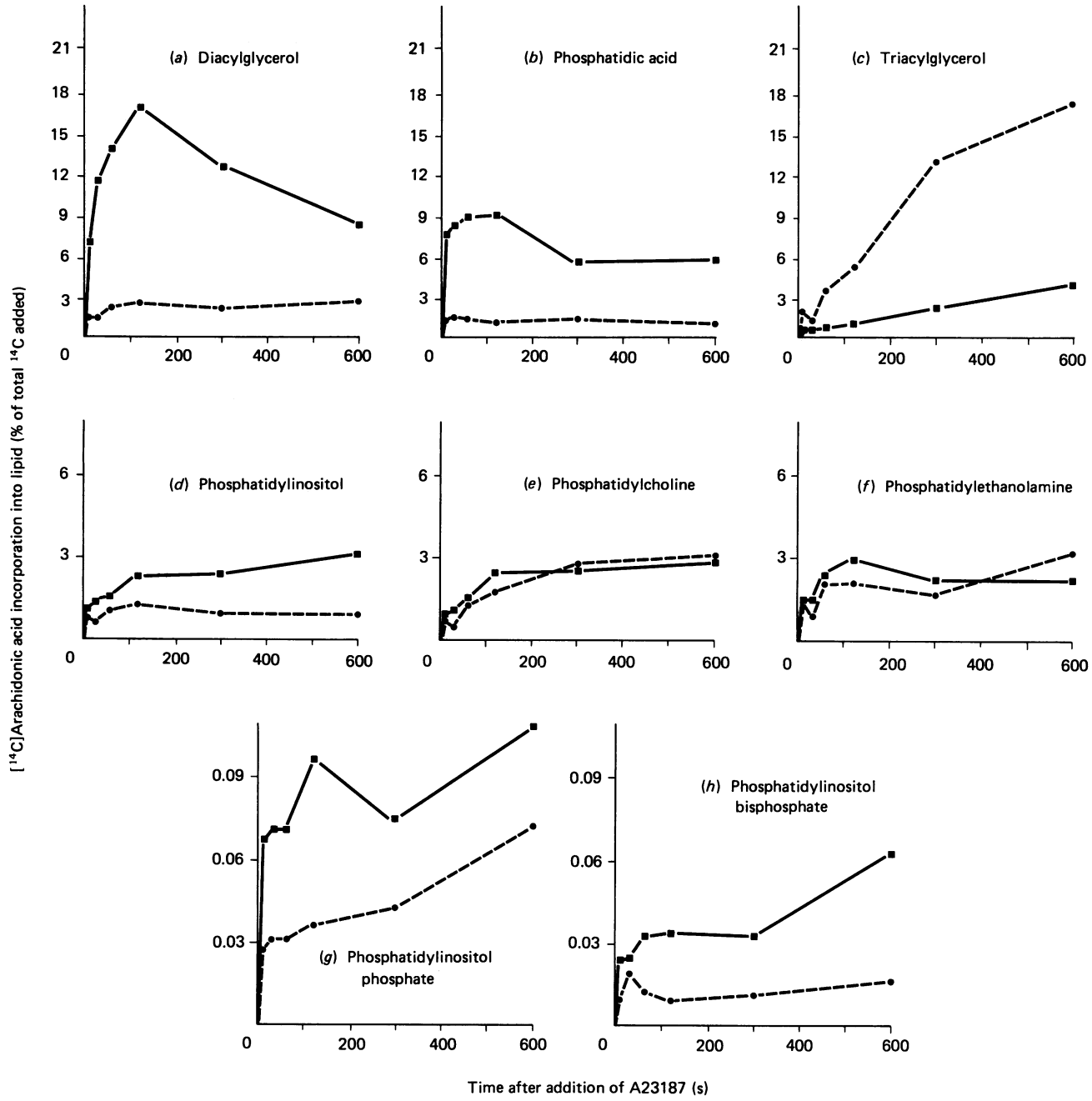

Fig. 2. [^{14}C]Arachidonic acid incorporation into various lipids at different times after exposure of cells to A23187 (0.5 μg/ml)

Labelled arachidonic acid and A23187 were added together, and after periods between 10 and 600 s lipids were extracted with chloroform/methanol (1:2, v/v) and separated as described in the Experimental section. Each point is the pooled mean of eight measurements made on two cell preparations (four measurements for each preparation). ■——■, A23187-treated cells; ●---●, control cells treated with an equivalent concentration of DMSO vehicle. All results are expressed as a percentage of the total amount of ^{14}C label present immediately before stimulation.

(0.18 ± 0.02 nmol/10^7 cells). In short, A23187-stimulated release of arachidonic acid for HETEs production occurred more slowly than the changes in diacylglycerol and phosphatidic acid turnover. The kinetics of HETEs production instead paralleled closely the release of radiolabelled arachidonic acid from phosphatidylcholine. Fig. 3(*e*) shows the release of radiolabel from phosphatidylcholine when polymorphonuclear leucocytes were prelabelled with [^{14}C]arachidonic acid for 1 h, washed and stimulated in the absence of label. Phosphatidylcholine was the major source of released radiolabel, a finding in agreement with earlier studies (Walsh *et al.*, 1981; Takenawa *et al.*, 1983, Foster & Rush, 1986). The other lipid showing significant loss of radiolabel was phosphatidylinositol [as found by Walsh *et al.* (1981) and Takenawa *et al.*, (1983) but not by Foster & Rush (1986)]. Loss of radiolabel from phosphatidylinositol occurred more rapidly than could be measured by our techniques (i.e. within seconds). Thereafter the input of ^{14}C label into the phosphatidylinositol pool balanced any loss of radiolabel from that pool, so that there was no net change (see Fig. 3*d*).

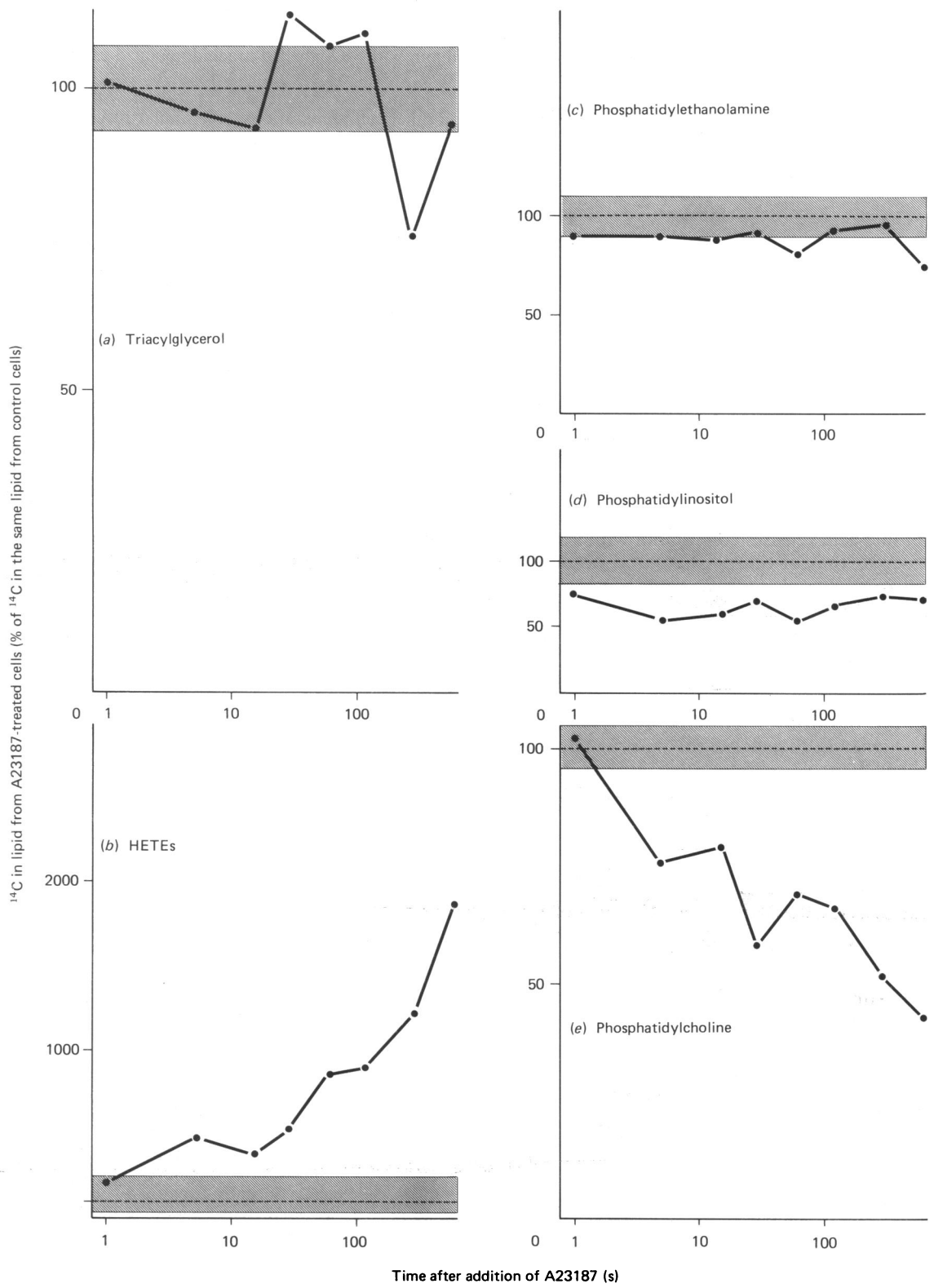

Fig. 3. Loss of [^{14}C]arachidonic acid from different lipid pools after exposure of prelabelled cells to A23187 (0.5 μg/ml)

DMSO-treated control cells were harvested at the same time as those exposed to A23187 and both groups were extracted with chloroform/methanol (2:1, v/v). Results are expressed as the amount of radiolabel in each lipid pool for A23187-treated cells, as a percentage of the amount of radiolabel in the same pool from control cells (measurements made in triplicate on a single cell preparation; similar results were obtained in time course experiments on two different cell preparations). Shaded areas show 95% confidence limits of the 100% value. Time is shown on a logarithmic scale. In order to make comparisons between different lipids easier, the distance between 0 and 100% on the scale of percentage radioactivity has been adjusted to correspond to the relative amounts of label in each lipid at the time of addition of A23187. These relative amounts were: triacylglycerol, 39%; phosphatidylcholine, 30%; phosphatidylethanolamine, 13% phosphatidylinositol, 8%; HETEs, 1%.

Table 3. Identification of the lipid pool which provides the arachidonic acid used for HETEs production

Polymorphonuclear leucocytes (> 99% pure) were labelled for 60 min with ^{14}C-labelled arachidonic acid and for 5 min with ^{3}H-labelled arachidonic acid, then washed and exposed for 5 min to A23187 (0.5 μg/ml) or DMSO (20 μl/ml) (control). Ratios of ^{3}H/^{14}C in different lipid pools are expressed as means of four measurements on a single cell preparation, with 95% confidence limits calculated by using Fieller's theorem. Repeat experiments on other cell preparations gave similar results.

Lipid	^{3}H/^{14}C	
	In control cells	In A23187-treated cells
Free arachidonic acid	3.63 (3.05–4.44)	3.40 (3.24–3.61)
Diacylglycerol	4.84 (3.82–6.56)	3.59 (2.99–4.46)
HETEs	1.39 (1.22–1.59)	1.25 (1.20–1.30)
Phosphatidylinositol	0.93 (0.84–1.02)	1.01 (0.85–1.20)
Phosphatidylcholine	0.64 (0.50–0.80)	0.74 (0.60–0.89)
Phosphatidylethanolamine	1.16 (0.95–1.42)	1.24 (1.03–1.50)
Triacylglycerol	0.24 (0.06–0.43)	0.22 (0.17–0.29)

Table 3 describes an experiment which supported the concept that HETEs were predominantly synthesized from arachidonic acid release from a pool with a rate of turnover closer to that of the phospholipid pools than the diacylglycerol pool. Cells were exposed to [^{14}C]arachidonic acid for a long period (1 h) and [^{3}H]arachidonic acid for a short period (5 min), before being washed and stimulated with ionophore. This labelling protocol caused pools with rapid turnover of arachidonic acid to have a higher ^{3}H/^{14}C ratio than pools with a slower rate of turnover. Thus the ^{3}H/^{14}C ratio was considerably higher in diacylglycerol than in phospholipids or triacylglycerol, reflecting the greater rate of arachidonic acid turnover in this lipid. The ^{3}H/^{14}C ratio in HETEs was considerably lower than the ^{3}H/^{14}C ratio in diacylglycerol, making it unlikely that diacylglycerol was a major immediate source of arachidonic acid metabolized to HETEs. The ^{3}H/^{14}C ratio in HETEs was slightly higher than the ^{3}H/^{14}C ratio in either phosphatidylinositol or phosphatidylcholine. If, however, it is assumed that about one-fifth of the arachidonic acid used for HETEs production was originally present as free acid before A23187 stimulation (a value compatible with our measurements of free arachidonic acid and HETEs in resting and stimulated cells) then the ^{3}H/^{14}C ratio of the HETEs is exactly what would be expected if the arachidonic acid released from esterified lipids was derived only from the phospholipid pool.

There was no evidence that triacylglycerol acted as a major direct source of arachidonic acid for conversion to HETEs. Using cells prelabelled with [^{14}C]arachidonic acid and stimulated by our standard protocol, no marked loss of [^{14}C]arachidonic acid from triacylglycerol was observed (Fig. 3*a*). In the experiment with ^{3}H/^{14}C double-labelled cells, the ratio of label in triacylglycerol was very much lower than the ratio of label in HETEs (Table 3). However, A23187 stimulation did markedly inhibit the uptake of arachidonic acid into triacylglycerol (Fig. 2). Because of this, in the presence of competing acylation reactions, e.g. into phosphatidylcholine, even a low level of lipase activity would eventually cause an A23187-stimulated shift of arachidonic acid out of the triacylglycerol pool and into, for example, the phosphatidylcholine pool, as described by Elsbach & Farrow (1969).

Can release of arachidonic acid from the phospholipid pool regulate HETEs production?

The low level of free arachidonic acid in polymorphonuclear leucocytes makes it likely that availability of arachidonic acid limits production of HETEs. To provide confirmation of this, loss of radiolabelled

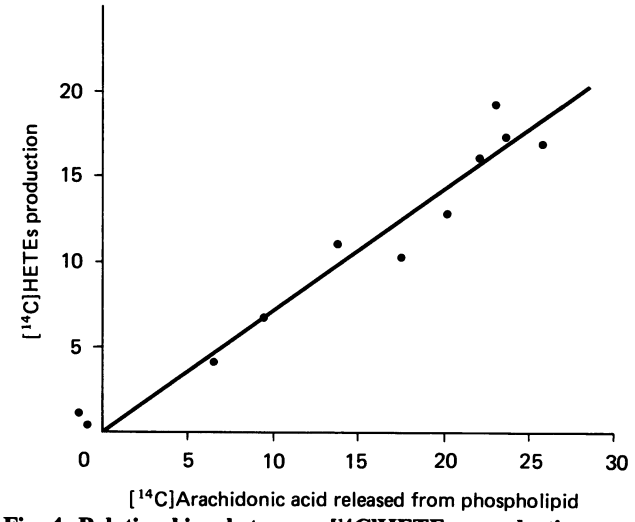

Fig. 4. Relationship between [^{14}C]HETEs production and [^{14}C]arachidonic acid release from phospholipid

Prelabelled cells were stimulated with ionophore in the presence of concentrations of mepacrine between zero and 1000 μM. Such concentrations had no effect on cell viability as assessed by release of lactate dehydrogenase (release < 10%). [^{14}C]Arachidonic acid release from phospholipid was calculated as the difference in radioactivity in the phospholipid pool between cells exposed to A23187 or to DMSO vehicle for 5 min. Similarly, the small amount of [^{14}C]HETEs produced in the absence of ionophore has been deducted from the total [^{14}C]HETEs present in A23187-treated cells to give [^{14}C]HETEs production. Each point is the mean of three determinations on a single cell preparation. Experiments on three other cell preparations gave similar results. The best fit regression line, calculated by the method of least squares, is shown. Radioactivity is expressed as a percentage of all the label present in the system immediately before cell stimulation.

Table 4. Effect of mepacrine on production of labelled 5-HETE and 5,12-diHETE (*a*) from [^{3}H]arachidonic acid added after cell stimulation or (*b*) from [^{14}C]arachidonic acid used to prelabel the cells prior to stimulation

All numbers are the mean (with S.D.) of three determinations on a single cell preparation, and are expressed as a percentage of the total amount of ^{14}C- or ^{3}H-labelled material present immediately prior to stimulation.

Treatment	Production of radiolabelled 5-HETE and 5,12-diHETE as a percentage of all label in system	
	^{3}H	^{14}C
Control	9.7±1.0	3.4±0.9
A23187 (0.5 μg/ml)	33.0±1.1	20.1±0.4
Mepacrine (500 μM)	11.5±1.3	2.3±0.4
Mepacrine (500 μM) and A23187 (0.5 μg/ml)	41.6±4.2	8.3±1.5

arachidonic acid from the phospholipid pool was assayed in the presence of different concentrations of the phospholipase inhibitor, mepacrine. The concentrations of mepacrine required to give half-maximal inhibition of labelled arachidonic acid release from phospholipids, and to give half-maximal inhibition of HETEs production, were similar (approx. 100 μM). There was a linear correlation between arachidonic acid release from phospholipids and HETEs production (Fig. 4). Regression analysis gave a best fit equation approximating to:

$$[\text{HETEs}] = 0.73 \times [\text{arachidonic acid released from phospholipid}]$$

The correlation coefficient, r^2, was 0.94. To ensure that correlation between arachidonic acid release and HETEs production was not merely due to mepacrine acting individually on lipoxygenase and phospholipase enzymes, cells prelabelled for 1 h with [^{14}C]arachidonic acid were exposed to ionophore in the presence or absence of mepacrine, then provided with [^{3}H]arachidonic acid for a very short period (2 min). In these conditions, ^{14}C label was almost all in the esterified pool and ^{3}H label largely in the free fatty acid pool. Mepacrine (500 μM) blocked conversion of ^{14}C, but not ^{3}H, label to HETEs (Table 4). Hence mepacrine was acting to inhibit HETEs production indirectly, by limiting release of arachidonic acid from phospholipid, and not directly, by preventing conversion of released arachidonic acid to HETEs.

Mechanism of arachidonic acid release from phosphatidylinositol and phosphatidylcholine

When cells were labelled with both arachidonic acid and stearic acid, 5 min exposure to A23187 caused significant loss of only the former fatty acid from phosphatidylcholine or phosphatidylinositol (results not shown). This confirmed, using polymorphonuclear leucocytes of rabbit origin, earlier observations on human cells made by Walsh *et al.* (1981). Stearic acid preferentially occupies the 1-position, and arachidonic acid the 2-position, of phospholipids. These results are therefore compatible with phospholipase A_2 mediated phospholipid breakdown. They are not proof of such breakdown because the ability of arachidonic, but not stearic, acid to be converted to metabolites (e.g. leukotriene B_4) which cannot be reacylated could also, if reacylation processes were fast enough, give an apparent specificity of release.

Inhibitors provide another approach to identifying the enzyme causing arachidonic acid release from phosphatidylinositol and phosphatidylcholine. Mepacrine is a widely used phospholipase A_2 inhibitor, and in other systems has been reported to inhibit phospholipase A_2 without inhibiting generation of phosphatidic acid from phosphatidylinositol, presumably via phospholipase C (Lapetina & Billah, 1981). In our hands, mepacrine inhibited release of radiolabelled arachidonic acid from both phosphatidylcholine and phosphatidylinositol (IC_{50} approx. 100 μM in both cases), but interpretation of this finding was complicated by our observation that our isolated polymorphonuclear leucocyte phosphoinositide-hydrolysing phospholipase C preparation was also inhibited by mepacrine with an IC_{50} approx. 200 μM.

Manoalide is a novel phospholipase A_2 inhibitor structurally very different from mepacrine (Glaser & Jacobs, 1986; Lombardo & Dennis, 1985). It was 15–70-fold more potent than mepacrine when tested against preparations of polymorphonuclear leucocyte phospholipase A_2, yet reduced the activity of an isolated

Table 5. Distribution of radiolabel following addition of lysophosphatidylcholine (1-[1-^{14}C]palmitoyl) to unstimulated and A23187-stimulated cells

Polymorphonuclear leucocytes (> 99% pure) were incubated for 5 min with lysophosphatidylcholine (1-[1-^{14}C]palmitoyl) in the presence of either A23187 (0.5 μg/ml) or an equivalent dilution of DMSO vehicle. Lipids were extracted with butanol and separated by three-stage t.l.c. (Irvine *et al.*, 1984). All results are expressed as a percentage of the total radioactivity added to each culture, and each value is the mean (±S.D.) of seven determinations using two different cell preparations. Student's unpaired *t* test showed a significant effect of A23187 (compared with DMSO) in increasing the incorporation of label into both the phosphatidylcholine and free fatty acid pools ($0.05 > P$).

Lipid	Radioactivity associated with different lipids (%)		
	At time zero	After 5 min exposure to control cells	After 5 min exposure to A23187-treated cells
Lysophosphatidylcholine	99±2	55±6	24±8
Phosphatidylcholine	0	24±3	42±8
Free fatty acid	0	11±2	28±6
Triacylglycerol	0	9±7	5±5

Table 6. Distribution of radiolabel following addition of lysophosphatidyl[U-^{14}C]inositol to unstimulated and A23187-stimulated cells

Polymorphonuclear leucocytes (> 99% pure) were incubated for 5 min with lysophosphatidyl[U-^{14}C]inositol in the presence of either A23187 (0.5 μg/ml) or an equivalent dilution of DMSO vehicle. Lipids were extracted with butanol and the organic phase was separated on silica gel H (Gonzalez-Sastre & Folch-Pi, 1968), whereas the aqueous phase was separated on Dowex 1-X8 (Bijsterbosch *et al.*, 1985). Early eluted Dowex fractions were further resolved by t.l.c. (Koch-Kallnbach & Diringer, 1977). Standard radiolabelled inositol derivatives were subjected to the same extraction procedure and observed radioactivity measurements are corrected for different extraction efficiencies. All results are expressed as a percentage of the total radioactivity added to each culture, and each figure is the mean (±S.D.) of four determinations using a single cell preparation.

	Radioactivity associated with different inositol derivatives (%)		
Derivative	At time zero	After 5 min exposure to control cells	After 5 min exposure to A23187-treated cells
Lysophosphatidylinositol	92±5	34±5	35±2
Glycerophosphoinositol	2±1	53±1	53±2
Phosphatidylinositol	2±1	4±1	5±1
Inositol phosphates	0	1±1	1±1
Inositol	0	3±1	3±1
Unaccounted for	4±1	5±1	3±1

polymorphonuclear leucocyte phospholipase C preparation by less than 30% at 200 μM. At 10 μM it had no effect on polymorphonuclear leucocyte viability as assessed by lactate dehydrogenase release, yet when added to whole cells inhibited labelled arachidonic acid release from both phosphatidylcholine and phosphatidylinositol with an IC_{50} of approx. 2 μM. This suggests that arachidonic acid release from both phosphatidylcholine and phosphatidylinositol is phospholipase A_2 mediated, but we are cautious in such an interpretation because the relationship of results with isolated enzymes to the behaviour and role of these enzymes in whole cells remains to be defined.

Fate of lysophospholipids generated by phospholipase A_2 action

If phospholipase A_2 is involved in release of arachidonic acid from cell phospholipids, can the other products of enzyme action, lysophospholipids, be detected? Repeated attempts to demonstrate generation of lysophosphatidylcholine or lysophosphatidic acid (using [^{14}C]stearate-labelled cells) or lysophosphatidylinositol (using [^{3}H]inositol-labelled cells) were unsuccessful, although labelled lysophospholipids added to cells immediately prior to extraction with butanol were extracted with greater than 75% efficiency. Many other workers have had difficulty in extracting lysophospholipids from stimulated polymorphonuclear leucocytes, and the explanation usually proposed is that such compounds, if generated, are rapidly reacylated (Walsh *et al.*, 1981). To test this hypothesis, labelled lysophosphatidylcholine and lysophosphatidylinositol were added, in turn, to unstimulated and A23187-stimulated rabbit polymorphonuclear leucocytes. As Table 5 shows, lysophosphatidylcholine was indeed acylated by these cells, and the rate of acylation was enhanced by A23187. Formation of labelled free fatty acid and triacylglycerol suggest phospholipase A_1 activity also increased in A23187-stimulated cells. By comparison, the major pathway of removal of lysophosphatidylinositol appeared not to be acylation but conversion to glycerophosphoinositol (Table 6). Many workers, including ourselves, have observed that when [^{3}H]inositol is supplied to cells, glycerophosphoinositol becomes labelled (Koch-Kallnbach & Diringer, 1977; Berridge *et al.*, 1983, Bijsterbosch *et al.*, 1985). In unstimulated cells, with our standard [^{3}H]inositol labelling protocol, glycerophosphoinositol accounted for 15.5% of phosphorylated inositol derivatives in the water-soluble pool (range 9.1–22.9%, $n = 7$). By contrast with inositol phosphates, labelling of glycerophosphoinositol increased only modestly following A23187 stimulation (see Fig. 1).

The role played by glycerophosphoinositol in inositol metabolism is unclear. Our labelling data are in favour of a pathway in which phosphatidylinositol is not directly broken down by phospholipase C, but can instead be broken down by the phospholipase A enzymes to lysophosphatidylinositol and eventually glycerophosphoinositol. Studies in other cell types have suggested that deacylation of phosphatidylinositol may influence susceptibility to phosphorylation (Baker & Thompson, 1973) or to attack by a membrane-bound phospholipase C (Murase & Okuyama, 1985). Possibly, our understanding of the phosphoinositide cycle is incomplete, and account should be taken of possible interactions between A and C enzymes in the control of both diacylglycerol and inositol trisphosphate formation.

We have found no evidence in our studies for phospholipase D mediated breakdown of phosphatidylinositol as recently proposed by Cockcroft (1984), although much of Cockcroft's data is in accord with ours and can be reinterpreted in terms of the dual metabolic pathways to phosphatidic acid suggested by our results.

How good a model is A23187 for 'physiological stimuli' of arachidonic acid release?

A23187 was used in this study because of its effectiveness both in activating arachidonic acid release, and in stimulating eicosanoid production. It is, however unrelated in structure to putative physiological stimuli of arachidonic acid release such as the fifth component of complement (C5a), or the bacterial chemotactic factors, for which fMLP serves as a synthetic analogue. In many literature reports, C5a or fMLP alone are described as ineffective in mobilizing the endogenous arachidonic acid of polymorphonuclear leucocytes for eicosanoid production, and this has led to doubts being thrown both on the validity of A23187 as a model, and on the significance of

Table 7. Synergy between fMLP and a product of A23187-stimulated cells in enhancement of arachidonic acid release

Polymorphonuclear leucocytes, prelabelled for 1 h with [^{14}C]arachidonic acid, were dispensed in Dulbecco's saline containing twice the normal concentration of Ca^{2+} and Mg^{2+}. Unlabelled cells, exposed briefly to A23187 (0.5 μg/ml) in Dulbecco's saline lacking Ca^{2+} and Mg^{2+}, were immediately washed twice in $Ca^{2+}+Mg^{2+}$-free Dulbecco's saline, then added to the labelled cells at the same time as the fMLP. The final mixture, volume 1 ml, contained the normal amount of Ca^{2+} and Mg^{2+}, 5×10^6 labelled cells and 5×10^6 unlabelled cells. After 5 min at 37 °C, lipids were extracted with chloroform/methanol (2:1, v/v) and separated as described in the Experimental section. Each value is the mean (±S.D.) of four measurements on a single cell preparation. A23187 (0.5 μg) added to the cells reduced the amount of radiolabel in the phospholipid pool to 22.0±1.9% of total radiolabel (positive control). The effect of fMLP+unlabelled A23187-treated cells in stimulating loss of radiolabel from the phospholipid pool was significantly greater than the effect of either fMLP or A23187-pretreated cells alone (Duncan's multiple range test, $0.05 > P$). A similar experiment on a second cell preparation gave the same result.

Stimulating agent		Unlabelled cells		[^{14}C]Arachidonic acid incorporated into the phospholipid pool (% of total radiolabel added)
fMLP (1 μM)	DMSO (control)	Pretreated with A23187	Pretreated with DMSO	
−	+	−	+	28.1±1.8
+	−	−	+	28.9±1.2
−	+	+	−	25.2±5.5
+	−	+	−	17.8±1.9

phospholipase activation as a source of arachidonic acid for eicosanoid production (Clancy *et al.*, 1983).

Testing at ten concentrations between 0.0003 μM and 10 μM, we confirmed that addition of fMLP alone to prelabelled polymorphonuclear leucocytes was unable to cause significant release of [^{14}C]arachidonic acid from phosphatidylcholine (less than 6% release at all concentrations tested). By contrast, 0.5 μg of A23187/ml added to the same cells caused 21% release (significant $0.05 > P$, Dunnett's test). fMLP has been reported to activate phospholipase A_2 in isolated polymorphonuclear leucocyte membrane preparations (Bormann *et al.*, 1984). One possible way of reconciling these observations is to suggest that fMLP does activate phospholipase A_2, but that reacylation of arachidonic acid is occurring so rapidly in our system that label is being reincorporated into phospholipid before conversion to HETEs can take place. This is considered unlikely, since addition of 1% defatted bovine serum albumin to the incubation medium, to bind released arachidonic acid and prevent its reacylation, caused only a marginal increase in release of [^{14}C]arachidonic acid from phosphatidylcholine.

Bennett *et al.* (1980), Cockcroft *et al.* (1981), and others, have described how pretreatment of polymorphonuclear leucocytes with cytochalasin B can enhance responsiveness to fMLP, and we too observed that after pretreatment with 5 μg of cytochalasin B/ml concentrations of fMLP down to 0.01 μM were able to cause arachidonic acid release. The pattern of [^{14}C]arachidonic acid loss from different lipids was similar whether fMLP (plus cytochalasin B) or A23187 was the stimulus. In both cases, the major source of released radiolabel was phosphatidylcholine.

One interpretation of these results is that activating cells for arachidonic acid release requires two signals, only one of which is provided by fMLP (cytochalasin B presumably being able to provide the other). Induction of the arachidonic acid cascade in macrophages is already known to require synergistic action of multiple signals (Aderem *et al.*, 1986). In polymorphs, activation of phospholipase A_2 is associated with phosphorylation of an inhibitory peptide variously known as macrocortin or lipomodulin (Hirata, 1981). Many other cell activation processes are also thought to be regulated by phosphorylation of peptides (cf. Nishizuka, 1984). The requirement for more than one cofactor (e.g. diacylglycerol, Ca^{2+}) by certain protein-phosphorylating kinases provides one explanation for the requirement for synergistic action of multiple signals.

Cytochalasin B, if it is providing a 'second signal', is certainly not the 'physiological' second signal. One hypothesis considered was that A23187 alone stimulated arachidonic acid release because it caused polymorphonuclear leucocytes to release a putative 'physiological' second signal. In favour of this, addition of unlabelled polymorphonuclear leucocytes, preincubated with A23187 then washed, allowed [^{14}C]arachidonic acid-labelled polymorphonuclear leucocytes to respond to fMLP (Table 7). The nature of the second signal provided by the unlabelled A23187-treated cells is unknown. Preliminary data, showing that the ability of the unlabelled cells to synergize with fMLP is reduced if the cells are treated with 10 μg of 5,8,11,14-eicosatetraynoic acid/ml, as well as A23187, implies involvement of an arachidonic acid metabolite, but more work is required to identify this.

We suggest that our knowledge of the normal, physiological stimuli for arachidonic acid mobilization is incomplete. Indeed, under different conditions different combinations of signals may come together to trigger arachidonic acid release. Under such circumstances, A23187 seems a reasonable model for 'physiological' stimulation, delineating pathways with at least the potential for being activated during the inflammatory process *in vivo*.

Comparison of A23187-stimulated arachidonic acid release in the polymorphonuclear leucocyte and the platelet

A great deal of work on the mechanism of arachidonic acid release has used platelets. It is therefore valuable to compare our knowledge of this much-studied system with the results reported in this paper. Points of similarity are as follows.

1. A23187 is able to stimulate platelet arachidonic acid release on its own (Rittenhouse-Simmons & Deykin,

1977) although more 'physiological' stimuli may require a second signal (Rittenhouse & Allen, 1982).

2. Phospholipase C mediated breakdown of polyphosphoinositides occurs as an early event (Rittenhouse, 1984). An earlier, negative report (Billah & Lapetina, 1982*c*) may reflect the total dependence of this breakdown upon cyclo-oxygenase products and ADP.

3. There is evidence for phospholipase A_2 activation, including breakdown of phosphatidylinositol to lysophosphatidylinositol (Billah & Lapetina, 1982*a*,*b*; Rittenhouse, 1984).

4. Action of phospholipase A_2 on phosphatidylcholine and phosphatidylinositol is likely to be a major source of released arachidonic acid, although the relative importance, in the platelet, of phospholipase A_2 and diacylglycerol lipase-mediated release remains a topic of considerably controversy (for a brief discussion, see Rittenhouse, 1985).

5. The level of free arachidonic acid in the platelet is low, and in the absence of cyclo-oxygenase or lipoxygenase inhibitors, most of the fatty acid released after platelet activation is rapidly metabolized (Smith *et al.*, 1985). This suggests availability of free arachidonic acid can serve as a factor controlling formation of metabolites.

The suggestion, from our results, of early activation of a pathway to diacylglycerol separate from (poly)phosphoinositide breakdown has yet to be investigated in platelets.

We are grateful to Mrs. P. Shrubsall and Miss E. M. Hall for help with h.p.l.c. and to Dr. J. M. Smith who carried out the g.l.c. The phospholipase A_2 isolated enzyme assays were performed by Mr. A. Scott. Immobilized neomycin was prepared by Dr. M. Finch. We are also grateful to Dr. R. F. Irvine who checked the identity of some of our labelled inositol derivatives by high voltage electrophoresis, and to Sue Donovan for help in preparing this manuscript.

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Received 28 November 1985/11 April 1986; accepted 2 May 1986

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