Stimulation of phosphorylcholine turnover and diacylglycerol production in human polymorphonuclear leukocytes

Novel assay for phosphorylcholine

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Receptor-bypassing stimulants of human polymorphonuclear leukocytes (PMNLs), such as ionomycin or phorbol 12-myristate 13-acetate (PMA), generate an increase in diacylglycerol (DAG) which is independent of a phospholipase C specific for phosphatidylinositol 4,5-bisphosphate (PIP₂). Activation of a phospholipase C specific for phosphatidylcholine (PC) has been implicated as a source of DAG in other cells by measuring the release of radiolabelled phosphorylcholine. However, since PMNLs could not be labelled sufficiently with [³H]choline, we developed an h.p.l.c. assay to quantify mass levels of phosphorylcholine after enzymic conversion to [³²P]CDP-choline with CTP-phosphorylcholine (choline phosphate) cytidyltransferase (EC 2.7.7.15). This assay was linear to at least 20 nmol, and was sensitive to 10 pmol of phosphorylcholine. Baseline phosphorylcholine levels in unstimulated PMNLs were 2300 ± 510 pmol/10⁷ cells and were decreased by pretreatment with PMA (166 nM) or ionomycin (1 μM) for 10 min by 360 ± 130 and 600 ± 290 pmol/10⁷ cells respectively ($P < 0.05$). In contrast, baseline DAG levels were 147.6 ± 11.7 pmol/10⁷ cells in unstimulated PMNLs, and were increased by PMA or ionomycin by 1320 ± 222 and 1891 ± 264 pmol/10⁷ cells respectively ($P < 0.05$). Similarly, the chemoattractant fMet-Leu-Phe raised DAG levels by 731 ± 111 pmol/10⁷ cells and decreased phosphorylcholine levels by 180 ± 60 pmol/10⁷ cells. Activation of PMNLs by PMA, ionophore or fMet-Leu-Phe thus leads to the sustained production of DAG accompanied by the disappearance of phosphorylcholine. This suggests that these stimulants enhance PC turnover via a hydrolytic mechanism which is independent of phospholipase C, with activation of a PC-specific phospholipase D being a plausible mechanism.

INTRODUCTION

Activation of human polymorphonuclear leukocytes (PMNLs) by chemoattractant receptors is dependent upon a G-protein coupled to phospholipase C which is specific for phosphatidylinositol 4,5-bisphosphate (PIP₂) (Smith *et al.*, 1986). The hydrolysis of PIP₂ gives rise to the two intracellular messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Becker *et al.*, 1986; Snyderman *et al.*, 1986; Cockcroft, 1987). The increases in IP₃ and DAG result in mobilization of intracellular calcium (Berridge & Irvine, 1984) and activation of protein kinase C (Nishizuka, 1984) respectively. Previously, we demonstrated that these initial steps, if sufficiently sustained, lead to calcium influx resulting in a prolonged increase in intracellular calcium and subsequent generation of a second sustained rise in DAG (Truett *et al.*, 1988). The latter increase in DAG production appears necessary for optimal activation of the respiratory burst. Unlike the initial rise in DAG, however, the second increase is not derived from PIP₂ hydrolysis in PMNLs.

The production of DAG independent of inositol phospholipid hydrolysis has been documented in other cells in response to naturally occurring hormones, the

tumour promoter phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin (Irving & Exton, 1987; Takuwa *et al.*, 1987; Grillone *et al.*, 1988; Rider *et al.*, 1988). Phosphatidylcholine (PC) has been identified as a source of DAG in response to PMA in the pre-adipocyte cell line 3T3-L1 (Besterman *et al.*, 1986). Additional evidence that PC can serve as a source of DAG includes the identification of a PC-specific phospholipase C (Wolf & Gross, 1985), the release of phosphorylcholine in response to purinergic stimuli (Irving & Exton, 1987) and hydrolysis of PC in oncogene-transformed cell lines (Lacal *et al.*, 1987). We therefore sought to determine if the PIP₂-independent increase in DAG levels following stimulation of PMNLs was derived from hydrolysis of PC by a phospholipase C.

The small net decrease in the mass of PC required to account for the increase in DAG levels proved difficult to measure due to the large amount of this phospholipid, which accounts for 40 % of the total cellular phospholipid content (Serhan *et al.*, 1982). Therefore, quantification of the amount of radiolabelled phosphorylcholine released upon stimulation of cultured cells has been used to measure hydrolysis of PC by a phospholipase C (Besterman *et al.*, 1986). However, our attempts to label

Abbreviations used: PMNL, polymorphonuclear leukocyte; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PC, phosphatidylcholine.

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PC adequately in PMNLs were unsuccessful due to limited incorporation of [^3H]choline by these terminally differentiated cells. Using partially purified CTP-phosphorylcholine (choline phosphate) cytidyltransferase (EC 2.7.7.15) isolated from rat liver, total phosphorylcholine has been measured in liver by conversion to [^3H]CDP-choline with [^3H]CTP (Choy *et al.*, 1978). This assay was adapted for the increased sensitivity and reproducibility which was required to quantify the smaller amounts of phosphorylcholine present in human PMNLs.

EXPERIMENTAL

Materials

PMA was from Sigma Chemical Co.; and [γ - ^{32}P]ATP and [α - ^{32}P]CTP were from New England Nuclear. *sn*-1,2-Dioleoylglycerol (diolein) and *Escherichia coli* strain N4830/pJW10 membranes (Loomis *et al.*, 1985) were gifts from Dr. R. M. Bell and Dr. W. R. Bishop. Ionomycin was from CalBiochem. Scintillation fluid (Tru-Count) was from IN-US, Fairfield, NJ, U.S.A. Culture medium was from GIBCO.

Cell preparation

PMNLs (> 95% purity) were isolated from heparinized (10 units/ml) blood of healthy volunteers (Cox *et al.*, 1986). 3T3-L1 cells were a gift from Dr. P. Blackshear and were maintained as described (Besterman *et al.*, 1986).

Quantification of *sn*-1,2-diacylglycerols

DAG levels were determined by conversion of DAG into radiolabelled phosphatidic acid by DAG kinase (Truett *et al.*, 1988).

Quantification of phosphorylcholine

Attempts to quantify phosphorylcholine were made by measuring the amount of [^3H]phosphorylcholine released. PMNLs were suspended at 10^7 cells/ml in Medium 199 with 10 mM-Hepes and [^3H]choline (5 $\mu\text{Ci}/\text{ml}$). After incubating for 24 h at 37 °C, the cells were washed three times with Medium 199 and resuspended in Hanks' balanced salt solution containing 10 mM-Hepes and 4.2 mM- NaHCO_3 (pH 7.4). Cellular phospholipids were separated by t.l.c. (Serhan *et al.*, 1982) and PC was quantified (Gerlach & Deuticke, 1963).

Total phosphorylcholine was assayed by quantifying the enzymic conversion to CDP-choline with modification of a previous method (Choy *et al.*, 1978). CTP-phosphorylcholine cytidyltransferase was purified from rat liver through octyl D-glucoside extraction (Weinhold *et al.*, 1986) and stored at -70 °C. Aliquots of cells (190 μl of PMNLs at 1×10^8 cells/ml or 3T3-L1 cells at 5×10^6 cells/ml) were warmed at 37 °C for 5 min, and 10 μl of either stimulant or diluent was added for the indicated times prior to addition of a final concentration of 10% trichloroacetic acid. A standard curve was generated by adding exogenous phosphorylcholine (0.5–2 nmol) to trichloroacetic acid-treated aliquots of cells. After centrifugation (13 600 g, 10 min), 200 μl was removed and extracted four times with water-saturated diethyl ether. Tris/HCl (50 μl of 1 M, pH 7.7) and 30 μl of Tris/succinate (pH 7.4) were added followed by inorganic pyrophosphatase (10 μl of 0.8 unit/ml) which was added to the incubation mixture to prevent back-

inhibition of the cytidyltransferase reaction by the inorganic pyrophosphate generated. The reaction was initiated by addition of 50 μl of CTP-phosphorylcholine cytidyltransferase and 10 μl of 10 mM- $[\alpha$ - $^{32}\text{P}]$ CTP ($\sim 200 \mu\text{Ci}/\mu\text{mol}$) and incubated at 37 °C for 60 min. The reactions were terminated by boiling for 2 min and centrifugation (13 600 g, 10 min). Tris/glycine buffer (100 μl of 1 M, pH 10.5) and alkaline phosphatase (150 μl of 10 units/ml) were added to 150 μl of the supernatant and incubated at 37 °C for 60 min. After boiling for 2 min, the reaction mixture was centrifuged (13 600 g, 10 min) and the supernatant stored at -20 °C until analysed for $[\alpha$ - $^{32}\text{P}]$ CDP-choline formation.

The $[\alpha$ - $^{32}\text{P}]$ CDP-choline formed was separated by h.p.l.c. using a Whatman Partisil 10 SAX column (0.46 cm $\times$ 25 cm). The column was washed with water for 1 min, followed by a linear gradient to 0.03 M-potassium phosphate (pH 3.8) over 4 min and to 0.05 M over the next 50 min. The flow rate was held at 1 ml/min throughout. Retention times were determined by co-injection of known unlabelled standards with the incubations using u.v. detection at 254 nm. The eluate was monitored for radioactivity using an on-line detector (Ramona LS-4; IN/US).

Statistical analysis

As not all conditions (i.e. time points for the various stimuli) could be examined within each group of cells obtained from a single donor, the data are normalized as the net change from the basal level obtained for the experimental group in which the condition was examined. The basal levels obtained from all the experimental groups are expressed as the mean $\pm$ S.E.M., as are the net changes from the basal levels for the various conditions. Significance ($P < 0.05$) was determined by Student's paired *t* test comparing the actual levels of DAG or CDP-choline for each condition with the corresponding basal level obtained within that group.

RESULTS

Quantification of DAG levels in PMNLs

PMNLs were stimulated with PMA (166 nM), ionomycin (1 μM) or fMet-Leu-Phe (1 μM) in the presence of cytochalasin B (10 μM). PMA stimulated a slow rise in DAG (Fig. 1) increasing by $1320 \pm 222 \text{ pmol}/10^7$ cells ($P < 0.001$) after 10 min. In comparison, ionomycin initiated a more rapid and greater rise in DAG concentrations (Fig. 1) with $1891 \pm 264 \text{ pmol}$ of DAG/ 10^7 cells generated above baseline by 10 min ($P < 0.001$). As previously reported (Truett *et al.*, 1988), the peak DAG response to fMet-Leu-Phe occurred at ~ 2 min, producing $731 \pm 111 \text{ pmol}$ of DAG/ 10^7 cells above control levels ($P < 0.005$, $n = 3$).

Assay for PC hydrolysis

Approx. 60 000 pmol of PC/ 10^7 PMNLs were measured, requiring detection of around a 2% change in the net level of this phospholipid to account for the maximal DAG increase found in stimulated PMNLs. This change was below the level of sensitivity of the assays available for measuring total PC. Therefore, we labelled endogenous PC with [^3H]choline in order to detect release of [^3H]phosphorylcholine. However, the maximum cal-

Fig. 1. Time course of increase in DAG levels in PMNLs in response to PMA (●) (166 nM) or ionomycin (▲) (1 μ M)

The basal concentration of DAG was 147.6 ± 11.7 pmol/10⁷ cells ($n = 7$). * Indicates $P < 0.05$ when compared with baseline levels.

culated specific activity of the PC was only 0.32 Ci/mol ($n = 3$) after 24 h. This specific activity proved too low to measure changes in phosphorylcholine concentrations, as maximal stimulation would only generate ~ 1400 counts/10⁷ cells, with baseline background counts of ~ 7500 remaining even after chromatographic separation.

Enzymic assay of phosphorylcholine

In order to detect small changes in phosphorylcholine levels following cell stimulation, total phosphorylcholine

Fig. 2. Composite of radioactive h.p.l.c. profiles of [³²P]CDP-choline formation after the addition of exogenous phosphorylcholine to trichloroacetic-acid cell extracts

The retention time of CDP-choline was determined by addition of an unlabelled CDP-choline standard with u.v. monitoring at 254 nm. The radioactivity in the solvent front reflects material in the eluate not interacting with the column related to the ³²P released by the addition of alkaline phosphatase. The radioactivity of the compounds related to [α -³²P]CTP was removed by the alkaline phosphatase reaction. The retention time of CMP was slightly less than that of CDP-choline, while those of CDP and CTP were longer (results not shown). The assay was linear over a range of 20 nmol. The minimum sensitivity was 10 pmol. The standard curve for this assay (inset) had a regression coefficient of 0.99 and was typical of other experiments.

Fig. 3. Typical radioactive profiles of phosphorylcholine levels in (a) 3T3-L1 cells in response to PMA and (b) PMNLs in response to PMA and ionomycin

(a) Typical radioactive profile on h.p.l.c. of increased phosphorylcholine levels (as measured by [³²P]CDP-choline) at 60 s in 3T3-L1 cells in response to PMA (200 nM) compared with the overlaid profile of unstimulated cells. (b) Typical radioactive profile showing the decreased phosphorylcholine levels (as measured by [³²P]CDP-choline) at 10 min in PMNLs in response to PMA (166 nM) and ionomycin (1 μ M). The retention times of CDP-choline were determined by co-injection of an unlabelled standard monitored at 254 nm. The variation in retention between these two experiments was due to the different h.p.l.c. columns.

was assayed by modification of a previous method (Choy *et al.*, 1978). However, results between experiments were highly variable (results not shown). Further purification of CTP-phosphorylcholine cytidyltransferase (Weinhold *et al.*, 1986) resulted in more consistent inter-experiment reproducibility. Addition of alkaline phosphatase eliminated the labelled CMP peak, which was difficult to separate from the CDP-choline. The use of h.p.l.c. rather than t.l.c. provided more reliable separation of the product, and the use of ³²P rather than ³H increased the sensitivity. In every experiment (see Fig. 2), exogenous phosphorylcholine was added to unstimulated extracts in order to quantify conversion and ensure linearity. Conversion ranged from 22% to 65%, with a mean of 40%, during the 60 min incubation. Conversion in the presence of the cell extract was approximately half of that obtained for a standard curve performed in buffer alone, indicating the necessity of including the cell extract in the generation of a standard curve. The assay was linear during the addition of 20 nmol of phosphorylcholine (see Fig. 2).

Fig. 4. Time course of decrease in phosphorylcholine levels in PMNLs in response to PMA (●) (166 nM) and ionomycin (▲) (1 μ M)

The basal concentration of phosphorylcholine in PMNLs was found to be 2300 ± 510 pmol/ 10^7 cells ($n = 5$).

* Indicates $P < 0.05$ when compared with baseline levels.

To test the validity and applicability of the assay, phosphorylcholine levels were determined in 3T3-L1 cells (Fig. 3a). PMA (200 nM) has previously been shown to cause a rapid increase of [3 H]phosphorylcholine and DAG in 3T3-L1 cells labelled with [3 H]choline (Besterman *et al.*, 1986). Under similar conditions using the present enzymic assay, PMA released 1260 ± 420 pmol of phosphorylcholine at 60 s above a baseline concentration of 6310 ± 410 pmol/ 10^6 cells ($n = 2$) (see Fig. 3a). This increase was in agreement with the previous report. However, a lower baseline concentration of phosphorylcholine was reported (~ 2200 pmol/ 10^6 cells) and may reflect the fact that all pools of PC may not have been labelled to a uniform specific activity with [3 H]choline. Stimulated release of [3 H]phosphorylcholine may have come from a more highly labelled pool of PC.

Quantification of phosphorylcholine in PMNLs

Unlike 3T3-L1 cells, addition of PMA or ionomycin produced a significant decrease in the amount of phosphorylcholine in PMNLs as determined by h.p.l.c. quantification of [32 P]CDP-choline as demonstrated in representative radioactive profiles in Fig. 3(b). The phosphorylcholine concentrations in unstimulated PMNLs were 2300 ± 510 pmol/ 10^7 cells ($n = 5$) as measured by CDP-choline formation. Stimulation with PMA (166 nM) resulted in a slow decrease in phosphorylcholine concentration, whereas ionomycin (1 μ M) generated a more rapid and greater decline (Fig. 4). At 10 min, concentrations were decreased by 360 ± 130 pmol/ 10^7 cells ($P < 0.025$, $n = 5$) in response to PMA and 600 ± 290 pmol/ 10^7 cells ($P < 0.05$, $n = 4$) in response to ionomycin (Fig. 4). The magnitude of decrease of phosphorylcholine in response to fMet-Leu-Phe at the time of peak rise for DAG (2 min) was 180 ± 60 pmol/ 10^7 cells ($P < 0.05$, $n = 3$).

DISCUSSION

Activation of the respiratory burst in PMNLs has been associated with sustained production of DAG from

a source which is independent of PIP_2 . It was hypothesized that phospholipase C hydrolysis of PC could provide a source for this DAG. To measure PC utilization in PMNLs, a novel assay was required. Reliable and sensitive changes in the levels of phosphorylcholine could be detected using an assay involving the radionuclide [α - 32 P]CTP, partially purified CTP-phosphorylcholine cytidyltransferase, and h.p.l.c. with on-line radioactivity monitoring to quantify [α - 32 P]CDP-choline production. The enzymic assay of phosphorylcholine levels in 3T3-L1 cells were consistent with the results reported using the release of [3 H]phosphorylcholine in cells pre-labelled with [3 H]choline. Attempts to use this latter assay method proved unsuccessful in PMNLs due to the low level of specific activity despite a 24 h incubation with [3 H]choline.

Unlike 3T3-L1 cells, stimulation of PMNLs led to a decrease in phosphorylcholine. The time course and fall of phosphorylcholine corresponded well with the magnitude and rate of rise of the DAG levels when the cells were stimulated with PMA, ionomycin or fMet-Leu-Phe. Thus, unlike in 3T3-L1 cells, activation of a phospholipase C specific for PC does not appear to be a mechanism for generating the PI-independent DAG increase in stimulated PMNLs. However, these findings do support work in NG108-15 neuroblastoma cells (Liscovitch *et al.*, 1987) and GH3 pituitary cells (Kolesnick & Paley, 1987) showing that 1,2-diacylglycerols and phorbol esters stimulated PC synthesis via the CDP-choline pathway. These present data would therefore suggest that this latter pathway is a significant means by which DAG is metabolized in PMNLs. This raises the possibility that if phosphorylcholine is being released by phospholipase C hydrolysis of PC, it could be reincorporated too rapidly to detect significant release. However, even the very early changes in phosphorylcholine levels following stimulation did not suggest that there was an increased release of phosphorylcholine to account for the later decrease (Fig. 4).

The second rise of DAG in PMNLs does not appear to arise from phospholipase C hydrolysis of PC. Our previous results examining production of inositol phosphates (Truett *et al.*, 1988; results not shown) also discount the additional possibility of phospholipase D hydrolysis of PIP_2 or PIP .

The present data demonstrate that phosphorylcholine is utilized by stimulated PMNLs, suggesting that PC is being actively metabolized. Thus, a more likely mechanism for the generation of DAG in PMNLs, if it is derived from PC, is via the activity of a phospholipase D specific for PC. This latter enzyme activity would produce choline and phosphatidic acid with subsequent formation of DAG by dephosphorylation. Phospholipase D activity in HL60 cells has been suggested by time-course experiments showing rapid formation of phosphatidic acid with slower formation of DAG (Pai *et al.*, 1988) and by finding phosphatidic acid formation even in the presence of a DAG kinase inhibitor (Mahadevappa, 1988). Phospholipase D hydrolysis of phosphatidylinositol with formation of phosphatidic acid and inositol had been suggested to occur in response to fMet-Leu-Phe (Cockcroft, 1984). Our preliminary results in human PMNLs suggest that a phospholipase D specific for PC may generate DAG as well (Truett *et al.*, 1989). An alternative route of DAG formation is synthesis *de novo* via the Kennedy pathway. These hypotheses are now testable,

and in conjunction with the present assay for phosphorylcholine should lead to a better insight into PMNL activation.

J.J.M. is an R. J. R. Nabisco Scholar.

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