Quantification of contributions of phospholipid precursors to diradylglycerols in stimulated mononuclear phagocytes

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Phosphatidylcholine (PC) hydrolysis has been shown to occur in hormone-stimulated cells and represents a potential metabolic source, in addition to phosphoinositides, for the generation of diradylglycerols (DG). We performed studies in order to quantify the importance of this pathway in DG formation. We incubated murine peritoneal macrophages with platelet-activating factor (PAF), ionomycin, phorbol myristate acetate (PMA) or no stimulus in a series of timed incubations ranging from 15 s to 20 min. We quantified the profiles of the molecular species in the accumulated DG after extraction, specific radiolabelling to give [³²P]phosphatidic acid by DG kinase, and conversion to the dimethyl derivative. We used two independent methods for molecular species analysis: (1) reversed-phase h.p.l.c. separation with in-line β -radiation detection of peaks, and (2) an argentation-t.l.c. separation with scintillation counting of bands. Our results showed a clearly biphasic sequence in the composition of accumulated DG. The molecular species composition of early DG (up to 1 min stimulation time) was very similar to that of unstimulated DG, whereas the proportions of the species present in later DG were substantially altered. In the same experiments, we extracted native phospholipids from unstimulated macrophages, separated phosphatidylinositol (PI), PC, phosphatidylethanolamine (PE) and phosphatidylserine (PS), converted them to the corresponding DGs by using phospholipase C, and determined their molecular species compositions as above. In comparison with the diradyl compositions of stimulated DG, the diradyl composition of PI closely matched that of early DG, the differences between the PC and PI compositions matched the differences between early and late DG very closely, and the compositions of PE and PS were unique and unrelated. We quantified these relationships more precisely by multilinear regression analysis to calculate the theoretical best mix of five molecular species compositions (PI, PC, PE, PS and unstimulated DG) that would most closely replicate the early and late accumulated DG compositions. We found that by both h.p.l.c. and t.l.c. analyses, 15–30% (PAF) or 25–50% (ionomycin and PMA) of the later DG could be accounted for by PC hydrolysis. These results represent quantifications of phospholipid class contributions to stimulated DG formation, and demonstrate the potential importance of PC hydrolysis in phagocytic leucocytes.

INTRODUCTION

Various studies have elucidated several cellular mechanisms for the generation of diradylglycerols (DG) which are activated in response to diverse stimuli (see [1–3] for reviews). The data suggest that distinct phospholipid classes may serve as precursors for DG accumulation. In response to Ca^{2+} -mobilizing stimuli, pathways of hydrolysis of both polyphosphoinositides (PPI) and phosphatidylcholine (PC) are activated and contribute to DG production. In response to chemoattractants in phagocytic leucocytes, receptor/GTP-binding protein/phospholipase C coupling results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), with the production of DG and inositol 1,4,5-trisphosphate [4,5]. Phospholipase D activity towards PC has been demonstrated in response to chemoattractants, pharmacological elevation of cytosolic Ca^{2+} and the activation of protein kinase C (PKC) [6–8]. Accumulation of DG in response to chemoattractants is biphasic [8,9], which suggests that sequential mechanisms are operating. The second, sustained, phase of DG accumulation has been attributed to the stimulated influx of extracellular Ca^{2+} and the activation of PKC [8,9]. The involvement of PKC in cellular responses is itself tightly regulated by intracellular products of stimulated phospholipid metabolism, including Ca^{2+} [mobilized as a result of phosphatidylinositol (PI)

metabolism] and DG and arachidonate (formed from phospholipid precursors) [1,10,11]. Previous data have suggested that the relative contributions of specific mediators may be determined by the PKC isoenzyme(s) present in the specific cell type [11]. The relative quantitative contributions of specific phospholipid precursors to the DG production that is induced by chemoattractants have not previously been established.

Mononuclear phagocytes represent an excellent model system in which to examine the molecular mechanisms involved in the regulation of PKC. The activation of PKC in phagocytic leucocytes in response to chemoattractants has been well characterized, and is correlated with the rapid stimulation of PI hydrolysis [4,5]. Mononuclear phagocytes also exhibit phospholipase D activity towards PC in response to appropriate stimuli [8,12]. These cells are also a major physiological site of arachidonate release and a source of eicosanoid production [13].

We have previously reported that platelet-activating factor (PAF), a chemoattractant for murine macrophages [14], stimulates PIP_2 hydrolysis, DG accumulation and phospholipase D activation [8,12]. The pharmacological effects of either cytosolic Ca^{2+} elevation or PKC activation resulted in the stimulation of phospholipase D activity and the accumulation of DG. The effects of both the Ca^{2+} ionophore ionomycin and the phorbol ester phorbol myristate acetate (PMA) could be attributed to the

Abbreviations used: DG, diradylglycerols; PA, phosphatidic acid; DMPA, dimethylphosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PPI, polyphosphoinositides; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PAF, platelet-activating factor; PMA, phorbol myristate acetate; HHBSS, Hanks balanced salt solution containing 10 mM-Hepes, pH 7.4.

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activation of PKC [8]. Activation of phospholipase D and accumulation of DG is further potentiated by cytokine priming [12].

In the present studies, we have examined the relationship of the diradyl composition of the major murine macrophage phospholipids with that of cellular DG under a series of conditions. Using methods developed by Kennerly [15,16], our results indicate that DG in unstimulated cells are similar in diradyl composition to PI. This suggests that the maintenance of basal DG and PKC activity in murine macrophages may depend on the metabolism of PPI. The composition of DG remains similar after short (≤ 1 min) exposures of macrophages to PAF, ionomycin or PMA. However, after longer exposures, the diradyl composition of DG changes substantially towards that of PC, consistent with PC hydrolysis being an important source contributing to DG formation. Quantification of this compositional shift suggests that 15–30% of total DG after PAF and 25–50% after ionomycin or PMA is derived from PC hydrolysis. We conclude from these results that different cellular mechanisms control the early and the sustained formation of DG in phagocytic leucocytes.

EXPERIMENTAL

Materials

Culture media components were from GIBCO and Fisher. Plastic culture dishes were from Falcon. [32 P]ATP and [32 P] H_3PO_4 were from Dupont–NEN. Dioleoylglycerol and PAF were from Avanti Polar Lipids. Ionomycin and PMA were from Calbiochem. Solvents were from Fisher.

Cell culture and stimulation

Pathogen-free C57BL/6 mice, 6 weeks old, were obtained from Charles River Laboratories (Raleigh, NC, U.S.A.). Thio-glycollate-elicited macrophages were obtained by peritoneal lavage ($> 90\%$ macrophages) and enriched by adherence ($> 98\%$ macrophages) as previously described [17]. Macrophages (15×10^6 per 28.4 cm^2 well) were cultured for 16 h in RPMI 1640 medium containing 2.5% fetal calf serum, 2 mM-glutamine, 125 units of penicillin/ml and 6 μg of streptomycin/ml at 37°C in 5% CO_2 . Wells were washed twice with Hanks balanced salt solution containing 10 mM-Hepes, pH 7.4 (HHBSS), and incubated in a water bath at 37°C in 2.0 ml of the same buffer. Stimulants (0.4 ml) were added, and after the indicated times, ranging from 15 s to 20 min, the reaction was stopped by the addition of methanol. Stimulants and final concentrations were: PMA (0.1 $\mu\text{g}/\text{ml}$), ionomycin (10 μM) and PAF (0.1 μM), as previously described [12]. All experiments included baseline (HHBSS added) control wells for determination of unstimulated DG, and similar wells, cultured as above and with [32 P] H_3PO_4 (3 $\mu\text{Ci}/\text{ml}$), for determination of native phospholipids. All conditions were repeated in triplicate wells.

Extraction of DG and conversion into [32 P]dimethylphosphatidic acid (DMPA)

DG were extracted by a neutral (1 M-NaCl aqueous phase) Bligh & Dyer [18] procedure and quantitatively converted to [32 P]PA as described by Preiss *et al.* [19]. The method was modified to give a 120-fold increase in the specific radioactivity of the [32 P]PA by using six times more [32 P]ATP and 20 times less ATP (0.05 mM) in the reaction mixture, and incubating for 2.5 h instead of 30 min. The recovery and extent of conversion by this modification was verified using a known amount of dioleoylglycerol, and was found to be $> 80\%$. After t.l.c. separation from sphingolipids as described [19], the area corresponding to

PA by radioautography was scraped, extracted and derivatized to [32 P]DMPA as described by Kennerly [15].

Extraction and separation of cellular phospholipids and conversion to DG

Wells were washed four times to remove [32 P] H_3PO_4 , and HHBSS was added as above. Methanol was added as above, and phospholipids were extracted by an acidic (0.1 M-HCl aqueous phase) Bligh & Dyer [18] procedure and separated by the two-dimensional t.l.c. method, as described by Abdel-Latif *et al.* [20]. This gave reliable separation of all phospholipid classes. The locations corresponding on radioautographs to authentic PC, phosphatidylethanolamine (PE), phosphatidylserine (PS) and PI were separately scraped, extracted, ultrasonically dispersed in 50 mM-potassium phosphate buffer, pH 7.0, and converted to DG by treatment for 1 h at 20°C with 24 units of phospholipase C (Sigma P-7147)/0.3 ml. DG was extracted by a neutral Bligh & Dyer procedure, passed through chloroform-equilibrated 1 ml silica columns to remove residual ^{32}P -labelled products, and derivatized to [32 P]DMPA as described above. Release of the ^{32}P label into the aqueous phase of the Bligh & Dyer extract demonstrated that the conversion of PC and PE was $> 95\%$ complete, and that of PS and PI was $> 90\%$ complete.

Molecular species analysis of DMPA

Molecular species of DMPA were analysed by both h.p.l.c. and t.l.c. methods [15,16], which yielded distinct separations into 12 and seven fractions respectively. The species analyses obtained for the DMPA derived from the four phospholipid classes were clearly distinguishable and independent, which permitted multi-linear regression analysis to be performed as described below. This analysis, rather than identification of all individual species present in each fraction, was the purpose of our studies. We used 95% of each DMPA sample for the h.p.l.c. analysis and the remaining 5% for the t.l.c. analysis.

(a) **Molecular species analysis by reversed-phase h.p.l.c.** H.p.l.c. was performed as described by Kennerly [16], but revealed more of the small ($< 5\%$) component peaks and yielded smoother raw radiochromatograms because of the greater specific labelling we employed. Briefly, samples of DMPA were dissolved in 25 μl of acetonitrile and separated on a 4.6 mm $\times$ 150 mm, 4 μm C_{18} Ultrasphere h.p.l.c. column (Beckman) using methanol/water (98.75:1.25, v/v) at 0.9 ml/min as the mobile phase. Effluent radioactivity was monitored directly without co-added scintillant by an in-line β -radiation detector (Berthold), using a 0.5 ml detector cell loop. The total counts measured in successive 5 s time windows were recorded into computer disc files. Using the Fig.P program version 6.0 (Biosoft), running on an IBM PC computer, the radiochromatograms were digitally reconstructed, smoothed without peak height distortion by the Savitzky & Golay moving window least-squares technique [21] and, as needed, normalized to constant area (1000 c.p.m./min), combined for averaging of replicate samples, proportionally mixed to simulate admixtures, and printed out. All chromatograms yielded 12 characteristic peaks (Fig. 1a), with radioactive counts in one peak typically in the range 25000–500000 cpm. The locations in the chromatogram of five molecular species standards were confirmed to agree with those determined by Kennerly [16]: $\text{C}_{18:0-20:4}$ (1-stearoyl-2-arachidonoyl) in peak 6, $\text{C}_{16:0-20:4}$ (1-palmitoyl-2-arachidonoyl) in peak 2, $\text{C}_{18:0-18:1}$ (1-stearoyl-2-oleoyl) in peak 10, $\text{C}_{16:0-18:1}$ (1-palmitoyl-2-oleoyl) in peak 5, and $\text{C}_{18:1-18:1}$ (1,2-dioleoyl) in peak 6. For further data analysis, chromatograms were reduced to a set of 12 numbers representing the 12 peak heights in order, each re-expressed as a percentage of their total (Fig. 1b).

(b) **Molecular species analysis by argentation-t.l.c.** T.l.c. was

Fig. 1. H.p.l.c. analysis of DG from murine macrophages

(a) Typical h.p.l.c. radiochromatogram of the molecular species of DG extracted from unstimulated macrophages. As described in the Experimental section, cells were elicited, harvested and incubated, and without further stimulation DG was extracted, radiolabelled and derivatized. The 12 characteristic peaks used to quantify DG composition are shown, labelled 0–11. (b) Composition of DG from unstimulated macrophages expressed as the percentage contribution of each h.p.l.c. peak to the total DG. The 12 peak heights in each radiochromatogram were measured; these were totalled and each was expressed as a percentage of the total, as described in the Experimental section. Data shown are means of 14 experiments.

performed by a modification [15] of the method of Renkonen [22]. Briefly, samples were spotted on to heat-activated plastic-backed 20 cm silica t.l.c. plates previously impregnated with 5% aqueous silver nitrate. A mobile phase of chloroform/methanol (24:1, v/v) was run to 7 cm, followed by drying of the plates and a second development in the same direction to 20 cm using chloroform/methanol (98.5:1.5, v/v). The seven characteristic bands corresponding to totals of 0–6 double bonds in the two fatty acid chains were located by radioautography (Fig. 2a), cut out for scintillation counting, and their c.p.m. re-expressed as a percentage of the total (Fig. 2b). Typical radioactive counts in one band ranged from 2000 to 20000 c.p.m. Five molecular species standards (see above) were confirmed to migrate with the bands expected from their numbers of double bonds.

Multilinear regression analysis of molecular species profiles of stimulated DG

For each species profile of stimulated DG (separately for the h.p.l.c. and t.l.c. analyses), we used multilinear regression analysis of the five species profiles (PC, PE, PS, PI and unstimulated DG), determined in the same experiment, to calculate their optimum mixing proportions that would most closely reproduce the DG

Fig. 2. T.l.c. analysis of DG from murine macrophages

(a) Typical autoradiogram of a t.l.c. separation of the molecular species of DG extracted from unstimulated macrophages. As described in the Experimental section, cells were elicited, harvested and incubated, and without further stimulation DG was extracted, radiolabelled and derivatized. The seven characteristic bands used to quantify DG composition are shown, labelled 0–6. (b) Composition of DG from unstimulated macrophages, expressed as the percentage contribution of each t.l.c. band to the total DG. The seven bands in each lane were separately scraped, and their radioactivity was counted and expressed as a percentage of the total, as described in the Experimental section. Data shown are means of 14 experiments.

profile. The Fig.P computer program was used to find these proportions, and statistical weighting of the data was used (weights proportional to the reciprocals of the square roots of the peak height or band count data).

RESULTS

Molecular species analyses of DG in unstimulated macrophages

These were determined simultaneously with each time course for the experiments presented below. In previous studies we have found that unstimulated murine peritoneal macrophages contain a total DG mass of 25–30 pmol/ 10^6 cells [8,12].

Molecular species analysis of this unstimulated DG by the h.p.l.c. method yielded highly characteristic radiochromatograms similar to that shown in Fig. 1(a). The average proportions of the 12 peak heights over 14 experiments are shown in Fig. 1(b). Peak 6, with which $C_{18:0-20:4}$ co-migrates, was the tallest peak present for DG from unstimulated macrophages, accounting for 38–46% of the total of the 12 peak heights. Peaks 2, 3 and 7 together accounted for another 35% of the total.

Molecular species analysis of unstimulated DG by t.l.c. displayed an equally typical distribution of bands. A typical profile is shown in Fig. 2(a). The average proportions of the seven bands over 14 experiments are shown in Fig. 2(b). Band 4, with which $C_{18:0-20:4}$ co-migrates, was the most prominent band and accounted for 36–43% of the total radioactivity for the seven bands. Bands 1 and 2 together contained another 28% of the total.

Fig. 3. Typical series of eight h.p.l.c. radiochromatograms of DG molecular species obtained in macrophages stimulated with PMA (0.1 $\mu\text{g/ml}$)

Stimulation times were 0, 0.25, 0.5, 1, 2, 5, 10 and 20 min, as described in the Experimental section. Each chromatogram is redrawn as in Fig. 1(b), and the bold left-most bars in the time series shown for each h.p.l.c. peak represent the zero-time control (unstimulated DG). Beginning at 5 min, there was a substantial decline in the proportion of peak 6, while peaks 1, 2 and 3 increased.

Time course of the molecular species analyses of PAF-, ionomycin- and PMA-stimulated DG

In previous studies we found that the macrophage DG mass is increased by 30–130 % above baseline values in a time-dependent manner [8,12] in response to each of a series of stimuli, including the chemotactic agent PAF, the Ca^{2+} ionophore ionomycin, and the PKC activator PMA.

In each of a series of experiments, cells were exposed to one of these stimulants for periods of 15 s to 20 min, or left unstimulated (HHBSS added control). Molecular species analysis by h.p.l.c. for a typical experiment using PMA as the stimulant is shown in Fig. 3, which shows eight aligned chromatograms, each one normalized to a 12-peak total height of 100 %. The left-most, bolder, 12 bars show the chromatogram of unstimulated DG, similar to that in Fig. 1(b). Each of the other sets of 12 bars shows the chromatogram obtained with one of the increasing durations of stimulation with PMA. After the shorter intervals of stimulation there was, at most, a small change in the molecular species profile, with a small increase in the proportion of peak 6 at 15–30 s. After the longer stimulation periods of 5–20 min, there was a 24 % decrease (from 42 to 32 % of the total) in the contribution of peak 6 to the total chromatogram, and a substantial increase in many of the other peaks, particularly peaks 2 and 3. Molecular species analysis by t.l.c. for the same PMA experiment is shown in Fig. 4. As in Fig. 3, each of the eight chromatograms is normalized to a seven-band total radioactivity count of 100 %. After brief stimulation there was little or no change in the profile. After stimulation of 5–20 min, there was a 24 % decline (from 41 % to 31 % of total radioactivity) in the proportion of band 4 (species with four double bonds), a similar decline in band 5, and a marked rise in bands 0, 1 and 2.

We performed a series of time course experiments similar to the above using PMA ($n = 6$), ionomycin ($n = 5$) and PAF ($n = 7$) as stimulants. DG molecular species analyses were obtained as described above and the percentage of the total in each h.p.l.c. peak or t.l.c. band was calculated and re-expressed as the difference from the baseline value (the corresponding percentage of the total in unstimulated DG that was determined

Fig. 4. Typical series of eight t.l.c. separations of DG molecular species obtained in macrophages stimulated with PMA (0.1 $\mu\text{g/ml}$)

Stimulation times were 0, 0.25, 0.5, 1, 2, 5, 10 and 20 min, as described in the Experimental section. Each separation is drawn as in Fig. 2(b), and the bold left-most bars in the time series shown for each t.l.c. band represent the zero-time control (unstimulated DG). Beginning at 5 min, there was a substantial decline in the proportions of bands 4 and 5, while bands 0, 1 and 2 increased.

in the same experiment). The data obtained by averaging these difference traces over all experiments are shown in Figs. 5 and 6 for the h.p.l.c. and t.l.c. analyses respectively. Average results after PMA stimulation were similar to those described for the typical experiment above, and stimulation for 5–20 min with ionomycin or PAF likewise resulted in decreases in h.p.l.c. peak 6 and t.l.c. band 4, and increases in h.p.l.c. peaks 2 and 3 and t.l.c. bands 0, 1 and 2. After brief stimulation for only 15–30 s, there were smaller and precisely opposite changes with ionomycin and PAF, particularly in the h.p.l.c. analyses, which imparted a biphasic appearance to the difference traces. As discussed below, this observation is probably related to earlier observations which have shown that Ca^{2+} ionophores and chemoattractants, but not phorbol esters, stimulate PPI hydrolysis [5].

Molecular species analyses of PC, PE, PS and PI in unstimulated macrophages

These were determined simultaneously with each time course experiment presented above. Complete separation of PC, PE, PS and PI was obtained by two-dimensional t.l.c., with results similar to those reported by Abdel-Latif (Fig. 1 of ref. [20]). No significant spot representing lyso-PE was found, suggesting that during the preceding extractions, hydrolysis of the 1-alkenyl-linked species of PE, which constitute approx. 40 % of total PE in our cell type [23], was negligible. Each phospholipid was separately extracted and converted to DG using phospholipase C as detailed in the Experimental section. The h.p.l.c. molecular species analyses of the four phospholipids are shown in the left panel of Fig. 7. The 12 peaks in the profile of unstimulated (Fig. 1) and stimulated (Fig. 3) DG are again present, but in different proportions. The right panel of Fig. 7 shows the percentage contribution of each peak to the total of the peak heights relative to its proportion in unstimulated DG. The profile of native PI is almost indistinguishable from that of unstimulated DG. Peak 6

Fig. 5. Effect of PMA, ionomycin and PAF on the molecular species composition of DG determined by h.p.l.c.

Macrophages were stimulated for 0.25, 0.5, 1, 2, 5, 10 or 20 min. Data are shown as the sequence of differences relative to unstimulated DG of the proportional contribution to total DG of each h.p.l.c. radiochromatogram peak. (a) Means of six experiments using $0.1 \mu\text{g}$ of PMA/ml. There was a large decline of peak 6 at 5, 10 and 20 min, and rises in the proportions of peaks 1, 2 and 3. (b) Means of five experiments using $10 \mu\text{M}$ -ionomycin. Late changes similar to those induced by PMA were observed. In addition, at early times (0.25, 0.5 and 1 min), there were opposite changes (rise in peak 6; decline in peaks 2 and 3). (c) Means of seven experiments using $0.1 \mu\text{M}$ -PAF. Both early and late changes were observed which were similar to but smaller than those induced by ionomycin.

is dominant and contributes 46–54% of the total profile. In the PC profile, peaks 2 and 3 are more prominent than in PI, while peak 6 accounted for only 27–35% of the total. The pattern of the major h.p.l.c. peaks 2, 3, 6, 7 and 10, taken together, is distinctive and unique for each phospholipid class. The t.l.c. species analyses of the four phospholipids are shown in Fig. 8 in an analogous form. Again, the PI profile is almost indistinguishable from that of unstimulated DG. Band 4 is prominent and contributes 36–41% of the total profile. In the PC profile, bands

Fig. 6. Effect of PMA, ionomycin and PAF on the molecular species composition of DG determined by t.l.c.

Macrophages were stimulated for 0.25, 0.5, 1, 2, 5, 10 and 20 min. Data are shown as the sequence of differences relative to unstimulated DG of the proportional contribution to total DG of each t.l.c. band. (a) Means of six experiments using PMA ($0.1 \mu\text{g}/\text{ml}$). There was a large decline in band 4 at 5, 10 and 20 min and rises in bands 0, 1 and 2. (b) Means of five experiments using $10 \mu\text{M}$ -ionomycin. Changes similar to those induced by PMA were observed. (c) Means of seven experiments using $0.1 \mu\text{M}$ -PAF. Similar but less marked changes were observed.

0, 1 and 2 are much more prominent than in PI or unstimulated DG, while band 4 accounts for only about 14–20% of the total. Despite a superficial resemblance of the PI and PE profiles, and of the PC and PS profiles, sufficiently characteristic features remain, such as the ratios of peaks 5 to 6 (PI versus PE) and of peaks 0 to 6 (PC versus PS).

Dominance of the $C_{18:0-20:4}$ molecular species in PI is expected from the known composition of PI in murine macrophages, and our obtaining a similar result rules out the possibility that

Fig. 7. H.p.l.c. radiochromatograms of molecular species of DG derived from the native phospholipid classes extracted from unstimulated macrophages

Phospholipids were separated by two-dimensional t.l.c. as described in the Experimental section and converted to their corresponding DG molecular species by incubation with phospholipase C (from *B. cereus*). Radiolabelling and derivatization were performed as for DG. (a) The 12 characteristic peaks, labelled 0–11, are present in widely differing proportions in the four phospholipid classes. Each trace is the average of eight chromatograms. (b) Compositions from (a), calculated as in Fig. 1(b) and displayed as differences from the composition of unstimulated native DG that is shown in Fig. 1(b).

significant oxidation of unsaturated species might be occurring during radioautography in either of the preceding t.l.c. steps.

Simulated proportional admixtures of the diradyl molecular species compositions of unstimulated DG with those of PI and PC

For both the h.p.l.c. and t.l.c. analyses above, there was a striking similarity between (a) the differences in the diradyl compositional analyses when going from unstimulated DG to PC (Figs. 7 and 8), namely declines in h.p.l.c. peak 6 and t.l.c. band 4 and rises in h.p.l.c. peaks 2 and 3 and t.l.c. bands 0, 1 and 2, and (b) the changes that occur in the diradyl analyses of total cellular DG when going from initial unstimulated DG to DG stimulated for 5–20 min by PAF, ionomycin or PMA (Figs. 5 and 6). We wished to quantify this observation further.

Fig. 9 (upper panels) compares the diradyl compositions of unstimulated DG determined by h.p.l.c. and t.l.c. (left-most bars of each triplet) with two representative simulated proportional admixtures of diradyl compositions, namely equimolar mixtures of unstimulated DG with PI (middle bars) or with PC (right-most bars) respectively. The lower panels of Fig. 9 show the corresponding difference tracings.

The similarity of this series of synthetic mixtures (Fig. 9) with the sequences of species analyses we obtained for time courses of stimulated DG (Figs. 3–6) suggested the possibility of further analysing the compositions of stimulated DG, as follows. Each sample of total DG obtained after a period of stimulation might be thought of as a mixture of DG derived partly from the original unstimulated DG and partly from hydrolysis of PI, PC, PE and/or PS. To calculate the most likely (best fitting) proportional contributions of each of the five possible sources to the total DG, we used multilinear regression. This allowed us to use all of the 12 peak data or seven band data obtained for each h.p.l.c. chromatogram or t.l.c. profile respectively.

For each unstimulated (control) sample of DG, regression analysis verified the mathematically expected 100% contribution from unstimulated DG and no contribution from any phospholipid class. Table 1 shows averaged results for DG after 15 s and 10 min of stimulation by PMA, ionomycin and PAF with respect to the contribution of PC to total DG. After stimulation for 15 s there was little or no evidence for a contribution of PC hydrolysis. After stimulation for 10 min, a substantial contribution from PC to total DG is apparent (15–30% after PAF and 25–50% after ionomycin or PMA), an effect which begins to

Fig. 8. T.l.c. separations of molecular species of DG derived from the native phospholipid classes extracted from unstimulated macrophages

Phospholipids were processed as in Fig. 7 and [32 P]DMPA was separated by argentation-t.l.c. as described in the Experimental section. (a) The seven characteristic bands are present in clearly differing proportions in the four phospholipid classes. Each set is the average of eight determinations. (b) Compositions from (a), displayed as the differences from the composition of unstimulated native DG (shown in Fig. 2b).

be seen in the data for 5 min stimulations and persists after 20 min (results not shown). Results for PI indicate a contribution to total DG that peaks at 20–30% after 15 s of stimulation with PAF and one that peaks later (after 1 min) at 5–10% with ionomycin. Due to the close similarity of the diradyl compositions of PI and unstimulated DG (see above), these results for estimated PI contributions may be less accurate. The origin of the remainder of total DG was the unstimulated DG. There was no evidence for a contribution of PS to DG, although the analysis did indicate small (< 11%) contributions of PE to DG in several cases at longer stimulation times. We found, however, that if the PE contribution was replaced by one of PC, the mathematical best fit that had been determined was worsened only slightly, as judged by finding only moderate increases (worsening) in the sum of the squared residuals. To address the possibility of PE hydrolysis directly, we radiolabelled macrophages with [14 C]-ethanolamine overnight prior to stimulation. There was no significant increase in radiolabelled aqueous metabolites (R. J. Uhing, P. W. Hollenbach & R. J. Sebaldt, unpublished work), making it unlikely that PE hydrolysis contributes to DG formation.

DISCUSSION

Membrane phospholipids in mononuclear phagocytes are precursors for important intracellularly and extracellularly acting lipid mediators. These cells are an important site of eicosanoid

formation from free arachidonate (reviewed in [13]), which is released by phospholipase A_2 from one or more pools, where it is normally esterified primarily in the *sn*-2 position of PC, PE and PI. Eicosanoids act extracellularly to regulate a variety of autocrine and paracrine functions. Free arachidonate [3] and some of its metabolites (reviewed in [11]) may also act intracellularly to activate PKC. PA is a potentially important intracellular mediator formed by phospholipase D-catalysed cleavage of PC and by phosphorylation of DG. DG, formed from phospholipids by activated phospholipase C and perhaps from PA phosphohydrolase, is a potent activator of PKC. To date, molecular mechanisms of phospholipid metabolism have been best defined in leucocytes, and we undertook the present studies in order to quantify the relative contributions of hydrolysis of the major phospholipid classes in these cells to formation of DG under representative conditions.

The present studies examined the DG accumulated in macrophages after timed stimulations with the potent chemoattractant PAF, and other stimuli, in relation to the cellular phospholipid precursors from which the DG might be formed. We used reversed-phase h.p.l.c. and argentation-t.l.c. analyses of molecular species of derivatized DG [15,16] and multilinear regression analyses to quantify the results. Based on our compositional findings, we found that PPI hydrolysis is the major pathway that maintains basal macrophage DG. It also accounts for initial DG accumulation (< 30 s) in response to PAF or to the Ca^{2+} ionophore ionomycin. Our data further indicate that PC

Fig. 9. Theoretical predictions for (a) h.p.l.c. and (b) t.l.c. separations of molecular species of DG, consisting of precise mixtures of native DG and DG derived from PI or PC

In each triplet, the left bar corresponds to native DG only, the centre bar shows the prediction for an admixture of equal proportions of DG from PI and native DG and the right bar that for equal proportions of DG from PC and native DG. The lower panels show these predictions as differences from the composition of native DG. The changes predicted for an admixture of native DG with DG from PC (h.p.l.c. peak 6 decreased and peaks 2 and 3 increased; t.l.c. band 4 decreased and bands 0, 1 and 2 increased) correspond precisely to the changes that were actually found with stimulation for 5 min or more by 0.1 μg of PMA/ml, 10 μM -ionomycin or 0.1 μM -PAF (Figs. 5 and 6).

Table 1. Contribution of PC-derived DG to total cellular DG

The contribution of PC-derived DG to total DG was calculated by multilinear regression analysis of h.p.l.c. and t.l.c. data respectively.

Stimulus	DG from PC (% of total DG)	
	H.p.l.c.	T.l.c.
PMA (15 s)	3	7
PMA (10 min)	30	52
Ionomycin (15 s)	0	0
Ionomycin (10 min)	25	51
PAF (15 s)	3	13
PAF (10 min)	14	28

is probably the major precursor for the sustained DG accumulation that occurs when macrophages are exposed to PAF, ionomycin or PMA for > 2 min. This is consistent with our previous studies [8] and those of others [9,24–26], which have shown that DG accumulation exhibits a biphasic response to chemoattractants, and with our previous demonstration of the transient nature of inositol phosphate production [8].

A significant body of data indicates that intracellular messengers derived from stimulated PIP_2 hydrolysis may participate in the generation of DG, and it is possible that the sustained phase of macrophage DG accumulation may be a result of these signals. Both pharmacological elevation of intracellular Ca^{2+} and activation of PKC regulate transduction events that result in DG accumulation in a variety of cells (reviewed in [1–3,10]). The ability of a series of chemoattractants such as PAF to stimulate DG accumulation and superoxide production in neutrophils correlates with their ability to stimulate a sustained elevation of

cytosolic Ca^{2+} [9]. We have previously reported [8] that PKC activation is involved in the ability of PAF to stimulate sustained DG accumulation in macrophages, and that the role of elevated cytosolic Ca^{2+} in PAF-induced DG accumulation is likely to be activation of this enzyme.

Our results for compositional analysis of DG accumulated in response to PAF, ionomycin and PMA suggest that sustained chemoattractant-induced DG accumulation is due in large part to PC hydrolysis. In the multilinear regression analyses that calculated the best-fit combinations of potential phospholipid precursors that would explain the observed cellular DG species analyses (see the Experimental section for details), we found that the exactness of fit, as measured by the sum of the squared residuals, was somewhat less for DG diradyl compositions from longer periods of stimulation than for compositions from brief stimulations. The proportional contributions determined for the later times may thus be less accurate. We also found that substitution of a small amount of one of the precursors by another would result in only moderate worsening of the mathematical best fit that had been determined. This was especially true for the substitution of PE for PC when analysing DG compositions from longer stimulations. Our results indicate that PC metabolism is a major contributor to DG formation for longer stimulations, but the decreased goodness of fit at these times may indicate the occurrence of subcellular localization of specific PC pools or species-specific DG metabolism.

In mononuclear phagocytes, PC contains approximately equal amounts of diacyl and alkylacyl substituents [27]. In formyl-peptide-stimulated neutrophils, accumulation of diacylglycerols has been shown to precede that of alkylacylglycerols [24,26]. Both diacyl and alkylacyl phospholipids can serve as precursors for eicosanoid production in mononuclear phagocytes (reviewed in [13]). Activation of phospholipase A_2 by external stimuli has been clearly implicated in this process, but a contribution from the metabolism of DG by DG lipase has not been ruled out.

Arachidonate-containing DG, especially the C_{18:0-20:4} species (which migrates in h.p.l.c. peak 6 and t.l.c. band 4), constitutes a particularly large percentage of both unstimulated and early PAF-stimulated DG, reflecting the exceptional enrichment of PI in this species. It is of interest that the three stimuli examined here have similar potencies for stimulating eicosanoid secretion in macrophages. Whether this implies a role for DG lipase in eicosanoid formation in these cells requires further investigation.

Previous studies in phagocytic leucocytes have suggested that hydrolysis both of PIP₂ by a phospholipase C and of PC by a phospholipase D is likely to contribute to chemoattractant-induced DG accumulation. Our studies confirm the importance of both of these phospholipids as DG precursors. A variety of data indicates that chemoattractant-induced PPI hydrolysis is mediated by a receptor-coupled GTP-binding protein which activates a phospholipase C (reviewed in [4,5]). Less is currently known about the molecular mechanisms by which chemoattractants activate phospholipase D activity towards PC, although participation of a GTP-binding protein in this response has also been suggested [28].

In summary, these results indicate that PC hydrolysis is an important pathway in the sustained (> 2 min) DG response to PAF in macrophages, whereas PPI hydrolysis is largely responsible both for maintaining basal cellular DG levels and for generating the initial rapid (< 30 s) DG response to PAF. The molecular mechanisms controlling these events are likely to be important in the regulation of many of the physiological functions and responses of mononuclear phagocytes, including antigen processing, immune regulation, phagocytosis and chronic inflammation.

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