Basal and phorbol-ester-stimulated turnover of phosphatidylcholine in HeLa cells involve different pathways

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HeLa cells prelabelled with [³H]lysoplatelet-activating factor (lyso-PAF) accumulated phosphatidic acid (PtdOH) when incubated in the presence of either propranolol (an inhibitor of PtdOH phosphohydrolase) or phorbol ester. In the presence of ethanol, phorbol ester but not propranolol stimulated the accumulation of phosphatidylethanol, an index of phospholipase D activity. Incubation of cells with [³H]lyso-PAF led to a rapid accumulation of label in diacylglycerol (DG) followed by a delayed accumulation in PtdOH. It is concluded that propranolol-induced PtdOH accumulation is derived from DG by DG kinase and does not involve phospholipase D.

INTRODUCTION

In many animal cells interaction of surface receptors with agonists which leads to activation of protein kinase C, or with phorbol esters which activates protein kinase C directly, results in the hydrolysis of phosphatidylcholine (PtdCho) by phospholipase D [1,2]. This can be detected as the release of [³H]choline from cells in which phospholipid pools have been prelabelled with [³H]choline [3–5], or by measuring the phospholipase D-catalysed accumulation of phosphatidylalcohol in the presence of a primary alcohol [6–9]. Much less is known about the turnover of PtdCho in unstimulated cells. In a recent report we suggested that this turnover involves the sequential actions of phospholipase C, diacylglycerol (DG) kinase and PtdOH phosphohydrolase [5]. Incubation of HeLa cells with propranolol, an inhibitor of PtdOH phosphohydrolase [10], resulted in the accumulation of PtdOH. Isotopic experiments and mass determinations of phospholipids were consistent with the proposal that the PtdOH arose from pre-formed lipid by a pathway not involving phospholipase D. However, in these experiments it was not possible to rule out the possibility of some contribution by *de novo* synthesis to the accumulation of PtdOH. In the present paper we have prelabelled HeLa cells with an ether derivative ([³H]lyso-PAF) and have obtained additional evidence that the turnover of PtdCho is rapid and does not involve phospholipase D.

MATERIALS AND METHODS

Materials

1-O-[³H]Octadecyl-*sn*-glycerophosphocholine (lyso-PAF; 163 Ci/mmol) was obtained from Amersham Corp. (Amersham, Bucks., U.K.). Phospholipase D (extracted from cabbage) was from Sigma and was used to prepare PtdEth standard [11].

Cell culture and labelling

HeLa cells were maintained in 100 mm diam. Petri dishes as described [5]. To prelabel cells, confluent cultures were incubated in fresh medium with [³H]lyso-PAF (80 or 100 min). After incubation the medium was removed and the cells incubated with fresh medium minus label for 20–30 min before the addition of test substances. In other experiments cultures were incubated with [³H]lyso-PAF for the times indicated. Incubations were

terminated by removal of the medium and the addition of 2 ml of cold methanol. Lipids were extracted [12] and PtdOH and PtdEth separated in a solvent consisting of the upper phase of ethyl acetate/trimethylpentane/acetic acid/H₂O (13:2:3:10, by vol.). For the simultaneous separation of PtdOH and DG, plates (20 cm) were developed for 12 cm in the above solvent to separate PtdOH. The plates were air-dried, cut 2 cm below the solvent front and neutral lipids separated in a solvent system containing hexane/diethyl ether/acetic acid/methanol (65:35:2:4.5, by vol.). The media used during the prelabelling period and subsequent incubations were: (i) Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (Table 1); (ii) medium containing 127 mM-NaCl, 5 mM-KCl, 31 mM-NaHCO₃, 1.03 mM-MgCl₂, 0.22 mM-KH₂PO₄, 0.28 mM-MgSO₄,

Table 1. Effect of PMA and propranolol on PtdOH and PtdEth formation in HeLa cells prelabelled with [³H]lyso-PAF

Cells were prelabelled with [³H]lyso-PAF (experiment 1, 2.02×10^6 d.p.m.; experiment 2, 1.93×10^6 d.p.m.) for 100 min as described in the Materials and methods section. In experiment 1 propranolol (500 μ M), PMA (100 nM) or dimethylsulphoxide (0.1%) were added and cultures incubated for 20 min. In experiment 2 cultures were incubated for 30 min with ethanol (85 mM) plus dimethylsulphoxide (0.1%), ethanol plus PMA (100 nM) or ethanol plus propranolol (500 μ M). Radioactivity associated with PtdOH and PtdEth was determined as described in the Materials and methods section. Results are expressed as the mean ($\pm$ S.E.M.) of four (experiment 1) or three (experiment 2) replicate determinations. After prelabelling with [³H]lyso-PAF radioactivity associated with PtdCho was 34337 ± 418 and 25158 ± 2177 d.p.m. in experiments 1 and 2 respectively. * $P < 0.05$; ** $P < 0.001$.

Treatment	Radioactivity (d.p.m./dish)	
	PtdOH	PtdEth
Experiment 1		
Control	93 $\pm$ 11	–
Propranolol	492 $\pm$ 26**	–
PMA	249 $\pm$ 15**	–
Experiment 2		
Ethanol	129 $\pm$ 5	Not detectable
Ethanol + PMA	266 $\pm$ 62*	422 $\pm$ 57
Ethanol + propranolol	316 $\pm$ 19**	7 $\pm$ 7

Abbreviations used: DG, diacylglycerol; lyso-PAF, lysoplatelet-activating factor; PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanol; PMA, phorbol 12-myristate 13-acetate.

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Table 2. Accumulation of labelled DG and PtdOH in HeLa cells incubated with [³H]lyso-PAF

Cells were incubated with 500 μ M-propranolol for 20 min. [³H]Lyso-PAF (1 μ Ci) was added and incubation continued for 30 s, 2 min or 5 min, and incorporation into PtdOH, DG and PtdCho determined as described in the Materials and methods section. Results are expressed as mean ($\pm$ S.E.M.) of three replicate determinations. Similar results were obtained in two separate experiments.

Time (min)	Radioactivity (d.p.m./dish)		
	PtdOH	DG	PtdCho
0.5	22 $\pm$ 13	451*	4440 $\pm$ 1109
2	38 $\pm$ 13	698 $\pm$ 138	13444 $\pm$ 898
5	209 $\pm$ 49	1070 $\pm$ 55	10858 $\pm$ 1076

* $n = 2$.

Table 3. Effect of propranolol on PtdOH and DG radioactivity in cells prelabelled with [³H]lyso-PAF

Cells were prelabelled with [³H]lyso-PAF (2.85×10^6 d.p.m.) for 80 min as described in the Materials and methods section. Propranolol (500 μ M) was added and incubation continued for the time indicated. Radioactivity associated with PtdOH and DG was determined as described in the Materials and methods section. Results are the mean ($\pm$ S.E.M.) of three (control) or four (propranolol-treated) replicate determinations. After prelabelling, radioactivity associated with PtdCho was 161812 ± 11393 d.p.m. Similar results were obtained in a separate experiment. * $P < 0.05$; ** $P < 0.001$; N.S. not significant when compared with control.

Time (min)	Radioactivity (d.p.m./dish)			
	Control		Propranolol	
	PtdOH	DG	PtdOH	DG
0	899 $\pm$ 84	4886 $\pm$ 228	—	—
5	699 $\pm$ 43	5156 $\pm$ 143	1057 $\pm$ 50**	4289 $\pm$ 266*
10	790 $\pm$ 35	5033 $\pm$ 495	1407 $\pm$ 49**	4138 $\pm$ 94 N.S.
20	842 $\pm$ 31	4784 $\pm$ 152	1474 $\pm$ 79**	3483 $\pm$ 173**

0.63 mM- Na_2HPO_4 , 16 mM-Hepes, 1 mM- CaCl_2 , and 5.5 mM-glucose, pH 7.4 (Tables 2 and 3).

Statistics

Results were analysed using Student's unpaired t -test.

RESULTS AND DISCUSSION

[³H]Lyso-PAF was rapidly acylated to PtdCho by HeLa cells as has been reported for other cell lines ([13]; see Table 2). Incubation of the prelabelled cells with propranolol or with phorbol 12-myristate 13-acetate (PMA) resulted in the accumulation of labelled PtdOH (experiment 1, Table 1). The accumulated PtdOH could arise directly by phospholipase D hydrolysis of [³H]PtdCho, or indirectly from the sequential actions of phospholipase C and DG kinase. To distinguish between these pathways, prelabelled cells were incubated with PMA or propranolol in the presence of ethanol. Phospholipase D catalyses a transphosphatidyl reaction in the presence of ethanol, resulting in the formation of PtdEth [14]. The accumulation of this relatively stable derivative has been widely used as an index of phospholipase D activity. As shown in Table

1 (experiment 2), propranolol did not stimulate PtdEth accumulation. On the other hand PtdEth accumulation was stimulated by PMA, a known activator of protein kinase C, a phenomenon which has been extensively documented [2].

The above results indicate that PtdOH formation from preformed PtdCho involves an indirect pathway. Evidence that PtdOH formation was preceded by the accumulation of DG was obtained in an experiment in which radioactivity associated with both products was followed with time of incubation in the presence of [³H]lyso-PAF (Table 2). Propranolol was included in the incubations to inhibit PtdOH phosphohydrolase activity and limit further metabolism of the accumulated PtdOH. It is clear that incubation with [³H]lyso-PAF resulted in the rapid appearance of label in DG, but that labelled PtdOH could only be detected after a lag period of 2–5 min. The effect of propranolol on DG- and PtdOH-labelling in cells prelabelled with [³H]lyso-PAF was also determined (Table 3). Incubation with propranolol resulted in the accumulation of radioactive PtdOH and at the same time a decrease in DG labelling. These results also support the conclusion that PtdCho turnover involves both DG kinase and PtdOH phosphohydrolase.

The present results support the proposal that PtdCho turnover in confluent HeLa cells is initiated by phospholipase C, resulting in the rapid formation of DG which is then converted into PtdOH by DG kinase. An important assumption made in reaching this conclusion is that the ether derivatives (lyso-PAF and PtdCho) are metabolically stable, and can not be converted into breakdown products which could be incorporated into the ester form of PtdOH by *de novo* biosynthesis. In particular, some cells contain an alkylglycerol mono-oxygenase [15] which catalyses the oxidative cleavage of the alkyl group to a fatty aldehyde. Alkylglycerol could be formed from lyso-PAF by the sequential actions of lysophospholipase D and lyso-PtdOH phosphohydrolase [16] or from PtdCho by the actions of phospholipase C and DG lipase. In the present experiments, after prelabelling the cells with lyso-PAF, 5% of the radioactivity associated with PtdCho was ester-linked (results not shown; determined as described previously [9]), indicating that some metabolism of the ether phospholipid had occurred. This was further accentuated in PtdOH where about 40% of the radioactivity which accumulated in the presence of propranolol was in the ester form with the remainder as the ether derivative (results not shown). While this result clearly establishes that a significant proportion of the accumulated PtdOH was derived from preformed phospholipid, it stresses the dangers of assuming that lyso-PAF is metabolically stable in all cells.

Given the prelabelling protocol used in the present experiments it is likely that labelled PtdCho would initially accumulate in the plasma membrane. However, previous studies have indicated that when Chinese hamster fibroblasts were incubated with a fluorescent derivative of PtdCho at 37 °C a significant fraction of the preformed lipid was retained in the plasma membrane, but that some lipid rapidly migrated to intracellular membranes [17]. If a similar situation exists in HeLa cells, it would be predicted that cells prelabelled with lyso-PAF at 37 °C will accumulate PtdCho in both the plasma membrane and in other membrane systems. Consequently, it is uncertain which cellular phospholipid pools are involved in the pathways described in the present report. This is clearly of importance and the subcellular distributions of PtdCho metabolites generated as a consequence of turnover, or following activation of phospholipase D with phorbol esters should be compared.

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