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ANTIGEN-INDUCED BIPHASIC DIACYLGLYCEROL FORMATION IN RBL-2H3 CELLS: THE LATE SUSTAINED PHASE DUE TO PHOSPHATIDYLCHOLINE HYDROLYSIS IS DEPENDENT ON PROTEIN KINASE C

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Summary: Exposure to antigen (Ag) caused a biphasic 1,2-diacylglycerol (DG) production in [^{3}H]myristic acid-labeled RBL-2H3 cells; the early, small transient phase and the second large sustained phase. The accumulation of phosphatidic acid (PA) or phosphatidylethanol (PEt) in the presence of ethanol was paralleled by the second-phase DG generation. Ag-induced formation of phosphocholine and choline in [^{3}H]choline-labeled cells suggested the hydrolysis of phosphatidylcholine (PC) by phospholipases C and D. Treatment with phorbol myristate (PMA) or A23187 caused increases in [^{3}H]DG and water-soluble [^{3}H]choline metabolites. In protein kinase C (PKC) down-regulated cells, PEt formation was markedly reduced. In these cells DG production induced by Ag and A23187 was largely suppressed, thus indicating that PKC would play an important regulatory role for PC hydrolysis. However, because the A23187 treatment showed significant accumulation of water-soluble choline metabolites in PKC down-regulated cells, an increase in intracellular Ca^{2+} is another factor regulating PC hydrolysis. Taken together, these results may indicate that PC hydrolysis in response to Ag is dependent on PKC and Ca^{2+}.

Antigen (Ag)-induced activation of mast cells and RBL-2H3 cells is known to be closely coupled with hydrolysis of phosphoinositides (1, 2). Although many previous studies (for review see Ref. 3) demonstrated that the major DG source was phosphoinositides, recent reports have described that agonist-induced hydrolysis of phosphatidylcholine (PC) also produces DG in various

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Abbreviations: DG, 1,2-diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; TG, triacylglycerol; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; 4α-PDD, 4α-phorbol 12,13-didecanoate.

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cell types (for review see Ref. 4). PC can be hydrolyzed by phospholipase C (PLC) distinct from PLC acting on phosphoinositides and phospholipase D (PLD) to yield DG and phosphatidic acid (PA), respectively. PA is subsequently converted to DG by PA phosphohydrolase. The regulatory mechanism of PC hydrolysis varies depending on the cell type and agonist (5, 6, 7, 8).

Recently, Kennerly and colleagues (9, 10) have described the role of PC for Ag-induced DG production in rat peritoneal mast cells. More recently, Ag-induced activation of PLD has been reported in mast cells (11). However, the regulatory mechanisms underlying PC hydrolysis are not well understood in mast cells. PMA treatment stimulates PC hydrolysis in many cell types (6, 12), suggesting evidence for the involvement of PKC. In platelets, erythroleukemia cells and neutrophils (13, 14), the increase in intracellular Ca^{2+} concentration appears to contribute to PC hydrolysis by PLC and/or PLD. RBL-2H3 cells, culture model of mast cells, are advantageous to gain further insight into the implication of PKC, because the PKC activity can be down-regulated by a prolonged incubation with PMA (15, 16). Moreover, longer incubation with radioactive compounds enables sufficient radiolabeling. In the present study, we have examined the contribution of PC to the DG production in Ag-stimulated RBL-2H3 cells and the regulatory mechanism of PC hydrolysis was discussed.

MATERIALS AND METHODS

Materials: Monoclonal mouse anti-DNP IgE was obtained from Seikagaku Corp. [Methyl-^{3}H]choline chloride was from American Radiochemicals. [^{3}H]Arachidonic acid, [^{3}H]inositol and blotting detection kit were from Amersham. [^{3}H]Myristic acid was from New England Nuclear. DNP-ascaris antigen was produced as described previously (2). Other chemicals were of reagent grade.

Cell treatment and lipid analysis: The 2H3 subline of RBL cells was maintained as previously described (17). Cells were prelabeled with [^{3}H]myristic acid (0.5 μCi/ml) for 6 h. For analysis of choline metabolites, the cultures were incubated with either [^{3}H]choline (0.5 μCi/ml) for 36 h. Cells were sensitized with monoclonal mouse anti-DNP IgE (0.5 μg/ml) for the last 2 h of radiolabeling.

After removal of the radiolabeling medium, cultures were rinsed three times with Tyrode-HEPES-gelatin (THG) buffer. Cells were equilibrated in THG for 5 min at 37°C prior to stimulation. Incubations were stopped by removing medium and immediately by adding 1 ml of ice-cold PBS/methanol (2:5, v/v) mixture. Cells were released from culture dishes with an additional 1 ml of PBS/methanol mixture, and transferred to a tube containing 700 μl of chloroform. Lipids were extracted according to the method of Bligh and Dyer (18). Phospholipids including PEt were separated by two dimensional thin-layer chromatography (19). The areas corresponding to lipid fractions were scraped into vials and the radioactivity was determined in a liquid scintillation counter.

Analysis of [^{3}H]choline metabolites: Cultures were washed twice with PBS and incubated for an additional 1 h with the medium containing 1 mM unlabeled choline chloride. The cultures were washed three times with THG, and the reaction and lipid extraction were carried out as described above. The aqueous phase of the chloroform/methanol extract was dried and resolved in 50% (v/v) ethanol. A portion of each sample was analyzed as previously described (20).

Immunoblotting and down-regulation of PKC: 2H3 cells were treated with 100 nM PMA for various times. The cells were broken by sonication in buffer A (5 mM EGTA, 0.5 mM PMSF, 5 mM 2-ME, 2 μg/ml leupeptin and 10 mM Tris-HCl, pH 7.5). The lysate was centrifuged at 1000 g for 5 min to remove unbroken cells. The resulting supernatant was solubilized and proteins were resolved by SDS-PAGE, and electrophoretically transferred to GVHP filter. The PKC band was analyzed by blotting with monoclonal antibodies (21). To down-regulate PKC activity, cultures were incubated with 100 nM PMA for 16 h. These conditions did not affect cell viability or radiolabeling.

RESULTS

Stimulation of [^{3}H]myristic acid-labeled cells with Ag caused a biphasic accumulation of DG (Fig. 1). The first peak was small and transient, whereas the second phase was a much larger sustained plateau to 10 min. In the presence of 1% ethanol, the first peak was not significantly changed. However, the second phase of DG was decreased by about 30%. Generation of PA was evident at 2 min after Ag stimulation (Fig. 2, left). In the presence of 1% ethanol, Ag-stimulated RBL-2H3 cells produced PEt which is a specific marker of PLD activation (Fig. 2, right). Increasing ethanol concentration caused a concentration-dependent PEt formation and the maximal formation occurred at 1% ethanol (data not shown). PEt formation was not observed within the first 2 min, but it reached a nearly maximal level at 3 min and maintained its level thereafter. The increase in PEt was accompanied by a reduced PA accumulation, suggesting that the loss of radioactivity from PA is accounted for by the production of [^{3}H]PEt.

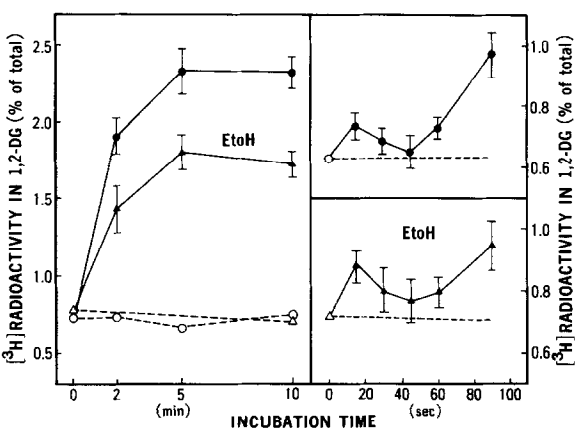

Figure 1. Ag-induced DG formation in [^{3}H]myristic acid-labeled RBL-2H3 cells. After labeling for 6 h with [^{3}H]myristic acid, cells were incubated for 5 min in the presence (△, ▲) or absence (○, ●) of 1% ethanol, and then stimulated with 0.5 μg/ml of DNP-ascaris (▲, ●) for the times indicated. Lipid extraction and determination of radioactivity were performed as described in Materials and Methods. Results are expressed as percentage of total radioactivity in the lipid fraction. Data presented are means±SE from three experiments performed in duplicate.

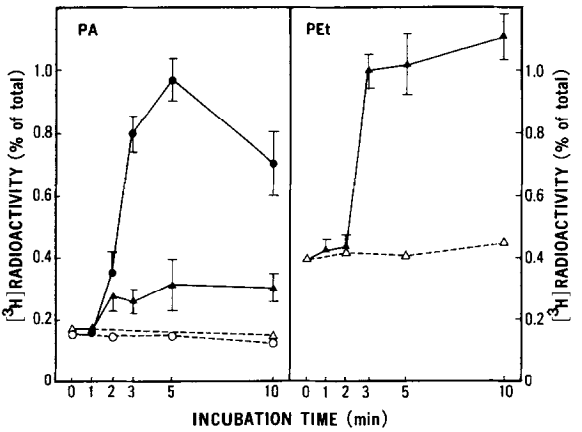

Figure 2. Ag-induced PA and PEt formation in [^{3}H]myristic acid-labeled RBL-2H3 cells.
Labeled cells were preincubated for 5 min in the presence or absence of 1% ethanol, and then stimulated with 0.5 μg/ml of DNP-ascaris. Other experimental conditions are described in Fig. 1. Data presented are means±SE from three experiments performed in duplicate.

In [^{3}H]myristic acid-labeled cells, the decrease of radioactivity in PC corresponded to 80% of the sum of increases in DG and PA (data not shown). Ag caused PC decrease and produced water-soluble [^{3}H]choline metabolites, [^{3}H]choline and [^{3}H]phosphocholine (Fig. 3). The time courses of the accumulation of these choline metabolites were markedly different (Fig. 4). The level of [^{3}H]choline was not changed within 2 min after Ag stimulation and reached the maximal level at 3 min. In contrast, [^{3}H]phosphocholine showed a progressive increase following Ag addition.

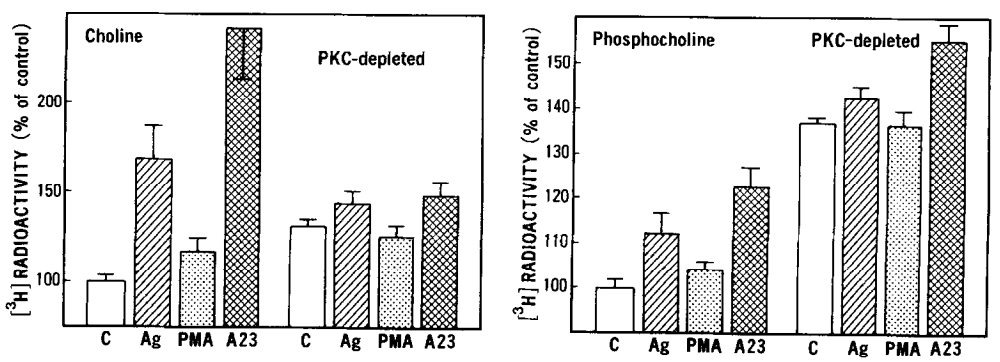

Figure 3. Ag-induced PC breakdown in [^{3}H]choline-labeled RBL-2H3 cells.
Cells were labeled with [^{3}H]choline for 36 h. For down-regulation of PKC, cells were incubated for the last 16 h with 100 nM PMA. After washing, cells were stimulated with Ag (0.5 μg/ml), PMA (100 nM) or A23187 (1 μM) for 10 min. Choline metabolites were analyzed as described in Materials and Methods. The radioactivity (dpm) in choline (1245) and phosphocholine (7525) in unstimulated control was designated as 100%. Data are presented as means from three experiments. Bars indicate SD.

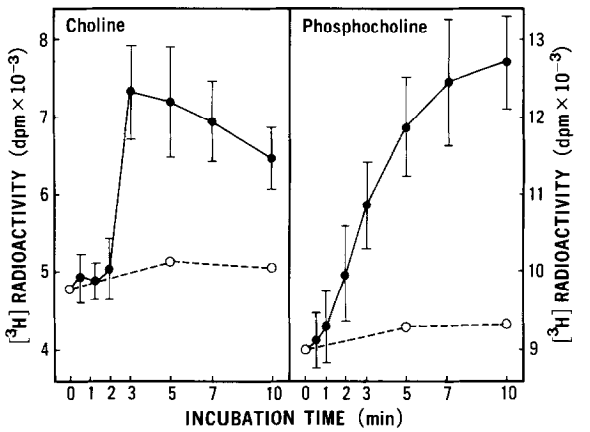

Figure 4. Time courses of Ag-induced accumulation of choline and phosphocholine in [^{3}H]choline-labeled RBL-2H3 cells.
[^{3}H]Choline-labeled cells were washed and exposed to Ag (0.5 μg/ml) for the times indicated. Choline metabolites were analyzed as described in Materials and Methods. Data presented are means±S.E. of quadruplicate determinations. Two other experiments gave similar results.

Addition of either PMA or A23187 gave rise to accumulation of DG and PA (Table I). Inactive phorbol ester, 4α-phorbol didecanoate (4α-PDD) had no effects on DG and PA formation. In the presence of ethanol, PEt formation was

TABLE I. Effects of down-regulation of PKC on DG, PA and PEt formation in RBL-2H3 cells treated with Ag, PMA or A23187

	Control cells			PKC down-regulated cells		
	DG	PA	PEt	DG	PA	PEt
	percent of total					
Control	0.72 ±0.06	0.16 ±0.04	0.62 ±0.08	0.84 ±0.10	0.18 ±0.02	0.68 ±0.06
Ag (0.5 μg/ml)	2.37 ±0.34	0.76 ±0.14	1.08 ±0.24	1.80 ±0.34	0.30 ±0.08	0.86 ±0.08
PMA (100 nM)	1.44 ±0.22	0.28 ±0.08	1.48 ±0.18	0.92 ±0.12	0.16 ±0.05	0.70 ±0.12
A23187 (1 μM)	2.64 ±0.35	0.98 ±0.25	4.91 ±0.78	1.47 ±0.29	0.32 ±0.09	0.84 ±0.06

RBL-2H3 cells were pretreated for 16 h with or without 100 nM PMA and labeled with [^{3}H]myristic acid for the last 6 h. After washing, cells were incubated for 5 min in the presence of 1% ethanol, and then stimulated with 0.5 μg/ml Ag, 100 nM PMA or 1 μM A23187 for 10 min. Other experimental conditions are described in Materials and Methods. Data presented are means±SD of four experiments.

observed in response to either PMA or A23187, indicating the activation of PLD. The accumulation of water-soluble [^{3}H]choline metabolites (Fig. 3) indicated the hydrolysis of PC by PMA and A23187. By incubation of RBL-2H3 cells with 100 nM PMA for 16 h, the contents of PKC (type II and III) were markedly reduced to less than 10% and Ag-induced secretory response was almost completely prevented (data not shown). In these PKC down-regulated cells, exposure to PMA no longer stimulated accumulation of DG and PA, and PEt in the presence of ethanol (Table I). Ag- and A23187-induced formation of DG and PA was still significant but greatly reduced, implying that PC hydrolysis was largely dependent on PKC. These findings were confirmed in [^{3}H]choline-labeled cells (Fig. 3).

DISCUSSION

Ag caused a biphasic DG production in [^{3}H]myristic acid-labeled RBL-2H3 cells. The transient IP_3 peak in Ag-stimulated 2H3 cells was observed at 15 s following Ag addition (22), which was parallel with the early first DG peak (Fig. 1), suggesting that the first phase of DG accumulation is due to PIP_2 hydrolysis by PLC and the late sustained phase is derived from other sources than PIP_2. Recently, Kennerly (10) has demonstrated that, although PI hydrolysis takes some but not main part, PC hydrolysis plays a greater role for DG accumulation in Ag-stimulated rat peritoneal mast cells.

PEt formation at the expense of PA (Fig. 2) indicates the activation of PLD in response to Ag. PA formed by the action of PLD can be dephosphorylated by PA phosphohydrolase to produce DG (4, 5). Furthermore, the greater and earlier accumulation of [^{3}H]phosphocholine compared to [^{3}H]choline suggested the activation of PC-PLC (Fig. 4). In ethanol-treated cells, as Ag-induced formation of PA was reduced to the nearly basal level, DG formation via the PC-PLD pathway should be expected to be mostly suppressed. However, DG formation was relatively resistant to ethanol treatment (about 30% decrease) (Fig. 1), which leads us to assume that the PC-PLC pathway makes a larger contribution to DG formation from PC.

Recent reports indicate that agonist-induced hydrolysis of PC by PLC and/or PLD is regulated by several factors such as Ca^{2+}, PKC and GTP-binding proteins. PMA but not 4α-PDD stimulated DG formation and accumulation of water-soluble choline metabolites in RBL-2H3 cells. In PKC down-regulated cells, PMA did not affect PC breakdown and the effects of Ag and A23187 were markedly reduced, suggesting that PKC plays an important regulatory role for PC hydrolysis and DG formation in RBL cells. The effect of Ca^{2+} was largely mediated by PKC activation since A23187-induced PC breakdown was reduced in PKC down-regulated. However, A23187 caused significant accumulation of DG and water-soluble choline metabolites in PKC down-regulated cells, implying that increase in intracellular Ca^{2+} is another factor

regulating PC hydrolysis. The Ag-induced PC hydrolysis occurred following the initial DG peak associated with the transient breakdown of PIP_2. These results lead us to consider that the second phase of DG is secondary to PIP_2 breakdown and that Ca^{2+} mobilized by IP_3 and PKC activation by DG facilitate PC hydrolysis.

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