DIACYLGLYCEROL METABOLISM IN MAST CELLS:
A POTENTIAL ROLE IN MEMBRANE FUSION AND
ARACHIDONIC ACID RELEASE*

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A substantial body of evidence has been presented recently that indicates that the
release of mediators from rat mast cells is closely linked to changes in phospholipid
metabolism (1-3). Selectively increased incorporation of 32PO4 into mast-cell phos-
phatidic acid (PA), phosphatidylcholine (PC), and phosphatidylinositol (PI) has been
shown to occur after stimulation by anti-IgE, concanavalin A, the polycationic
histamine-releasing agent compound 48/80, or the calcium ionophore A23187. Studies
of the kinetic and dose-response relationships (1), as well as the pharmacologic
modulation of these biochemical and functional responses (3), strongly suggest that
they are intrinsically related processes. Because 1,2-diacylglycerol (DAG) is a precursor
for each of these phospholipid molecules, a possible central role for DAG has been
postulated to account for the stimulated changes in mast-cell lipid metabolism (1).
The experiments described in this report were designed to determine the levels of
DAG during mediator release from mast cells and to probe the metabolic fate of
newly formed DAG.

Materials and Methods

Materials and Their Sources. Materials were obtained as described previously (1-4), except:
lipid-poor bovine serum albumin (BSA) from Calbiochem-Behring Corp., American Hoechst
Corp., San Diego, Calif.) and Clostridium perfringens phospholipase C from Worthington Bio-
chemical Corp., Freehold, N. J. 2-[1-14C]arachidonoyl-phosphatidylcholine was synthesized by
the method of Robertson and Lands (5). 2-[1-14C]arachidonoyl-DAG was obtained by phos-
pholipase C hydrolysis of labeled PC. All organic solvents except those used for thin-layer
chromatography (TLC) were redistilled before use.

Isolation and Purification of Mast Cells. Mast cells were obtained from the thoracic and
peritoneal cavities of male Sprague-Dawley (Camn Research Institute, Inc., Wayne, N. J.) rats
and were purified using a BSA density-gradient method which has been described in detail (6).
Mast cell preparations (at least 95% pure and at least 98% viable as assessed by trypan blue
exclusion [7]) were suspended in mast cell medium with phosphate (MCM-PO4) (150 mM
NaCl, 3.7 mM KCl, 1 mM CaCl2, 5.55 mM glucose, 5 mM sodium phosphate, 2 mM
piperazine-N,N'-bis(2-ethane sulfonic acid), 1 mg/ml lipid-poor BSA, and 1 U/ml heparin, pH
6.8) unless otherwise indicated.

Labeling Experiments. Purified mast cells (3 × 10^6 cells/ml) were prelabeled by incubation

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with [3H]arachidonic acid (10 µCi/10⁶ cells) for 30 min at 37°C. The labeled cells were washed twice, resuspended (1.5 × 10⁶/ml), and 3 × 10⁵ cells were aliquoted into polypropylene tubes. Mast-cell lipids were extracted at varying times after stimulation with compound 48/80 (1 µg/ml final concentration in a final vol of 0.25 ml) by the addition of 1.65 ml of chloroform/methanol/0.267 N HCl (1:2:0.3). After 30 min at room temperature, insoluble material was removed from the extract by centrifugation. The supernates were added to tubes containing unlabeled marker lipids (20 µg each of monolein, 1,2-diolein, arachidonic acid, triolein, and phosphatidic acid) and after thorough mixing, two phases were obtained by centrifugation and the upper, aqueous phase was discarded. The organic phase was dried and the lipids (dissolved in 0.05 ml of chloroform) were separated by TLC (hexane/diethylether/acetic acid; 30:70:1). Lipid-containing areas (detected by iodine staining) were cut out of the TLC plate, placed in Minivials (Fisher Scientific Co., Pittsburgh, Pa.), and the radioactivity assessed after the addition of Scintiverse (Fisher Scientific Co.).

**Isolation and Quantitation of Mast-Cell DAG.** Cellular lipids were extracted by the addition of 1.5 ml of chloroform/methanol/12 N HCl (50:100:0.33) at varying times after mast cells (10⁶ cells in 0.4 ml) were stimulated by compound 48/80. Insoluble material was removed by centrifugation after 30 min at room temperature. After the addition of 0.5 ml chloroform and 0.5 ml of 0.1 N HCl and mixing, two phases were obtained by centrifugation. No detectable acid-catalyzed hydrolysis of phospholipids to DAG was detected. The lipid residue obtained by drying the lower phase was dissolved in 0.10 ml of 5% (vol/vol) ether in hexane and applied to a 0.075-ml silicic acid column equilibrated in the same solvent. Contaminating neutral lipids were eluted by the addition of two 0.20-ml vol of 12.5% (vol/vol) ether in hexane. The DAG-containing fraction was eluted by the addition of two 0.20-ml vol of 50% (vol/vol) ether in hexane. After evaporating the solvent, DAG was quantitated by DAG kinase-catalyzed phosphorylation as described in detail in a separate communication (4). Briefly, DAG kinase (Escherichia coli) in the presence of [γ-32P]ATP, catalyzes the formation of [32P]phosphatidic acid in linear proportion to the quantity of DAG present from 10 to 1,000 pmol (4).

**Analysis of DAG Metabolites Formed by Mast Cells.** Unless otherwise indicated, 5 nmol of 2-[1-14C]arachidonoyl-DAG were dried onto the bottom of 12-× 75-mm polypropylene tubes. Mast cells (10⁶) suspended in 0.10 ml of MCM-PO₄ (without BSA) were added. The tubes were brought to 37°C and the reaction initiated by ultrasonic dispersion of the labeled substrate and mast cell enzymes (Biosonik microprobe, VWR Scientific, Inc., San Francisco, Calif., 30 W, 5 s). The ensuing reaction was halted by the addition of 1.8 ml chloroform/methanol/0.1 N HCl (0.5:1.0:0.3). After 15 min at room temperature, this extract was separated into two phases and the lipids separated by TLC as described above for labeling experiments.

**Analysis of Data.** All experiments were performed in triplicate and data are presented as mean ± SEM. Significance was assessed using the independent Student's t test (two-tailed).

### Results

**DAG in Mast Cells.** Mast cells were prelabeled with [3H]arachidonic acid to label the endogenous lipids that might act as precursors in the production of DAG. The data presented in Fig. 1 demonstrate that stimulation of mast cells with compound 48/80 resulted in a very substantial and rapid increase in the amount of labeled DAG. No differences were noted within 15 s after the addition of compound 48/80, but significant elevation of labeled DAG was detected within 1 min after stimulation. The relatively small absolute label accumulation in DAG (250–300 cpm) when compared to prestimulation levels of labeled PI, PC, PE, and triglyceride (7,600, 21,000, 4,800, and 4,300 cpm respectively) precluded demonstration of an absolute loss of label in any of these potential precursor lipids.

Although these results suggested that levels of DAG increase in stimulated mast cells, the elevated labeling of DAG could have been the result of an increase in the specific activity of DAG. To resolve this issue, levels of DAG in mast cells were assayed directly. The absolute levels of DAG increased in stimulated mast cells in parallel
Fig. 1. Accumulation of DAG in stimulated mast cells. Mast cells were stimulated either with compound 48/80 (open symbols, 1 μg/ml final concentration) or medium alone (closed symbols) for the time indicated before extraction, separation, and quantitation of lipids as described in Methods. Data obtained in labeling studies are indicated by open and closed circles while those obtained by direct quantitation are indicated by open and closed triangles. Data represent mean ± SEM of triplicate values obtained in one of three similar experiments. Stimulation ratios (± SEM) were obtained when data from all labeling experiments were pooled: 1 min, 3.15 ± 0.64; 5 min, 2.44 ± 0.36; for direct quantitation studies: 1 min, 2.54 ± 0.17; 3 min, 3.70 ± 0.71; 10 min, 3.13 ± 0.14.

TABLE I

| Lipid class      | Percentage of labeled products (%) |
|------------------|------------------------------------|
| Fatty acids      | 43.3 ± 7.2                         |
| Phospholipids    | 29.2 ± 15.2                        |
| Monoacylglycerol | 19.5 ± 11.5                        |
| Triglyceride     | 8.1 ± 2.7                          |

Labeled DAG was dispersed and 10⁵ mast cells broken by sonication. After 15 min at 37°C, lipid-soluble products were obtained by extraction and separated by TLC as described in Methods. The amount of label comigrating with authentic standards was determined and the percentage of the contribution of each lipid class to the total quantity of newly formed labeled products was calculated for each experiment. Data represent mean ± SEM of means obtained in three different experiments (total labeled products: 542 ± 67 pmol/15 min per 10⁵ cells).

with the increase in DAG labeling seen with [³H]arachidonate-labeled cells (Fig. 1). Significantly elevated DAG levels were observed 1, 3, and 10 min after the addition of compound 48/80 (stimulation ratios of 2.2, 2.29, and 3.07, respectively; P<0.001).

Fate of DAG in Mast-Cell Homogenates. The metabolism of DAG was examined in broken-cell preparations of mast cells as described in Methods. The major metabolic product obtained from labeled DAG was free arachidonic acid (Table I). Substantial formation of labeled triglycerides, phospholipids, and monoacylglycerol also was detected. Further analysis of free arachidonic acid formation was assessed as illustrated in Fig. 2. The data presented in Fig. 2A demonstrate that the production of unesterified arachidonate was linearly dependent upon the amount of mast-cell homogenate present during the incubation. When mast cells were heated (100°C for 5 min) before their disruption, no arachidonate was released from labeled DAG (not shown). Fig. 2B illustrates the rate of free arachidonic acid formation under these experimental conditions. The release of free arachidonate was constant for the first 5 min (220 pmol/min per 10⁵ mast cells), declining steadily thereafter. Interestingly, when 2-[¹⁴C]arachidonyl-phosphatidylcholine was used as substrate, instead of
labeled DAG derived from this same PC, no release of labeled arachidonic acid was detected at any homogenate concentration examined.

Discussion

The results of this study demonstrate that DAG levels are significantly elevated in stimulated mast cells. When mast cells were prelabeled with \(^{3}{H}\)arachidonic acid, washed, and stimulated, levels of labeled DAG were found to rise ~threefold (Fig. 1) coincident with active secretion (8). Quantitative analysis of mast-cell DAG demonstrated that whole-cell levels of DAG rise two- to threefold during secretion with kinetics parallel to those observed in the labeling studies (Fig. 1).

Because DAG has been found to be fusogenic (i.e., to cause or enhance membrane fusion) (9) and is associated with membrane fusion in erythrocytes (10, 11), the increased levels of DAG in stimulated mast-cell membranes may play a role in the fusion process that leads to the release of preformed mediators during secretion. These observations are in accord with the hypothesis (1) that elevated \(^{32}\text{P}O_4\) incorporation into PI, PA, and PC in mast cells occurs as a result of enhanced DAG production and may in part reflect negative regulation of secretion by reducing DAG levels.

The origin of this newly formed DAG is not yet established. De novo synthesis of DAG seems unlikely because radiolabeled glucose or glycerol are not acutely incorporated into this newly formed DAG (D. A. Kennerly, B. Tung, and T. J. Sullivan. Manuscript in preparation.). The actions of phospholipase C, phosphatidate phosphohydrolase, or triglyceride lipase on their respective substrates appear to be the most likely mechanisms of DAG formation.

The absence of a rise in DAG levels within 3–8 s after stimulation is interesting because increased \(^{32}\text{P}O_4\) incorporation into PA is detectable at that time (1). Early PA formation may occur as a result of phospholipase D action on phospholipids.
followed by a secondary conversion to DAG by the action of a phosphatidate phosphohydrolase. Alternatively, mast-cell DAG kinase may initially remove newly formed DAG (by phosphorylation to form PA) until stimulated DAG production exceeds this enzyme's capacity, thereby resulting in abruptly increased levels of DAG. Preliminary data (not shown) demonstrate the existence of a mast-cell DAG kinase, but information regarding the mast-cell metabolic pathways that utilize DAG is incomplete.

Studies of the fate of 2-[1-14C]arachidonoyl-DAG in broken-cell preparations of mast cells demonstrated that free arachidonate is a major product of the action of mast-cell enzymes on arachidonic acid-containing DAG. This hydrolytic activity was time dependent, proportional to the quantity of sonicated mast-cell suspension present (Fig. 2), and was abolished by heating the mast-cell preparations to 100°C, indicating that the hydrolysis is probably an enzymatic process. The absence of labeled arachidonate production when labeled phosphatidylcholine was used as substrate (Fig. 2 A) indicates that the lipolytic activity in mast-cell homogenates preferentially cleaved fatty acids from DAG and suggests the presence of more DAG-lipase activity than phospholipase A₂ activity. A systematic evaluation of mast-cell phospholipase A₂ activity would be valuable to determine the relative importance of these two arachidonate-generating pathways. The ability of mast cells to convert DAG to fatty acid and monoacylglycerol may be of considerable importance in the membrane fusion process, because these moieties are more fusogenic than DAG itself (9).

In the studies of Lewis et al. (12), A23187-stimulated mast cells produced ~200 pmol of arachidonate products/10⁶ cells over a 30-min period (12). In this study, DAG lipase activity in mast-cell homogenates was capable of hydrolyzing 220 pmol arachidonic acid/min per 10⁶ cells from DAG. The DAG lipase activity demonstrated in these studies would be sufficient to account for the level of arachidonate-metabolite formation known to occur.

Summary

Purified rat peritoneal mast cells stimulated with the polycationic histamine-releasing agent compound 48/80 demonstrated a two- to fourfold increase in cellular levels of 1,2-diacylglycerol (DAG) within 1 min as detected by radioactive labeling and direct quantitation experiments. When 2-[1-14C]arachidonoyl-DAG was incubated in the presence of mast-cell homogenates, a rapid conversion to free arachidonate, and to a lesser extent, to monoacylglycerol, triglyceride, and phospholipid was observed. The release of arachidonate was proportional to the amount of broken-cell preparation added and the time of incubation, was prevented by preheating mast-cell preparations, and did not occur when 1-[1-14C]arachidonoyl-phosphatidylcholine was used as substrate, suggesting that the degradation was mediated by an enzyme with DAG-lipase activity. Although much work remains to be done to clarify the precise role of DAG in mast cells, DAG metabolism may be involved in secretion by generating substances which may facilitate membrane fusion and also in arachidonic acid-derived mediator formation by liberating esterified arachidonic acid from mast-cell lipids. Taken together, these studies indicate that the formation of DAG may play a central role in mast-cell function.

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References

1. Kennerly, D. A., T. J. Sullivan, and C. W. Parker. 1979. Activation of phospholipid metabolism during mediator release from stimulated rat mast cells. *J. Immunol.* 122:152.

2. Kennerly, D. A., C. W. Parker, and T. J. Sullivan. 1979. Increased levels of 1,2-diacylglycerol (DG) during mediator release from mast cells. *Fed. Proc.* 38:1018.

3. Kennerly, D. A., C. J. Secosan, C. W. Parker, and T. J. Sullivan. 1979. Modulation of stimulated phospholipid metabolism in mast cells by pharmacologic agents which increase cyclic 3',5' adenosine monophosphate levels. *J. Immunol.* In press.

4. Kennerly, D. A., C. W. Parker, and T. J. Sullivan. 1979. Use of diacylglycerol kinase to quantitate picomole levels of 1,2-diacylglycerol. *Anal. Biochem.* In press.

5. Robertson, A. F., and W. E. M. Lands. 1962. Positional specificities in phospholipid hydrolysis. *Biochemistry.* 1:804.

6. Sullivan, T., K. Parker, W. Stenson, and C. W. Parker. 1975. Modulation of cyclic AMP in purified mast cells. I. Responses to pharmacologic, metabolic, and physical stimuli. *J. Immunol.* 114:1473.

7. Boyse, E., L. Old, and I. Chouroulinkou. 1964. Cytotoxicity test for demonstration of mouse antibody. *Methods Med. Res.* 10:39.

8. Sullivan, T., K. Parker, S. Eisen, and C. W. Parker. 1975. Modulation of cyclic AMP in purified rat mast cells. II. Studies on the relationship between intracellular cyclic AMP concentrations and histamine release. *J. Immunol.* 114:1480.

9. Ahkong, Q., D. Fisher, W. Tampion, and J. Lucy. 1973. The fusion of erythrocytes by fatty acids, esters, retinol, and α-tocopherol. *Biochem. J.* 136:147.

10. Allan, D., M. Billah, J. Finean, and R. Michell. 1976. Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular [Ca++]*. *Nature (Lond.)* 261:58.

11. Allan, D., M. Low, J. Finean, and R. Michell. 1975. Changes in lipid metabolism and cell morphology following attack by phospholipase-C (*Clostridium perfringens*) on red cells or lymphocytes. *Biochim. Biophys. Acta.* 413:309.

12. Lewis, R., L. Roberts, J. Lawson, K. F. Austen, and J. Oates. 1979. Generation of oxidative metabolites of arachidonic acid from rat serosal mast cells. *J. Allergy Clin. Immunol.* 63:220.