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An Enhanced Incorporation of Fatty Acid into Phosphatidyl Choline that Parallels Histamine Discharge in Mast Cells

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Summary. Purified rat peritoneal and pleural mast cells preincubated briefly with radioactively labeled fatty acid were treated with A23187, which bypasses primary receptors in stimulating exocytosis. An enhanced incorporation of fatty acid into phosphatidyl choline (PC) that occurred in parallel with histamine release at 24–25 °C was observed and was initially proportional to the total amount of histamine discharged. Enhanced PC labeling and histamine secretion were also proportional at temperatures ranging from 17–37 °C. Both radioactive linoleic and palmitic acids were incorporated selectively at the β-position of the glycerol backbone of PC. PC labeling by [^{3}H]choline was not detectably different in control and stimulated cells, and phosphatidic acid did not exhibit selectively enhanced β-acylation. Thus, the stimulated labeling in A23187-treated cells may occur secondary to the action of a phospholipase A_2 that favors PC as a substrate.

Other peritoneal cell types exhibit a very similar A23187-stimulated selective labeling of PC. Therefore, autoradiography has been used to provide a direct demonstration that in purified preparations, mast cells are the principal cell type engaged in A23187-elicited incorporation of fatty acid into PC. The efficacy of this approach has relied on special procedures devised to obtain significantly different autoradiographic grain densities between control and stimulated preparations that can be attributed to differences in the level of [^{3}H]palmitate-labeled PC.

Preliminary tests using compound 48/80 as a secretory stimulus for mast cells have identified a similar selectively enhanced PC labeling. In either case, however, consideration of possible relationships between PC metabolism and the secretory process are premature since they have not been tested directly.

Key Words mast cells · exocytosis · calcium ionophore · phospholipid labeling · autoradiography

Introduction

The mast cell is highly specialized for releasing stored macromolecules by exocytosis, and, in large part through its secretory activity, it is presumed to play an integral role in processes related to host defense [34]. Further, it is potentially a favorable experimental system for conducting studies of the mechanism of exocytosis for two reasons: (1) Membranes involved in discharge constitute a large fraction of total cell membrane [23], and (2) appropriate secretory stimuli lead to complete discharge in less than 2 min such that related metabolic events may be amplified and compressed in time to a sufficient extent to enable detection above the basal metabolic and biosynthetic activities of the cell.

A number of metabolic processes, especially those involving amplified phospholipid metabolism, have been studied in a variety of cell types, including mast cells stimulated by agents known to induce secretion. These processes include the enhanced incorporation and turnover of inorganic phosphate and inositol in phosphatidyl inositol (PI) [5, 10, 13, 28, 40], a transient increase in diglyceride (DG) levels possibly related to PI metabolism [32, 45], the liberation of arachidonic acid and its subsequent metabolism via the cyclooxygenase and lipoxygenase pathways [4], and the enhanced N-methylation of phosphatidyl ethanolamine (PE) [25, 26, 29, 38, 39, 43]. In most cases, however, these processes have been shown either to be more intimately related to the proximal interaction of primary stimuli with the cell surface than with the terminal discharge of secretion [40] or to be dissociable from membrane fusion events such as exocytosis [24, 31, 40].

In the studies reported in this paper we have sought to focus on phospholipid effects that might relate to the actual interaction of secretion granule and plasma membranes during exocytosis in mast cells. At the outset we have used experimental conditions designed to avoid alterations in lipids that appear to require stimulation of primary surface receptors. Our working hypothesis, similar to that of others [e.g., 32, 38, 49] is that phospholipase A_2 action may induce focal perturbations of membrane structure and thereby facilitate reorganization and establishment of continuity between bilayers in regions where they are closely apposed. The follow-

ing strategies and findings directly influenced the design of the studies conducted.

1) Cell stimulation has been performed initially and most extensively using the calcium ionophore A23187. Although analogies between ionophore action and physiological processes leading to increased intracellular calcium activities are questionable, it has been shown, first, that A23187 will induce exocytosis [12] and, second, that discharge is obtained without enhanced N-methylation of PE (25), a process observed when mast cells are stimulated with immunoglobulin E (IgE) [29].

2) Cells have been incubated during experiments at 24–25 °C as a further attempt to reduce the complexity of lipid metabolic events occurring with stimulation. Under these conditions amplified ^{32}P-inorganic phosphate incorporation in mast cell phospholipids treated with IgE, compound 48/80, concanavalin A, or A23187 is either reduced (for phosphatidic acid (PA)) or largely disappears (PI, phosphatidyl choline (PC)) [31] even though discharge of histamine is readily observed [33, 60].

3) Due to the apparent inability to detect increased levels of lysophosphatide upon secretory stimulation (in exocrine pancreas [52], parotid and mast cells (our own unpublished findings)), we have assumed that there may be rapid repair of phospholipids by reacylation and have sought to detect this activity. We show that the labeling of PC at the β-position of the glycerol backbone by either radioactive linoleic or palmitic acids is selectively enhanced and parallels histamine discharge in stimulated mast cells.

4) Although arachidonic acid has been studied widely as a fatty acid that is incorporated by exchange into phospholipids [64] and liberated in response to stimulation [34, 46], we sought in characterizing the enhanced acylation to avoid possible complications that might arise from secondary metabolism of arachidonate-labeled phospholipid.

5) The observation that enhanced incorporation of palmitic acid also is selective for the β-position of PC has made possible an autoradiographic demonstration that ionophore-stimulated labeling occurs in mast cells. We show that palmitate-labeled PC can be selectively retained during processing of specimens for microscopy to such an extent that grain density differences observed between control and stimulated preparations can be inferred to reflect differences in the cellular content of labeled PC.

6) Finally, we present preliminary evidence indicating that compound 48/80, a secretory agonist operating at the mast cell surface [41], induces an enhanced selective acylation of PC analogous to that observed with A23187.

Materials and Methods

Preparation of Cells

Cell suspensions obtained by peritoneal and pleural lavage (pH 6, phosphate-buffered saline [59]) of male Sprague-Dawley rats 225–250 g (Charles River Suppliers, Wilmington, MA) sacrificed by decapitation were used either for mast cell purification or as an unfractionated population. Mast cells were purified on Ficoll discontinuous gradients according to Uvnas [59] and were washed free of Ficoll and stored (0 °C; <2 hr) in a solution containing 4 mg/ml glucose, 1 mg/ml bovine plasma albumin (BPA), 145 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 6.6 mM Sorensen phosphate buffer pH 7. Mast cell yield and purity determined using a hemocytometer were 1.2–1.7 × 10^6 cells per animal and 90 ± 7%, respectively. Unfractionated cells used in some experiments were harvested by centrifugation (100 × g, 3 min) and washed and stored in the same medium; cell density and mast cell content (5–10%) were determined using a hemocytometer.

Prior to experimental incubations cell suspensions were washed free of albumin by centrifugation (3 times at 350 × g, 2 min) in 145 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 6.6 mM Sorensen phosphate buffer at pH 7.0, 4 mg/ml glucose, and 1 mg/ml gelatin (referred to as suspension medium). The cells were resuspended in this medium at a concentration of 7.5–9.0 × 10^6 cells/ml.

Preparation of Fatty Acid Containing Media

Labeled fatty acids (0.4 mM [^{14}C] or 2 mM [^{3}H] in ethanol) were diluted to a final concentration of 2.3 × 10^{-6} M in 145 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 6.6 mM Sorensen phosphate buffer at pH 7.0 containing 4 mg/ml glucose and 0.048 mg/ml defatted bovine serum albumin (~3 : 1 molar ratio of fatty acid to albumin). This medium was used in studies examining the extent of association of fatty acid with cells, the incorporation of fatty acid into specific lipids, and the autoradiographic detection of stimulus-enhanced labeling of PC. Unlabeled fatty acids were substituted at the same concentration in cell samples used for measuring release of histamine and leakage of lactate dehydrogenase.

Incubation and Processing of Cell Suspensions

Incubations were carried out at 24–25 °C (except where temperature was an experimental variable) in a constant temperature recirculating water bath. Aliquots of cell suspension (55 µl, 4.5–5 × 10^5 cells) were preincubated 2 min in 1.5 ml polypropylene tubes; 44 µl fatty acid medium was then added followed 1 min later by 1 µl ethanol (control) or 1 µl A23187 (7 × 10^{-5} M in ethanol; stimulated) to start incubations that ranged in time from 10 sec to 9 min. Where compound 48/80 was used as a secretory stimulus, suspension medium alone (control) or supplemented with 48/80 (stimulated, final concentration 4 µg/ml[1]) replaced eth-

[1] The dose of compound 48/80 used (4 µg/ml) was chosen based on a preliminary dose-response experiment where histamine release was tested for mast cell preparations incubated at 25 °C. Stimulation has no apparent effect on cell integrity as determined by examining the leakage of LDH.

anol or A23187. The preparation of specimens for autoradiography and the parallel evaluation of the selective retention of labeled PC required larger samples. In this case cell suspensions contained 2.0–2.2 × 10^6 mast cells in 220 μl to which 178 μl fatty acid medium and 2 μl either ethanol or 1.4 × 10^{-4} M A23187 were added.

Incubations were terminated according to the ultimate use of each sample.

a) Estimation of Total Cell-Associated Fatty Acid

Samples were cooled to 0 °C, and the cells were rapidly sedimented by centrifugation (350 × *g*, 2 min). 50 μl of supernatant fluid was removed and saved at −20 °C for scintillation counting along with the cell pellet suspended in the remaining 50 μl of medium.

b) Fatty Acid Incorporation into Specific Lipids

2 μl of 200 mM EDTA was added, and the sample was immediately frozen in liquid nitrogen. Samples were stored at −20 °C until subjected to lipid extraction and thin-layer chromatography (TLC).

c) Autoradiographic Detection of Labeled PC

The procedure developed is discussed in a separate section of Methods below.

d) Lactate Dehydrogenase (LDH) Leakage

Processing by cooling and centrifugation was the same as for *b*. 80 μl of the supernatant fluid was removed for determination of released LDH activity. Cell-associated activity was calculated from the total activity of the cell pellet resuspended in the remaining supernate.

e) Release of Histamine

50 μl of each incubation sample was pipetted onto a microfiltration unit (prepared individually for each sample) outfitted with a Millipore filter (0.45 μm porosity). A cell-free filtrate averaging 35 μl was recovered in 400 μl polypropylene tubes within 5 sec under reduced pressure. 25 μl of filtrate and the remaining 50 μl of unfiltered cell suspension were both diluted to 1 ml with 0.1 N HCl and assayed for released and total histamine, respectively. Independent tests using standardized histamine solution indicated that the filtration devices caused no detectable change in histamine concentration. The filtration procedure did not result in detectable damage to cells since the activity of LDH in the filtrate was the same as that found in the supernatant fluid when mast cells were processed as in *d*.

In more than half of the experiments reported (reflecting the availability of adequate numbers of cells) incubations testing the incorporation of fatty acid and the release of histamine were carried out in duplicate; the duplicates in most cases showed agreement within 10%.

For studies of choline incorporation into PC, mast cells were preincubated 1 hr in suspension medium containing 2 mM [^{3}H]choline (specific radioactivity 30 μCi/μmol). After prelabeling, the cells were transferred directly to 25 °C and following a 3-min temperature equilibration were incubated for 0–15 min in the presence and absence of A23187. At timepoints samples were taken for lipid extraction and TLC and for measuring the extent of histamine release.

Extraction of Lipids and Thin Layer Chromatography

Lipids were extracted into organic solvent from cell suspensions that were adjusted to 0.2 ml with 0.15 M NaCl [7]. The methanol-aqueous phase was re-extracted with chloroform and unlabeled standards (LPC, SPH, PC, PE, PS, PI, PA and linoleic acid for phospholipid chromatography or mono-, di-, triglyceride, linoleic acid and cholesterol for chromatography of neutral lipids) were added to the pooled extracts. Extracts were dried under reduced pressure, and the residues were dissolved in 20 μl of N_2-saturated chloroform and spotted for TLC.

Phospholipids were resolved by two-dimensional TLC on silica gel 60 plates (10 × 10 cm, E. Merck) using $CHCl_3/CH_3OH/H_2O/NH_3$ (90 : 54 : 5.5 : 5.5) followed by $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (90 : 40 : 12 : 2) [47]. Neutral lipids were resolved by one-dimensional TLC on silica gel G (10 × 20 cm plates, hard layer; Analtech) in $CHCl_3/(CH_3)_2CO$ (9 : 1) [18]. Lipids were visualized by exposure to I_2 vapors, and the spots were either scraped directly for scintillation counting or scraped following autoradiography on X-ray film. Scrapings were mixed with 200 μl of water and 4.8 ml Liquiscint (National Diagnostics), stored >12 hr for lipid elution from the silica, and counted in a Beckman LS-250 liquid scintillation spectrometer. Using this protocol, it was possible to recover 95% of the radioactivity present in a standard sample of [^{14}C(U)] oleate-labeled PC.

Processing of Mast Cell Suspensions for Analysis of the Positionality of Glycerol Acylation in PC and PA

Samples containing ≥2.5 × 10^6 mast cells incubated with radioactive fatty acid in the presence or absence of A23187 were subjected to lipid extraction [6, 7]. Small samples were saved for two-dimensional TLC to provide estimates of recovery of the individual phospholipid species. The remaining extracts were supplemented with 30 nmol of the appropriate nonradioactive phospholipid (PC or PA) and subjected to two-dimensional TLC. PC and PA were located by a 5-sec exposure to I_2 vapor [16], eluted (4 hr, 25 °C with shaking) into 1 ml of either chloroform/methanol/water (60 : 20 : 4) for PC or chloroform/methanol/acetic acid/water (50 : 20 : 7 : 3.5) for PA, dried, and redissolved in chloroform. 30 nmol nonradioactive PA was added to the radioactive PC eluate and ~30 nmol nonradioactive PC added to the labeled PA in order to promote approximately uniform hydrolysis during phospholipase digestion. Traces of silica gel were removed by centrifugation, and the samples were dried, and each was resuspended in 200 μl 75 mM MOPS at pH 7.2 containing 5 mM $CaCl_2$. 18 μg of phospholipase A_2 (E.C.3.1.1.4, purified from rabbit parotid gland and shown to have absolute specificity of cleavage at the glycerol β-position [11]) and 300 μl of diethyl ether [65] were added, and incubation at 27 °C was carried out for 12 hr. Following evaporation of ether, the digests were extracted and lysophosphatides, residual phospholipids, and free fatty acid were resolved by TLC [44] and the distribution of radioactivity was determined. Radioactivity reflecting α-position acylation of the original PC (or PA) comigrates with LPC (or LPA), whereas that representing β-position acylation migrates with free fatty

acid. Together with radioactivity remaining as unhydrolyzed phospholipid, these values provide an estimate of extent of hydrolysis. By taking into account the overall recovery of radioactive phospholipid subjected to positional analysis and the extent of hydrolysis for each sample, control and stimulated preparations were compared for enhanced labeling at each of the acylation positions on the glycerol backbone.

Processing of Incubated Mast Cells for Autoradiography

The following series of steps has been used to prepare [^{3}H]palmitate-labeled mast cell suspensions incubated 1.0 or 1.5 min at 24 °C in the presence or absence of 0.7 μM A23187 for autoradiography to detect cells exhibiting enhanced labeling of PC. For steps 1–7 the temperature was maintained at −3 to −5 °C in an ice-salt water slurry; steps 9–13 were carried out at 0 °C.

1. Incubation was terminated by dilution with 3 ml suspension medium containing 20 mg/ml defatted BSA.
2. Within 1 min, the diluted suspension was layered in conical glass tubes over 1 ml 6% Ficoll 400 in suspension medium containing 10 mg/ml defatted BSA and spun in a centrifuge (3 min, 400 × *g*) to completely pellet the cells.
3. The pellets were resuspended in 6% glutaraldehyde, 2% formaldehyde, 0.1 M sodium cacodylate (pH 7.3) for 90 min.
4. The cells were pelleted 2 min at 350 × *g* (these centrifugation conditions are used in all subsequent steps where solutions are changed).
5. Unreacted aldehydes were quenched by resuspending the cells for 20 min in 0.15 M Tris, pH 7.4.
6. The cells were treated with eight successive cycles, each involving resuspension for 10 min in 10 mg/ml fatty acid poor BSA in 0.1 M cacodylate, pH 7.3, followed by resedimentation.
7. The cells were washed free of BSA in 0.1 M cacodylate, pH 7.3.
8. The samples were suspended in cacodylate-buffered 1.5% (wt/vol) tannic acid (pH adjusted to 7.3) and treated for 2.5 hr.
9. Tannic acid was removed by seven successive washes with 1 ml 0.1 M cacodylate buffer, pH 7.3.
10. The samples were suspended and post-fixed for 60 min in 2% osmium tetroxide in 0.1 M cacodylate.
11. The cells were washed three times (1 ml each) in 0.15 M sodium acetate, pH 7.2, and subsequently stained 1 hr in 0.5% (wt/vol) magnesium uranyl acetate in the same buffer.
12. Following two washes in 1 ml acetate buffer the samples were dehydrated in acetone: 2 × 10 min in 70% aqueous acetone; 1 × 10 min in 95% acetone; 2 × 10 min in 100% acetone.
13. The cells were suspended and maintained 1 hr in a 1 : 1 (vol/vol) mixture of acetone and Epon 812 [36].
14. The cells were suspended in Epon 812; after 15 min they were packed as a loose pellet by centrifugation and maintained 6 hr at 4 °C.
15. The cells were repelleted, resuspended in fresh Epon, maintained 60 min at room temperature, and spun to the tips of embedding capsules by centrifugation (2 min, 8000 × *g*).
16. The embedded samples were polymerized for 48 hr at 70 °C.

For autoradiography, sections (0.25–0.5 μm) of embedded cell pellets were mounted on glass slides, coated with photographic emulsion [51] and stored (1–4 weeks at 4 °C) in desiccated boxes. The autoradiograms were developed in Kodak D-19 [9] and briefly stained with 1% methylene blue in sodium borate [44]. In each autoradiographic experiment sections from three different samples of both control and A23187-treated preparations were photographed using a Zeiss photomicroscope and printed for analysis at a final magnification of ×1125. For quantitation the surface area of each cell section was measured and the number of overlaying autoradiographic grains was counted.

Other Assays

Histamine was assayed according to a modification [48] of the fluorometric procedure of Shore et al. [54].

Lactate dehydrogenase (LDH) was determined on aliquots of cell-free medium and mast cell suspension both dissolved and diluted with 0.25% Triton X-100 in 0.1 M potassium phosphate, pH 7.2, using as substrate 0.3 mM reduced nicotinamide adenine dinucleotide and 4 mM sodium pyruvate [52].

Materials

Ficoll 400 was obtained from Pharmacia; gelatin from Difco Laboratories; bovine plasma albumin Fraction V from Reheis Chemical Co.; defatted bovine serum albumin from Miles Laboratories; unlabeled fatty acids were obtained from Nuchek and Sigma Companies; compound 48/80 was purchased from Sigma and tannic acid from Mallinkrodt. Radioactively labeled fatty acids ([^{14}C]linoleic acid, 900 mCi/mmol; [^{14}C(U)]oleic acid, 990 mCi/mmol; [^{14}C(U)]palmitic acid, 610 mCi/mmol; [9,10-^{3}H]palmitic acid, 12.3 Ci/mmol; and [^{3}H]choline, 80 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerel)) were purchased from New England Nuclear. The calcium ionophore A23187 was the generous gift of Dr. Robert L. Hamill, Eli Lilly Research Laboratories. All other chemicals and solvents were reagent grade.

Results

The Discharge of Histamine by Mast Cells at 25 °C

A23187-dependent discharge of histamine by purified mast cells typically observed at 24–25 °C is shown in Fig. 1. Release increases by 1–2% in controls over the 9-min incubation and ranges between 3 and 8% of the total histamine. Discharge by stimulated preparations characteristically reaches a level of 25–30% above control (substantially lower than that customarily observed at 37 °C [19]). The initial rate of discharge and the extent to which the rate decreases after the first 3 min is variable from experiment to experiment.

In order to confirm that the treatments—ethanol and A23187 in the presence of ethanol—cause minimal damage to mast cells, parallel samples were assayed for leakage of cytosolic LDH activity. The control and stimulated preparations were indistinguishable; in each case >96% of the LDH was cell-associated at the onset of incubation while

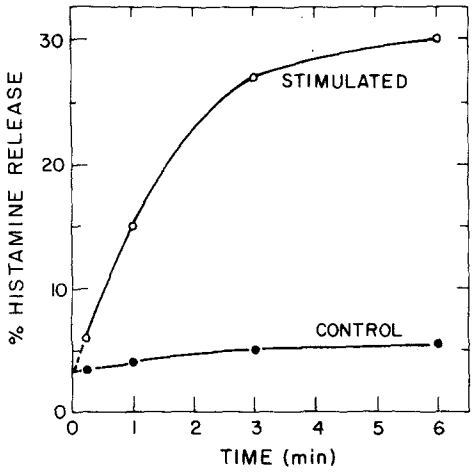

Fig. 1. The discharge of histamine by purified mast cells in the presence (*stimulated*) and absence (*control*) of 0.7 μM A23187 at 24 °C. These data constitute one of a series of six experiments in which progressive histamine release and phospholipid labeling were examined in parallel

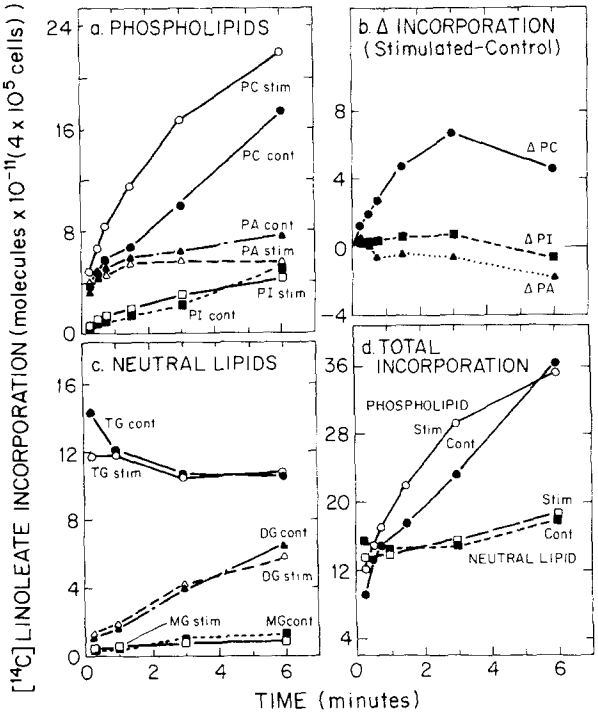

Fig. 2. Time course of labeling of mast cell lipids with [^{14}C]linoleate in the presence (*stim*) and absence (*cont*) of A23187 at 24 °C. (*a*): Incorporation into the phospholipid species that show the most significant labeling with time. PE (not shown) generally labels to a much lower level and its labeling in stimulated samples decreases with time relative to that in controls. (*b*): Plot of the difference in absolute levels of labeling between stimulated and control preparations as a function of time. (*c*): Incorporation of [^{14}C]linoleate into neutral lipids. (*d*): Incorporation of ^{14}C]linoleate into total phospholipid and total neutral lipid as a function of time

>95% of the activity was retained by cells after 8 min.

The Incorporation of Radioactive Fatty Acid into Cellular Lipids

When samples of purified mast cells (each containing 4 × 10^5 cells) were incubated in the presence of 1 μM [^{14}C]linoleic acid, ~40% of the label was bound and thus sedimentable by centrifugation. Binding did not vary significantly as a function of cell stimulation or time of incubation. Based on the known average surface area of the plasma membrane of a rat peritoneal mast cell [23], we estimate that the level of cell-associated exogenous fatty acid corresponds to one molecule per 30 phospholipid molecules present in the plasma membrane. This level of fatty acid constitutes a probe rather than a perturbant of membrane properties especially since the fatty acid is not expected to remain at the plasma membrane but to distribute rapidly among cellular membranes [56].

The incorporation of [^{14}C]linoleate into cellular lipids has been followed for periods of time ranging from 10 sec to, in most cases, 6 min after the addition of ethanol or A23187. In all cases incorporation into phospholipid and neutral lipid combined was less than 10% of the linoleate added. Autoradiography of thin-layer chromatograms identified all lipid species for which labeling was detectable. Quantitative results (one of six experiments) focusing on phospholipids and neutral lipids that exhibit either substantial labeling or notable changes in labeling with stimulation are presented in Fig. 2. The incorporation is expressed as total molecules per sample (calculated from the measured disintegrations per minute and the specific radioactivity of the fatty acid) according to the assumption that existing fatty acid pools in the cells are negligibly small relative to the amount of exogenously added fatty acid [56]. This assumption is supported by the observation that the levels of incorporation of fatty acid into individual phospholipids are essentially unchanged when the linoleate concentration is doubled or halved (not shown). The major effect of the ionophore is to stimulate selectively the labeling of PC. In control preparations the labeling of PC increases linearly, represents 39–43% of the total radioactive phospholipid (calculated from Fig. 2*a* and *d*), and is presumed to reflect turnover. By contrast, incorporation into PC in A23187-treated samples initially increases relative to control (Fig. 2*a*

Table 1. Labeling of mast cell phospholipids ± A23187

A. [^{3}H]choline incorporation into phosphatidyl choline[a]

Incubation time	DPM control	DPM stimulated
(min)		
0	12830	13740
0.5	11680	11630
1	12700	12400
3	12180	13100

B. Fatty acid incorporation into individual phospholipids

Species	[^{14}C]palmitate		[^{14}C]linoleate	
	Control	Δ^b	Control	Δ^b
LPC	47	42	33	13
SPH	678	114	380	3
PC	456	2020	1522	1824
PS, PI	99	31	275	218
PA	684	227	719	182
PE	422	63	332	86

C. Position specificity of acylation in PC and PA

Sample, fatty acid	Phosphatidyl choline				
	% Label[c]			DPM stim LPC[d]	DPM stim FFA[d]
	LPC	FFA	SD	DPM cont LPC	DPM cont FFA
Control, palmitate	38.7	61.3	3.8	2.3 ± 1.1	5.5 ± 1.9
Stimulated, palmitate	20.0	80.0	3.0		
Control, linoleate	17.1	82.9	4.7	1.3 ± 0.3	1.7 ± 0.4
Stimulated, linoleate	13.5	86.5	3.7		
Sample, fatty acid	Phosphatidic acid				
	% Label[c]			DPM stim LPA[d]	DPM stim FFA[d]
	LPA	FFA	SD	DPM cont LPA	DPM cont FFA
Control, palmitate	37.1	62.9	2.0	1.0 ± 0.1	1.4 ± 0.4
Stimulated, palmitate	30.0	70.0	6.3		
Control, linoleate	50.0	50.0	8.6	1.4 ± 0.4	1.7 ± 0.6
Stimulated, linoleate	45.8	54.2	5.9		

[a] Data in *A* are values obtained for samples containing equal numbers of cells. In the absence of information concerning the choline and CDP-choline pools of mast cells, values are given as DPM.

[b] Δ signifies the difference stimulated-control. All values shown in *A* represent molecules $\times 10^{-9}$ for 4.0×10^5 cells as calculated from the specific radioactivity of the fatty acid used.

[c] Label in *C* expresses the distribution of radioactivity between the hydrolysis products of phospholipase A_2 action and thereby the α- and β-hydroxyl groups of the parent phospholipids. Standard deviations (SD) are given for three determinations of positional labeling of PC and two for PA. Note that labeling of PC and PA by palmitate in control preparations slightly favors the β-position. The molecular turnover presumed to lead to labeling probably involves not only the entire phospholipid molecule but also a more rapid exchange of individual fatty acids especially at the β-position.

[d] DPM stim LPC/DPM cont LPC, DPM stim FFA/DPM cont FFA for PC and DPM stim LPA/DPM cont LPA, DPM stim FFA/DPM cont FFA for PA are ratios of radioactivity calculated for stimulated and control samples after correcting for differences in % enzymatic hydrolysis and recovery of radioactivity. % enzymatic hydrolysis was 82 ± 18 (x ± SD) and recovery was 80 ± 11 (x ± SD) for 20 experimental samples.

and *b*), signifying enhanced labeling. Between 15 sec and 6 min radioactive PC increases from 40 to 63% of the total phospholipid label. Based on the chemical content of PC per mast cell (~3.6×10^9 molecules, determined by lipid extraction and quantitative TLC of a known number of cells) it is possible to estimate that the maximal level of enhanced incorporation (~1.7×10^6 molecules/cell) involves ≤0.05% of the total cellular PC. Among the other labeled phospholipids, PI exhibits slightly enhanced incorporation in stimulated preparations, whereas PA labeling is more variable and usually shows a small decrease in the presence of A23187. The results obtained from time points up to 3 min are quite reproducible from experiment to experiment with the exception of the initial level of labeling of PA which fluctuated between the level shown in Fig. 2*a* and half that level. For incubations extending beyond 3 min, some variability was observed in the level of labeling of all phospholipid species in stimulated preparations. In most cases (four out of five experiments in which incubation was extended to 6 min or longer) differences between control and stimulated samples either stabilized or decreased (Fig. 2*b* and *d*) past 3 min.

Figure 2*c* shows that labeled triglyceride is maintained at a uniform, rather high level throughout the incubation (presumably following a rapid initial acylation of diglyceride) and that large differences in labeling of neutral lipids between stimulated and control preparations are not observed.

Preparations were also checked for radioactivity that did not partition into the chloroform phase during lipid extraction. The small amount of label found in the combined methanol-aqueous phase and protein interface solely constituted free fatty acid (identified by TLC after inducing its partition into $CHCl_3$ by mild acidification).

Metabolic Pathways Related to the Enhanced Labeling of PC

Two studies have been carried out in evaluating the possible pathways for enhanced labeling of PC. First, we tested whether radioactive choline incorporation into PC was increased in A23187-treated cells as a way of estimating the contribution made by the reaction of diglyceride with CDP-choline. Mast cell suspensions were preincubated 60 min at 37 °C with 2 mM [^{3}H]choline since previous studies suggested that maximal labeling of the soluble choline pools may be obtained under such conditions [57]. Subsequently, incubations in the presence and absence of A23187 were performed at 25 °C as in other lipid labeling experiments. As shown in Table

1*A*, there was no consistent or significant difference in the amount of labeled PC between control and stimulated samples over the same 3-min period where enhanced fatty acid incorporation was consistently observed. Although experimental approaches in which there is a substantial baseline of PC labeling (as in the present case) may preclude detection of minor changes in PC labeling, the inability to observe any consistent trend suggests that the synthesis of PC from CDP-choline and DG is not amplified. Consequently, other mechanisms should be considered to explain the stimulated PC labeling observed with [^{14}C]linoleate.

In the second study, we compared the positional specificity of esterification of the glycerol backbone of PC and PA (the latter, a general precursor in phospholipid biosynthesis) using [^{14}C]linoleate and [^{14}C]palmitate. Linoleate constitutes 11% of the total detectable fatty acid of PC from mast cells [58] and is incorporated primarily into the glycerol β-position of mammalian phospholipids [1]. Palmitic acid constitutes 27% of the fatty acid of PC from mast cells [58] and is typically located at the glycerol α-position [2]. The results are presented in Table 1*B* and *C*. Part *B* shows the distribution of both labeled palmitate and linoleate among the phospholipids that was obtained in one of three similar experiments. The basic finding that A23187 selectively stimulates incorporation into PC (as in Fig. 2) is confirmed for both fatty acids. In each of the three experiments, the Δ incorporation (stimulated minus control) for palmitate *vs.* linoleate into PC were very similar although the level of labeling of PC in the control was always lower for palmitate. The absolute magnitude of Δ incorporation into PC varied between the three experiments; however, the variations were paralleled by variations in net (stimulated-control) histamine release.

Part *C* of Table 1 summarizes the results of the three analyses of positional incorporation of palmitate and linoleate into PC and PA. In each case the data are presented in two forms. First, the percentage distribution of label has been calculated, taking 100% to be the sum of radioactivity associated with the hydrolysis products LPC (or LPA) and free fatty acid (FFA). The extent of hydrolysis averaged 82% for all samples analyzed; consequently, the label distribution between the products should generally reflect the positional labeling of the parent phospholipid. Second, the ratios $\frac{\text{DPM stimulated LPC}}{\text{DPM control LPC}}$ and $\frac{\text{DPM stimulated FFA}}{\text{DPM control FFA}}$ have been determined following corrections for differences in percent hydrolysis and recovery of radioactivity. Values significantly >1.0 indicate enhanced incorporation in stimulated samples, while the relative magnitudes of the lysophosphatide and fatty acid ratios aid in the evaluation of selective positional labeling. From the percentage distribution (% label) figures, it is evident that A23187 treatment leads to little, if any, significant change in the positional labeling of PA with either fatty acid whereas PC exhibits a slight enhancement in β-position labeling by linoleate and a dramatically enhanced incorporation into this position with palmitate. In the case of linoleate, increased β-position labeling of PC would be expected regardless of whether the stimulation mechanism entailed selective acylation or amplification of *de novo* synthesis [1, 8, 63]. However, the major shift in distribution for PC labeling observed using palmitate indicates, first, that the ionophore-enhanced incorporation strongly favors the β-position and, second, that the putative acyl transferase appears to use the most abundant substrate [8]. The stimulated/control labeling ratios for lysophosphatide and fatty acid shown in Table 1 support these observations, since for PC $\frac{\text{DPM stimulated FFA}}{\text{DPM control FFA}} > \frac{\text{DPM stimulated LPC}}{\text{DPM control LPC}}$, and further illustrate two additional features of the labeling process. First, since the ratios observed for products of PA hydrolysis are not significantly different and both close to 1.0, A23187-induced changes in PA labeling typified by Fig. 2 probably involve the whole PA molecule. Second, in the case of PC, although the FFA ratio is significantly higher than that for LPC, the LPC ratio is >1.0, suggesting that α-position labeling may have been stimulated to a small extent. α-position labeling may reflect either the biosynthesis of PC from PA through DG, which went undetected in the choline incorporation experiment, or a minor direct α-acylation of β-lysophosphatidyl choline. Overall, this second study provides the strongest evidence that A23187-enhanced β-position labeling of PC occurs by a mechanism distinct from pathways involving PA as a biosynthetic precursor.

Correlation of Histamine Release and PC Labeling

Comparison of Figs. 1 and 2 indicate that histamine release and ionophore-enhanced labeling of PC proceed in parallel. To check whether these processes could be correlated quantitatively, a plot was constructured of histamine release *vs.* Δ incorporation of linoleate in PC using the data of six separate experiments. The results shown in Fig. 3 appear to fall into two categories. First, data from samples incubated up to and including 3 min appear linearly related; linear regression analysis gives a line (shown in the figure) with a correlation coefficient of 0.89. Data obtained from samples incubated longer than 3 min form the second category and are more scat-

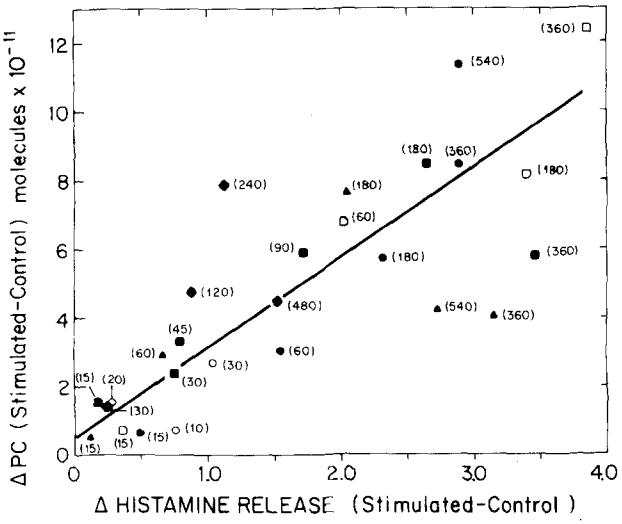

Fig. 3. Correlation plot of enhanced (stimulated minus control) incorporation of [^{14}C]linoleate into phosphatidyl choline *vs.* the net (stimulated minus control) histamine release for mast cell preparations. The data expressed as molecules of labeled PC and μg of released histamine for samples of identical size have not been normalized either per cell or on a percent population basis in order to avoid any implicit assumptions that the cells are uniformly responsive to the stimulus. Data were obtained in six separate experiments (each indicated by a different symbol) in which mast cell preparations exhibited different degrees of purity: ●, 87%; ▲, 82%; ■, 86%; ○, 91%; ♦, 95%; □, 95%. The numbers next to each data point indicate the time of incubation in seconds after addition of A23187 or ethanol. Linear regression analysis of all results obtained for ≤3 min incubation (n = 19) yielded the solid line (correlation coefficient = 0.89)

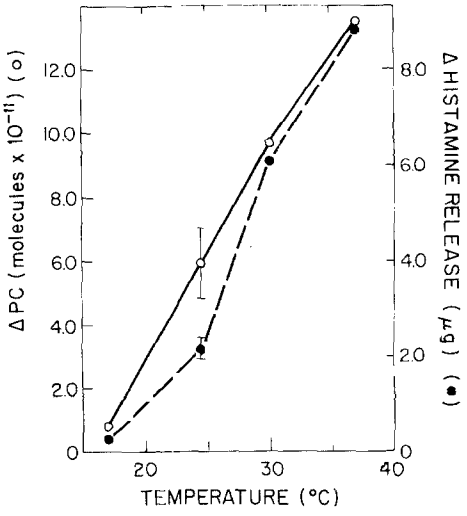

Fig. 4. Comparison of enhanced PC labeling with [^{14}C]linoleate and stimulated histamine release during 1 min incubation as a function of temperature. The values plotted represent the differences observed between control and A23187-treated cells where duplicate samples were tested both for histamine secretion and fatty acid incorporation. The maximum discrepancy in the duplicates was observed for the samples incubated at 24 °C, and the range of the Δ values is indicated by vertical lines

tered about the correlation curve. At these more extended timepoints, we have observed that the overall incorporation of fatty acids in stimulated preparations is variably sustained or decreased and that the rate of histamine release declines to different extents from experiment to experiment; no systematic change in the relationships of two processes is apparent.

To test the correlation further, histamine release and enhanced linoleate incorporation into PC were compared at different temperatures—17, 25, 30 and 37 °C. An incubation time of 1 min was chosen to insure that the rapid phase of secretory discharge would be examined at each temperature. The results shown in Fig. 4 indicate that the two processes proceed to proportionate extents over the entire temperature range.

Enhanced PC Labeling in A23187-Treated Peritoneal Cells

The data examined by correlation in Fig. 3 were obtained in experiments in which mast cell purity varied from 82–95%. Since the ionophore can stimulate calcium-mediated events in a variety of cell types, the question arises as to the extent that cellular contaminants of the preparation could contribute to the observed labeling. Although the slope of the correlation curve is insensitive to mast cell purity over the range studied (suggesting that contaminating cells considered as a group are not exclusively involved in the process), the data cannot rule out the presence of an individual cell type that as a persistent contaminant could be responsible for the enhanced fatty acid incorporation. Therefore, we examined fatty acid incorporation using unfractionated peritoneal cells (containing only 5–10% mast cells). The results obtained using 1.5 μM palmitic acid or 1 μM linoleic acid and 3 min incubation at 25 °C in the presence and absence of A23187 are shown in Table 2*A*. This experiment represents one of four such studies and was carried out in parallel with that for purified mast cells (Table 1*B* and *C*). Enhanced selective labeling especially of PC is very similar in magnitude to that obtained for purified mast cells. Part *B* of Table 2 represents an analysis of the positional specificity of acylation of PC; as in the case of purified mast cells, stimulated incorporation is highly specific for the β-position.

The Localization of Enhanced PC Labeling to Mast Cells

Since the preceding studies suggested that cell types other than mast cells could be responsible for the observed PC labeling, we sought direct evidence of mast cell participation. For this purpose we have devised a multiple-step processing procedure applied post-incubation (presented under Methods) that leads to a selective retention of PC in cell samples, thereby enabling visualization by autoradiography of PC-dependent differences in labeling density between control and A23187-treated samples.

[^{3}H]palmitate was used for fatty acid labeling because it has a specific radioactivity suitably high for autoradiographic studies and especially because A23187 causes at least a fourfold increase in radioactive PC over the levels observed in control samples. However, since most naturally occurring phospholipids contain saturated fatty acid esterified at the glycerol α-position [2], palmitate labeled phospholipids in stimulated cells are expected to contain two saturated fatty acid chains. Osmium tetroxide will not crosslink and immobilize saturated fatty acids; consequently, other means for retaining PC had to be explored.

The approach taken to preserve labeled PC in fixed cells is based on a procedure developed by Saffitz et al. [50] that employs tannic acid as a selective mordant for choline-containing phospholipids [30]. Modifications have been introduced to insure removal of labeled species other than PC to a level sufficient to create a difference in autoradiographic grain density between control and stimulated preparations ascribable to enhanced PC labeling. The procedure has been validated by monitoring all solutions for released radioactivity and by TLC of lipid extracts of both partially processed cells and processing solutions that contained significant amounts of extracted label.

The Retention of Labeled PC during Autoradiographic Processing

Table 3 shows the extent of extraction of total radioactivity (>98% free fatty acid) in the series of processing solutions used to prepare cell samples for autoradiography. Evidently the bulk (~90%) of radioactivity is removed by step 1. In combination principally with step 6, the cell-associated radioactivity is reduced to ~2% of initial levels. 70% acetone removes most of the remaining extractable radioactivity, and the considerably smaller amounts of label extracted in more concentrated acetone suggests that cell-associated free fatty acid had been reduced to very low levels.

Table 2. Phospholipid labeling in peritoneal cells (7% mast cells)

A. Fatty acid incorporation in the absence and presence of A23187

Species	[^{14}C]palmitate 1.5 μM		[^{14}C]linoleate 1 μM	
	Control	Δ[a]	Control	Δ[a]
LPC	106	6	105	131
SPH	591	134	338	34
PC	614	2516	900	1512
PS, PI	82	25	150	526
PA	162	66	162	64
PE	518	407	241	−1

B. Position specificity of acylation in PC

	% Label[b]			DPM stim LPC[c]	DPM stim FFA[c]
	LPC	FFA	SD	DPM cont LPC	DPM cont FFA
Control, palmitate	29.4	70.6	2.5	0.9 ± 0.1	5.9 ± 1.9
Stimulated, palmitate	5.9	94.1	0.1		
Control, linoleate	11.8	88.2	0.2	1.1 ± 0.1	2.8 ± 0.7
Stimulated, linoleate	5.0	95.0	1.5		

[a] Δ represents the difference stimulated-control. All values shown in *A* represent molecules × 10^{-9} for 4.0 × 10^5 peritoneal cells as calculated from the specific radioactivity of the fatty acid used.
[b] % Label in *B* refers to the distribution of radioactivity between the hydrolysis products of phospholipase A_2 action and thereby the α- and β-hydroxyl groups of the parent phospholipids. Standard deviations (SD) are given for two determinations of positional labeling of PC.
[c] DPM stim LPC/DPM cont LPC, DPM stim FFA/DPM cont FFA are ratios of radioactivity calculated for stimulated and control samples after correcting for differences in % enzymatic hydrolysis and recovery of radioactivity. For 8 experimental samples, % enzymatic hydrolysis was 94 ± 8 (x ± SD) and recovery was 82 ± 6 (x ± SD). As in Table 1*C* a ratio significantly >1.0 indicates enhanced positional labeling.

Table 4*A* compares the distribution of [^{3}H]palmitic acid among lipid species resolved by TLC from control and A23187-treated mast cell preparations both initially (frozen in liquid nitrogen before any processing) and just prior to tannic acid treatment (step 8). The results (corresponding to the study reported in Table 3) were obtained with cell preparations that had been incubated in 0.3 μM [^{3}H]palmitate; higher concentrations of fatty acid (0.5, 0.8 μM) used in two separate experiments gave very similar distributions of radioactivity. Evidently, the retention by the cell samples of labeled PC is excellent while other phospholipids are variably retained. The loss of PA, which constitutes the largest contribution to phospholipid labeling in control samples, is negligible. Radioactivity associated

Table 3. The distribution of radioactivity in processing solutions during preparation of mast cells for autoradiograhy

Processing step[a]	Solution	Percent total label	
		Control	Stimulated
1, 2	Incubation medium + 20 mg/ml BSA diluent + 6% ficoll solution	89.7	89.7
3	Aldehyde fixative	3.3	3.1
5	Tris quench	0.4	0.4
6	BSA (10 mg/ml) washes	3.9	3.6
7–9	Cacodylate buffer + tannic acid	0.5	0.3
11	Acetate buffer + magnesium uranyl acetate	0.4	0.4
12	70% Acetone	0.8	0.7
12	95% Acetone	0.1	0.1
12	100% Acetone	0.08	0.08
13	Acetone-Epon 812	0.05	0.08
	Label retained by mast cells[b]	<0.7	<1.6

[a] Step numbers correspond to those listed in Methods. OsO_4 fixative (step 10) contained a negligible amount of radioactivity.
[b] Label retained by mast cells is a projected value for specimens at the end of processing. The measurement of cell-associated radioactivity was not directly determined past step 8.

Table 4.

A. Distribution of [^{3}H]palmitic acid among mast cell lipids[a]

Species	1.5 min incubation			
	Control		Stimulated	
	Initial	Pre-tannic acid	Initial	Pre-tannic acid
Origin	328	402	348	282
LPC	342	102	863	116
SPH	8160	488	8440	787
PC	5046	4907 (97%)	32714	30135 (92%)
PS, PI	805	688	988	699
PA	8696	8896	9058	8110
PE	3155	112	3338	113
Fatty acid + Neutral lipid	1353908	24821 (1.8%)	1296498	24286 (1.9%)

B. Projected label distribution for mast cell samples post-processing based on TLC of processing solutions (steps 9–12)

Species	Control	Stimulated
Origin	296	214
LPC	68	44
SPH	339	634
PC	4476 (89%)	28609 (87%)
PS, PI	565	627
PA	8495	7922
PE	88	62
Fatty acid + Neutral lipid	<1000	<2000
Expected % PC	29	71
DPM Stimulated / DPM Control	2.6	

[a] All values represent average disintegrations per minute for duplicate samples from the same experiment. The duplicates agreed with each other within 10–15%. Values in parentheses indicate the extent of retention of the species relative to the initial sample. Based on the efficiency of TLC extraction for fatty acid (average = 70%), the values for fatty acid shown in *A* are ~70% of what is actually expected in cells at this stage of processing. An accurate estimate of the distribution of radioactivity among lipid species is critical in establishing whether autoradiography can be used to detect enhanced PC labeling in mast cells. We have carried out the following procedure in completing this projection, especially with regard to the final level of radioactive fatty acid associated with cellular specimens. (1) Processing solution generated by steps 9–12 were subjected to lipid extraction and TLC; (2) radioactivity associated with individual lipid species was determined; (3) the results were summed for all species and the recovery of the total radioactivity present in the initial processing solution was calculated. Although incomplete recoveries are likely to reflect selective loss of free fatty acid, the missing radioactivity was divided among individual species in proportion to their levels assayed from the TLC plate. In this way the projection shown reflects the maximal expected loss of phospholipids and the minimal expected loss of fatty acid.

with PS + PI and at the origin are only slightly reduced, whereas major decreases are observed for label at the level of SPH and PE and for LPC (although in the latter case the total radioactivity in question is rather small). In three different experiments we observed that most of the radioactivity associated with SPH was removed from the cells (and could be identified in extracts of the processing solutions by TLC) during step 6. Thus the level observed in the pre-tannic acid sample probably accurately reflects the actual amount of this species remaining with the cells[2]. For PE approximately half the amount appearing to be lost from the cells is recovered in the dilution medium of the first step. The remainder may have been crosslinked during aldehyde fixation to other cellular components and thereby rendered not extractable into $CHCl_3$.

The label distribution shown in Table 4*A* is considered to be comprehensive since systematic scraping, elution, and scintillation counting of the entire silica surface of chromatograms failed to identify radioactivity in unexpected regions. However, the recovery of radioactivity through TLC of the extracts of processing solutions averaged only

[2] We cannot provide an explanation for the extraction into BSA-containing solutions of cell-associated radioactivity tentatively identified as SPH. Intact phospholipids such as SPH have very low solubilities in aqueous media and characteristically do not exhibit strong interactions with BSA. For our present purposes, the extraction is fortunate since it reduces the level of labeled species other than PC remaining associated with the cells.

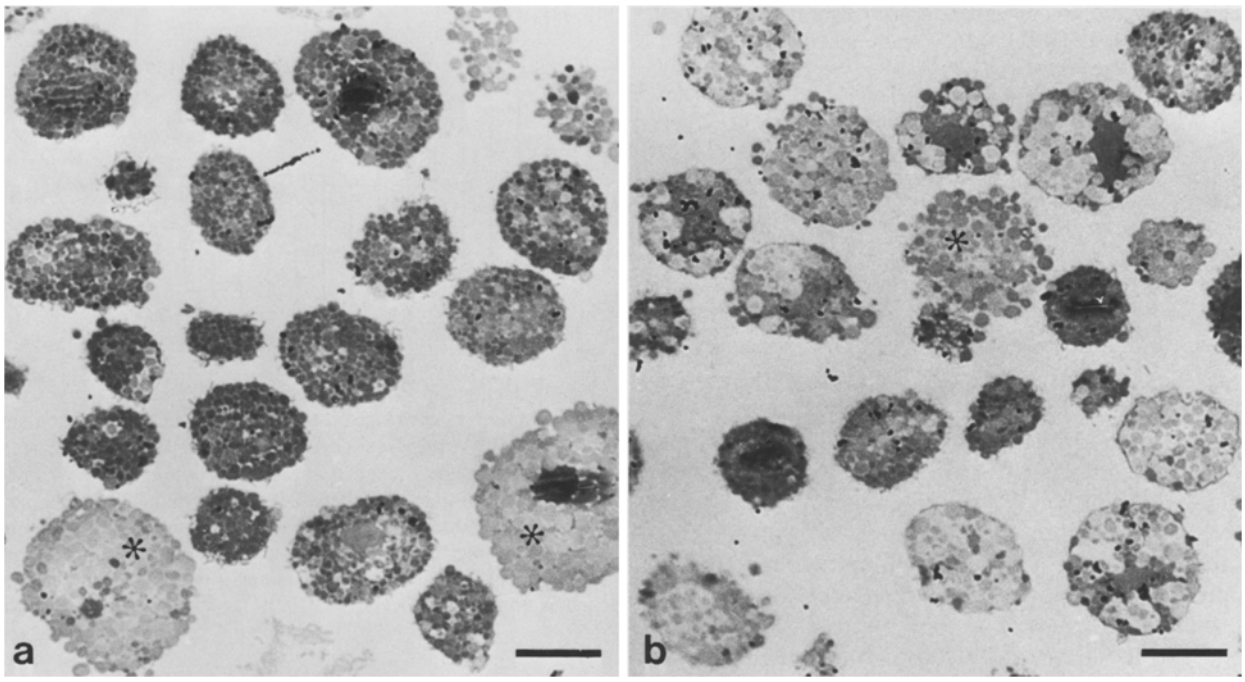

Fig. 5. Light microscope autoradiograms of mast cells labeled with [^{3}H]palmitate and exposed for 14 days. (*a*): Control preparation predominantly showing intact mast cells exhibiting little evidence of exocytosis and generally low levels of overlying autoradiographic grains. Two enlarged cells (*) (referred to as damaged or swollen in the text) can be seen. (*b*): Preparation that has been stimulated with A23187. Evidence of discharge indicated by numerous images of partially dispersed granule content within membrane-bounded pockets can be seen in many cells. Generally the autoradiographic grain density is higher than in *a*, especially over many of the discharging cells. Again, expanded and broken (*) cells can be seen. Magnification *a*,*b* is × 1,100 (bar corresponds to 10 μm)

~70%, due in large part to the incomplete extraction of free fatty acid into $CHCl_3$; consequently, the values of radioactivity shown for fatty acid and neutral lipid in Table 4*A* are likely to be underestimates. These losses have also been taken into account in Table 4*B* where the label distribution expected for fully processed mast cell samples has been projected. Table 4 indicates that almost 90% of the radioactive PC has been retained in both control and stimulated samples. Labeled PA is also very efficiently retained (≥90%), and we have not identified ways of suppressing its labeling during incubation or achieving its selective removal during processing. Despite the retention of PA, the procedure leads to a relative enrichment of labeled PC to the extent that it represents an estimated 29 and 71% of the total radioactivity, respectively, of control and stimulated cells. Since we have shown that radioactive fatty acid (specifically palmitate) is detected nearly exclusively in species that are $CHCl_3$-soluble, the enrichment allows the prediction that autoradiographic grains overlying mast cells in stimulated preparations will be 2.6-fold higher in density than those overlying cells in control samples if mast cells are responsible for the enhanced PC labeling.

Autoradiographic Studies

Photomicrographs (from one of two experiments) typifying the autoradiographic samples prepared in parallel with the samples used for the TLC analyses described above are presented in Fig. 5. Cells of controls are replete with secretion granules (Fig. 5*a*) while those in stimulated preparations show images of extensive compound exocytosis (Fig. 5*b*). Autoradiographic grains overlay cell profiles almost exclusively with very little background (extracellular) labeling. The number of grains associated with A23187-treated cells is apparently higher than that for unstimulated cells, thus supporting the contention that mast cells are responsible for stimulated fatty acid incorporation into PC. Contaminating cell types were rare in preparations used in the autoradiographic studies. Where observed, such cells (primarily lymphocytes and macrophages) occurred as aggregates, and no obvious difference was visualized in their degree of labeling between control and stimulated preparations. In both types of samples shown in Fig. 5 damaged cells constitute ~15% of the overall population; they show either extensive swelling or partially dispersed granules and are gen-

Table 5. Overall autoradiographic grain density for mast cell preparations

Sample	Cells counted	Total grains	Total area (mm^2)	Grains/area
Control	194	719	27.85	25.8
Stimulated	113	955	18.41	51.9
			$\frac{\text{Stimulated}}{\text{Control}}$ =	2.0

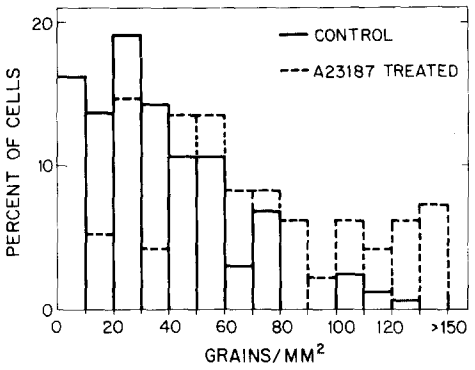

Fig. 6. Histogram of the autoradiographic grain density for [^{3}H]palmitate-labeled mast cells incubated in the absence and presence of A23187. Densities have been tabulated in increments of 10 grains/mm^2 except at the extreme right of the abscissa where all cells exhibiting densities greater than 150 grains/mm^2 have been grouped. The latter grouping was not done for statistical calculations in which all intervals were of equal size. Evidently stimulated preparations are enriched in mast cells having higher grain densities.

erally less heavily labeled compared to the rest of the cells[3].

We carried out two types of quantitative measurements on the autoradiograms. First, the numbers of overlying grains and surface areas were measured for a large population of cell profiles (including those which were either swollen or disrupted since many contain detectable labeling above background). The total number of grains and total surface area were used to compute an overall grain density, which is expected to be proportional to radioactivity per cell volume. The ratio of densities obtained for stimulated and control samples (2.0 in Table 5) agrees reasonably well with the ratio of DPM (stimulated/control) predicted on the basis of TLC analysis (2.6 in Table 4).

In the second type of measurement, we sought to verify statistically that mast cells in control and stimulated preparations are labeled at significantly different levels. In this case, the ~15% swollen and disrupted cells present in each sample were excluded from consideration. Distributions of grain densities were created by grouping the results tabulated for individual cells in increments of 10 grains/mm^2 in order to plot a histogram (Fig. 6). The distributions appear substantially different with A23187-treated preparations displaying a larger number of cells having higher grain densities; the average grain density for control cells (n = 161) was 34.2 grains/mm^2 compared to 68.9 grains/mm^2 for stimulated cells (n = 96). For statistical analysis we could not assume a normal distribution of grain density especially for A23187-treated cells since after 1.5 min stimulation the labeling response is not only partial but also may involve a fraction of the cells treated. Thus, we tested statistically whether the results obtained for control and stimulated preparations could have arisen from the same population of cells using the nonparametric two-sample test of Kolmogorov and Smirnov [55]. The analysis indicated that the probability is less than 0.1% that such a hypothesis is appropriate. Therefore we consider the data to provide strong support that A23187 causes the stimulated labeling of PC in mast cells.

PC Labeling in Peritoneal Cells Stimulated with Compound 48/80

The direct demonstration that A23817 causes enhanced β-position acylation in PC in mast cells provides a basis for comparing responses to more selective secretory stimuli that operate via cell surface receptors. A preliminary experiment of this kind has been carried out on unfractionated peritoneal cells using compound 48/80, a selective mast cell agonist [41]. The results are shown in Fig. 7 using [^{14}C]oleate which exhibits very similar labeling patterns to linoleate. As in Fig. 2*b*, an enhanced selective incorporation of fatty acid into PC is observed. The Δ incorporation (stimulated-control) for 4×10^5 peritoneal cells is an order of magnitude smaller than that obtained when A23187 is added to the same number of purified mast cells. However, mast cells constitute 10% of the peritoneal population used and are expected to be uniquely responsive to compound 48/80 [41]. The ordinate in Fig. 7 then reflects net incorporation by 4×10^4 mast cells, and the magnitude and distribution of labeling

[3] When mast cells were processed by procedures not involving fatty acid removal, damaged cells having a similar appearance were not detected. In the present case these cells never exhibited exocytotic figures, and we suspect that they represent a small population of unresponsive cells that is especially susceptible to damage initiated by exposure to large amounts of defatted BSA.

among phospholipid species is quite similar to that observed using A23187.

Discussion

The original strategy of using the calcium ionophore A23187 as a secretory stimulus for mast cells was to reduce the complexity of the supposed cascade of events that result in exocytosis [14, 24, 29] by bypassing interactions with primary surface receptors. In this way we hoped to identify processes possibly closely related to the mechanism of secretory discharge. The discovery that fatty acid labeling of PC is selectively amplified in stimulated cells has prompted us to probe in detail the nature of the enhanced incorporation and the degree to which it is commensurate with the discharge of histamine.

Fatty Acid Labeling

Since fatty acid rapidly equilibrates into cell membranes and exocytosis in mast cells did not influence the level of fatty acid binding to cells, we felt justified in considering mechanisms by which the calcium ionophore could affect reactions directly related to PC production and/or utilization and not merely substrate availability for PC synthesis. In this respect, the results of the analysis of position specificity of incorporation into the glycerol backbone of PC and PA were most definitive, consistently indicating that enhanced labeling was to a large extent specific for the glycerol β-hydroxyl group of PC and that, at most, only a small portion of stimulated PC labeling could reflect PC biosynthesis.

The possible ways leading to enhanced fatty acid incorporation include amplified fatty acid activation, increase in the concentration or availability of the acceptor, α-lysophosphatidyl choline, or stimulation of the putative acyltransferase. The apparent absence of a calcium requirement for both fatty acid activation [22] and acylation [17, 62] coupled with the β-position specificity of incorporation and the established Ca^{2+} dependence of most phospholipases A_2 [3, 15, 20, 21, 61] suggest that the presence of the ionophore may activate phospholipase A_2, thereby increasing the available acceptor.

Correlation with Stimulated Histamine Release

The data presented in Fig. 3 showing an apparent quantitative relationship between stimulated incorporation into PC and net rapid release of histamine from mast cell preparations treated with A23187 are in agreement with the initial hypothesis that the action of a phospholipase A_2 could be involved in

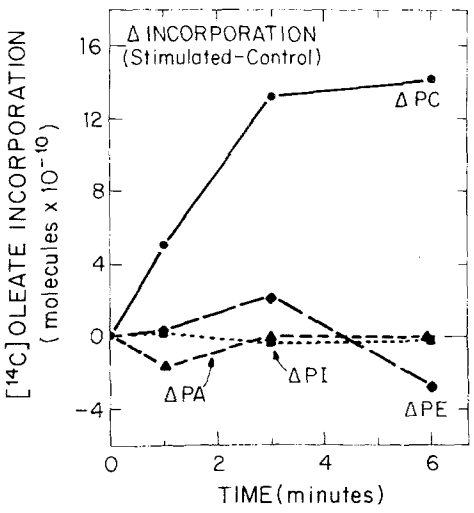

Fig. 7. The difference (Δ) in incorporation (stimulated-control) of [^{14}C]oleate into specific phospholipids of 4.0×10^5 unfractionated peritoneal cells in response to 4 μg/ml compound 48/80. Incubation time at 24 °C is plotted on the abscissa; since the peritoneal population used contained 10% mast cells, the values given on the ordinate relate to a population of 4.0×10^4 mast cells. ●——●, ΔPC; ♦— —♦, ΔPE; ▲— —▲, ΔPA; ■----■, ΔPI

the discharge process. Similarly, the parallelism observed for 1-min incubations at temperatures ranging from 17 to 37°C (Fig. 4) is also supportive, although the principal intention of the latter study was to check that the correlation is not lost as in the case of histamine release *vs.* enhanced incorporation of inorganic phosphate into PA, PI, and PC of mast cell preparations [31]. The limitations of such correlations must be stressed especially because our assay is indirect (phospholipase action has only been inferred) and because of the known diversity of effects of A23187 on eukaryotic cell structures and function. It is not possible at present to distinguish whether the processes are functionally independent expressions of cellular stimulation or whether enhanced incorporation could be related to secretory discharge.

Two studies already can be viewed as necessary in attempting to relate enhanced PC labeling in mast cells to stimulated cellular processes. First, its kinetics and magnitude must be carefully checked in relation to the release of prostaglandins and other mediators of inflammation and local homeostasis under the same experimental conditions since acylation may reflect utilization of lysophosphatides generated during liberation of precursor arachidonic acid. Prostaglandin production by mast cells in response to IgE and A23187 has been reported [35, 46]; at 37 °C it lags in large part behind secretory discharge and apparently involves considerably

larger quantities of fatty acid (a minimum of threefold more over the period of exocytosis (Fig. 4 in [35]) than are involved in acylation of PC (shown herein for 37 °C in Fig. 4).

The second study involves more direct approaches to characterizing the phospholipase(s) A_2 whose stimulation is presumed to lead to enhanced PC labeling. Two recent reports examined phospholipase A_2 action in rat mast cells and basophilic leukemia cells and suggest its possible involvement in histamine release. N-substituted derivatives of PS have been identified as potent inhibitors of phospholipase A_2 activity of mast cells and their application leads to parallel inhibition of phospholipase action and histamine release not only in the presence of a primary stimulus, concanavalin A (supplemented with PS as a potentiator), but also with A23187 [37]. An analogous inhibition of fatty acid release and histamine secretion by phospholipase inhibitors *p*-bromophenacyl bromide and mepacine has been characterized using basophilic leukemia cells [38]. Additionally, the latter study suggests that A23187 may elicit the release of a spectrum of fatty acids from briefly prelabeled cells whereas IgE causes selective release of arachidonate [14]. Evidently these lipolytic processes should be subjected to a detailed investigation in which the entire spectrum of substrates and products of uniformly prelabeled cells are qualitatively and quantitatively identified.

PC Labeling in Peritoneal Cells

Our results showing that unfractionated peritoneal cells carry out an acylation reaction analogous to that observed with purified mast cells underscore our hesitancy in suggesting that functional relationships might exist between stimulated phospholipase action and the secretory process. Regardless of the apparent wide variation in the degree of specialization of these cells for the extracellular discharge of stored products, we are not dissuaded from examining more closely the possible role of phospholipase action in exocytosis for a number of reasons. First, as may be the case for basophilic leukemia cells, A23187 may amplify multiple phospholipase activities, some of which are not closely related to secretory discharge. Second, it may be possible to rationalize all or a part of the enhanced labeling of other cell types as related to membrane interactions involved in endocytosis as well as covert [42] secretion. Third, adrenal cortical cells, which do not appear to use exocytosis for releasing accumulated steroids, exhibit no enhanced fatty acid incorporation into PC in the presence of A23187 when examined in experiments very similar in design to our own [53].

Autoradiographic Studies

The abilities to use palmitic acid as a label for the β-position of PC in stimulated cells and to obtain a selective, thorough retention of labeled PC during processing of specimens for autoradiography have made possible the direct demonstration that amplified PC labeling occurs in mast cells. However, the extent of labeling of individual cells in both stimulated and control preparations is variable. Heterogeneity is particularly notable in stimulated samples where there is a very strong but not absolute correlation of grain density and visual evidence of compound exocytosis. These heterogeneities have led us to express quantitative results concerning labeling and discharge in relation to the total mast cell population rather than per individual cell. Although many of the autoradiographic grains formed over discharging cells (Fig. 5*b*) appear close to regions where compound exocytosis is evident, studies conducted at the electron microscopic level will be necessary to check whether stimulation increases the grain density over specific organelles.

Compound 48/80-Stimulated Labeling of PC

The results obtained with compound 48/80 can be viewed as a first step toward our ultimate objective of testing for analogous behavior by mast cells in response to primary stimuli. It will be most interesting to examine responses mediated by surface interactions of IgE not only because this method of triggering histamine discharge appears to express the main physiologic response of mast cells [34], but also because Ho and Orange [27] have reported a 120–200% increase over control in [^{3}H]oleic acid labeling of PE (rather than PC) in sensitized rat peritoneal mast cells challenged with antiIgE. Absolute levels of labeling were not reported; consequently, comparisons to the extent of PC labeling as well as the relatively low levels of PE labeling that we have observed ±A23187 is currently not possible.

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