Biochemical Pharmacology, Vol. 35, No 15, pp. 2535–2544, 1986.
Printed in Great Britain

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

IgE-DEPENDENT ACTIVATION OF MAST CELLS IS NOT ASSOCIATED WITH ENHANCED PHOSPHOLIPID METHYLATION

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(*Received* 5 *December* 1985; *accepted* 7 *February* 1986)

Abstract—Enhanced phospholipid methylation has been suggested to be an obligatory event in activation–secretion coupling in mast cells. However, we have consistently failed to demonstrate increased [^{3}H]methyl incorporation into either whole-lipid extracts or separated lipids to accompany IgE-dependent induction of histamine release from rat peritoneal mast cells despite variation of the experimental protocol, animals and animal diet. Although the presence of 3-deazaadenosine and homocysteine thiolactone reduced IgE-dependent histamine release, treatment with these compounds elevated mast cell cyclic AMP levels. We conclude that IgE-dependent activation of mast cells is not associated with enhanced phospholipid methylation and that inhibition of histamine release by methylation inhibitors may be due to other effects of the drugs.

Activation of mast cells and basophils by cross-linkage of plasma membrane-bound IgE and subsequent bridging of IgE-Fc receptors [1] initiates a complex series of membrane and cytoplasmic events which culminate in the non-cytotoxic secretion of preformed and newly generated mediators. Amongst these are an increase in calcium influx [2–6], stimulation of adenylate cyclase [5, 7–11], fusion of perigranular and cell membranes to facilitate extrusion of preformed mediators [12] and increased arachidonate production as evidenced by the release of newly generated cyclooxygenase and lipoxygenase products [13–15]. Two distinct pathways of membrane phospholipid metabolism have been suggested to be involved in activation–secretion coupling. The first, cleavage of polyphosphoinositides is initiated when mast cells are stimulated by either immunological or non-immunological mechanisms [16, 17]. Stimulation of this pathway has been shown to be associated with calcium mobilization following cell activation in many systems (reviewed in ref. 18). The second, enhanced phospholipid methylation, has been associated with only IgE-dependent mast cell stimulation and, as such, has been suggested to be one of the earliest events in mast cell activation [19]. However, the participation of phospholipid methylation in mast cell activation-secretion coupling has recently been questioned.

Experiments using rat erythrocyte membranes have indicated that the sequential methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) is catalyzed by two separate *S*-adenosylmethionine-dependent methyltransferases [20]. Methyltransferase I, which is associated with the cytoplasmic surface of the membrane, catalyses the methylation of PE to phosphatidyl-*N*-monomethylethanolamine (PMME). Following its reorientation towards the external surface of the membrane, PMME becomes methylated to phosphatidyl-*N*,*N*-dimethylethanolamine (PDME) and PC [21]. Phospholipid methylation has been suggested to be involved in signal transduction across membranes and has been associated with increases in membrane fluidity [20, 21], promotion of transmembrane calcium fluxes [22] and the facilitation of coupling of β-adrenoceptors to adenylate cyclase [23]. Furthermore, cleavage of PC by phospholipase A_2 has been suggested to generate lysophosphatidylcholine (lyso PC) and provide arachidonic acid for the synthesis of prostaglandins and leukotrienes [2, 24, 25]. The participation of membrane phospholipid methylation in mast cell activation–secretion coupling would, therefore, present an attractive hypothesis.

Evidence for enhanced phospholipid methylation accompanying mast cell activation was initially reported by Hirata *et al.* [26], who found that stimulation of rat mast cells for secretion with concanavalin A stimulated decarboxylation of phosphatidylserine (PS) to PE and subsequent methylation of this phospholipid to PC. More recent experiments performed by Ishizaka and coworkers have consistently demonstrated a transient increase in phospholipid methylation of up to 120-fold to be associated with IgE-dependent activation of rat mast cells [3, 9], human purified lung mast cells [5], mouse mast cells [4, 27] and cultured human cord-derived basophils [28]. The peak of [^{3}H]methyl incorporation into phospholipids, ranging from 10 to 15 sec in rodent mast cells to 30 sec in human lung mast cells, always precedes changes in cyclic AMP levels, [^{45}Ca]calcium influx and mediator secretion.

An obligatory role for phospholipid methylation in IgE-dependent activation of mast cells and basophils is suggested from the finding that inhibition of phospholipid methylation with inhibitors such as 3-deazaadenosine (DZA) or 3-deaza-*S*-isobutyryladenosine (3-deaza-SIBA) is associated with inhi-

bition of calcium ion influx and histamine secretion [2, 3, 5, 19, 28]. A close relationship between adenylate cyclase activation and phospholipid methylation is suggested by inhibition of both events in parallel by methylation inhibitors [5, 9, 19]. Further evidence of an obligatory role of phospholipid methylation is suggested by the observations that genetic deletion of methyltransferase I or II from rat basophil leukaemia cells renders them incapable of mediator secretion following IgE-dependent stimulation but not by stimulation with ionophore A23187 which bypasses these early events [29].

However, several recent publications have questioned the obligatory role of phospholipid methylation in IgE-dependent mast cell activation–secretion coupling. Boam *et al.* [30] and Moore *et al.* [31] have failed to confirm increased PE methylation following IgE-dependent or concanavalin A stimulation of rat mast cells. Vance and de Kruijff [32] have calculated that the extremely small changes in methylation reported in cell membranes are of insufficient magnitude to account for the many physiological responses attributed to them whilst Mio *et al.* [33] could find no evidence that *N*-methylation of PE increases the fluidity of phospholipid bilayers. In addition, we have shown that DZA may inhibit histamine release from human lung mast cells [34] and potentiate release from human basophils activated with A23187 [35], a secretagogue not associated with activation of phospholipid methylation [2, 29].

This paper describes our unsuccessful attempts to demonstrate increased lipid methylation following IgE-dependent stimulation of rat mast cells and examines the effects of DZA on the cyclic AMP response to immunological challenge.

MATERIALS AND METHODS

Materials. The following materials were used: L-α-Phosphatidylserine (PS), phosphatidylethanolamine (PE), monomethylphosphatidylethanolamine (PMME), dimethylphosphatidylethanolamine (PDME), phosphatidylcholine (PC), lysophosphatidylcholine (lyso PC), L-methionine, *S*-adenosylmethionine iodide (AdoMet), L-homocysteine thiolactone (Hcy), cyclic AMP (free acid), polyethylene glycol (molecular weight 8000) (Sigma Chemical Co., Poole, Dorset, U.K.); chloroform, methanol, *n*-propanol, propionic acid, ethanol, *n*-butanol, acetic acid, acetic anhydride, triethylamine, hydrochloric acid, trichloroacetic acid (analytical grade, BDH Chemicals, Eastleigh, Hampshire, U.K.); propan-2-ol, hexane (HPLC grade, Rathburn Chemical Ltd., Walkerburn, Scotland, U.K.); optiphase scintillant (LKB, Loughborough, Leicestershire, U.K.); LK5DF silica-gel plates (Whatman, Maidstone, Kent, U.K.); Metrizamide (Nyegaard, Oslo, Norway); anti-rat IgE (Miles Yeda, Slough, U.K.); 3-deazaadenosine (DZA) (Southern Research Institute, Birmingham, Alabama, U.S.A.); L-[^{3}H]methyl-methionine (12 Ci/mmol or 80 Ci/mmol) (New England Nuclear, Southampton, Hampshire, U.K.); [^{3}H-methyl]AdoMet (70–80 Ci/mmol), [^{14}C-methyl]choline chloride (50 mCi/mmol), ^{125}I-cyclic AMP (~2000 Ci/mmol) (Amersham International, Amersham, Buckinghamshire., U.K.). Mouse monoclonal anti-dinitrophenyl (DNP) IgE (prepared by the method of Liu *et al.* [36]) and dinitrophenyl-human serum albumin conjugate ($DNP_{13}HSA$, prepared by the method of Eisen [37]) were generous gifts of Dr. Teruko Ishizaka, Johns Hopkins University, Baltimore, MD, U.S.A.

HEPES-buffered salt solution (HBSS(−)) contained 137 mM NaCl, 5.5 mM glucose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 5 mM morpholinoethanesulphonic acid, 2.7 mM KCl, 0.4 mM NaH_2PO_4 and 0.05% gelatin. Buffer designated HBSS in addition contained 1.8 mM $CaCl_2.2H_2O$, 0.9 mM $MgCl_2.6H_2O$ and 30 μg/ml L-α-phosphatidylserine. Buffer pH was adjusted to 7.0 by addition of 10 M NaOH. Tris–A-EDTA buffer contained 120 mM NaCl, 25 mM Tris, 10 mM sodium ethylenediaminetetraacetic acid (EDTA), 5 mM KCl and 0.05% human serum albumin. The pH was adjusted to 7.6 using 10 M NaOH. All chemicals used in these buffers were of analytical grade.

Animals and diet. Male Sprague–Dawley or Wistar strain rats weighing 275–350 g were obtained from Charles River, Kent, U.K. They were maintained on Rat and Mouse No. 1 (Modified) diet (Special Diet Services, Witham, Essex, U.K.). American male Sprague–Dawley rats (275–350 g), supplied by Dr. T. Ishizaka, were obtained from Holtzman, Madison, WI, and were maintained on the diet used at her laboratory in Baltimore.

Mast cell purification and passive sensitization. Mast cells were harvested and purified as described by Ishizaka *et al.* [3]. Mast cells obtained by peritoneal lavage of rats using 2 × 10 ml Tris–A-EDTA were purified to >90% purity by centrifugation (500 *g*, 10 min) through 22.7% metrizamide. After washing in HBSS(−), mast cells were passively sensitized by incubation (37°, 1 hr) with 100 μg/ml mouse monoclonal anti-DNP IgE in 0.25 ml HBSS(−)/10^6 cells. Cells were then washed three times with HBSS(−) and finally in HBSS.

Histamine release. Duplicate 45 μl aliquots containing 1–2 × 10^5 mast cells were incubated for 10 min at 37° prior to challenge with 5 μl of antigen, anti-IgE or HBSS. Release reactions were allowed to proceed for 15 min prior to addition of 950 μl cold HBSS(−) and centrifugation (500 *g*, 5 min, 4°). Supernatant and cell pellet histamine content was assayed by spectrophotofluorimetry [38] and histamine release expressed as a percentage of cellular total corrected for spontaneous release measured in the absence of secretagogue.

In studies where the effects of DZA and Hcy on IgE-dependent histamine release were examined, these compounds were incubated with cells for 60 min prior to challenge. Spontaneous histamine release was unaffected by these compounds at the concentrations used.

Lipid methylation. Aliquots of mast cells in HBSS (2–4 × 10^6/ml) were incubated (25 min, 37°) with L-[^{3}H]methyl-methionine (4 μM, 12 Ci/mmole). Duplicate 45 μl aliquots of the suspension were transferred to 5 ml polypropylene tubes and incubation continued for 5 min. At various times following

addition of 5 μl of either antigen (final concentration 0.1 μg/ml), anti-IgE (final concentration 1%) or HBSS, the reaction was stopped by addition of 900 μl of ice-cold trichloroacetic acid (TCA) containing 10 mM methionine. Following centrifugation and washing of the precipitate with 1 ml 10% TCA (12,000 *g*, 10 min, 4°), cell lipids were extracted by agitation for 10 min with 3 ml of chloroform/methanol (2/1 v/v) [39]. To remove polar contaminants the lipid extract was washed twice by mixing with 1.5 ml of 100 mM aqueous KCl/methanol (1/1 v/v). One-millilitre aliquots of the lower chloroform phase were removed to glass scintillation vials and evaporated (3 hr, 80°) before addition of 10 ml of Optiphase scintillant. Radioactivity in the extract was measured by β-scintillation spectrometry using a Packard Tricarb 300 CD β-scintillation counter (Packard Instruments, Reading, Berkshire, U.K.) and expressed as pmole [^{3}H] by reference to the specific activity of [^{3}H]methyl-methionine used for cell labelling (12 Ci//mmol = 26,664 dpm/pmol).

In two experiments, the efficiency of the 10 min lipid extraction was demonstrated by >94% recovery of [^{3}H]dipalmitoyl-phosphatidylcholine in the chloroform organic phase following washing with methanol/KCl. Furthermore, in mast cells prelabelled with [^{3}H]methyl-methionine (4 μM) as described, increasing the time of extraction with chloroform/methanol up to 120 min did not increase the recovery of labelled lipid. No increase in recovery was obtained by increasing the temperature of extraction from ambient temperature to 37° or by sonicating during extraction. These findings therefore indicate that the routine 10 min lipid extraction was sufficient to recover all radiolabelled mast cell phospholipid, and that any changes in lipid labelling would not be masked by variable recoveries between samples.

Thin-layer chromatography of methylated lipids. Mast cells (5–10 × 10^6/ml in HBSS) were labelled by incubation for 30 min with L-[^{3}H]methyl-methionine (4 μM, 80 Ci/mmol). Duplicate 180 μl aliquots were challenged with 20 μl HBSS or antigen, 1.0 μg/ml, and following 10–120 sec incubation lipid extracts prepared as described above. One millilitre aliquots of chloroform phase were evaporated under N_2 and the residue redissolved in 50 μl of chloroform/methanol (2/1) containing authentic standards of PE, PMME, PDME, PC and lyso PC. Samples were applied to LK5DF silica gel plates and developed for four hours with *n*-propanol/propionic acid/chloroform/water (3/2/2/1). Lipid spots identified by iodine staining and corresponding with the known Rf value of authentic standards (PMME, 0.65; PDME, 0.59; PC, 0.48; lyso PC, 0.26) were scraped into scintillation vials, dispersed by vortexing with 400 μl methanol and 3 ml of Optiphase scintillant added. [^{3}H]methyl-label was quantified by β-scintillation spectrometry and incorporation calculated in pmol [^{3}H] as described above.

High-performance liquid chromatography of methylated lipids. Mast cells (10–20 × 10^6/ml) were dual-labelled by incubation for 30 min with L-[^{3}H]methyl-methionine (4 μM, 80 Ci/mmol) and [^{14}C]methyl-choline chloride (100 μM, 50 m Ci/mmol). Aliquots of 180 μl were then challenged with 20 μl of antigen, 1 μg/ml, and following 15–60 sec incubation, lipid extracts were prepared as described for TLC analysis. One-millilitre aliquots of chloroform phase were evaporated under N_2 gas and the residue redissolved in 25 μl of solvent A (propan-2-ol/hexane/H_2O 6/8/0.75) containing authentic standards of PE, PMME, PDME and PC. Lipid separation was performed by the method of Geurts Van Kessel *et al.* [40]. Following injection onto a silica-gel column (Waters Associates, μ Porasil), samples were eluted using solvent A at a flow rate of 1 ml/min. Five minutes following injection, a 15 min linear gradient was introduced which varied between 100% solvent A and 100% solvent B (propan-2-ol/hexane/H_2O 6/8/1.4). Eluted lipids were detected by u.v. absorption (206 nm) using a flow-cell spectrometer (Waters Associates, Model 450) the retention times of standards being PMME, 14 min, PDME, 22 min and PC, 28 min. Eluted material was collected at 30-sec intervals in scintillation vials and following addition of Optiphase scintillant radioactivity was measured by β-scintillation spectrometry. Results were corrected for different post-column recoveries of [^{3}H] between samples by reference to the recovery of [^{14}C]PC.

S-adenosylmethionine labelling. Aliquots of mast cells in HBSS (4–8 × 10^6/ml) were incubated (30 min, 37°) with L-[^{3}H]methyl-methionine (4 μM, 12 Ci/mmol). Following centrifugation and two washes of the cell pellet with 2 ml HBSS (1000 *g*, 1 min, 4°), cells were resuspended in HBSS to a density of 4–8 × 10^6/ml. After 5 min incubation at 37°, duplicate 45 μl aliquots of mast cells were activated with 5 μl of anti-IgE, 1%. At various times thereafter, reactions were stopped by addition of 200 μl ice-cold ethanol/100 mM HCl (9/1) and centrifugation (10,000 *g*, 1 min, 4°). Aliquots of 150 μl of supernatants were evaporated under N_2 prior to resuspension in 30 μl of water containing 20 μg of authentic AdoMet. Following chromatography on silica-gel plates (LK5DF) using a solvent of *n*-butanol/acetic acid/H_2O (12/3/5), plates were dried and AdoMet detected by u.v. absorption at 254 nm, ninhydrin staining and with reference to the R_f value of authentic L-[^{3}H]methyl-*S*-adenosylmethionine. These complementary methods of detection were necessary due to contamination of commercially-available authentic unlabelled AdoMet with other u.v.-absorbing and ninhydrin-staining material. Following elution from the gel by vortexing with 400 μl H_2O and addition of scintillant, [^{3}H]methyl-label in AdoMet was quantified by β-scintillation spectrometry and expressed as pmol/10^6 mast cells.

Cyclic-AMP determination. Duplicate 200 μl aliquots containing about 2 × 10^5 mast cells were incubated (60 min, 37°) with HBSS or DZA, 100 μM, and Hcy, 100 μM. At various times following challenge with 50 μl of 1/60 dilution of anti-rat IgE (Miles Yeda, Slough, Buckinghamshire, U.K.), the reaction was stopped by addition of 900 μl of ice-cold ethanol with vortex mixing. The tubes were then centrifuged (2500 *g*, 20 min, 4°), aliquots of the supernatants evaporated and cyclic AMP quantified by radioimmunoassay following acetylation [41]. All samples were on the linear part of the assay standard curve (30–1000 fmol cyclic AMP). Under the exper-

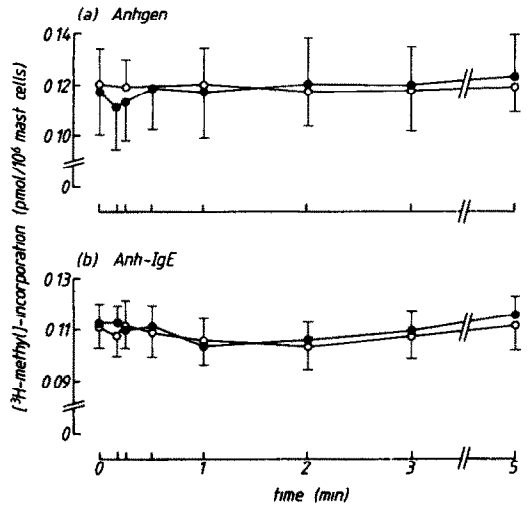

Fig. 1. [^{3}H]methyl incorporation into lipids of unstimulated (○) rat mast cells and mast cells challenged (●) with (a) antigen or (b) anti-IgE. Results are mean ± S.E.M. of duplicate samples of six experiments. In cells challenged with 0.1 μg/ml $DNP_{13}HSA$ antigen, spontaneous and net stimulus-induced histamine releases were 8.8 ± 3.7% and 18.1 ± 2.2% respectively. In cells challenged with 1% anti-rat IgE, spontaneous and net stimulus-induced histamine releases were 4.5 ± 0.5% and 25.6 ± 3.9% respectively.

imental conditions none of the drugs used interfered with the assay.

RESULTS

The effect of IgE-dependent activation on mast cell lipid methylation

The effect of $DNP_{13}HSA$ antigen activation on [^{3}H]methyl incorporation into chloroform/methanol extractable lipids was examined in six experiments in which baseline label incorporation was 0.12 ± 0.01 pmol/10^6 mast cells (Fig. 1a). Although antigen challenge resulted in 18.1 ± 2.2% histamine release, no changes in lipid labelling were observed to occur within 5 min of challenge. Similarly, challenge of mast cells with anti-IgE, which released 25.6 ± 3.9% histamine, did not significantly alter lipid labelling from a baseline of 0.11 ± 0.01 pmol/10^6 mast cells in six experiments (Fig. 1b). The use of mast cells from Wistar rats, which incorporated 0.086 ± 0.005 pmol label/10^6 mast cells, also failed to demonstrate enhanced lipid labelling to accompany the 13.5 ± 2.1% stimulation of histamine release by $DNP_{13}HSA$ antigen in three experiments.

To determine the distribution of [^{3}H]methyl labelling within chloroform/methanol-extractable lipids of unstimulated and stimulated mast cells, lipids were separated by silica gel thin-layer chromatography (TLC) in three experiments. Of the total radiolabel in the extract only 26.4 ± 4.7% was demonstrable in PMME and PDME, PC and lyso PC (Table 1). The major identifiable fraction of the label, 35.1 ± 2.5% was associated with the neutral lipids running at the solvent front which may represent methyl-esterification of fatty acids [42, 43]. Challenge of passively sensitized mast cells with $DNP_{13}HSA$ antigen (0.1 μg/ml) released 22.4 ± 3.6% histamine but caused no changes in labelling of phospho- or neutral lipids measured in samples at 10, 15, 30, 60 or 120 sec after challenge. Table 1 shows the results obtained 15 sec after challenge, the time of maximum increase in lipid labelling reported by Ishizaka *et al.* [3].

Results similar to those obtained using TLC were obtained in two experiments in which [^{3}H]methyl labelled lipids from unstimulated and stimulated mast cells were separated by high performance liquid chromatography (HPLC). In unstimulated cells (Fig. 2), 40.6% of the total lipid label (0.17 pmol/10^6 mast cells) was associated with neutral lipids at the solvent front, whereas only 14.3% was associated with methylated derivatives of PE. Of these PMME constituted 3.7%, PDME 5.3% and PC 5.3%. Despite inducing 39.3% net histamine secretion, challenge of cells with $DNP_{13}HSA$ antigen (0.1 μg/ml) produced no increase in [^{3}H]methyl incorporation into phospholipids at 15, 30 or 60 sec after challenge. At 15 sec after challenge the 12.1% of total lipid label (0.16 pmol/10^6 mast cells) identified in PE deriva-

Table 1. Distribution of [^{3}H]methyl label in mast cell lipid extracts

	Radiolabel (fmol/10^6 mast cells)	
	Unchallenged cells	15 sec post challenge
PMME	6.0 ± 1.5 (4.8 ± 1.2%)	5.8 ± 2.0 (4.8 ± 1.6%)
PDME	11.9 ± 1.2 (9.6 ± 1.0%)	11.8 ± 1.4 (9.7 ± 1.1%)
PC	13.2 ± 1.1 (10.6 ± 0.9%)	12.2 ± 1.6 (10.0 ± 1.3%)
lyso PC	1.7 ± 0.4 (1.4 ± 0.3%)	1.6 ± 0.2 (1.3 ± 0.2%)
Neutral lipid (solvent front)	43.6 ± 3.1 (35.1 ± 2.5%)	44.4 ± 3.4 (36.3 ± 2.8%)
Total applied	124.2 ± 7.3 (100%)	122.0 ± 6.9 (100%)

Total lipid extracts of unchallenged or antigen-activated cells were prepared and lipids separated by thin-layer chromatography as described. Results are mean ± S.E.M. of three experiments performed in duplicate in which spontaneous and net antigen ($DNP_{13}HSA$, 0.1 μg/ml)-induced histamine release was 8.5 ± 1.3% and 22.4 ± 3.6% respectively.

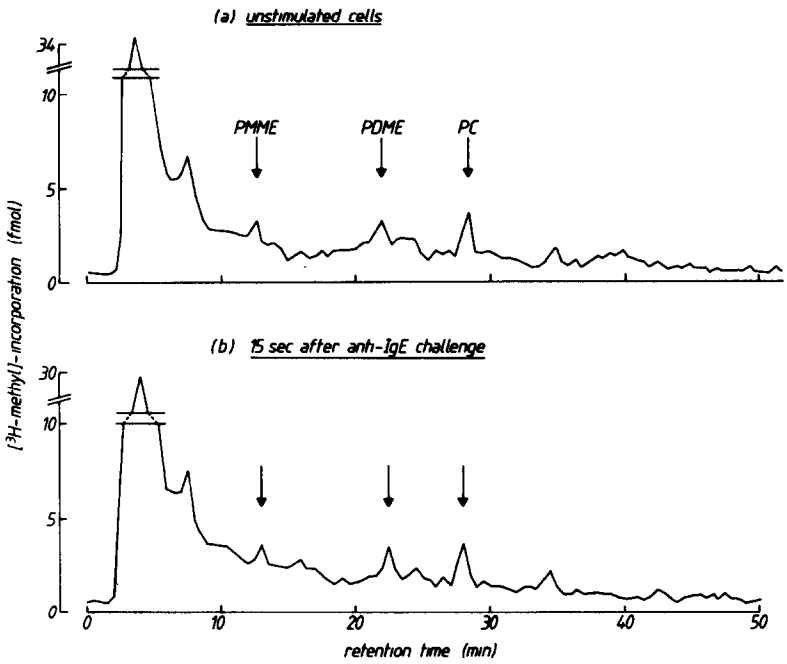

Fig. 2. High-pressure liquid chromatography of [^{3}H]methyl-labelled lipids of rat mast cells. Results are from one experiment in which lipid extracts were prepared 15 sec following addition of (a) buffer or (b) $DNP_{13}HSA$, 0.1 μg/ml. Incubation of cells with [^{14}C]methyl-choline as an internal standard resulted in incorporation of 253 and 229 fmol [^{14}C]methyl into PC in unstimulated and antigen-activated cells respectively. Spontaneous and net antigen-induced histamine release was 4.3% and 39.3% respectively. A further experiment of the same design gave similar results.

tives comprised 4.2% in PMME, 4.3% in PDME and 3.6% in PC.

As it has been reported that donation of methyl groups from AdoMet to phospholipids results in a reciprocal fall in AdoMet labelling following immunological activation of rat mast cells [3], the radioactivity associated with both chloroform/methanol extractable lipids and AdoMet was measured in three experiments. In unstimulated cells the label in the lipid fraction was 0.11 ± 0.02 pmol and that in AdoMet 1.25 ± 0.16 pmol/10^6 mast cells. Challenge with $DNP_{13}HSA$ antigen resulted in 23.1 ± 3.0% histamine release but no significant changes in [^{3}H]methyl incorporation into lipids or loss from AdoMet during the subsequent 5 min (Fig. 3).

Variations of experimental conditions

As we were unable to demonstrate increased lipid methylation during IgE-dependent activation of rat mast cells by adherence as closely as possible to published techniques [3], the experimental techniques were varied in an attempt to detect the transfer of [^{3}H]methyl groups to phospholipids.

The concentration of [^{3}H]methyl-methionine used to label the AdoMet pool was varied between 0.25 and 25 μM in one experiment. Although this resulted in a concentration-related increase in radio-labelling of both AdoMet and chloroform/methanol extractable lipids (0.16 pmol to 2.16 pmol and 0.09 pmol to 0.35 pmol/10^6 mast cells respectively), no enhancement of lipid methylation was observed at 10, 15, 30, 60 or 120 sec after challenge with sufficient anti-IgE to induce 41.0% histamine release.

The time of preincubation of cells with [^{3}H]methyl-methionine (4 μM) was altered over the range 30–120 min in two experiments. Labelling of the intra-

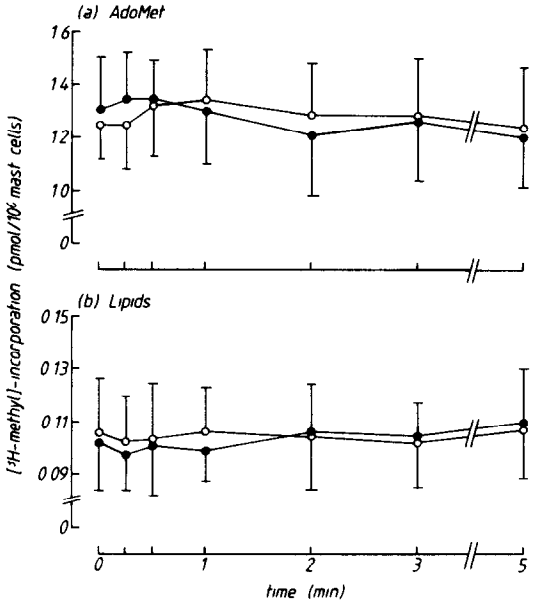

Fig. 3. [^{3}H]methyl Incorporation into (a) AdoMet and (b) total lipids of unstimulated (○) and antigen-activated (●) rat mast cells. Results are mean ± S.E.M. of duplicate samples of three experiments in which spontaneous and net $DNP_{13}HSA$ (0.1 μg/ml)-induced histamine release was 5.2 ± 0.8% and 23.1 ± 3.0% respectively.

cellular AdoMet pool increased hyperbolically with time up to a maximum of 2.21 pmol/10^6 mast cells at 90 min whilst lipid labelling increased approximately linearly throughout the incubation period, incorporation at 120 min being 0.38 pmol/10^6 cells. Despite inducing 32.3% histamine release, anti-IgE (1%) challenge did not increase the [^{3}H]methyl content of chloroform/methanol extractable lipids in any of the cell preparations at 10, 15, 30, 60 or 120 sec after challenge.

As prolonged storage of methionine results in its radiolytic conversion to contaminant compounds such as methionine sulphone and sulphoxide, the possibility that such compounds may be differentially incorporated into lipids of activated mast cells was tested in one experiment in which cells were incubated for 30 min with freshly synthesized [^{3}H]methylmethionine or a sample which had been stored at −20° for 1 year, conditions under which approximately 30% decomposition would be expected to have occurred. It was observed that in preparations incubated with old methionine, there was a 15-fold greater blank incorporation of [^{3}H]label into chloroform/methanol extracts as assessed by extraction of cells which had been incubated for <10 sec with radiolabel. This was presumably due to the presence of a radiolabelled contaminant, possibly methionine sulphone or sulphoxide, with a higher solubility than methionine in chloroform. In unstimulated cells, radiolabel associated with lipids was similar, being 0.11 pmol/10^6 mast cells with fresh [^{3}H]methionine and 0.13 pmol/10^6 mast cells with old methionine. In neither cell preparation was increased lipid labelling seen to accompany anti-IgE-induced histamine release of 50.0%.

In all experiments 30 μg/ml of L-α-phosphatidylserine (PS) was used to enhance IgE-dependent histamine release. It has been reported [26] that, following incorporation into the cell membrane, PS is decarboxylated to PE which becomes a substrate for methyltransferase-dependent generation of PMME, PDME and PC. As the fatty acid composition of PS may influence its utilization by the putative decarboxylases and methyltransferases involved in mast cell activation-secretion coupling, results obtained using PS from three manufacturers (Sigma, Lipid Products and Calbiochem-Behring) were compared in three experiments. Anti-IgE-induced histamine release varied between 28 and 40% with the three PS samples and no increases in [^{3}H]methyl incorporation into lipids were observed.

Variations in animals and diet

Because of our consistent failure to reproduce the observations of increased phospholipid methylation during IgE-dependent mast cell activation reported by Ishizaka *et al.* [3], it was considered that genetic differences or differences in diet between our rats and those used by these workers may influence the phospholipid composition of mast cell membranes and thereby affect experimental results. We have, therefore, performed a number of collaborative experiments with Dr. Ishizaka in which she supplied us with animals and diet from her own laboratory. For brevity, purified peritoneal mast cells isolated from these animals are referred to as American RPMC while those from our own Sprague–Dawley rats are called British RPMC.

Labelling of the AdoMet pool of American RPMC by 30 min incubation with 4 μM L-[^{3}H]methyl-methionine was 1.06 pmol/10^6 mast cells in one experiment (cf. 1.25 ± 0.16 pmol/10^6 mast cells (N = 3) for British RPMC). In four experiments, label incorporation into extractable lipids from unstimulated American RPMC was 0.14 ± 0.01 pmol/10^6 mast cells, similar to that observed in British RPMC. Challenge of American RPMC with $DNP_{13}HSA$ antigen produced 9.17 ± 1.09% histamine release but produced no significant increase in lipid methylation (Fig. 4a). In two further experiments, stimulation with anti-IgE resulted in 6% histamine release without increasing total lipid labelling (Fig. 4b).

TLC analysis of the lipid extracts from American RPMC (Table 2) showed similar results to those previously described for British RPMC (Table 1). In unstimulated cells, 25.6% of the total label was associated with neutral lipids at the solvent front and only 23.9% with derivatives of PE. Stimulation of the cells with 1% anti-IgE induced 26.4% histamine release but no increase in phospholipid labelling at 10, 15, 30, 60 or 120 sec after challenge. The results at 15 sec showed 29.1% of the extracted label to be associated with neutral lipids and 22.3% with PE derivatives.

A possible reciprocal relationship between lipid and AdoMet [^{3}H]methyl content following immunological activation was sought in American RPMC.

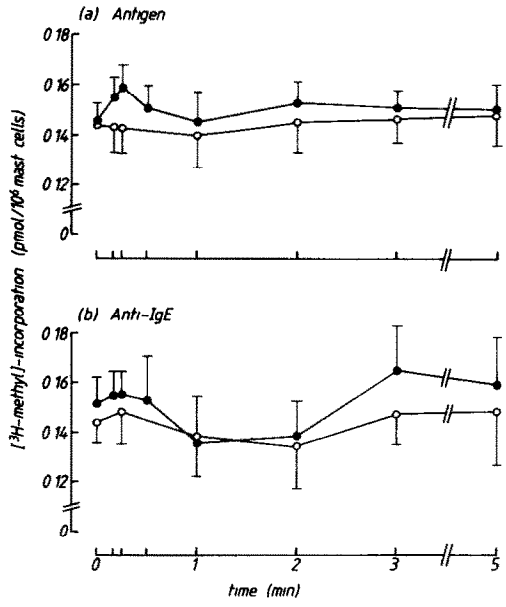

Fig. 4. [^{3}H]methyl incorporation into unstimulated (○) American rat mast cells and mast cells challenged (●) with (a) antigen or (b) anti-IgE. Results are mean ± S.E.M. of duplicate samples of (a) four experiments and (b) two experiments. In cells challenged with 0.1 μg/ml $DNP_{13}HSA$ antigen, spontaneous and net stimulus-induced histamine releases were 6.1 ± 1.6% and 9.17 ± 1.09% respectively. In cells challenged with 1% anti-IgE, spontaneous and net stimulus-induced histamine releases were 4.7% and 6.0% respectively.

Table 2. Distribution of [^{3}H]methyl label in lipid extracts from mast cells of American rats

	Radiolabel (fmol/10^6 mast cells)	
	Unchallenged cells	15 sec post challenge
PMME + PDME	20.7 (13.8%)	19.8 (12.4%)
PC	11.9 (7.9%)	12.4 (7.8%)
lyso PC	3.3 (2.2%)	3.4 (2.1%)
Neutral lipid (solvent front)	38.5 (25.6%)	46.3 (29.1%)
Total applied	150.2 (100%)	159.1 (100%)

Total lipid extracts of unchallenged or anti-IgE-activated cells were prepared and lipids separated by thin-layer chromatography as described. Results are mean of duplicate determinations in one experiment in which spontaneous and net anti-IgE-induced histamine release was 10.9% and 26.4% respectively.

In unstimulated cells, 0.14 pmol of label/10^6 mast cells was associated with extracted lipids and 1.06 pmol with AdoMet. Challenge with anti-IgE induced 26.4% histamine but caused no demonstrable change in either lipid or AdoMet associated label (Fig. 5).

Effect of methylation inhibitors on mast cell cyclic AMP levels

A close relationship has been suggested between phospholipid methylation and cyclic AMP production during the first 15–30 sec after immunological activation of mast cells, both events being reduced in parallel by inhibitors of phospholipid methylation [19]. In view of our failure to obtain direct evidence for increased methylation we have examined the effects on cyclic AMP changes of preincubation of purified mast cells for 1 hr at 37° with 3-deazaadenosine (DZA, 100 μM) in the presence of homocysteine thiolactone (Hcy, 100 μM) in 5 experiments. In untreated cells, baseline levels of cyclic AMP of 0.96 ± 0.05 pmol/10^6 mast cells increased to a maximum of 1.46 ± 0.10 pmol/10^6 cells 15 sec after challenge with anti-IgE (Fig. 6). This increase was statistically significant ($P < 0.01$). Histamine release in these experiments was 36.5 ± 8.6%. Preincubation of mast cells with DZA + Hcy significantly ($P < 0.05$) increased the resting cyclic AMP level of unstimulated cells to 1.18 ± 0.06 pmol/10^6 mast cells. Whilst DZA + Hcy inhibited anti-IgE-induced histamine secretion by 66.5 ± 5.2%, the cyclic AMP response to challenge

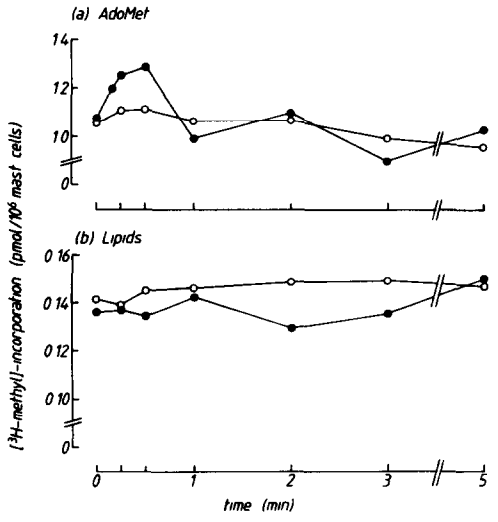

Fig. 5. [^{3}H]methyl Incorporation into (a) AdoMet and (b) lipid extracts of unstimulated (○) and anti-IgE-activated (●) mast cells from American rats. Results are mean of duplicate determinations in one experiment in which spontaneous and net anti-IgE (1%)-induced histamine release was 10.9% and 26.4% respectively.

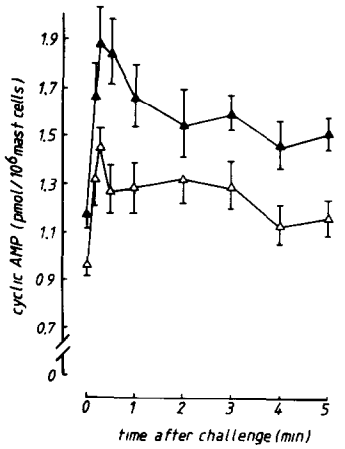

Fig. 6. Effect of the combination of DZA (100 μM) and Hcy (100 μM) on anti-IgE-dependent increase in mast cell cyclic AMP. Mast cells were incubated for 60 minutes with buffer (△) or DZA + Hcy (▲) prior to challenge with 1% anti-IgE. Results are mean ± S.E.M. of five experiments performed in duplicate in which spontaneous and net anti-IgE-induced histamine secretion was 7.8 ± 1.2% and 36.5 ± 8.6% respectively.

was both elevated and prolonged, levels at 15 sec and later being significantly ($P < 0.05$) higher than control. Similar results were obtained in a single experiment using American RPMC.

DISCUSSION

Workers in two laboratories [3–5, 26] have reported that a transient 2–120-fold rise in phospholipid methylation precedes histamine secretion from mast cells following IgE-dependent stimulation. This has led to the hypothesis that phospholipid methylation is an obligatory event in mast cell activation–secretion coupling. However, using identical methods to these workers, we have been consistently unable to detect enhanced phospholipid methylation to be associated with rat mast cell histamine release activated by antigen or anti-IgE. We therefore conclude that this event has no role in stimulus–secretion coupling in these cells.

A transient enhancement of phospholipid methylation has been reported by Ishizaka *et al.* [3] in rat peritoneal mast cells activated with anti-IgE-receptor antibodies (anti-RBL) or anti-IgE. In these experiments, phospholipid methylation was assessed by incorporation of [^{3}H]methyl-groups from [^{3}H]methyl-methionine into chloroform/methanol-extractable lipids without their further separation. Transient increases in [^{3}H]methyl incorporation of 120- and 32-fold were detected at 15 sec after cell activation with anti-RBL and anti-IgE respectively. These lipid changes were associated with release of approximately 18% of net cellular histamine. In our experiments, we have analysed changes in lipid and phospholipid [^{3}H]methyl incorporation in mast cells activated with either specific antigen or anti-IgE. Although obtaining similar amounts of histamine release to Ishizaka *et al.*, in no experiments have we observed increased lipid or phospholipid methylation. In our hands, [^{3}H]methyl-incorporation into lipids of 0.086–0.12 pmol/10^6 unstimulated mast cells is higher than the value obtained by Ishizaka and coworkers (0.014–0.027 pmol/10^6 mast cells [3]) but similar to that obtained by Boam *et al.* (0.15 pmol/10^6 cells) [30]. Analysis of the lipid extracts by TLC or HPLC showed only 14–22% of the total radioactivity to be associated with methylated derivatives of PE, with neutral lipids containing the major fraction. These results strongly suggest that measurement of [^{3}H]methyl incorporation into chloroform/methanol extracts without further separation of phospholipids would not give a true assessment of phospholipid methylation.

Although in our studies antigen challenge induced 23–39% net histamine release, we found no evidence of a transient increase in [^{3}H]methyl incorporation into PE using TLC or HPLC to separate methylated products. The HPLC results are in agreement with those reported by Moore *et al.* [31].

As phospholipid methylation is dependent on the donation of methyl groups from intracellular AdoMet, it is a necessary prerequisite to confirm the [^{3}H]methyl labelling of the AdoMet pool. TLC analysis showed the [^{3}H]methyl incorporation into mast cell AdoMet to be 1.25 ± 0.16 pmol/10^6 cells, some 10-fold higher than that associated with extractable lipids and similar to the ratio reported by Ishizaka *et al.* [3]. These workers reported the transference of 92% of AdoMet-associated radiolabel into phospholipids following IgE-receptor activation, severely depleting the pool of radiolabelled methyl donor. Assuming a similar conversion in our cell preparations, the [^{3}H]methyl-AdoMet levels in our experiments would have been sufficient to allow a 12-fold increase in lipid methylation following challenge to be demonstrable. Despite releasing 23% histamine, anti-IgE challenge induced no changes in the [^{3}H]methyl content of either AdoMet or extractable lipids, confirming the observations of Moore *et al.* [31].

As we were consistently unable to demonstrate stimulus-dependent enhancement of phospholipid methylation using published techniques, the effects of varying some of the experimental conditions were examined. It was considered possible that under the standard conditions used there was insufficient labelling of [^{3}H]methyl-AdoMet to allow the detection of phospholipid methylation. Therefore, in some experiments the concentration of [^{3}H]methyl-methionine and the time of its incubation with mast cells were altered. Increasing the concentration of methionine from 0.25 μM to 25 μM during the standard 30 min incubation allowed a concentration-dependent increase in the labelling of intracellular AdoMet as did increasing the time of cell incubation up to 120 min with a fixed concentration (4 μM) of [^{3}H]methyl-methionine. However, under neither of these conditions was enhancement of lipid methylation observed following IgE-dependent activation.

Vance and de Kruijff [32], albeit on theoretical grounds, have suggested that small amounts of apparent [^{3}H]methyl incorporation into lipids from [^{3}H]methyl-AdoMet could be due to the presence of a radiolabelled contaminant. This possibility was examined in our preparations by comparing the incorporation of [^{3}H]methyl groups into mast cell lipids following cell incubation with fresh stocks of radiolabelled [^{3}H]methyl-methionine and with [^{3}H]methyl-methionine which had been subject to prolonged storage. Storage increases the proportion of sulphone and sulphoxide oxidation products of methionine, derivatives which are readily extracted into chloroform [44]. In support of this, we observed that there was a greater non-lipid-associated carryover of [^{3}H] label into washed chloroform extracts of cells preincubated with partially decomposed [^{3}H]methyl-methionine. However, when allowance was made for this increased carryover, the incorporation of label into mast cell lipids was similar to that with fresh stocks of methionine. In neither preparations were any IgE-dependent increases in lipid labelling observed.

Possible variations in the fatty acid composition of phosphatidylserine (PS) used to enhance histamine secretion are also unlikely to account for the differences between our results and those of Ishizaka *et al.* [3] as we have observed no stimulus-increased lipid methylation in cells activated in the presence of PS from three different sources. Furthermore, genetic or dietary differences between our experimental animals and those of Ishizaka *et al.* [3] are not the reason for our widely differing findings as the results of experiments using animals and diet

supplied by Dr. Ishizaka were almost identical to those using our own rats.

Although we have confirmed previous reports [3, 5, 19, 34, 45] that the methylation inhibitor DZA in combination with Hcy decreases histamine release from immunologically activated mast cells, this does not necessarily support a role for phospholipid methylation in the secretory process as these compounds also inhibit other AdoMet-dependent methyltransferases. Protein carboxymethylation [46] is involved in exocytosis in rat chromaffin cells [47, 48] and pancreatic acinar cells [49] and the possible involvement of this event in mast cell activation has not been investigated. Additionally, in human lung mast cells where IgE-dependent phospholipid methylation has also been demonstrated by Ishizaka *et al.* [5], methylation inhibitors decrease histamine release activated by calcium ionophore A23187 [34], a secretagogue which by-passes many of the membrane-associated events coupling IgE-receptor activation to mediator secretion.

It has been reported by Ishizaka *et al.* [5, 9] that inhibitors of phospholipid methylation decrease the early rise in cyclic AMP associated with IgE-dependent activation of mast cells. This has been interpreted as supporting a role for phospholipid methylation in the immunological activation of adenylate cyclase. In contrast, Zimmerman *et al.* [50] have demonstrated that DZA in combination with Hcy elevates intracellular cyclic AMP by inhibiting cyclic AMP phosphodiesterase and activating adenylate cyclase. Our observations that pretreatment of rat mast cells with methylation inhibitors enhances both the basal and IgE-stimulated rises in cyclic AMP would support the observations of Zimmerman *et al.* [50]. Indeed, the enhancement of intracellular cyclic AMP levels by DZA, an effect also seen in human lung mast cells and basophils [51], may represent an alternative mechanism of action of purported methylation inhibitors in preventing IgE-dependent histamine secretion.

To conclude, although we can offer no explanation for the differences between our results and those reported by Ishizaka *et al.* [3], our consistent failure to demonstrate enhanced phospholipid methylation to accompany IgE-dependent stimulation of histamine secretion from rat mast cells indicates that this event is not an essential component of the activation–secretion coupling mechanism in mast cells.

Acknowledgements—We wish to thank Dr Teruko Ishizaka for her constructive comments and for supplying us with animals and materials used in this study. We acknowledge the technical assistance of Dr A. D. Postle and Mr A. Hunt of Child Health, Faculty of Medicine, University of Southampton. R.C.B. was supported by an SERC CASE award in collaboration with Fisons Plc.

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