Alteration of Lipid Composition Modulates FcεRI Signaling in RBL-2H3 Cells†

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ABSTRACT: We have used sonicated liposomes of phosphatidylcholine (PC), sphingomyelin (SM), or a mixture of cholesterol (chol) and PC to investigate the role of cellular lipid composition in FcεRI-mediated stimulation of RBL-2H3 cells. Overnight treatment with either PC or SM liposomes causes a substantial enhancement of antigen-stimulated degranulation and phospholipase A_2 activity, whereas treatment with a PC/chol mixture results in partial inhibition of the antigen-stimulated response. The most consistent change in the cellular lipid composition that results from the PC and SM liposome treatments is a ~40% decrease in the chol/phospholipid (PL) ratio. The lipid treatments do not alter degranulation stimulated by AlF_4^- or by Ca^{2+} ionophore in the presence or absence of PMA, suggesting that lipid alteration affects a receptor-specific signaling process. The lipid treatments do not appear to alter antigen-stimulated tyrosine phosphorylation or Ca^{2+} mobilization. Possible involvement of protein kinase C (PKC) activation in the signal-enhancing effect of the PL treatments was investigated by using calphostin C and phorbol-12-myristol-13-acetate (PMA) to inhibit PKC activity and degranulation in RBL-2H3 cells. Both SM and PC treatment restore the antigen-mediated degranulation response that is inhibited by long-term treatment (≥16 h) with 100 nM PMA or short-term treatment (10 min) with 5 μM calphostin C. The results indicate that a decreased chol/PL ratio facilitates or enhances the receptor-mediated activation of a PKC-like pathway that plays an important role in FcεRI-stimulated degranulation. Our results support the hypothesis that membrane structure plays an important role in regulating the activation of downstream signaling pathways by activated receptor complexes.

In many cell systems, lipid supplementation during culture has revealed that membrane lipids modulate cellular function (Barrantes, 1993; Leray et al., 1993), enzymatic activity (Bolen & Sando, 1992; Orr et al., 1992), tumor development (Shinitzky et al., 1988), viral infection (Skornick et al., 1992), aging (Hübner et al., 1988; Moscona-Amir et al., 1989; Yechiel & Barenholz, 1986), and other biological properties of the cells (Yeagle, 1989; Spector & Yorek, 1985). Although results from these studies appear to be diverse and system specific, it is clear that the activity of cell surface receptors can be regulated by membrane lipids. We have now investigated these effects for FcεRI, the high affinity receptor for immunoglobulin E (IgE).[1]

FcεRI is found on the surface of mast cells and basophils and consists of three subunits, α, β, and a pair of disulfide-linked γ subunits (Ravetch & Kinet, 1991). Aggregation of IgE receptor complexes triggers a number of events that lead to the release of histamine and other inflammatory mediators (Beaven & Metzger, 1993; Siraganian, 1988). Because of its importance as a model immune receptor, FcεRI-mediated signaling has been the focus of research interest for many laboratories. Despite a large amount of research on this system, the role of the lipid environment in receptor-mediated signaling has been little explored. In the present study we have used defined liposomes to investigate the relationship between the lipid composition and FcεRI function. The results suggest an important role for the chol/PL ratio in regulating FcεRI-mediated degranulation and arachidonic acid production.

MATERIALS AND METHODS

Reagents. Synthetic PC (1,2-dioleoyl-*sn*-glycerol-3-phosphocholine) and bovine brain SM were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Egg PC, cholesterol, PMA, and the Ca^{2+} ionophore A23187 were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-5-Hydroxytryptamine binoxalate ([^{3}H]5HT) and [^{3}H]arachidonic acid ([^{3}H]AA) were purchased from Dupont NEN (Boston, MA). Indo-1 was purchased from Molecular Probes (Eugene, OR). Calphostin C was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). Antiphosphotyrosine antibody PY20 conjugated to horseradish peroxidase was purchased from ICN (Costa Mesa, CA). Murine monoclonal anti-DNP IgE was obtained and purified as previously described (Liu et al., 1980; Holowka & Metzger, 1982). Bovine serum albumin (BSA), conjugated with an average of 16 DNP/mol of protein (DNP-BSA), was prepared as previously described (Eisen et al., 1956). Fiske−SubbaRow reagent (Fiske & SubbaRow, 1925) was prepared by adding 0.25 g of 1-amino-2-naphthol-4-sulfonic acid (Eastman Organic Chemical, Rochester, NY) into 100 mL of freshly prepared 15% (w/v)

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[1] Abbreviations: IgE, immunoglobulin E; PKC, protein kinase C; PMA, phorbol-12-myristol-13-acetate; PLA_2, phospholipase A_2; DNP, 2,4-dinitrophenyl; DNP-BSA, multivalent antigen consisting of an average of 16 molecules of 2,4-dinitrophenol conjugated to 1 molecule of bovine serum albumin; chol, cholesterol; PC, phosphatidylcholine; SM, sphingomyelin; AA, arachidonic acid; LPL, lysophospholipid; SDS−PAGE, sodium dodecyl sulfate−polyacryamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; BSS, buffered saline solution; PLC, phospholipase C.

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sodium bisulfite with shaking, followed by 1 g of sodium sulfite. The reagent was then filtered and stored in the dark. The H_2O used in sample preparation was distilled and deionized.

Liposome Preparation. PL or PL/chol mixture in chloroform was blown dry in a glass vial with nitrogen gas. A buffered saline solution (BSS) containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, and 20 mM HEPES, pH 7.4, was then added (1 mL per 10 mg of lipid), and the mixture was sonicated with a Vibra Cell model ASI ultrasonic disruptor (Sonics & Materials Inc., Danbury, CT) tuned at maximum setting at 40 °C until the solution became clear (typically 0.5−1 h). The liposome suspension was then sterilized by filtration through a 0.2 μm syringe filter (Acrodisc, Gelman Sciences, Ann Arbor, MI) and added to the cells to give a final concentration of 2.5 mM for both PL and chol.

Cell Preparation. RBL-2H3 cells (Barsumian et al., 1981) were grown adherent in either 75 or 150 cm^2 flasks and harvested in 135 mM NaCl, 5 mM KCl, 20 mM HEPES, and 1.5 mM EDTA, pH 7.4, for 10 min and then centrifuged at 200g for 10 min and resuspended in its original culture medium at a final concentration of 2×10^6 cells/mL. For cells that were to be used for [^{3}H]5HT or [^{3}H]AA assays, 20 μg IgE was added to sensitize a 20 mL aliquot of cells together with either [^{3}H]5HT (1 μCi/mL) or [^{3}H]AA (0.1 μCi/mL) before cells were plated out into 24-well plates (Corning Glass Works, Corning, NY) at a density of 1×10^6 cells/well. For most experiments, the liposome suspension or BSS (0.1 mL/well) was added 1−8 h after cell plating. For PKC depletion experiments, 100 nM PMA was added either to cells in suspension or to the wells before cell plating. All cell samples were incubated a total of ≥16 h at 37 °C in a 5% CO_2 incubator. After 8 h of culture, some wells received sonicated liposomes of PC, SM, or a combination of PC and cholesterol as above, and then these cells were continued in culture along with non-lipid-treated cells. For cells that were to be analyzed for lipid composition, 20 mL of cells with IgE were incubated in a 75 cm^2 flask and treated in parallel with the cells in 24-well plates. For lipid treatment of cells assayed in suspension, 4 mL of sonicated lipid together with IgE and [^{3}H]5HT was added to cells in 20 mL of medium in a 75 cm^2 flask ≥16 h before the experiment.

Release Assays. After the 16 h culture period described above, cells in the 24−well plates were washed three times with BSS containing 0.1% gelatin and incubated with different doses of DNP-BSA in 0.5 mL BSS at 37 °C (1 h for [^{3}H]5HT release and 30 min for [^{3}H]AA metabolite release). Total radioactivity incorporated per well was determined by adding 500 μL of 1% Triton X-100 detergent instead of antigen. For some samples, the cells were treated with 5 μM calphostin C for 10 min before addition of DNP-BSA. A stock solution of 10 mM calphostin C in DMSO was used, and no effect of the solvent alone on degranulation was observed. Aliquots (300 μL) of the supernatant from each well were centrifuged at 6600g for 1 min and ^{3}H radioactivity from 100 μL aliquot was counted in a Beckman LS #1801 liquid scintillation counter as previously described (Baird et al., 1983). For some experiments, [^{3}H]5HT-labeled, IgE-sensitized cells that had been cultured in flasks were harvested and resupended in BSS to a final concentration of 4×10^6 cells/mL. Aliquots 100 μL of these cells were added to 100 μL aliquots of BSS containing 0.2% gelatin and different doses of antigen that had been deposited into a 96-well U-bottom microtiter assay plate (Falcon). The cells were then incubated at 37 °C for 1 h, the supernatants were removed with a filter collection system (Skatron Inc., Sterling, VA), and the amount of radioactivity released into the supernatant was measured as above.

Lipid Compositional Analyses. Cells that had been incubated with or without liposomes in 75 cm^2 flasks were harvested as described above and resuspended in BSS. Cells were counted with a hemocytometer and pelleted at 200g for 10 min. The lipids were then extracted and analyzed according to previously published methods (Folch et al., 1957; Yavin & Zutra, 1977; Bartlett, 1959). For this procedure, the cell pellet (about 2×10^7 cells) was resuspended in 0.25 mL of BSS, and cells were lysed immediately by adding 2.6 mL of MeOH and vortexing for a few seconds, and then 3.2 mL of chloroform was added. The resulting lysate was centrifuged at 850g for 20 min at 4 °C in a Sorvall GLC-2B centrifuge (DuPont, Wilmington, DE) to spin down nonsolubilized material (protein, cytoskeleton, nucleus, etc.). The supernatant was collected with a glass pipette. The solid pellet was rinsed once with 1 mL of chloroform/MeOH (2:1, v/v) followed by 0.2 mL of MeOH. The mixture was centrifuged again, and the supernatant was combined with the previously collected extract. This crude extract was washed with 2.2 mL H_2O, and another centrifugation at 850g was performed to separate the organic and aqueous phases. The upper aqueous phase was withdrawn and discarded. The tube wall was rinsed three times with water saturated with 2:1 chloroform/MeOH without disturbing the organic phase. MeOH was added drop-wise after the last rinse to bring the residual washing fluid and the chloroform layer into one phase.

The resulting extract was diluted to a final volume of 10 mL with 2:1 chloroform/MeOH. 0.5 mL (sample 1) and 1.5 mL (sample 2) of the above extract were blown dry in two separate tubes with N_2 gas. Sample 2 was dissolved in a minimum volume of chloroform and spotted on to the lower left corner of a 10 × 10 cm silica gel 60 HPTLC plate (Merck, Darmstadt) with a glass pipette tip. This process was repeated until most of the lipid had been transferred on to the TLC plate. The residual lipid remaining on the pipette tip and in the tube of sample 2 was then assayed for phosphorus content (see below) in parallel with sample 1 in order to calculate a corrected total amount of phospholipid that was spotted onto the TLC plate. The composition of the lipid spot was then analyzed with 2-D TLC according to Yavin and Zutra (1977), and the separated lipid spots were visualized and identified after exposing the TLC plate to iodine vapor. The lipid spots were scraped off the plate into glass centrifuge tubes and, in parallel with other samples, hydrolyzed in 18 N H_2SO_4 and assayed for phosphorus according to Bartlett (1959).

Cholesterol Content Analyses. Cholesterol was analyzed by sonicating dried lipid extract from 1×10^6 cells in 1 mL of H_2O at 40 °C for 10 min. The total cholesterol content was then determined with a cholesterol detection kit (Sigma Chemical Co). In this method, any cholesterol esters are first hydrolyzed to free cholesterol by cholesterol esterase. Free cholesterol are then oxidized by cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide. This hydrogen peroxide is then coupled with the chromogen,

4-aminoantipyrine, and *p*-hydroxybenzenesulfonate in the presence of peroxidase to yield a quinoneimine dye which has an absorbance maximum of 500 nm ($\epsilon = 5.676$ cm^{-1} μM^{-1}, determined from standard purchased from Sigma Co.). The chol/PL ratio was determined by sonicating dried lipid from 2×10^6 cells into 2 mL H_2O. One milliliter and 0.5 mL portions of this sonicate were used for cholesterol assay and phosphorus analysis, respectively.

Antigen-Stimulated Tyrosine Phosphorylation. RBL cells were sensitized with IgE and incubated with liposomes for 16−24 h as described above. Cells were harvested, washed once with BSS, and then resuspended in BSS plus 0.5% gelatin at a concentration of 4.0×10^6 cells/mL. 2.0×10^6 cells were incubated at 37 °C for 2 min in the presence or absence of different doses of DNP-BSA. Cells were then pelleted by centrifugation at 9500*g* for 1 min in a Beckman Microfuge 11 (Beckman Instruments Inc., Palo Alto, CA). The cell pellet was lysed immediately in 200 μL of reducing SDS−PAGE sample buffer containing 1% SDS, 0.04 M Tris pH 6.8, 10% glycerol, 1% 2-mercaptoethanol, and 200 μM sodium vanadate and placed in boiling water for 5 min. Proteins were separated by SDS-PAGE on 10% polyacrylamide gel (40 μl or 4×10^5 cell equivalents lysate per lane) and subsequently transferred onto nitrocellulose (TM-NC4-ROLL, Hoefer Scientific, San Fransisco, CA) with an electroblotter (Enprotech model IMM-1, Hyde Park, MA). The membrane was blocked with 4% BSA in TBS (25 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, pH 7.4) and 0.05% Tween-20 at 37 °C for 1 h. This was followed by probing with horseradish peroxidase-conjugated anti-phosphotyrosine antibody PY20 (1 μg/5 mL in blocking solution) for 1 h. Excess antibody was removed by three washes with blocking solution and then by one wash with TBS. PY20-bound proteins were detected with an ECL chemiluminescence detection system from Amersham Corp. (Arlington Heights, IL).

Ca^{2+} Measurements. Cells were loaded with indo-1 as previously described (Weetal et al., 1993), and measurements were made with an SLM 8000 spectrofluorimeter operated in ratio mode with excitation and emission wavelengths of 330 and 400 nm, respectively. Suspensions (2 mL) of indo-1-loaded cells ($1-2 \times 10^6$ cells/mL) in acrylic cuvettes (Sarstedt Inc., Princeton NJ) were maintained at 37 °C with constant stirring during measurement.

RESULTS

Initial experiments tested the effect of overnight (>16 h) treatment of RBL cells with sonicated egg PC liposomes on the ability of multivalent antigen, DNP-BSA, to stimulate degranulation ([^{3}H]5HT release) from IgE-sensitized RBL cells in suspension. Figure 1 shows the results from a typical experiment, in which this pretreatment caused a substantial enhancement of stimulated degranulation over a wide range of antigen doses. In 17 experiments of this design the average enhancement of stimulated degranulation was ~70% at an antigen dose of 10 ng/mL (Table 1). As seen in Figure 1, including chol at a 1:1 molar ratio with PC inhibits the degranulation response. Under these conditions, the average inhibition of degranulation stimulated by 10 ng/mL DNP-BSA was ~45% in 10 experiments (Table 1).

These results suggested the involvement of cholesterol modulation on the enhancement of stimulated degranulation due to PC treatment, as this treatment is known to reduce

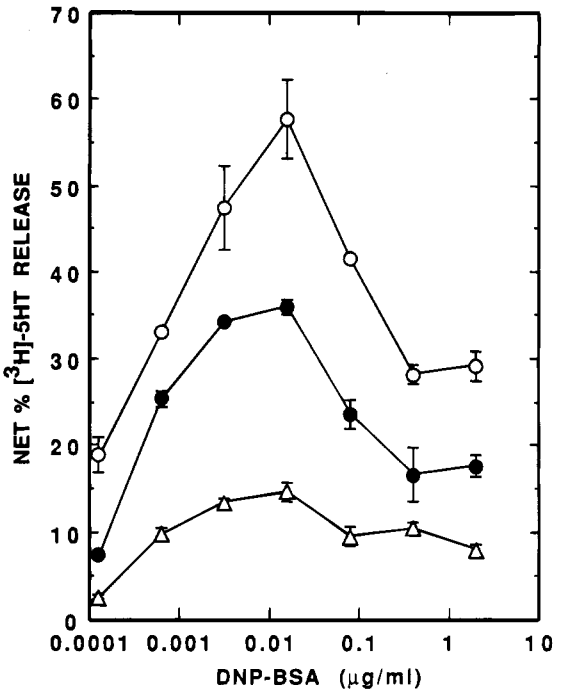

FIGURE 1: Effect of liposome treatments on antigen stimulated exocytosis. RBL cells were treated with PC (○), PC/chol (△), or medium alone (●) during an overnight incubation with [^{3}H]5HT and IgE before they were harvested, washed, and stimulated in suspension with the indicated doses of antigen for 1 h at 37 °C. Points show averaged values of duplicate samples with spontaneous release subtracted. Error bars indicate the range of values when greater than the width of the data point. Spontaneous release for this experiment was 16, 13, and 14% for control, PC, and PC/chol treated samples, respectively.

Table 1. Summary of Effects of Liposome Treatments on Antigen-Stimuated Secretion from RBL Cells

treatment[a]	assay conditions	relative secretion ± SD[b]
PC	suspended cells	171 ± 35 (17)[c]
chol/PC	suspended cells	55 ± 22 (10)
PC	adherent cells	157 ± 41 (15)
SM	adherent cells	141 ± 26 (13)

[a] Lipids were sonicated in buffered saline solution and filtered as described in Materials and Methods before being added to RBL cells for overnight treatment. The final concentration for each PL or cholesterol was 2.5 mM. [b] Response to 10 ng/mL DNP-BSA expressed as percent of response observed with control cells that were not treated with lipids. Control responses ranged from 20 to 70% of total [^{3}H]5HT released, and spontaneous (unstimulated) release was <20% in these experiments. [c] Numbers in parentheses represent the numbers of experiments used for calculating average values and standard deviation for relative [^{3}H]5HT secretion.

the cholesterol content of mammalian cells (Spector & Yorek, 1985; Cooper et al., 1978). Sphingomyelin treatment also reduces the cholesterol content of cells, so we examined the effect of this PL and found an average of 40% enhancement in stimulated degranulation in 13 experiments with an optimal dose of DNP-BSA (Table 1). The [^{3}H]5HT release experiments with SM treatment were carried out on adherent cells in order to compare them to the [^{3}H]AA release experiments described below. In these experiments, PC treatment causes a similar but somewhat reduced enhancement of [^{3}H]5HT release compared to its effects on suspended cells (Table 1).

We next tested the effects of these treatments on PLA_2-catalyzed release of ^{3}[H]-AA metabolites that is stimulated

Table 2. Effects of Lipid Treatments on Cellular Responses and Lipid Composition[a]

treatment[b]	[3H]AA release (%) spontaneous	[3H]AA release (%) stimulated[c]	[3H]5HT release (%) spontaneous	[3H]5HT release (%) stimulated[c]	PC[d] (%)	PE (%)	PLE (%)	PS (%)	SM + PI (%)	chol (nmol per 10^6 cells)	chol/PL[e]
experiment A											
control	0.5	2.5	11	42	44	25	8	10	14	16	0.49
PC	1.0	5.1	10	55	52	22	7	6	12	7	0.27
chol + PC	0.8	2.3	9	37	47	8	14	11	20	24	0.64
experiment B											
control	0.9	3.6	6	68	47	18	10	9	16	16	0.46
SM (1.3)	2.8	5.9	7	80	45	18	11	7	19	14	0.32
SM (2.5)	3.0	5.6	9	84	41	20	6	8	26	12	0.30

[a] Samples within the same experiment were prepared in parallel for lipid analyses, [3H]AA, and [3H]5HT assays. [b] Cells were incubated with final concentrations of 2.5 mM for cholesterol and each lipid, except for the case of SM (1.3), where cells were incubated with 1.3 mM SM. [c] Percent release stimulated by 20 ng/mL DNP_{16}-BSA as determined by subtraction of percent spontaneous release in column 1 from percent of release in the presence of antigen. [d] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLE, phosphatidyllysoethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PI, inositol phospholipids; chol, cholesterol. Amount given as percent of total PL recovered from 2-D TLC analysis in terms of phosphorus content. [e] Cholesterol/total phospholipid molar ratio from separate determination of cholesterol and phosphorus content.

by FcεRI aggregation (Garcia-Gil & Siraganian, 1986; Narasimhan et al., 1990). For these experiments, we incubated RBL cells overnight with either [3H]AA or [3H]-5HT in parallel and measured release of ^3H in cell supernatants after stimulation of washed adherent cells with DNP-BSA. The first two columns in Table 2 shows two representative examples of [3H]AA metabolite release (experiments A and B). In these experiments, PC treatment caused a 2.0-fold increase (experiment A) and SM treatment caused a 1.6-fold increase (experiment B) in the stimulated release of AA metabolites. In the same experiments, stimulated [3H]5HT release was enhanced after PC or SM treatment (Table 2, column 4), as expected from the experiments described above, whereas treatment with 1:1 chol/PC caused a small inhibition of stimulated release of both [3H]5HT and [3H]AA metabolites (Table 2, experiment A). Treatment of RBL cells with PC or SM also consistently caused an increase in the spontaneous release of [3H]AA metabolites, as seen in Table 2, column 1. In experiment A, a 2.0-fold increase in spontaneous release of AA metabolites was seen in the PC treated cells, and, in experiment B, a 3.2-fold increase in this parameter was seen for the SM treated cells. Similar effects of liposome treatments on [3H]AA metabolite release as those in Table 2 were seen in 12 other experiments (data not shown). No significant effect of PC or SM treatment was detected on the spontaneous release of [3H]5HT (Table 2 and data not shown).

The experiments in Table 2 were designed to investigate the relationship between cellular PL content and stimulated release by analyzing the lipid content of control and lipid treated cells in parallel with the release assays. We used a protocol based on TLC separation to investigate changes in phospholipid composition and cholesterol content under conditions of functional responses altered by the liposome treatments. For the two experiments shown in Table 2, we obtained consistent results for the compositional analysis for the non-lipid-treated control cells. They contain PC as the major PL (40−50%), and they contain phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidyllysoethanolamine (PLE), SM, and PI as minor phospholipids. Cholesterol exists in a large amount; its molar ratio to total PL is about 0.5 (last column, Table 2), similar to results reported for other hematopoetic cells (Van Blitterswijk, 1984). After overnight liposome treatment, the total amount of phospholipids per cell remained relatively constant compared to control cells (data not shown), whereas the amounts of individual phospholipids were altered to varying extents (Table 2). Most notably, chol + PC caused a substantial reduction in PE and a concomitant increase in PLE. The most consistent change due to liposome treatment, however, was in the cholesterol content of the cells. PC and SM treatments substantially reduced chol/PL molar ratios from ~0.5 in the control cells to ~0.3 in the lipid treated cells, whereas chol/PC treatment increased the ratio to >0.6 (Table 2, last column). Similar changes in the chol/PL ratio after the lipid treatments were seen in five other experiments (data not shown). Thus, there is a correlation between the antigen-stimulated responses and the chol/PL ratio such that the smaller the ratio the better the responses.

To test whether the effects of liposome treatments on degranulation are receptor-specific, we attempted to bypass FcεRI and directly stimulate the cells through G proteins and Ca^{2+}/PKC activation. To stimulate the cells via heterotrimeric G proteins, we treated adherent cells with AlF_4^- at doses causing [3H]5HT release comparable to that stimulated by 20 ng/mL DNP-BSA. As shown in Figure 2, pretreatment of the cells with PC or PC + chol caused no significant effect on AlF_4^- stimulated release, even though PC treatment of cells caused significantly more release after antigen stimulation than seen in the control. Similar results were obtained in three other experiments (data not shown). As noted above, the enhancement of antigen stimulated degranulation of adherent cells due to PC treatment is less than that observed for suspended cells. Treatment of cells with PC + cholesterol shows little or no inhibition of antigen-stimulated degranulation if the cells are adherent when stimulated. It is possible that signals mediated by adherence proteins can overcome the inhibition caused by increased cholesterol content.

Figure 3 shows another attempt to bypass FcεRI by treating adherent cells with the combination of 50 nM PMA and 100 nM A23187 which stimulates [3H]5HT release at a level comparable to 20 ng/mL DNP-BSA. Pretreatment of the cells with PC or PC + chol had no significant effect on A23187 plus PMA-stimulated [3H]5HT release from adherent cells, even though PC-treated cells released significantly more [3H]5HT due to antigen stimulation than control cells stimulated with antigen. Also shown in Figure 3, treatment of cells with either PMA or A23187 alone causes only a

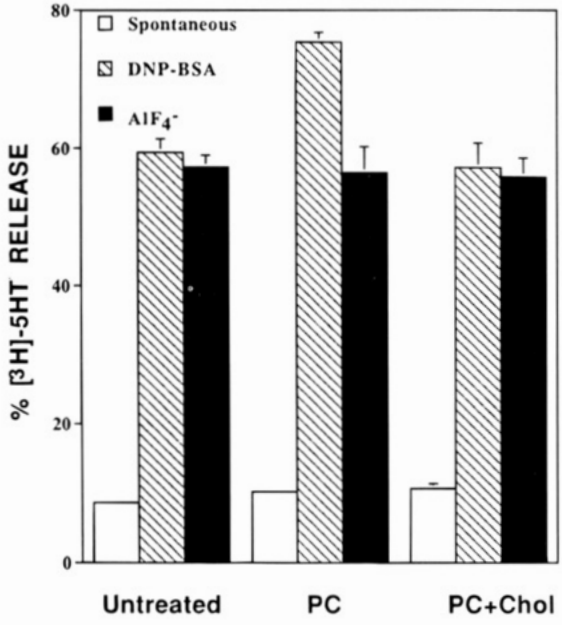

FIGURE 2: Lipid treatments do not alter exocytosis stimulated by AlF_4^-. RBL cells were sensitized with anti-DNP IgE and labeled with [^{3}H]5HT in 24-well assay plates; some cells were incubated with liposomes overnight as described in Materials and Methods. Adherent cells were then triggered with either 20 ng/mL DNP-BSA (hatched bars) or 20 mM NaF and 100 μM $AlCl_3$ (filled bars). Spontaneous release is shown as open bars. Error bars represent standard deviations from triplicate samples.

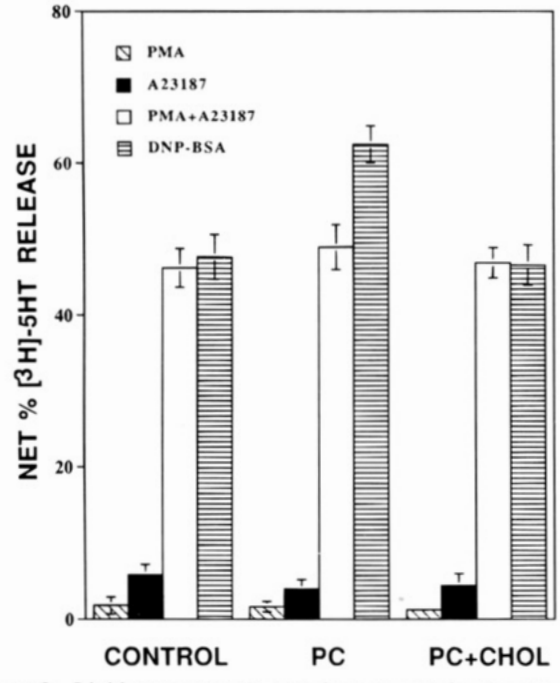

FIGURE 3: Lipid treatments do not alter exocytosis stimulated by the Ca^{2+} ionophore A23187 in the presence or absence of PMA. Cells were prepared as in Figure 2 and then stimulated with 20 ng/mL DNP-BSA, 50 nM PMA, 100 nM ionophore, or a combination of 50 nM PMA and 100 nM ionophore. Spontaneous release in these samples ranged between 12 and 16%, and this was subtracted to yield the net release. Error bars represent standard deviations from triplicate samples.

small amount of [^{3}H]5HT release above spontaneous, and pretreatment of the cells with either PC or PC + chol has no significant effect on these responses.

The results of Figures 2 and 3 suggested that the effect of liposome treatments on [^{3}H]5HT and [^{3}H]AA release are due

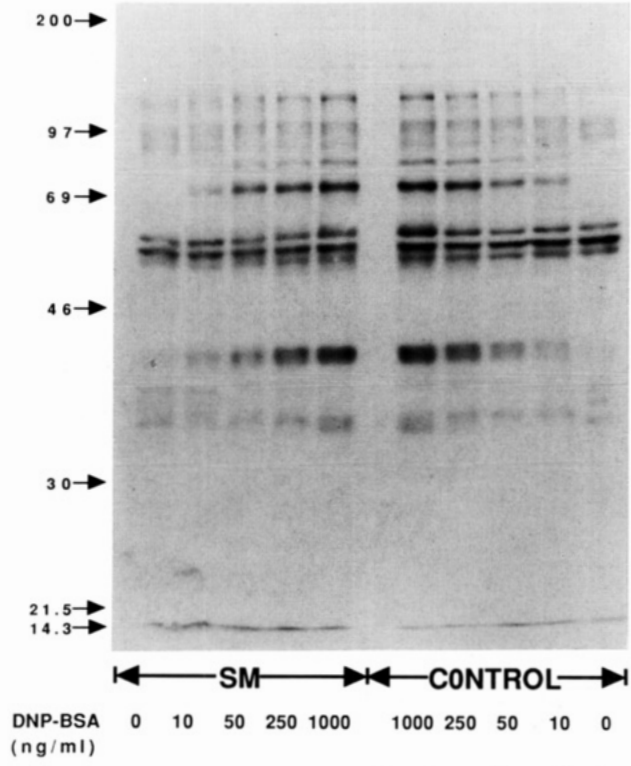

FIGURE 4: Effect of sphingomyelin treatment on antigen-stimulated tyrosine phosphorylation. Cells were sensitized with IgE and incubated with or without liposomes overnight before they were stimulated with indicated doses of antigen and subsequently lysed and analyzed for tyrosine phosphorylation of cellular proteins as described in Materials and Methods. Numbers on the left indicate mass of protein standards (kDa).

to receptor-proximal steps in signaling; we therefore investigated the effects of these treatments on several early events that are stimulated by FcεRI aggregation. Figure 4 shows the dose dependence for antigen-stimulated tyrosine phosphorylation of RBL cell proteins from either SM-treated or control cells. As previously reported, there are several different protein substrates that become tyrosine-phosphorylated in a dose-dependent fashion, including major bands at 72 kDa (Benhamou et al., 1990) and 42−44 kDa (Santini & Beaven, 1993). In this and five other similar experiments, no significant effects of either SM, PC, or chol/PC treatment were detected on stimulated tyrosine phosphorylation of the major cellular substrates. Small differences in the amounts of some bands at suboptimal doses in Figure 4 were not consistently seen in several different experiments under these conditions (data not shown). However, we cannot rule out the possibility that other conditions or other methods of analysis of stimulated tyrosine phosphorylation might reveal some systematic alterations in liposome-treated cells.

We also investigated possible effects of the liposome treatments on antigen-stimulated Ca^{2+} responses in the RBL cells using the intracellular fluorescent indicator indo-1. Recent experiments in our laboratory have shown that Ca^{2+} responses to antigen in suspended RBL cells are insensitive to several different tyrosine kinase inhibitors that block stimulated tyrosine phosphorylation and degranulation in RBL cells (M. A. Liotta, D. Holowka, and B. Baird, manuscript in preparation). These results suggest that Ca^{2+} mobilization can be mediated by a pathway that does not require tyrosine phosphorylation. Figure 5 shows that treatment of cells with either PC (experiment 1) or SM

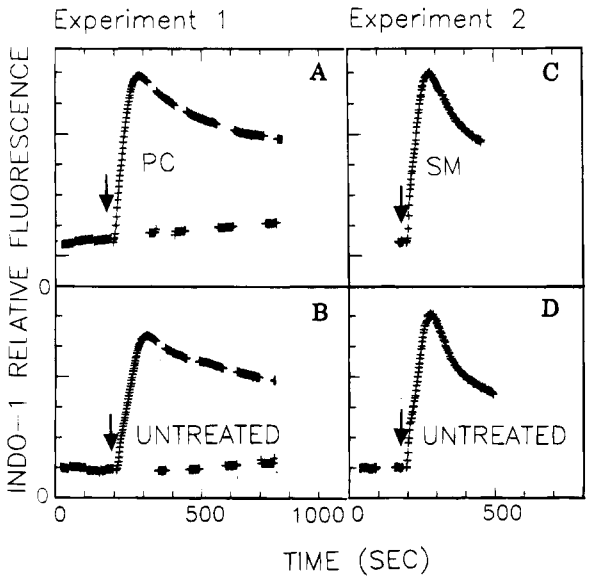

FIGURE 5: Effects of lipid treatments on antigen-stimulated $[Ca^{2+}]_i$ responses in RBL cells monitored by changes in intracellular indo-1 fluorescence intensity (ex 330 nm; em 400 nm). Arrows indicate addition of 20 ng/mL DNP-BSA. Bottom curves (B, D) correspond to non-lipid-treated control cells. Upper curves correspond to PC-treated cells (A) or SM-treated cells (C) as indicated. Left and right panels show experiments from two different days.

(experiment 2) causes little or no change in antigen-stimulated Ca^{2+} mobilization. The small enhancement of Ca^{2+} responses seen in these experiments in the lipid-treated cells were not observed consistently, and, in at least one experiment, no changes in Ca^{2+} responses were observed in the same cells that showed typical enhancement in antigen-stimulated degranulation due to PC pretreatment (data not shown).

Besides Ca^{2+} mobilization and tyrosine kinase activation, the other major pathway known to play an role in antigen-stimulated degranulation is activation of PKC (White & Metzger, 1988; Ozawa et al., 1993a,b). We tested the ability of our liposome treatment to compensate for the inhibition of antigen-stimulated [^{3}H]5HT release caused by PMA-induced down regulation of Ca^{2+}-dependent isoforms of PKC [α and β (Ozawa et al., 1993a)]. Figure 6B shows that overnight treatment with 100 nM PMA causes ~90% inhibition of antigen-stimulated degranulation (spontaneous release subtracted) from these adherent cells. In cells treated with PC or SM subsequent to an 8 h pretreatment with PMA, antigen-stimulated degranulation was increased 7−9−fold compared with PMA-treated cells that did not receive the liposome treatments (Figure 6B). This PL-enhanced response is 66−86% of the value for control cells that were not treated with PMA or liposomes (Figure 6A). As expected from previous results with adherent cells (Table 1), PC or SM treatment in the absence of PKC inhibition showed a 1.3−1.4-fold enhancement of stimulated release compared with the control cells (Figure 6A). No significant effects of PMA or liposome treatment are seen on unstimulated (spontaneous) release in these experiments.

We also investigated the ability of PC and SM treatment to restore the degranulation response that is inhibited by the PKC inhibitor calphostin C. This microbial metabolite selectively inhibits phorbol ester-activatable forms of PKC and appears to do so by binding to the regulatory domain of these isoforms (Kobayashi et al., 1989). As shown in Figure 6C, brief treatment with 5 μM calphostin C causes 60% inhibition of antigen-stimulated [^{3}H]5HT release in control cells, but prior overnight treatment with PC or SM almost entirely alleviates inhibition by calphostin C, as indicated by comparison with the control in Figure 6A. Results similar to those shown in Figure 6 were obtained in five additional experiments (data not shown). These results indicate that a decrease in the chol/PL ratio enhances activation of an FcεRI-stimulated signaling pathway that can compensate for the loss of stimulated PKC activity which is normally important for this receptor-mediated degranulation.

DISCUSSION

This study shows that treatment of RBL cells with PC or SM liposomes can effectively reduce the amount of cholesterol in cell membranes and, conversely, that chol/PC liposomes can be used to increase the amount of cholesterol in RBL cell membranes. Furthermore, a change in an FcεRI-mediated signaling pathway accompanies the change in cholesterol content. For suspended RBL cells, the reduction in chol/PL ratio that accompanies PC treatment correlates with an increase in antigen-stimulated release of [^{3}H]-5HT, whereas increasing this ratio by treatment with chol + PC correlates with inhibition of this response (Figure 1 and Table 1). The liposome treatments also affect antigen-stimulated [^{3}H]5HT release with adherent RBL cells (Tables 1 and 2), although the enhancement due to PC treatment is somewhat reduced and the inhibition due to chol + PC is reduced (Table 2) and sometimes not detected (Figures 2 and 3). Possibly, the component of enhanced signaling caused by adherent cells' interactions with the extracellular matrix (Hamawy et al., 1992) is not sensitive to the chol/PC ratio; this would reduce the apparent magnitude of the lipid modulation effects.

[^{3}H]AA metabolite release measured with adherent cells is also sensitive to changes in the cell membrane lipid composition and shows an inverse correlation with the chol/PC ratio similar to that seen with [^{3}H]5HT release (Table 2). For [^{3}H]AA metabolite release, significant changes in the unstimulated cells due to PC or SM treatment were detected, and these suggest that alterations in membrane structure due to the lipid treatments can affect PLA_2 signaling pathways independent of FcεRI involvement. In contrast, the lack of lipid treatments effects on spontaneous [^{3}H]5HT release or on [^{3}H]5HT release stimulated by AlF_4^- (Figure 2) or by Ca^{2+} ionophore and phorbol ester (Figure 3) suggests that the lipid treatments effects observed for FcεRI-mediated degranulation are specific for receptor-proximal events.

From the lipid compositional analyses, it appears that the liposome treatments act on FcεRI-mediated signaling by inserting or withdrawing cholesterol from the cell membrane, and the observed effects possibly result from perturbation of membrane structure or from alteration of direct interaction of chol with relevant proteins. Both mechanisms have been found to influence the activity of membrane enzymes such as the Na^+,K^+-ATPase (Yeagle, 1991). Although it seems unlikely that an antigen-mediated change in the conformation of FcεRI is important in the receptor mediated signaling (Metzger, 1992), the perturbation caused by lipid alteration could affect the structural arrangement of FcεRI in a way that alters its interaction with other membrane components. We attempted to determine whether the local environment

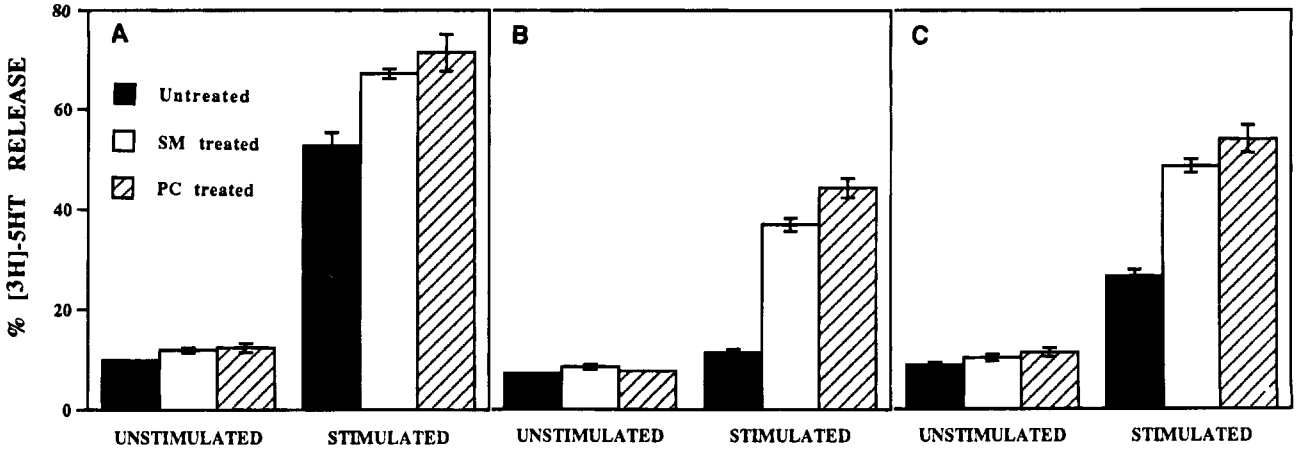

FIGURE 6: Lipid treatments restore antigen-stimulated degranulation that is inhibited by long-term PMA treatment or short-term calphostin C treatment. [^{3}H]5HT-loaded, IgE-sensitized cells were plated into 24-well plates and incubated with (panel B) or without (panels A and C)100 nM PMA for 8 h. Lipids were then added to some wells, and the incubation was continued for another 10 h. Cells were then washed and incubated with (panel C) or without (panels A and B) 5 μM calphostin C at 37 °C for 10 min before they were stimulated with 20 ng/mL DNP-BSA for 30 min. Error bars represent standard deviations from triplicate samples.

of FcϵRI is altered by lipid modulation with phosphorescence anisotropy measurements to measure the rotational diffusion of erythrosin-labeled IgE–receptor complexes (Myers et al., 1992); in preliminary experiments, no significant changes due to PC treatment were observed (data not shown).

The earliest signaling event activated by FcϵRI appears to be a tyrosine kinase casade that is initiated by the transphosphorylation of the β and γ subunits of one receptor by a src-family tyrosine kinase bound to a juxtaposed receptor in an antigen-mediated aggregate of IgE–receptor complexes (Paolini et al., 1991; Pribluda et al., 1994). This receptor phosphorylation event in RBL cells appears to recruit additional tyrosine kinases to the aggregate including the pp53/56^{lyn} (Eisenman & Bolen, 1992; Yamashita et al., 1994) and p72^{syk} (Hutchcroft et al., 1992; Benhamou et al., 1993). We did not detect any significant changes in antigen-activated tyrosine phosphorylation with anti-phosphotyrosine western blotting of whole cell lysates after liposome treatment, suggesting that the effects of lipid modulation on degranulation and PLA_2 activation are not not the result of changes in the activation of the tyrosine kinase cascade.

It is possible that changes in stimulated tyrosine phosphorylation of specific substrates important for signaling, such as the β and γ subunits of FcϵRI or phospholipase C γ-1 (PLC γ-1) would not be revealed by our analysis. Activation of p72^{syk} is dependent on β/γ phosphorylation (Jouvin et al., 1994), and it is believed that this activation is important for the increased tyrosine phosphorylation of proteins detected in whole cell lysates (Weiss & Littman, 1994). Also, it is generally believed that Ca^{2+} mobilization stimulated by FcϵRI aggregation is initiated by PLC γ-1 activation (Beaven & Metzger, 1993), but the lipid treatments do not cause a consistent alteration in the stimulated Ca^{2+} response. Thus, if lipid modulation alters antigen-stimulated tyrosine phosphorylation of the β/γ subunits of FcϵRI or of PLC γ-1, these are not sufficient to affect the downstream signaling pathways that depend on these phosphorylation events.

The pattern of altered PLA_2 and PKC activation by lipid modulation in the absence of detectable effects on tyrosine kinase activation or Ca^{2+} mobilization suggests that the altered cholesterol content primarily affects the ability of these early events stimulated by FcϵRI aggregation to connect with downstream signaling events. Although there is evidence that PKC activation by FcϵRI is mediated by diacylglycerol production (Beaven & Metzger, 1993), the regulation of this activation process is not well understood in cells, and the involvement of phospholipase D as well as PLC in diacylglycerol production has been indicated (Lin et al., 1991). Likewise, the mechanism by which receptors mediate PLA_2 activation is only partially understood (Beaven & Metzger, 1993). Our results are consistent with the hypothesis that FcϵRI-mediated activation of these *downstream* signaling pathways has membrane structural requirements that differ from those for activation by $AlF4^-$ or by Ca^{2+} ionophore plus phorbol ester.

The lipid treatment could possibly influence receptor-mediated signaling by altering membrane fluidity. We attempted to monitor such an alteration by measuring steady-state fluorescence anisotropy with the plasma membrane probe 1-[4-(tetramethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH; Bronner et al., 1986) which has been used successfully in model systems (Prendergast et al., 1981). However, no anisotropy change was detected after lipid treatment in the RBL cells (data not shown). Although anisotropy changes have often been reported to be associated with lipid treatment of cells (Shinitzky, 1984), this is usually observed with a probe such as DPH that does not remain confined to the plasma membrane. It is possible that the probe TMA-DPH that we used might not be sensitive to localized fluidity effects.

We recently observed that the fluorescent lipid probe DiI labels a membrane domain that redistributes with aggregated IgE–receptor complexes, whereas other probes, including TMA-DPH, do not co-redistribute (Thomas et al., 1994). A variety of evidence (including unpublished results) leads us to hypothesize that aggregated FcϵRI cause coaggregation of caveolae-like membrane structures that are rich in signaling proteins and depend on cholesterol for their structural integrity (Lisante et al., 1994; Anderson, 1993). Modulation of cellular cholesterol content might then be expected to affect the interaction of the aggregated FcϵRI with these caveolae-like structures and activation of downstream signaling, even though tyrosine kinase activity directly associated with the receptors might not be affected. Extensively aggregated IgE–receptors are known to become detergent

insoluble (Robertson et al. 1986), and preliminary experiments show that SM treatment, which enhances degranulation (Table 1), substantially reduces this induced insoluble state (unpublished results). This observation is consistent with the previous suggestion that the detergent-insoluble state characterizes receptor aggregates that are less able to activate downstream signaling events (Seagrave and Oliver, 1990). Treatment of cells with cytochalasin D also enhances downstream signaling while reducing detergent insolubility, and the combination of cytochalasin D and SM almost completely eliminates aggregation-dependent detergent insolubility of FcεRI (unpublished results). These results suggest that at some point aggregated receptors become associated with a detergent-insoluble membrane domain, and this restricts their ability to interact with caveolae-like structures and to activate downstream signaling pathways, without necessarily inhibiting early events. Cytochalasin-sensitive microfilaments apparently regulate the interaction of aggregated receptors with these insoluble domains, and cholesterol depletion by SM treatment also decreases the insolubility of these receptors, presumably by allowing a structural disruption of the domains.

The ability of PC or SM treatment to overcome the inhibition of degranulation by calphostin C or long-term treatment with PMA suggests that plasma membrane cholesterol can play a significant role in regulating receptor-mediated activation of PKC. RBL cells contain Ca^{2+}-dependent α and β and Ca^{2+}-independent δ, ϵ, and ζ isoforms of PKC, and all of these translocate to membranes to some extent upon stimulation by either antigen or PMA (Ozawa et al., 1993a,b). In addition to diacylglycerol, membrane-bound PKC isoforms generally also require the presence of negatively charged PL such as phosphatidylserine for maximal activity, but no direct role has been suggested for cholesterol in the function of PKC (Bolen & Sando, 1992; Orr et al., 1992). The lack of lipid modulation effects on degranulation stimulated by PMA plus Ca^{2+} ionophore suggests that PMA-activated PKC isoforms are not directly affected by the alteration of cholesterol content, and this is consistent with the hypothesis discussed above that lipid modulation plays a more basic role in altering the coupling of aggregated receptors and tyrosine phosphorylation to downstream signaling events. It is presently unclear whether the activation of a particular isoform of PKC is responsible for the ability of PL treatment to overcome the inhibition by calphostin or long-term PMA. PKC-ζ would appear to be the best candidate for this role, as this isoform does not have a true phorbol ester binding domain (Ono et al., 1989) and thus should not be directly affected by calphostin C or PMA. Preliminary attempts to determine whether antigen-stimulated PKC-ζ translocation to membranes is affected by lipid modulation yielded negative results (data not shown), and further experiments are necessary to resolve this question.

In summary, we have found that liposomes of defined composition can modulate FcεRI-mediated signaling leading to degranulation in RBL cells. The biochemical basis for these effects appears to involve alterations in cholesterol content of the plasma membrane, which in turn affects receptor-mediated activation of PLA_2 and a PKC-like pathway. The molecular mechanism for these effects remains to be determined, but the lack of detectable alteration of antigen-stimulated tyrosine phosphorylation or Ca^{2+} mobilization suggests that cholesterol plays an important role in regulating the coupling between the earliest signaling events and downstream signaling pathways.

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