Phospholipid Distribution in the Microenvironment of the Immunoglobulin E–Receptor from Rat Basophilic Leukemia Cell Membrane†

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ABSTRACT: It has been previously found that lipids were required to maintain intact the tetrameric structure of the receptor for immunoglobulin E (IgE) ($Fc_\epsilon R$) in detergent solutions [Rivnay, B., Rossi, G., Henkart, M., & Metzger, H. (1984) *J. Biol. Chem. 259*, 1212–1217, and references cited therein]. Failure of commercially obtained lipids to provide sufficient protection, however, underscored the necessity for development of additional analytical approaches. In order to identify the phospholipid distribution in the intimate natural environment of this receptor, both the plasma membrane vesicles and the ligand–receptor complex (IgE–$Fc_\epsilon R$) have been isolated by affinity chromatography. The phospholipids of both preparations were compared. After extensive washing with detergent lipid micelles, IgE–$Fc_\epsilon R$ retained 0.1–1% of the total phospholipids in the purified plasma membrane. The receptor-bound lipids were shown to contain phosphatidylcholine and sphingomyelin; the content of the latter lipid was enriched 2–5-fold compared with that in the plasma membranes. This pattern was observed with several detergents employed for purification and under a variety of experimental conditions. In light of the general distribution of choline phospholipids in the outer leaflet of plasma membranes, this enrichmemt may not be a characteristic of this particular receptor exclusively. These observations should be particularly helpful in studies on aggregation-induced functions of the isolated Fc_ϵ receptor. In general, the methods employed enable isolation of purified and lipid-protected integral proteins and also provide an appropriate reference source of intact membrane vesicles. These qualities render this approach useful in similar studies of other membrane proteins.

Rat basophilic leukemia cells, like normal basophils and mast cells, express on their surface a receptor with high affinity for immunoglobulin E (IgE).[1] Aggregation of this receptor by a variety of means initiates degranulation, thereby triggering the allergic reaction (Ishizaka & Ishizaka, 1984; Metzger, 1983). Initial attempts to isolate this receptor by immune precipitation or affinity chromatography directed toward the tightly bound ligand resulted in the isolation of a 45-kDa glycosylated polypeptide that retained, by definition, the IgE binding capacity (Conrad et al., 1976; Kulczycki et al., 1976; Kanellopoulos et al., 1979; Kumar & Metzger, 1982). This component, however, could not be reincorporated into a bilayer by using common reconstitution procedures of detergent removal. Efforts to elucidate this phenomenon as well as studies with chemical cross-linkers led to the finding that this receptor is in fact a tetramer containing the above-mentioned IgE-binding glycoprotein (now designated the α subunit), one β subunit of ~30 kDa, and two 9-kDa γ subunits linked via a disulfide bond(s) in the form of a dimer (Holowka et al., 1980, 1981; Holowka & Metzger, 1982; Rivnay et al., 1982, 1984; Peretz-Montfort et al., 1983; Rivnay & Metzger, 1982).

The successful isolation of the intact tetramer depended strictly on the lipids and detergents to which the receptor had been exposed during the solubilization and purification steps. This was shown both directly by PAGE analysis (Rivnay et al., 1982, 1984; Kinet et al., 1985a,b) and indirectly by attempts to reincorporate IgE–$Fc_\epsilon R$ complexes into liposomes, whereby the dissociated IgE–α subunit partial complex failed to become incorporated (Rivnay & Metzger, 1982). Endogenous RBL tumor lipids were efficiently protected from dissociation when maintained with CHAPS under solubilizing conditions at a minimal micellar detergent/lipid ratio (ρ). Further analysis of these conditions revealed that the tetramer was stable within the range of ρ = 2–5 (Rivnay et al., 1984; Kinet et al., 1985b). Purified receptors can be washed to remove the bulk lipids and remain soluble and intact at submicellar concentrations of CHAPS (Kinet et al., 1985b). Sufficient protection is therefore likely to be provided by the more tightly bound, residual lipids, associated with the purified receptor.

The observation that RBL-derived lipids had the maximal protective potential was indicative of some specificity. Identification of the required lipids is thus important for studies on the mechanism by which they prevent dissociation of the α subunit from the other subunits. It will also enable substitution of crude lipid extracts (used to date for purifying the receptor) with a well-defined set of lipids, more suitable for biochemical studies.

Attempts to identify the lipid classes required to support the integrity of the IgE receptor have been made earlier

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[1] Abbreviations: cmc, critical micelle concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; bv, column bed volume equivalent; HPLC, high-performance liquid chromatography; IgE, immunoglobulin E; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine (excluding plasmalogen); PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PL/R, phospholipid:receptor molar ratio; plasmalogen, 1-alk-1-enyl-2-acyl derivative of the specified class; PS, phosphatidylserine; RBL, rat basophilic leukemia, 2H3 sublime; Sph, sphingomyelin; TLC, thin-layer chromatography; DNP, dinitrophenyl; TNP, trinitrophenyl; kDa, kilodalton; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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(Rivnay et al., 1982; Kinet et al., 1985b). Indeed a few synthetic and natural phospholipids were found as partially effective in protecting against dissociation, but none of these lipids alone was as efficient as the crude lipid mixture extracted from RBL or from rabbit liver. It thus appears that the receptor for IgE requires either more than one lipid class or a single class that is less abundant in commercially available phospholipids. In this work we aimed to identify the composition of the phospholipid environment surrounding the IgE receptor on basophils.

Materials and Methods

Reagents and Chemicals. Mouse IgE was prepared from hybridoma cells (HI-DNP-ϵ-26.82) (Liu et al., 1980) as detailed elsewhere (Howlowka & Metzger, 1982). Rat IgE was a gift from Dr. H. Metzger (NIH). Iodination was performed as indicated (Rivnay & Metzger, 1982), yielding routinely a specific activity of (1–10) $\times$ 10^5 cpm/μg of IgE. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from Calbiochem (La Jolla, CA) or Sigma (St. Louis, MO) and stored as 0.5 M stock solution. Cholic acid (Na^+ salt) was purchased from Sigma. Triton X-100 was purified as detailed elsewhere (Chang & Bock, 1980). ϵ-*N*-DNP-L-lysine was purchased from Sigma. TNP-Sepharose was prepared by the cyanogen bromide coupling method (Jakoby & Wilchek, 1974). Crude RBL lipids were prepared from RBL-2H3 tumors grown in Sprague-Dawley weanling rats (Kanellopoulos et al., 1979). The lipids were extracted as described previously (Rivnay & Metzger, 1982; Renkonen et al., 1963).

Buffers. Two buffer mixtures were used: (A) Tris buffer, composed of 15 mM Tris-HCl, pH 7.2, 125 mM NaCl, and 5 mM KCl; (B) Tris–CHAPS–lipids, designated TCL and composed of the same Tris buffer, supplemented with RBL lipids (2 mM in phospholipid phosphate) and CHAPS (14.7 mM), to yield a micellar detergent:phopsholipid molar ratio (ρ; Rivnay & Metzger, 1982) of 5. All solutions were made with deionized and distilled water.

Cell Cultures. RBL-2H3 cells (Barsumian et al., 1981) were grown in tissue culture flasks (150 cm^2, Costar Mullingar-Ireland) as detailed elsewhere (Taurog et al., 1979). On day 0, 3 $\times$ 10^6 cells/flask were seeded, and on day 3 the medium was replaced by a fresh medium (Dulbecco modified Eagle's, GIBCO) containing [^{32}P]orthophosphate (Nuclear Research Centre, Negev, Israel), 15 μCi/mL. The latter medium was deficient in phosphate and contained only 0.1 mM ($^1/_{10}$ of the regular NaH_2PO_4 concentration). On day 5, 45 h after medium change, cells were washed once with Tris, incubated for 45 min at 37 °C, and washed again twice. Cells were detached by 8–10 min of exposure to 1.5 mM ethylenediaminetetraacetic acid (EDTA) (in 124 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, and 5.6 mM glucose) and washed twice with Tris.

Preparation of Microsomes. Cells were incubated with IgE (5 μg/10^7 cells) at ~5-20 $\times$ 10^6 cells/mL in culture medium. Following $1^1/_2$ h of incubation at room temperature (~23 °C) the cells were washed 3 times with Tris and suspended at 10^8/mL in Tris containing the following inhibitors of proteolysis: aprotinin (~30 TIU/mL), pepstatin A (6 μg/mL), leupeptin (2 μg/mL), phenylmethanesulfonyl fluoride (PMSF; 2 mM) (all from Sigma), and iodoacetamide (40 mM; Fluka). Batches of this cell suspension were diluted 1:1 with water for 5 min in a cold, 15-mL Correx test tube (15-mm i.d.) and minced with polytron PT 10-35 (Kinematika, Switzerland). Homogenates were prepared by 3 $\times$ 16 s cycles of the polytron set at frequency 6 (~14 700 rpm) in ice and spun immediately for 15 min at 3000 rpm (Sorvall rotor SS-34). Supernatants were spun through a 7% sucrose cushion for 1 h at 35 000 rpm (Beckman rotor 50Ti or 60Ti). The pellets were briefly homogenized in Tris (plus all five inhibitors at half of the above concentrations), sampled for protein (Bradford, 1976) and phospholipid (Rouser et al., 1966) analyses, and either used immediately or frozen in liquid air and stored at −80 °C.

Phospholipid Analysis. Phospholipids were analyzed colorimetrically (Rouser et al., 1966). Assessment of [^{32}P]-phospholipid specific activity was done for lipid extracts of the microsomal fractions. Specific activities ranged between 10 $\times$ 10^3 and 30 $\times$ 10^3 cpm/nmol. Under the employed labeling conditions, the specific activity of total phospholipid extracted from the plasma membrane fraction is identical with that of the phospholipid in unfractionated microsomes (Fischer & Rivnay, unpublished results). However, the specific activities of individual phospholipid classes (isolated on TLC plates) are scattered around the weighted average measured for the total phospholipid extracts. Thus, for molar quantification, the radioactive counts should be divided by the respective specific activities of the individual lipids. The specific activities of the major lipids (relative to the weighted average) are as follows: 125% for PC, 98% for PE, 80% for PE (plasmalogen), 62% for PS, 67% for PI, and 71% for Sph (Fischer & Rivnay, unpublished results).

Preparation of Columns and Loading. Columns were prepared as follows: 0.2 mL of resin (Sepharose or TNP-Sepharose) was packed in 1-mL syringes plugged with glass wool. Larger columns (0.5 mL of resin in a 3-mL syringe) were occasionally used. Columns were washed sequentially with water, 2 column bed volume equivalents (bv) of 0.2 M acetic acid, water, and Tris, and finally 1 bv of 1 mg/mL bovine serum albumin (BSA) in Tris. Sample processing and separation were performed in a cold room (4 °C). The loaded microsomal sample was made 1 mg/mL in BSA ~10 min before loading, soaked onto the column for 15 min, refluxed twice through the packed resin, and then washed with 3 bv of Tris. Further washing with either Tris or other solution (as indicated) was made as detailed in the text.

Analysis of Column-Bound Material. Assessment of bound IgE was made by directly counting the columns (after washing) in a γ counter (Packard Autogamma Model 5210). The resin was then transferrred quantitatively to a 15-mL glass tube, the lipids were extracted twice as described elsewhere (Rivnay & Metzger, 1982; Renkonen et al., 1963), and a sample was counted in a β scintillation counter (Packard). β counts were corrected for the ~1–3% ^{32}P γ counts. At the chosen specific activities corrections were of the order of up to 30% in the plasma membranes and negligible in the purified receptors.

Phospholipid Separations. Thin-layer chromatography (TLC) in two dimensions and high-performance liquid chromatography (HPLC) were performed as detailed previously (Yavin & Zutra, 1977; Guerts van Kessel et al., 1977; Rivnay, 1984).

Results

Three methodological approaches had been designed in this study to deal with quantitative and qualitative problems pertinent to analytical studies of the type handled in this communication: (1) Cells were labeled biosynthetically with [^{32}P]orthophosphate in phosphate-deficient growth medium to enable detection of picomolar quantities of phospholipids. (2) Plasma membrane vesicles and FC$_\epsilon$R complexes were purified by using a common criterion for purification—IgE-mediated affinity chromatography. This was considered to

Table I: Affinity Purification of RBL-2H3 Plasma Membranes

			recovered in washed column					
			^{125}I-IgE[b]		binding specificity[c]	[^{32}P]phospholipids		binding specificity[c]
expt	column[a]	washing volume	cpm(10^3)	% of input		cpm(10^3)	% of input	
1	TNP-Sepharose	3	247	55.6	"100"	245	17.2	"100"
	Sepharose		59	13.3	24	89.5	6.3	37
2	TNP-Sepharose	75[d]	293	48.5	"100"	409	12	"100"
	Sepharose		19	3.2	6.6	97	2.8	23
	TNP-Sepharose + hapten[e]		32	6.1		196	5.7	

[a] Each line represents one column of 150-μL resin in tuberculin syringes. [b] Specific activities of IgE were 14.7×10^5 and 2.7×10^5 cpm/μg in experiments 1 and 2, respectively. [c] Binding specificity index = 100(percent retained on Sepharose)/(percent retained on TNP-Sepharose). [d] The washing step was controlled to last 7.5 h at a constant flow rate. [e] Column, microsomal sample, and washing buffer of this control had all been equilibrated with 2 mM DNP-caproic acid (free hapten) 10 min before loading. The column was otherwise processed as the two other columns of this experiment.

provide a sound reference lipid mixture to which receptor-bound lipids should be compared. Failure to inappropriately remove impurities of other subcellular microsomal organelles that potentially differ from plasma membranes in lipid composition may lead to erroneous conclusions on the uniqueness and specificity of the receptor-bound lipids. (3) The protocols were designed to minimize exposure of $Fc_\epsilon R$ to pure micellar detergent to prevent receptor dissociation.

Affinity Purification of Plasma Membranes. In order to establish the specificity and efficiency of plasma membrane purification, using the IgE-mediated affinity to TNP-Sepharose, we have employed the following approach. Cells were grown on [^{32}P]orthophosphate, loaded with ^{125}I-IgE, and homogenized for subcellular fractionation. The crude microsomal fraction thereof was loaded onto the affinity column, washed with Tris, and the retained ^{125}I-IgE and [^{32}P]-phospholipids were quantified.

As seen in Table I, TNP-Sepharose columns with excessive binding capacity (100 μg of IgE/column) retain approximately half of the ^{125}I-IgE. The unbound fraction conceivably comprises inside-out plasma membrane vesicles. Underivatized Sepharose CL-4B, which was not expected to interact with IgE, retained considerably less of this marker.

The binding specificity was further examined in relation to the efficiency of washing. The nonspecific columns (Sepharose) washed with only 3 bv (Table I, expt 1) retained 24% of the amount of IgE bound to the TNP-Sepharose. In contrast only 6.6% of the IgE was retained when 75 bv were used (Table I, expt 2). To ensure a reasonable signal-to-background ratio, microsome equivalents of ≥ 1 μg of IgE were usually loaded. Washing was routinely performed with 25–75 bv, resulting in <5% nonspecific binding.

Unlike the ^{125}I-IgE that serves as a marker for plasma membranes only, extracted [^{32}P]phospholipids represent all microsomal membranes. Therefore, TNP-Sepharose columns retained only 12–25% of this marker as compared to ~6% on underivatized Sepharose. The differential between the control and haptenated columns was not markedly improved upon prolonged washing. Normalizing the percent ^{32}P recovery for the total IgE loaded on columns implies that the plasma membrane fraction comprises ~20–50% of the microsomal fraction. The remainder therein includes other nonplasma membrane vesicles (e.g., Golgi apparatus, endoplasmic reticulum, and lysosomes).

Another control for binding specificity was based on blocking the TNP binding sites by a soluble hapten (TNP-caproic acid). Retention of both tracers was largely diminished under these conditions (Table I, expt 2).

Preparation of Receptor-Associated Phospholipids. In order to isolate the receptor-associated lipids, columns were washed with TCL instead of Tris. Preliminary experiments

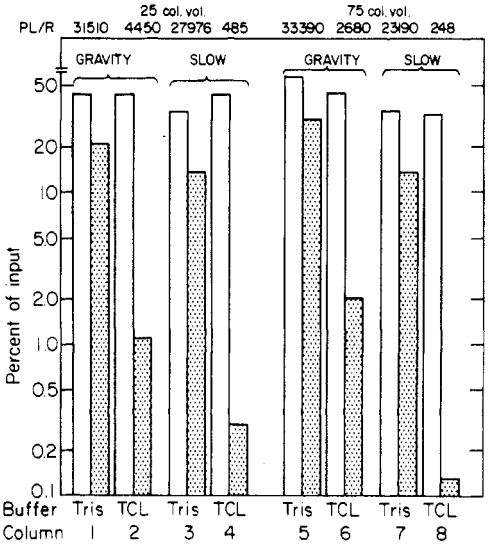

FIGURE 1: Effects of washing protocol on the extent of purification with TNP-Sepharose columns. Columns were loaded with identical samples of ^{32}P-labeled microsomes containing ~1.2 μg of ^{125}I-IgE. After 15 min of binding and quick washing with 1 mL of Tris, pairs of columns were processed in parallel to assess the role of two variables (washing volume and duration) on the purification of plasma membranes (Tris washing) or of solubilized and immobilized receptors (TCL washing). Washing volumes of 25 and 75% bv were compared in columns 1–4 and 5–8, respectively. A quick, gravity-controlled rate (~10–15 min) was compared with a slow, pump-controlled rate (7.5 h) in columns 1, 2, 5, and 6 and 3, 4, 7, and 8, respectively. Final column-bound IgE (open bars) and phospholipids (stippled bars) were quantified and expressed as percent of original input (histogram) or as the molar ratio (PL/R on top of the figure). TCL = 10 mM CHAPS and 2 mM phospholipids in Tris buffer.

indicated that removal of unbound lipids improved with the extent of column washing. Two alternative explanations could account for this difference: (a) more extensive removal of unbound micelles and (b) additional dissociation of labeled lipids from the bound receptor–lipid mixed micelles due to the prolonged washing. To examine these possibilities, eight separate columns of TNP-Sepharose (one per treatment) were loaded with identical samples of microsomes and processed in pairs wherein one column has been treated with Tris buffer and the other with TCL. The washing protocols in each pair varied such that the specific effect of the volume and duration of the washing on the final results could be mutually assessed. As shown in Figure 1, IgE retention on the TNP-Sepharose is not affected by the washing volume, by the rate of washing, or by the solubilization of the bound membranes (open bars). In contrast, the extent of phospholipid retention (stippled bars) is variable. Unbound membranes were more efficiently removed by the longer washing with Tris at both volumes (columns 3 vs. 1 and 7 vs. 5), reducing the amount of bound

Table II: Affinity Purification of Receptor-Associated [^{32}P]Phospholipids

expt	column[a]	pretreatment	loaded preparation	washing buffer[b]	retained (% of input)[c] IgE	retained (% of input)[c] phospholipid
1[d]	TNP-Sepharose		mouse IgE/intact microsomes	Tris	44.0	21.0
	TNP-Sepharose		mouse IgE/intact microsomes	TCL	43.5	1.0
	Sepharose		mouse IgE/intact microsomes	TCL	1.4	0.11
2[e]	TNP-Sepharose		rat IgE/detergent extract	TCL	1.0	0.035[f]
	TNP-Sepharose	cold, mouse IgE–receptor complexes	rat IgE/detergent extract	TCL	0.8	0.036

[a] See remark in Table I. [b] Washing lasted 7.5 h in both experiments. [c] See Materials and Methods for the mode of quantification. [d] ^{32}P-Labeled microsomes bearing mouse (anti-DNP) ^{125}I-IgE were loaded on TNP-Sepharose or on underivatized Sepharose and washed with 25 bv of Tris or TCL, as indicated, prior to lipid extraction. [e] Presolubilized ^{32}P-labeled microsomes, bearing rat IgE with no anti-DNP activity, were incubated with TNP-Sepharose or with TNP-Sepharose preloaded with 2 μg of cold IgE–Fc$_\epsilon$R complexes (cold IgE anti-DNP and cold lipids). The latter control was aimed at examining if the solubilized bulk lipids adhere nonspecifically to the bound IgE–Fc$_\epsilon$R complexes, rather than to the column matrix. Both columns were washed with TCL with 75 bv prior to extraction. [f] In spite of similarities in the percentages of nonspecific/specific retentions in IgE and in the phospholipids, the class distribution in this residual amount reflects the unfractionated microsomes rather than the purified IgE–Fc$_\epsilon$R.

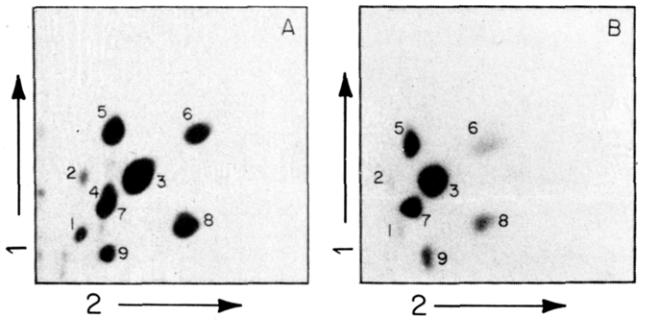

FIGURE 2: Autoradiogram of [^{32}P]phospholipids extracted from TNP-Sepharose affinity columns. Samples of the microsomal fraction (2 mg of total protein) prepared from RBL cells grown for 45 h on 15 μCi of [^{32}P]orthophosphate/mL were loaded on 250-μL TNP-Sepharose columns as detailed under Materials and Methods. Both columns were washing with 1 mL Tris and then treated differentially. One column was washed with Tris(75 bv) during 7.5 h (A). The other column was washed similarly but with Tris–CHAPS–lipid mixture (B) as detailed in the text. A sample of ~1 μg of IgE was associated with each column, comprising ~35% of the input. The extracted phospholipids yield phospholipid/receptor molar ratios of 27 500:1 and 210:1, respectively. TLC plates were loaded with 96 000 and 2360 cpm and exposed for 4 and 60 h, respectively, in mixture A and mixture B. Arrows 1 and 2 indicate the direction and the order of development in the basic and acidic solvents, respectively. The major spots are (1) β-lyso-PC, (2) α-lyso-PC (derived from plasmalogen-PC), (3) PC, (4) α-lyso-PE, (5) β-lyso-PE (derived from plasmalogen-PE), (6) PE, (7) sphingomyelin, (8) PS, and (9) PI.

Table III: ^{32}P Distribution in Column Extracts

spot[a]	phospholipids	intact cell[b] (%)	extracted lipids (A) intact plasma membranes cpm	(A) %	(B) receptor-associated cpm	(B) %
1	β-lyso-PC	0.6	1902	2.0	36	1.8
2	PC plasmalogen[c]	2.0	1656	1.7	20	1.0
3	PC 1,2-diacyl[d]	51.4	38526	40.2	864	42.4
4	β-lyso-PE	2.2	3466	3.6	nd[e]	
5	PE plasmalogen[c]	11.0	13288	13.9	355	17.4
6	PE 1,2-diacyl[d]	9.7	8561	8.9	106	5.2
7	Sph	1.6	7721	8.1	429	21.0
8	PS	4.0	8312	8.7	110	5.4
9	PI	7.8	3286	3.4	118	5.8
	choline PL/amine PL	2.08		1.47		2.38
	Sph/choline PL	0.03		0.15		0.31
	PS/PI	0.51		2.5		0.93
	% plasmalogen in PE	53		61		77

[a] Spot numbers refer to Figure 2. [b] G. Fischer and B. Rivnay (unpublished results). [c] Spot 2 is actually the acid-degradation breakdown product of plasmalogen, α-lyso-PC, but is representative of the original, intact molecule. The latter would have been indistinguishable from 1,2-diacyl-PC unless exposed to acid. The same is true for spot 5. [d] Traces of 1-alkyl-2-acyl subclass may be included. [e] nd, not detectable.

^{32}P 1.5–2-fold. More dramatic differences were obtained with TCL. Columns that were washed quickly ("gravity") with either volume retained ~1–2% of the input (columns 6 and 2). Thus, tripling the washing volumes under these conditions was not markedly more efficient. However, extending the washing time from ~10 min to 7.5 h with either volume resulted in a further decrease in residual lipids and in a detectable effect of the volume of washing (columns 4 vs. 2 and 8 vs. 6). Under the better conditions 0.1–0.3% of the initial ^{32}P load have been retained. The molar ratios of phospholipid/IgE–receptor complex calculated in these experiments (Figure 1, top) show that ~100-fold purification of the tightly bound, receptor-associated phospholipids can be achieved by using an efficient washing protocol. Appropriate controls were also made to examine the specificity of lipid retention in TCL-washed columns (i.e., in the solubilized and immobilized Fc$_\epsilon$R preparations). This was tested either on underivatized Sepharose, lacking TNP to be recognized by the mouse IgE (Table II, expt 1), or on TNP-Sepharose columns but with membranes bearing rat IgE, Which exhibits no specificity toward TNP (Table II, expt 2). These controls show that the nonspecific retention of lipid is 10–30-fold lower than the specific retention, namely, that the washing was comparably efficient for the lipids as for the IgE–Fc$_\epsilon$R.

Phospholipid Analysis. Assessment of the lipid composition was made on two-dimensional TLC by comparing the [^{32}P] lipid extracts to two TNP-Sepharose columns. One was washed with Tris only (plasma membrane lipids) and the other with TCL (receptor-associated lipids). Figure 2 illustrates the relative distribution of phospholipids in the two preparations (panels A and B, respectively). As seen in panel B, receptor-associated lipids overlap only partially with the pattern of plasma membranes (panel A). The former comprises three predominant classes: PC, sphingomyelin, and 1-alk-1-enyl-2-acyl-PE (plasmalogen PE). Since in these solvent systems sphingomyelin occasionally overlaps with 2-lyso-PE (1-PE), the two preparations have been reexamined in a combined analysis using both TLC and HPLC (Fischer & Rivnay, unpublished results; Rivnay, 1984). In addition the labeled sphingomyelin and PC were rerun on silica HPLC with isocratic separation with acetonitrile/methanol/85% H_3PO_4 (780:30:9 by volume). These confirmed that the intense spot marked 7 is largely sphingomyelin rather than 1-PE (not shown).

The quantitative analysis for the ^{32}P distribution is given in Table III. These data show that PC (spot 3) remains the major receptor-bound phospholipid. In addition, there is at least a 2-fold enrichment (in this particular experiment) of

Table IV: Distribution Parameters of Phospholipid Patterns in Various Fractions in the Procedure for Plasma Membrane and Receptor Preparation

distribution parameters	(A) intact RBL[b]	(B) microsomes	plasma membranes (C)[a] nonsonicated	plasma membranes (D) sonicated	(E) receptor
Sph (% of recovered)	1.58	4.55	8.36	8.94	34.9
Sph/choline PL[c]	0.028	0.086	0.148	0.179	0.659
choline PL/amine PL[c]	2.06	2.13	1.70	1.22	3.73
PS/PI	0.50	0.74	2.20	2.50	4.33
% plasmalogen in PE	53.2	55.6	62.0	65.0	55.6
IgE [^{32}P]PL (μg/10^6 cpm)	0.08–0.16[d]	0.61	1.75	2.30	~350[d]

[a] Each of the columns in C–E (one column per group) was loaded with 75 μL containing 217 μg of microsomal protein (~2 μg of IgE). The columns were slowly washed with Tris (C and D) or TCL (E) and then extracted. TLC plates were loaded with 10^5 cpm of extracted phospholipids, equivalent to ~8 μg of protein of the microsomal fraction (B), 10^5 cpm (C and D) and 2620 cpm E). Analysis was performed as detailed under Materials and Methods. [b] G. Fischer and B. Rivnay (unpublished results). [c] Choline PL includes 1,2-diacyl-PC, α-lyso-plasmalogen PC, β-lyso-PC, and Sph, detected as spots 3, 2, 1, and 7 in Figure 2. Amine PL includes 1,2-diacyl-PE, α-lyso-plasmalogen PE, β-lyso-PE, and PS, detected as spots 6, 5, 4, and 8 in Figure 2. [d] Estimated.

sphingomyelin (spot 7). Enrichment values as high as 3–4-fold were routinely obtained. Several distribution parameters are presented in the bottom of this table and indicate the following: (1) an increased ratio of choline phospholipids (PC + Sph) to amine phospholipids (PE + PS), (2) an increase in the relative contents of sphingomyelin in the choline phospholipids, (3) a decrease in the ratio of PS/PI (the two major negatively charged phospholipids components), and (4) a slight increase in the relative contents of plasmalogen (1-alk-1-enyl-2-acyl) in the PE class.

In order to further substantiate these trends, we have examined the above parameters obtained from 14 columns (7 experiments) wherein the phospholipid/receptor molar ratio (PL/R) could be evaluated more precisely. Averaging the data of the TCL-treated columns in these experiments was not straightforward, since the final PL/R varied among experiments in the range of 200:1 to 3400:1 due to the variable washing protocols or variability among experiments. Nevertheless, when the results were grouped according to PL/R, i.e., the results within a narrower range of PL/R were averaged (Figure 3), these parameters could be examined with confidence. Thus, the conspicuous rise found above for sphingomyelin was substantiated. Parallel increases were therefore seen both in the percentage of sphingomyelin within choline-containing phospholipids and in the ratio of choline PL to amine PL. In contrast the plasmalogen content in the PE class seems to remain constant throughout the procedure. The PS/PI ratio was more difficult to evaluate. It shows a significant rise when 90% of the lipids are removed (column B) followed by a drop when 99% of the lipid is removed (column C). Nevertheless, in some experiments, PS/PI ratios of as high as 4 were obtained (e.g., Table IV). Additional experiments suggested that neither of these lipids is enriched at the receptor-bound pool and that the occasional rise in this ratio results from an efficient removal of PI.

One potential source for the observed enrichment in sphingomyelin may have been a contamination of the plasma membrane (reference) preparation with other membrane fractions. To rule out this possibility, we first analyzed the phospholipid distribution in one microsomal preparation. Two additional samples were then separately processed on TNP-Sepharose columns, the first without any pretreatment and the other following a *5-min* sonication step. Sonic treatment was performed in order to dissociate possible aggregates of plasma membranes with other subcellular organelles. A similar sonicated sample was also processed for isolation of receptor-associated lipids (i.e., the column was washed with TCL rather than Tris). As seen in Table IV the distribution parameters that characterize the nonsonicated and sonicated microsome samples (C and D, respectively) are very similar.

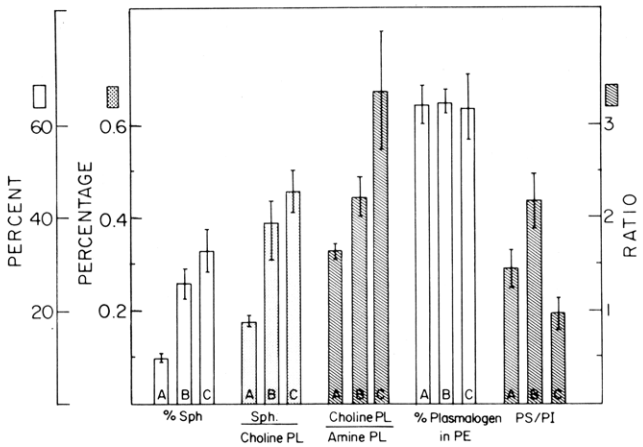

FIGURE 3: Interrelationship between phospholipid extracted from plasma membranes or partially purified IgE receptor complexes. Results from 14 columns that were loaded, processed, and extracted as described in text but with different washing duration and buffer volumes are evaluated. IgE of well-defined specific activity [(0.8–3.0) $\times$ 10^5 cpm/μg; >94% precipitable in 10% trichloroacetic acid] served as a quantitative marker for the receptor. Phospholipids were extracted from the crude microsomes and quantified colorimetrically, and the ^{32}P specific activity was determined (values ranged between 1 $\times$ 10^4 and 3 $\times$ 10^4 cpm/nmol). The distribution parameters, specified under the abscissa, were averaged (±SE) for (A) seven columns containing intact plasma membranes, (B) three columns washed with TCL but with apparently lower efficiency, and (C) four columns washed with TCL with high efficiency. The final PL/R obtained in the three groups was within the ranges of 22 000–35 000, 1600–3500, and 100–500 in A, B, and C, respectively.

The minor differences observed may indeed be ascribed to some contaminants of non-plasma-membrane organelles, since the nonsonicated values (C) are always intermediate between those of crude microsomes (B) and those of sonicated plasma membranes (D). Nevertheless, even the values obtained in column D, where contamination was minimized, are distinctly different from the respective values for the purified receptor in column E. The lower sphingomyelin content in the plasma membranes thus appears to be genuine, and the higher values associated with pure receptors indeed represent an enrichment.

Phospholipid analysis was also performed on the *eluted* IgE–Fc$_\epsilon$R (Figure 4) rather than on the resin-attached material. A pattern similar to that shown in Figure 2 was found, namely, retention of PC as the major class and enrichment in sphingomyelin (compare Figure 4 to Figures 2 and 3 and Table III). These results confirm the controls which showed that the lipid patterns had not been affected by nonspecific retention on the immobilizing resin. The elution protocol has not been employed routinely, due to incomplete IgE–Fc$_\epsilon$R elution that may have resulted in a biased lipid distribution.

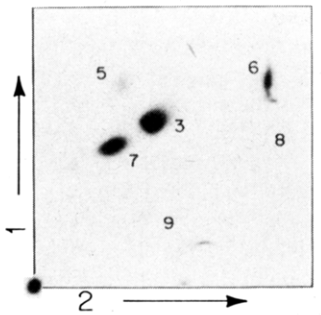

FIGURE 4: A TNP-Sepharose column (0.5 mL in a 3-mL syringe) was loaded with 5.2 mg of microsomes, washed with 2 mL of Tris and with 75 bv of TCL at a rate of 25 mL/h (90 min) at 4 °C. The column was then transferred to room temperature and quickly washed with 2 mL of 10 mM CHAPS and then with 1 mL of 3 mM CHAPS. IgE–Fc$_\epsilon$R complexes were eluted with 4 × 600 μL portions of 5 mM ϵ-*N*-DNP-L-lysine in 3 mM CHAPS. The final PL/R molar ratio in the eluate was 25. The chromatogram was loaded with 1600 cpm, developed, and exposed for 7 days. Distribution of the ^{32}P radioactivity in the major spots was as follows: 2.8% for PC-plasmalogen (spot 2, not seen in the figure), 35% for PC (3), 6.9% for PE-plasmalogen (5), 8.4% for PE (6), 19% for Sph (7), 1.3% for PS (8), and 2.4% for PI (9).

Effects of the Individual Components of the Washing Buffer. Since we used phospholipids in the washing buffer, we examined their effect on the distribution of the receptor-bound lipid preparations. A TNP-Sepharose column was washed with a buffer of higher CHAPS concentrations to yield ρ = 10 (2 mM RBL phospholipids and 24.6 mM CHAPS). To minimize dissociation of the receptor, anticipated under these conditions, this column was washed faster (~20 min) yet with the same 75 bv. The resulting preparation contained 527 phospholipid molecules per receptor molecule, and the following parameters were derived from TLC: % Sph = 44, Sph/choline PL = 0.54, choline PL/amine PL = 5.7, % plasmalogen in PE = 75%, and PS/PI = 0.59 [compare with Tables III and IV (E) and Figure 2]. These results indicated as expected that (a) the rate of removal of [^{32}P]phospholipids was faster at higher detergent-to-phospholipid ratios and (b) under these conditions the enrichment of sphingomyelin in the receptor-associated lipids becomes even more conspicuous.

To further examine the role of the lipids, we washed one column with CHAPS *alone* in order to completely eliminate the effect of the lipids. In the same experiment, we also tested two additional detergents: sodium cholate and Triton X-100. In all three preparations (Table V), sphingomyelin appears enriched as compared to its percentage in the plasma membrane [Table III (A), Table IV (C and D), Figure 2A]. Also apparent was the rise in choline PL/amine PL and the increased percentage of sphingomyelin within the choline PL. The plasmalogen contents within PE were not increased, in accord with Table IV and Figure 3. However, here, like in Table IV (E), the PS/PI ratio was considerably higher compared to plasma membrane values. The enhanced dissociation of $\beta\gamma_2$ from IgE–α (Kinet et al., 1985a) in the absence of lipids was not assessed in this experiment. We therefore chose to express PL/IgE and not PL/R. We assume, however, that under the present conditions (the higher cmc, the shorter time of exposure, and the higher receptor concentration) [cf. Figures 2A and 4 in Kinet et al. (1985a)], less than 50% of the receptor may have been dissociated.

Table V: Phospholipids Associated with Receptors That Were Partially Purified with Lipid-Free Detergent Solutions

	detergent		
	CHAPS	cholate	Triton X-100
washing			
detergent (mM)[a]	10.7	15.7	6.3
washing period (min.)	40	120	40
final PL/IgE (molar)[b]	57.2	363	150
phospholipids recovered[c] (%)			
β-lyso-PC	2.5	1.5	1.3
PC plasmalogen[d]	0.7	1.9	1.6
PC 1,2-diacyl[d]	34.6	44.4	45.5
PE plasmalogen[d]	2.9	12.5	3.3
PE 1,2-diacyl[d]	5.4	6.7	2.1
Sph	39.9	17.8	41.0
PS	8.9	9.4	4.5
PI	1.4	3.7	0.7
distribution parameters			
Sph/choline PL	0.51	0.27	0.46
choline PL/amine PL	4.51	2.29	9.1
% plasmalogen PE	35.4	64.9	61.4
PS/PI	6.25	2.55	6.4

[a] Detergents were designed to be 6 mM in the micellar form with values of 4.7, 9.7, and 0.3 mM as the cmc of the three detergents, respectively. [b] Twofold larger values may be a higher limit in this ratio since it is likely that only the undissociated $\beta + \gamma$ bind phospholipids (based on the tendency of the dissociated parts of the receptor to become incorporated into liposomes in reconstitution experiments (Rivnay et al., 1982). [c] Percent of each phospholipid in the extracted column lipids. [d] See remarks on these spots in Table II.

Discussion

Understanding the mechanism and forces that maintain the structural integrity of the IgE receptor is a prerequisite for future studies in which this receptor will be manipulated in its purified form. The earlier observations that implicated phospholipids as being involved in this mechanism (Rivnay et al., 1982, 1984; Perez-Montfort et al., 1983; Rivnay & Metzger, 1982) also suggested that commercially available, pure phosphospholipids (either synthetic or of natural sources) were not efficient in preserving the multisubunit structure of the receptor.

With the assumption in mind that the lipid components of the natural milieu of this receptor may provide the optimal composition to prevent dissociation, we have chosen to analyze the composition of the phospholipids that were tightly bound to the receptor complex. With the use of several methodological maneuvers it became possible in this study to label the endogenous phospholipids with ^{32}P with a high specific activity (>10^4 cpm/nmol). The receptor was then solubilized and applied to affinity columns, and most of the endogenous lipids were removed under conditions where dissociation was largely prevented.

Two classes of phospholipids remained tightly bound to the soluble receptor–IgE complexes. One was phophatidylcholine (PC), which maintained relative majority (~40%) similar to that found in the unfractionated plasma membrane lipids. The other was sphingomyelin, which was enriched in the receptor preparation 2–5-fold and comprised a similar portion of the receptor-bound lipids as PC. The preferred retention of sphingomyelin is unlikely to be an artifact; a large variety of experiments were performed to examine that possibility (e.g., Figure 4 and Tables II, IV, and V).

The sphingomyelin molecule possesses both an amide bond and a free hydroxyl group, which provide a higher potential for hydrogen bonding (Barenholz, 1984). The observed enrichment in sphingomyelin may thus have been promoted by this hydrogen-bonding capacity. Nevertheless this is not the sole cause for the retention of these lipids, since PC lacks the same H-bonding capacity and should have been washed away. Moreover, CHAPS, which also comprises several potential hydrogen-bonding groups, coexists, under the protocols em-

ployed, with sphingomyelin in the same micellar solution for prolonged periods at 1000-fold higher concentrations. Under these conditions it is logical that the detergent would have displaced the lipid from the receptor complex. Retention is unlikely to be primarily due to H bonding. It is difficult at this point to determine which portion of the sphingomyelin molecule is critical to the observed tight binding to the IgE receptor. Choline may be involved, since both sphingomyelin and PC are choline phospholipids.

The identification of receptor-bound phospholipids provides an answer to the insufficient protection of the IgE receptor by commercial lipid preparations: the latter lack sphingomyelin. Further analysis of the fatty acid composition of the two phospholipid classes may be helpful to establish the precise composition of the receptor's environment. Nevertheless, this may not be necessary since the earlier attempts to examine receptor stability with the commercial lipids did suggest that sphingomyelin, rather than PC, is the crucial lipid in that respect. These studies indicated that PC alone does not serve to adequately protect the receptor.

The results of the present analysis indeed provide a restricted list of potential phospholipid candidates to be examined for their effect on the structural integrity of the receptor. The question whether these lipids are bound uniquely to the Fc_ϵ receptor is secondary in importance. Nevertheless, regarding this question, it was surprising to find reports only on one other plasma membrane protein that binds sphingomyelin—the hepatic 5'-nucleotidase (Widnell & Unkeless, 1968; Merisko et al., 1981).

The common notion is that choline phospholipids (PC + sphingomyelin) reside at the outer leaflet of the plasma membrane whereas amino phospholipids (PS and PE) are largely confined to the inner leaflet. With this in mind, it is anticipated that a large number of plasma membrane proteins should bind PC and sphingomyelin as does $Fc_\epsilon R$. We have examined this possibility by analyzing the phospholipids bound to plasma membrane proteins (other than $Fc_\epsilon R$) that were retained on and eluted from Con A–Sepharose. As suspected, the two major phospholipids identified in this analysis were indeed PC and sphingomyelin. The scarcity in other sphingomyelin-associated plasma membrane proteins reported so far may apparently be due to the lower affinities between this lipid and many of the proteins embedded in the outer leaflet, so that the lipid does not survive the delipidation step.

The implication of an association between a phospholipid and a membrane protein has usually been derived either from direct analysis or from functional studies. In the few cases where both types of assessments (analytical and functional) were made [e.g., Widnell and Unkeless (1968), Merisko et al. (1981), and Deese et al. (1982)], the specific lipid found in the direct analyses were always necessary for the function. As a result it is also hoped that the lipid pattern found in the present study will indeed be the most optimal for future functional studies. As for the open questions on the structural level, this study advances or understanding of how to operate with the purified $Fc_\epsilon R$ in suggesting what should be an appropriate lipid mixture with which to preserve its integrity at micellar CHAPS. The interrelationship between these analytical data and the functional requirements needs to be analyzed directly. This however, will not be feasible in a reproducible and quantitative manner before one establishes conditions for obtaining preparations with a predetermined or at least controlled lipid:protein ratio. Attempts in this direction are currently under way. We are also performing further analyses of the species composition of sphingomyelin and PC derived from the unfractionated plasma membranes and from purified receptors.

The notion that the membrane lipids maintain functional proteins as an intact, undissociated complex is not unique for the receptor for IgE. It is gradually emerging as a characteristic of other systems, including phosphatidylserine decarboxylase (Rizzolo, 1981), the regulatory and catalytic units of the adenylate cyclase system (Arad et al., 1984), or in parts of the mitochondrial respiratory chain (Shimoura et al., 1984). In all these cases, proteins that were originally thought to exist as monomeric or as separate, unassociated proteins now appear to reside in multimers (Rizzolo, 1981) or to be complexed with other proteins under milder conditions. This notion merely indicates that in replacing membrane lipids, detergents cannot fully and optimally satisfy all aspects of lipid–protein interactions.

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Effect of Covalent Attachment of Immunoglobulin Fragments on Liposomal Integrity†

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ABSTRACT: Liposome stability during and after covalent coupling of Fab′ antibody fragments was investigated. Large unilamellar vesicles containing entrapped 5(6)-carboxyfluorescein (CF) as a marker for liposomal integrity were prepared by extrusion through polycarbonate membranes. *N*-[4-(*p*-Maleimidophenyl)-butyryl]phosphatidylethanolamine (MPB-PE) was employed as a liposomal anchor for the covalent coupling of Fab′ fragments. We observed that coupling of Fab′ fragments to liposomes containing 5 mol % MPB-PE caused a concentration-dependent increase in size and polydispersity of the liposomes. Dependent on the concentration of the MPB-PE anchor in the membrane and the concentration of Fab′ added, coupling was associated with the release of up to 95% of the entrapped CF. Rupture of the liposomes was identified as the primary mechanism of CF release during Fab′ coupling. Reduction of the MPB-PE concentration to 1 mol % resulted in liposomes that were stable during and after Fab′ coupling. The increased stability of these liposomes was due to the lower MPB-PE concentration and not to the lower number of attached Fab′ fragments. By proper adjustment of the experimental conditions for coupling, the number of Fab′ fragments attached to the 1 mol % MPB-PE liposomes could be increased without affecting the stability of the resulting liposomes. These stable liposomes, made by an extrusion method that avoids the use of organic solvents, detergents, or sonication, are therefore suitable for entrapment of labile compounds and can be used for immunotargeting or immunoassays.

Synthetic phospholipid bilayer vesicles (liposomes) with covalently attached proteins on their surface have been prepared by a number of investigators for a variety of purposes. Examples are liposomes coated with antibodies or their fragments for immunotargeting of entrapped drugs to cells [reviewed by Gregoriadis (1984)] or coated with protein antigens for use in liposome-based immunolytic assays (Ishimori et al., 1984; Bredehorst et al., 1985). Various coupling procedures have been reported with detailed analyses of coupling efficiency, preservation of antibody activity, and stability of the cross-link between antibody and liposome [reviewed by Gregoriadis (1984)]. However, very few studies have investigated the effect of covalent attachment of protein molecules on liposomes during and after the coupling reaction.

For this study, we have prepared large unilamellar liposomes under conditions that permit entrapment of molecules that are labile in the presence of organic solvents and detergents. As an anchor for the covalent attachment of Fab′ fragments through their free sulfhydryl group, these liposomes contained *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE)[1] (Martin & Papahadjopoulos, 1982). We have

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