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Structural Studies on the Membrane-Bound Immunoglobulin E-Receptor Complex. 1. Characterization of Large Plasma Membrane Vesicles from Rat Basophilic Leukemia Cells and Insertion of Amphipathic Fluorescent Probes†

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ABSTRACT: In order to investigate the properties of the membrane-bound IgE-receptor complex, a simple procedure has been adapted for preparing large plasma membrane vesicles from rat basophilic leukemia cells. These vesicles pinch off from the adherent cells after treatment with 2 mM *N*-ethylmaleimide or 50 mM formaldehyde and 1 mM dithiothreitol, and they are isolated from the supernatant after two centrifugation steps with yields of 20-25% of the initial cell-bound ^{125}I-IgE. With phase and fluorescence microscopy, micron-size vesicles are seen which are unilamellar and spherically shaped and devoid of intracellular organelles. On dextran gradients at least 70% of the ^{125}I-IgE is bound to membranes which band at low density, indicating large, intact vesicles that are impermeable to macromolecules. Between 60 and 75% of the bound ^{125}I-IgE is accessible to the external medium, showing the vesicles to be predominately right side out. This preparation was found to be suitable for resonance energy-transfer measurements. We have determined that amphipathic, fluorescent donor and acceptor probes partition into the vesicle bilayer in a randomly distributed, noninteracting manner. The densities of the probes can be ascertained directly from the amount of energy transfer that is observed as a function of acceptor concentration. This experimental system will allow energy-transfer measurements to determine distances between sites on receptor-bound IgE and the membrane surface.

Molecular details of the three-dimensional structure of mammalian cell surface receptor proteins are virtually unknown beyond basic information such as subunit composition and molecular weights. In particular little is known about the structure of receptors which bind immunoglobulins to mediate endocytosis or antigen-triggered transmembrane signaling (Unkless et al., 1981; Metzger et al., 1982). The receptor for immunoglobulin E (IgE)[1] may be the best characterized

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[1] Abbreviations: IgE, immunoglobulin E; RBL, rat basophilic leukemia; HAF, 5-(hexadecanoylamino)fluorescein; HAE, 5-(hexadecanoylamino)eosin; HHC, 3-hexadecanoyl-7-hydroxycoumarin; ORB, octadecylrhodamine B chloride; FITC, fluorescein 5-isothiocyanate; $DiOC_n$-(3), 3,3′-dialkyloxacarbocyanine; NEM, *N*-ethylmaleimide; HCHO, formaldehyde; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate.

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member of this group owing to the availability of a rat basophilic leukemia (RBL) cell line which contains this receptor in relatively large quantities (Barsumian et al., 1981) and to the very tight binding of rodent IgE (Kulczycki & Metzger, 1974; Sterk & Ishizaka, 1982) which provides an experimental handle in this system. This receptor mediates the degranulation of mast cells, basophils, and RBL cells when IgE–receptor complexes are cross-linked by multivalent antigen (Siraganian, 1981; Kagey-Sobotka et al., 1982; Fewtrell et al., 1979). Recent studies have shown that the receptor contains single copies of two different polypeptide chains: the α subunit is a glycoprotein of $M_r \approx 50\,000$ which binds IgE at the outside surface of the plasma membrane (Conrad & Froese, 1976; Kulczycki et al., 1976), and the β subunit is a nonglycosylated protein of $M_r \approx 35\,000$ which interacts directly with the hydrophobic region of the membrane bilayer (Holowka & Metzger, 1982; Holowka et al., 1981). Little is known about the molecular details of the interaction of the α subunit with IgE (Dorrington & Bennich, 1978; Perez-Montfort & Metzger, 1982) or the nature of the communication between the receptor and the interior of the cell during the transmembrane signaling event (Ishizaka, 1982; Fewtrell et al., 1982).

We have begun to employ quantitative fluorescence methods in order to obtain a more highly resolved picture of the IgE–receptor structure and its interaction with the plasma membrane. In the studies described in this and the following paper (Holowka & Baird, 1983) resonance energy transfer has been used to investigate how the receptor-bound IgE molecule is oriented with respect to the membrane surface by measuring the distance between donor and acceptor probes that are labeling specific sites in these two parts of the system. In the past energy transfer has been valuable in providing three-dimensional maps of sites within soluble proteins (Stryer, 1978), and recently the method has had some limited application in membrane systems that are considerably more complicated (e.g., Baird et al., 1979; Kimelman et al., 1979; Thomas & Stryer, 1982; Cerione et al., 1983). In order to apply energy transfer to the membrane associated IgE–receptor complex, large, oriented plasma membrane vesicles were prepared by adapting a method of chemically induced vesiculation (Scott, 1976) to RBL cells. Amphipathic probes which contain polar fluorescent head groups and nonpolar hydrocarbon tails insert into the membrane bilayer, and their distribution and density can be determined fluorometrically. The suitability of this experimental system for energy-transfer measurements has been examined in the studies presented herein.

Experimental Procedures

Chemicals. 5-(Hexadecanoylamino)fluorescein (HAF), 5-(hexadecanoylamino)eosin (HAE), 3-hexadecanoyl-7-hydroxycoumarin (HHC), octadecylrhodamine B chloride (ORB), and fluorescein 5-isothiocyanate (FITC) were obtained from Molecular Probes, Inc., and were used without further purification. The oxacarbocyanine probes with 6-carbon alkyl chains [$DiOC_6$-(3)] and 10-carbon alkyl chains [$DiOC_{10}$-(3)] were synthesized in the laboratory of A. Waggoner (Sims et al., 1974) and were gifts from the laboratory of W. W. Webb (Cornell University). Aprotinin and *N*-ethylmaleimide (NEM) were obtained from Sigma Chemical Co. Formaldehyde (HCHO) (37%) was purchased from Mallinckrodt, and dithiothreitol (DTT) was from Bio-Rad.

Immunoglobulin E. The murine monoclonal anti-2,4-dinitrophenyl-IgE was purified from ascitic fluid of CAF_1 mice bearing the tumor H 1 DNP-ϵ-26-82 (Liu et al., 1980) by a previously described procedure which uses affinity chromatography (Holowka & Metzger, 1982). ^{125}I-IgE was prepared as described for rat IgE (Kulczycki et al., 1974). Determination of specific activity and other quantitation of ^{125}I were carried out on a Beckman Gamma 4000 γ radiation spectrophotometer. FITC–IgE was prepared as described in the following paper (Holowka & Baird, 1983).

Table I: Preparation of IgE-Receptor-Containing Membrane Vesicles from RBL Cells

	cell-bound ^{125}I-IgE recovered[a] (%)	
	NEM	HCHO/DTT
(1) adherent RBL cells saturated with ^{125}I-IgE and washed	100	100
↓ 2 mM NEM or 50 mM HCHO and 1 mM DTT in 2 mM Ca^{2+}, 10 mM Hepes (pH 7.4); 0.15 M NaCl; 1 h, 37 °C		
(2) supernatant from treated flasks	74 ± 16	30 ± 3
↓ centrifugation, 135*g*, 10 min		
(3) supernatant from low-speed spin	34 ± 3	25 ± 1
↓ centrifugation, 25000*g*, 45 min		
(4) pelleted vesicles resuspended in PBS + protease inhibitors	20 ± 2	22 ± 4
↓ filtration with 5 μm Nucleopore polycarbonate filter; ↓ dialysis		
NEM membrane vesicles	18 ± 2	21 ± 3
HCHO/DTT membrane vesicles		

[a] Average values for eight preparations (NEM) or four preparations (HCHO/DTT).

RBL Cell Culture and Membrane Vesicle Preparation. Subline RBL-2H3 (Barsumian et al., 1981) was maintained in stationary cell culture as previously described (Taurog et al., 1979). Cells for preparation of membrane vesicles were grown adherent to large flasks (150–175-cm^2 surface area) and used 4–6 days after passage; typically six large flasks (about 2.5×10^8 cells) were used for a single preparation.

The procedure for isolation of plasma membrane vesicles from RBL cells is outlined in Table I. Nonadherent cells were decanted from the flasks, and then, if saturation of receptors with IgE was desired, minimal essential medium containing 10% calf serum, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) (pH 7.4), and ^{125}I-IgE in 5–10-fold molar excess over estimated receptor content was added (Kulczycki & Metzger, 1974; Barsumian et al., 1981). Flasks were incubated for 1 h at 37 °C with gentle rocking (≤40 cycles/s) in a large shaking water bath. The medium containing nonbound ^{125}I-IgE was then decanted, and the cells were washed twice with 5–10 mL of 10 mM Hepes (pH 7.4), 0.15 M NaCl, 2 mM $CaCl_2$, and 0.05% gelatin (Ca/Hepes/gel buffer). A 10 mL volume of the same buffer but containing either 2 mM NEM or 50 mM HCHO and 1 mM DTT was added to each flask, and these were rocked gently at 37 °C for 1 h as above. After the 1-h period, the flasks were gently

rapped several times to enhance vesicle release, then supernatants were decanted into tubes at 4 °C, and deoxyribonuclease I and $MgCl_2$ were added to final concentrations of 50 μg/mL and 2 mM, respectively. The suspension of vesicles was centrifuged at 135 g for 10 min to remove whole cells that had become detached, and then the supernatant was carefully removed and further centrifuged at 25000*g* for 45 min (4 °C) to pellet the vesicles. The membrane pellet was resuspended in 1 mL of 10 mM sodium phosphate (pH 7.5), 0.15 M NaCl, and 0.01% sodium azide (PBS/NaN_3) by using a syringe and 23-gauge needle. Butylated hydroxytoluene (10 μg/mL) and protease inhibitors were then added: either 1 mM phenylmethanesulfonyl fluoride or 0.5 unit/mL trypsin inhibitor of aprotinin was added to both types of vesicles; 10 μg/mL leupeptin and 5 μg/mL pepstatin A also were added to the HCHO/DTT vesicles. As a final step in the preparation, NEM vesicles were usually passed through a 5-μm Nucleopore polycarbonate filter to remove remaining cells, and HCHO/DTT vesicles were dialyzed for 2 days against two 50-mL volumes of the PBS/NaN_3 buffer containing the protease inhibitors in order to remove residual formaldehyde (Scott, 1976).

Vesicles were hypotonically lysed following the procedure of Schmidt-Ullrich et al. (1974): vesicles pelleted by centrifugation at 25000*g* for 30 min (4 °C) were resuspended in 10 mM Hepes (pH 7.4), then pelleted again, and resuspended in 1 mM Hepes (pH 7.4). For density graident analysis, lysed vesicles again were pelleted (25000*g*, 30 min, 4 °C) and then resuspended in 10 mM Hepes (pH 7.4) with protease inhibitors.

Microscopy. Membrane vesicles containing 7 pmol of IgE–receptor complexes/mL were incubated with 0.6 μM HAF for 15 min at room temperature. Aliquots were examined with a Nikon Optiphot epifluorescence microscope using a Nikon 100× oil immersion planachromat objective and a 2.5× ocular. Photomicrographs were recorded on Tri-X film, push-processed in Kodak D19 to ASA 1600.

Density Gradient Analysis. Samples 100–300 μL of the vesicle suspensions were layered on the top of 4 mL of a 1–25% w/w continuous dextran gradients (Dextran T-10, Pharmacia Fine Chemicals) which were prepared with either PBS/NaN_3 buffer for untreated vesicles or 10 mM Hepes for lysed vesicles. The gradients were then centrifuged at 110000*g* for 11–12 h at 4 °C in the SW-60 rotor of a Beckman L2-65B ultracentrifuge. Afterward fractions were withdrawn sequentially from the top with a needle and syringe. The density of each gradient fraction was determined from a refractive index measurement with reference to a calibration curve established by using known dextran concentrations and an extrapolated value of 1.40 g/mL for 100% dextran at 20 °C (Pharmacia technical information).

Analytical Methods. Determinations of protein content in the vesicle preparations were made according to the method of Bensadoun & Weinstein (1976) with bovine serum albumin as the standard. The concentration of IgE was measured by its OD at 280 nm by using $\epsilon_{0.1\%} = 1.62$ (Liu et al., 1980).

The binding of IgE to vesicles before and after membrane solubilization by 0.5% Triton X-100 was determined by using an ammonium sulfate precipitation assay (Rossi et al., 1977). Vesicles (about 10 pmol of receptor/mL) were incubated with saturating amounts of ^{125}I-IgE for 2 h at 4 °C, and then, if only externally accessible receptors were to be assessed, excess unlabeled IgE was added prior to solubilization and precipitation by 40% saturated ammonium sulfate. To determine the total number of receptors present, vesicles incubated with ^{125}I-IgE were solubilized without addition of unlabeled IgE, and the incubation was continued for another 1.5 h before precipitation.

Spectroscopy. Absorption spectra were recorded with a Cary 118 spectrophotometer. Light scattering contributions to the spectra of the vesicle-associated chromophores were subtracted by using either an appropriate vesicle control sample or an extrapolation of the Rayleigh scattering curve at the long wavelength side of the absorption transitions.

Fluorescence measurements were carried out at 20 °C in a Perkin-Elmer MPF-44B fluorescence spectrophotometer with corrected spectrum capability. Aliquots of the amphipathic probes in dimethyl sulfoxide stock solutions were added to the vesicles suspended in PBS (pH 7.5) in 1 × 1 cm or 3 × 3 mm quartz cuvettes, and the fluorescence was recorded after mixing. The final dimethyl sulfoxide concentration was always less than 2%, and no effect of dimethyl sulfoxide on the fluorescence or on the structure of the vesicles was detected. Light scattering contributions to the fluorescence signal were determined to be generally negligible under the conditions employed. The quantum yields of vesicle-bound donor fluorophores were determined as previously described (Cantley & Hammes, 1976) with sodium fluorescein in 0.1 N NaOH ($Q = 0.92$; Weber & Teale, 1957) as a standard for HAF and quinine sulfate in 0.1 N H_2SO_4 ($Q = 0.70$; Scott et al., 1970) as a standard for HHC. The absorbances of these probes at their exciting wavelengths (480 nm for HAF and 420 nm for HHC) were determined from appropriate dilutions of stock solutions with 0.5% Triton X-100 in PBS. The steady-state anisotropy, $\bar{A}$, was determined as defined as

$$\bar{A} = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}} \tag{1}$$

where $F_{\parallel}$ and $F_{\perp}$ are the fluorescence intensities measured when the emission polarizer is oriented in the vertical and the horizontal positions, respectively, and the excitation polarizer is oriented in the vertical position. Unequal transmission of vertically and horizontally polarized light by the emission monochromator was corrected as previously described (Chen & Bowman, 1965).

The efficiency of resonance energy transfer was calculated from the relationship

$$E = 1 - \frac{Q_{DA}}{Q_D} \tag{2}$$

where Q_{DA} and Q_D are the quantum yields of the donor molecule in the presence and the absence of the acceptor molecule. Relative values for Q_{DA} and Q_D were experimentally measured as the fluorescence intensity at a single donor emission wavelength, after it has been determined that the shape of the emission spectrum did not change significantly in the presence of acceptor molecules. For a single donor–acceptor pair, the critical transfer distance, R_0 (Å), at which the energy-transfer efficiency is 0.5 was calculated from eq 3 (Förster, 1959):

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_D n^{-4})^{1/6} \tag{3}$$

where J is an integral measuring spectral overlap of donor fluorescence emission and acceptor absorption; κ^2 is a factor characterizing the relative orientation of donor and acceptor dipoles, and n is the refractive index of the medium in which the energy transfer occurs. Values of $^2/_3$ for κ^2 and 1.4 for n were used; overlap integrals (J) were calculated as described previously (Cantley & Hammes, 1975).

The density of acceptor probes in the plane of the membrane was determined by measuring energy transfer from a donor probe present in the same bilayer as a function of the concentration of acceptor. These measurements yielded curves that could be fit by the two-exponential functions for the situation of energy transfer in two dimensions (Wolber & Hudson, 1979):

$$Q_{DA}/Q_D = 1 - E = 0.6463 \exp(-4.7497C) + 0.3537 \exp(-2.0618C) \quad (4)$$

$$Q_{DA}/Q_D = 1 - E = 0.6290 \exp(-4.5752C) + 0.3710 \exp(-1.9955C) \quad (5)$$

where C is the surface density in terms of the number of acceptors per R_0^2 when the distance of closest approach between donor and acceptor is $0.0R_0$ (eq 4) or $0.25R_0$ (eq 5). From physical considerations it is likely that the actual minimal distance lies somewhere in between these two values. In our application of these equations the molar concentration of acceptor at a single point in the titration curve was converted to the theoretical surface density which gave the same value for 1 − E, and the concentration units for the other data points in the titration curve were converted accordingly, assuming a linear correspondence. (This assumption is justified as based on fluorescence enhancement data such as shown in Figure 5.) The choice of the point on the data curve on which to base the conversion factor was determined by the best visual fit of the standard equations to all of the converted data points. We found that eq 4 and eq 5 gave nearly the same molar concentration to surface density conversion for all of the donor–acceptor pairs used in this study; this was expected since the efficiency of energy transfer in this situation is not sensitive to the distance of closest approach as long as it is small compared to the R_0 (Fung & Stryer, 1979; Snyder & Freire, 1982).

Results

Preparation and Microscopic Appearance of Membrane Vesicles. For an appropriate experimental system allowing quantitative fluorescence measurements of the membrane-bound IgE–receptor complex, we prepare large plasma membrane vesicles from RBL cells by an adaptation of the procedure developed by Scott for cultured fibroblasts and other cell lines (Scott et al., 1979). In this method vesicles pinch off after chemical induction of cell surface blistering or "blebbing" (Scott & Maercklein, 1979). As described under Experimental Procedures and outlined in Table I, we have used two different chemical treatments to induce the blebbing process, either 2 mM NEM or 50 mM HCHO with 1 mM DTT, and the yields of ^{125}I-IgE–receptor complexes associated with the vesicles have been consistently 20–25% of initial cell-bound ^{125}I-IgE under both sets of conditions. No significant ^{125}I-IgE is found associated with the membrane vesicles if a large (20-fold) excess of unlabeled IgE is incubated with the cells prior to the addition of ^{125}I-IgE. With both chemical treatments, the final preparations contain less than 0.2% residual whole cells compared to the initial amounts (Table II). There are some differences between the two preparations. As revealed by the numbers in Table I, the NEM treatment causes as many as 50% of the adherent cells to detach from the culture flask (these are subsequently removed by low-speed centrifugation), while the HCHO/DTT treatment causes very few of the cells to detach. Also, as monitored by trypan blue dye exclusion, the viability of the NEM-treated cells often falls below 90% by the end of the incubation period, while usually more than 95% of the cells treated with HCHO/DTT exclude the dye. These procedures were carried out with and without preincubation of the cells with IgE, and the observations were the same.

Table II: Composition of Isolated Membrane Vesicles from RBL Cells

component/parameter	quantity obtained from 10^8 cells: NEM treatment	HCHO/DTT treatment
(1) residual cells	$<2.5 \times 10^5$ (0.2%)	$<2.5 \times 10^5$ (0.2%)
(2) IgE receptor[b]	9.4 ± 3.8 pmol	11.0 ± 5.3 pmol
(3) protein, intact vesicles	0.47 ± 0.07 mg (3)[a]	0.46 mg (1)[a]
(4) protein, hypotonically lysed vesicles	0.23 ± 0.09 mg (2)[a]	0.25 ± 0.06 mg (2)[a]
(5) purification factor[c]	20-fold	21-fold

[a] Number of preparations analyzed in parentheses. [b] Determined from ^{125}I-IgE recovered and assuming a stoichiometry of 1:1 (Kannelopoulos et al., 1980). [c] Based on milligrams of protein per picomole of receptor for shocked vesicles vs. whole cells.

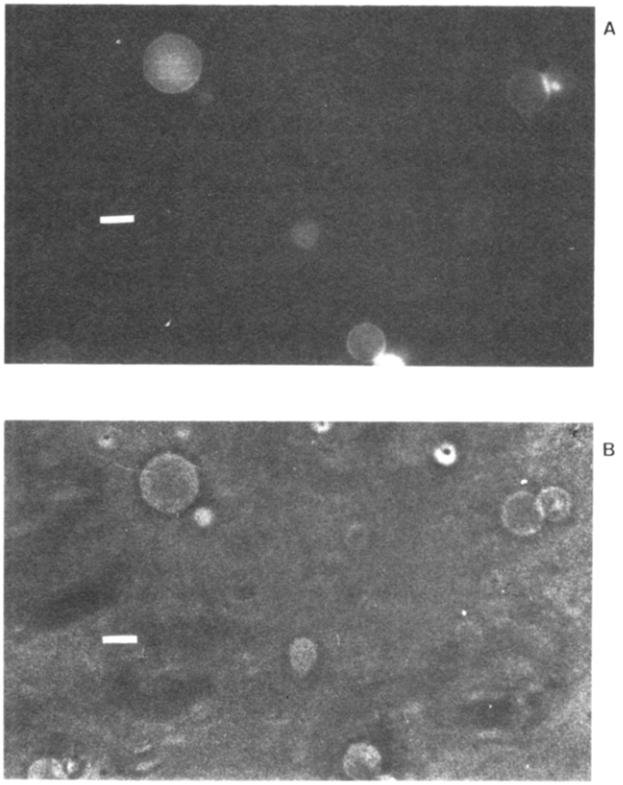

FIGURE 1: Micrographs of a HCHO/DTT vesicle preparation with bound HAF. (A) Fluorescence image of HAF bound to vesicles; (B) phase contrast image of same field as (A). Bar corresponds to 5 μM.

When these preparations are examined by phase microscopy, large spherical vesicles are seen which are semitransparent and often several microns in diameter (Figure 1B). NEM vesicles are generally smaller in size than HCHO/DTT vesicles, especially after ultrafiltration to remove residual cells in the former case (see Experimental Procedures); otherwise their appearance is quite similar. Fluorescence microscopy of these vesicles after they have been incubated with fluorescein isothiocyanate modified IgE (FITC–IgE) or with an amphipathic probe such as 5-(hexadecanoylamino)fluorescein (HAF) shows them to be unilamellar and to have a uniform ring stain which is diagnostic for surface labeling (Figure 1A). The addition of anti-IgE to vesicles labeled with FITC–IgE results in the accumulation of all of the fluorescence at a single pole on each

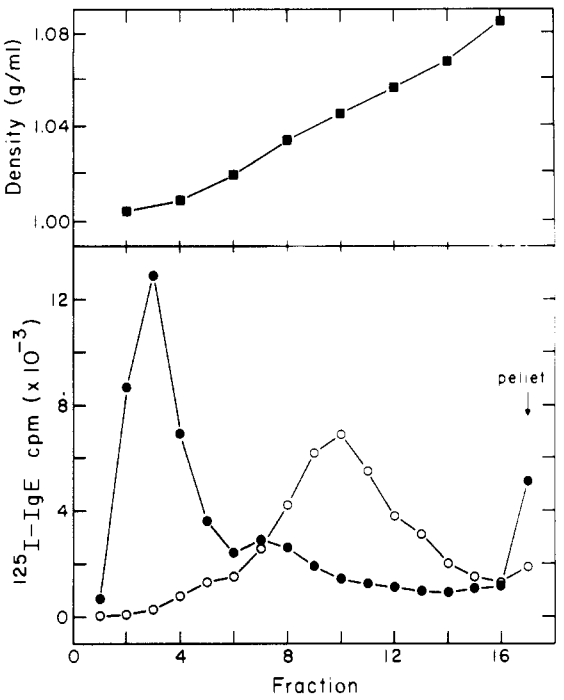

FIGURE 2: Dextran density gradient analysis of HCHO/DTT membrane vesicles with bound ^{125}I-IgE. (●) Intact vesicles in PBS/NaN_3 buffer; (○) hypotonically lysed vesicles in 10 mM Hepes buffer. Both gradients contained 0.05% gelatin and protease inhibitors.

vesicle's surface, suggesting highly mobile IgE–receptor complexes (data not shown). A distribution of vesicle sizes can be seen both by fluorescence microscopy and by electron microscopy of fixed negatively stained preparations (J. Telford, D. Holowka, and B. Baird, unpublished observations), but most of the membrane appears to be in vesicles greater than 0.1 μm in diameter. Although these vesicles appear to enclose trapped cytoplasm (Scott et al., 1979; also, see below), included organelles are generally not observed when fluorescent stains such as rhodamine 123 for mitochondria (Johnson et al., 1980) and acridine orange for histamine-containing granules (Love, 1979) are used.

Results summarized in Table II show that about half of the total protein in these preparations is lost when the vesicles are subjected to lysis by hypotonic shock in 1 mM Hepes (pH 7.4) and then separated by ultracentrifugation. The pelleted membranes retain all of the receptor-bound ^{125}I-IgE (data not shown), but phase microscopy reveals that they have been broken down into much smaller vesicles. The purification of receptor in these lysed vesicles relative to whole cells is generally about 20-fold, based on receptor-bound ^{125}I-IgE recovered per gram of protein (Table II).

Orientation of the Receptor in the Vesicles. The intact vesicles isolated without hypotonic lysis appear to be predominantly sealed and right side out. Figure 2 shows the profile of ^{125}I-IgE bound to HCHO/DTT vesicles, analyzed on a 1–25% gradient of Pharmacia Dextran T-10 (M_r 10500). At least 70% of the vesicles band at a density less than 1.03 g/mL, which suggests that they enclose some volume of fluid, while the large dextran molecules are excluded (Wallach & Schmidt-Ullrich, 1977). In a gradient of 1–30% sucrose (which osmotically reduces the volume of membrane vesicles) similarly prepared vesicles sediment as a broad band with its peak at 1.12 g/mL (data not shown). When the vesicles are lysed by hypotonic shock prior to dextran gradient analysis, the ^{125}I-IgE profile bands at a much greater density than the intact vesicles (Figure 2). This result is consistent with the

Table III: Release of ^{125}I-IgE from Membrane Vesicles by Treatment with pH 3.0 Buffer for 5 min at 4 °C[a]

sample	^{125}I-IgE trapped by filter, pH 7.2[b] (%)	^{125}I-IgE trapped after pH 3 treatment[c] (%)	^{125}I-IgE specifically released by pH 3 treatment[d] (%)
^{125}I-IgE bound to NEM vesicles	81 ± 6 (9)[e]	21 ± 4 (8)	74 ± 6 (8)
^{125}I-IgE bound to HCHO/DTT vesicles	76 ± 4 (5)	30 ± 5 (5)	60 ± 7 (5)
^{125}I-IgE unbound plus vesicles saturated by unlabeled IgE	4 (1)		

[a] Method adapted from Kulczycki & Metzger (1974). [b] A suspension of vesicles (0.5–1.0 mL) was filtered through a 13-mm diameter, 0.22-μm Millipore filter (GSWP) by application of positive pressure; effluent and filter were counted to determine percent bound. [c] Vesicle suspensions exposed to 50 mM glycine (pH 3.0) and 100 mM NaCl were neutralized before filtration by adding an equal volume of 50 mM tricine, (pH 8) and 100 mM NaCl containing excess unlabeled IgE. [d] Determined from data in column 3 after correction for percent of vesicles trapped from data in column 2. [e] Number of preparations tested in parentheses.

microscopic observation described above that the hypotonic treatment causes breakdown of the vesicles to a very small size which corresponds to a much larger surface-to-volume ratio. The dextran gradient profile for intact NEM vesicles has a minor band at about 1.01 g/mL and a major broad band at 1.04 g/mL (data not shown). This pattern is consistent with the generally smaller dimensions of the NEM vesicles compared to the HCHO/DTT vesicles and indicates a greater heterogeneity of sizes as well.

The orientation of the intact vesicles as revealed by the accessibility of the receptors to IgE has been assessed by two different methods. In initial studies, the degree to which receptor-bound ^{125}I-IgE could be dissociated by brief exposure of the vesicles to pH 3.0 was measured by using a membrane filtration assay. As summarized in Table III, the results indicate that about 74% of the receptor-bound ^{125}I-IgE can be released from the NEM vesicles, while about 60% is released from the HCHO/DTT preparation. After the exposure to pH 3.0, some aggregation of the HCHO/DTT vesicles can be detected microscopically, and this might cause sequestering of previously exposed IgE, reducing the amount released. Another explanation for the somewhat lower amount released is that the HCHO treatment used in preparing these vesicles causes some covalent cross-linking between IgE and the receptor. Autoradiographs of electrophoretic gels of ^{125}I-IgE-labeled HCHO/DTT vesicles run in the presence of Na-DodSO_4 and reducing agent show that about 10% of the IgE–ε-polypeptide migrates as higher molecular weight products. Some fractions of these aggregates also could contain some receptor, but this was not investigated further.

A second method for assessing receptor accessibility utilized the selective precipitation of Triton X-100 soluble IgE–receptor complexes by 40% ammonium sulfate (Rossi et al., 1977) and compared the binding of ^{125}I-IgE to unoccupied receptors on HCHO/DTT vesicles before and after membrane solubilization. The results in Table IV indicate that about 66% of the receptors are accessible on these vesicles. It can be seen that the total recovery of ^{125}I-IgE–receptors in the vesicles measured in this way (Table IV, column 3) is in excellent agreement with the total ^{125}I–IgE–receptors recovered in vesicles prepared from ^{125}I-IgE-saturated cells (Table II). Other experiments have

Table IV: Accessibility of Receptors from HCHO/DTT Membrane Vesicles before and after Solubilization with Triton X-100[a]

sample	^{125}I-IgE bound to receptor (pmol): before solubilization	after solubilization	accessible (%)
prep 1, 50 μL of vesicles +8 pmol of ^{125}I-IgE	0.92	1.27	72
prep 2, 50 μL of vesicles +8 pmol of ^{125}I-IgE	0.90	1.56	58
+4 pmol of ^{125}I-IgE	0.97	1.42	68

[a] Assessed by ammonium sulfate assay as described under Experimental Procedures. In both preparations 1 mL of vesicles was obtained from 2.5×10^8 cells that had not been preincubated with IgE.

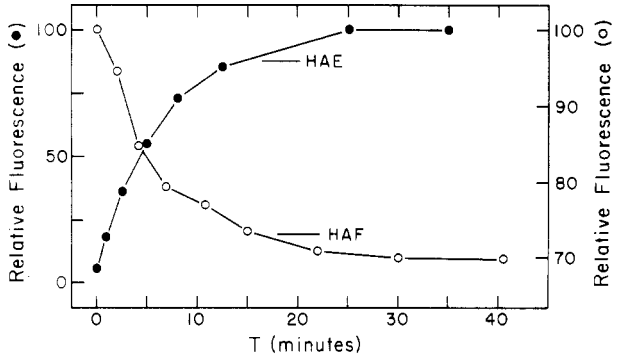

FIGURE 3: Time course of the binding of HAE (67 nM) to NEM vesicles (0.4 pmol of bound IgE/mL) containing bound HAF (53 nM). (●) HAE fluorescence (ex 525 nm, em 550 nm); (○) HAF fluorescence (ex 480 nm, em 515 nm). The fluorescence of HAE in the absence of membrane vesicles is indicated by the value at $T = 0$.

demonstrated that sealed HCHO/DTT vesicles isolated from dextran gradients show similar receptor accessibility, thus excluding the possibility that exposed receptors are primarily associated with unsealed vesicles.

Insertion of Amphipathic Fluorescent Molecules into the Vesicle Bilayer. From our preliminary studies, these vesicle preparations appeared to be suitable for investigating the structural relationship between receptor-bound IgE and the membrane surface. In order to interpret resonance energy-transfer experiments between donor probes on IgE and membrane acceptor probes, it was necessary to determine the nature of the interaction of the acceptor probes to be used with the vesicles. The fluorescent amphipathic molecules HAF and 5-(hexadecanoylamino)eosin (HAE) were used in the initial characterization. When HAE is added to a suspension of membrane vesicles, partitioning into the membrane bilayer can be monitored by the enhancement of fluorescence which is probably due to the relief of quenching that these fluorophores appear to experience in their aqueous micellar form (Baird et al., 1979; Sklar et al., 1980). The time course of this process is shown in Figure 3, where it is seen that the binding of this probe to the vesicles causes a 20-fold enhancement in fluorescence intensity at equilibrium. Under these conditions, about 90% of the HAE is associated with the vesicles as assessed by centrifugation (see Experimental Procedures). HAF also partitions into the vesicle bilayer under similar conditions,

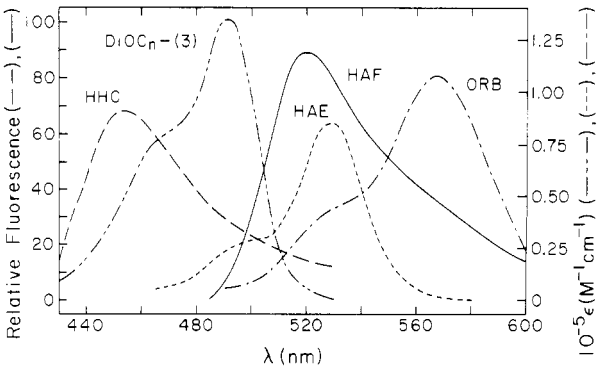

FIGURE 4: Spectral overlap of donor and acceptor amphipathic probes bound to membrane vesicles in PBS/NaN_3 buffer. Shown are the corrected fluorescence emission spectra for HHC [(– –) ex 420 nm] and HAF [(—) ex 480 nm] and the molar extinction coefficients (ϵ) for DiOC$_n$-(3) (– – – –), HAE (– – –), and ORB (– – –).

Table V: Spectral Properties of the Membrane-Bound Amphipathic Donor-Acceptor Pairs[a]

donor	Q_D[b]	$\bar{A}_D$[c]	acceptor	ϵ_{max} [λ_{max} (nm)]	$\bar{A}_A$[c]	R_0[d] (Å)
HAF	0.34	0.25	HAE	8.5×10^4[e] (528)	0.16	50.1
HAF	0.34	0.25	ORB	1.07×10^5[f] (538)	ND[h]	54.3
HHC	0.065	0.28	DiOC$_6$-(3)	1.40×10^5[g] (490)	ND	38.1
HHC	0.065	0.28	DiOC$_{10}$-(3)	1.40×10^5[g] (490)	ND	38.1

[a] Except where noted otherwise, values were determined as described under Experimental Procedures. [b] Quantum yield of membrane-bound donors in the absence of acceptor. [c] Steady-state anisotropy at wavelengths of maximum excitation and emission and 20 °C. [d] Distance between donor and acceptor corresponding to efficiency of energy transfer (E) = 0.50. [e] Value assumed to be equal to that for *N*-eosinyl-*N'*-phosphatidylethanolaminothiourea in phospholipid vesicles (Fung & Stryer, 1978). [f] Value determined for octadecylrhodamine B in 0.5% Triton X-100, and PBS, pH 7.4, by comparison with absorption spectrum in acidic ethanol ($\epsilon = 1.26 \times 10^4$ M^{-1} cm^{-1}, Kodak Publication No. JJ-169). [g] Value from Sims et al. (1974) for DiOC$_n$-(3) derivatives in octanol. [h] Not determined.

and greater than 95% of the amount added is associated with the vesicles at equilibrium, but the fluorescence enhancement observed is only about 2-fold in this case (data not shown). The spectral properties of both HAF and HAE indicate that they are substantially in their fully ionized states when bound to the vesicles in the buffer used (PBS/NaN_3, pH 7.5). The absorption spectrum for HAE (Figure 4) and its high quantum yield (Figure 3) are consistent with a net charge of 2– on that molecule (Zwicker & Grossweiner, 1963), while the corrected excitation spectrum of HAF (data not shown) is consistent with a partially ionized carboxyl group and a net charge of about 1.5– (Mercola et al., 1972).

When HAE is added to vesicles that have been preequilibrated with HAF, a time-dependent quenching of the HAF fluorescence is observed as the HAE fluorescence increases as shown in Figure 3. It is apparent that the time courses of the two processes shown in this figure are the same, indicating that the quenching of HAF fluorescence is due to the HAE molecules that have partitioned into the membrane. It was determined that trivial reabsorption of fluorescence or attenuation of exciting light by the probes do not contribute to the quenching under these conditions. Resonance energy transfer from HAF to HAE most likely accounts for the observed correlation: the spectral overlap for the HAF donor–HAE

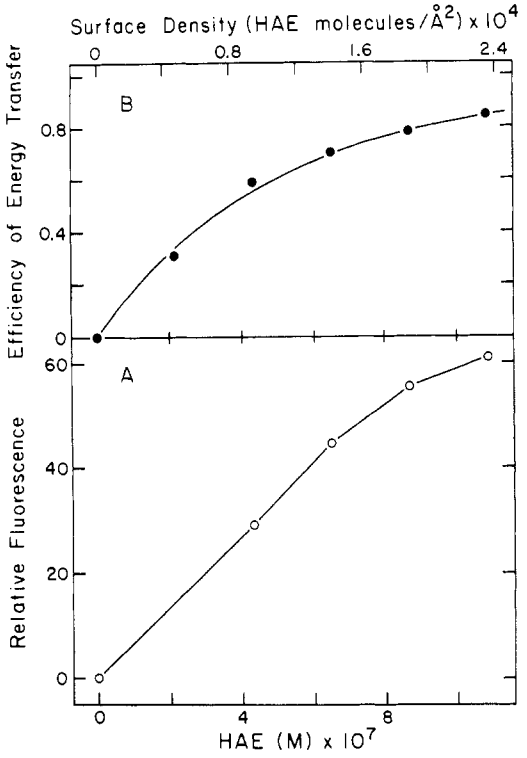

FIGURE 5: Titration of acceptor (HAE) into HCHO/DTT vesicles (1.6 pmol of bound IgE/mL) containing donor (320 nM HAF). (A) The fluorescence intensity of bound HAE (ex 520 nm, em 555 nm) as a function of HAE concentration at each addition (○). (B) Efficiency of energy transfer at each addition (●) as determined from the quenching of vesicle-bound HAF fluorescence (ex 480 nm, em 520 nm) by bound HAE. In (B) the solid line represents the two exponential solution to energy transfer in two dimensions derived by Wolber & Hudson (1979) which was scaled to the data as described under Experimental Procedures. The units on the upper abscissa are calculated from this fit together with the R_0 given in Table V.

acceptor pair shown in Figure 4 and the calculated R_0 = 50.1 Å (Table V) indicate that significant energy transfer will occur when the distance between these donor and acceptor molecules is less than 80 Å. Although quenching of HAF fluorescence was generally monitored for technical reasons in experiments such as that in Figure 3, sensitized emission of HAE associated with excitation of HAF was observed to correspond exactly to the HAF quenching which also indicates energy transfer is occurring. In another experiment (not shown), the donor molecule HAF was titrated into vesicles that already contained acceptor molecules, HAE, and the efficiency of energy transfer (0.72 at the concentrations employed) was found to be invariant over a 5-fold concentration range of the donor [(0.8–3.7) × 10^{-7} M]. This observation provides strong support to the conclusion that the quenching is due to energy transfer between randomly distributed, noninteracting donor and acceptor molecules and is not the result of associations between the molecules (Fung & Stryer, 1978).

The results shown in Figure 5 provide further evidence that the quenching observed is due primarily to energy transfer. Figure 5A shows that HAE fluorescence (at equilibrium) is proportional to the amount of HAE added to the vesicles at all except the highest concentrations employed in these experiments, which indicates that collisional quenching is not significant under these conditions. Figure 5B shows that the quenching of HAF fluorescence by increasing concentrations of HAE is a double-exponential function, and this is completely consistent with the predicted energy-transfer behavior for noninteracting donor and acceptor molecules that are randomly distributed in an infinite plane with the distance of closest approach much less than R_0 (Wolber & Hudson, 1979; Fung & Stryer, 1978). From plots such as this the density of HAE or any other acceptor in the plane of the membrane can be determined at any concentration of acceptor (see Experimental Procedures), and this calibration is indicated for HAE on the top abscissa in Figure 5.

Table V provides a summary of the appropriate spectral parameters and R_0 values for all of the donor–acceptor pairs that have been tested with the vesicles to characterize this experimental system for fluorescence energy-transfer studies. In addition to HAF, 3-hexadecanoyl-7-hydroxycoumarin (HHC) has been employed as a donor probe, and as shown in Figure 4 its fluorescence emission spectrum overlaps the absorption spectrum of the DiO-(3) carbocyanine derivatives (Sims et al., 1974) which have been used as the corresponding acceptors. Although the quantum yield of HHC when bound to the vesicles is very low, it is still about 20-fold enhanced over that of the aqueous micellar form, and the excitation and emission spectra (Figure 4 and unpublished observations) indicate that the 7-hydroxyl group is in a negatively ionized state (Alpes & Pohl, 1978). A total of four different acceptor probes have been tested in these studies (Table V); all have charged head groups, and all show large (20–60-fold) enhancement in quantum yield upon binding to the vesicles. All of the donor and acceptor probes used exhibit a linear increase in fluorescence intensity as a function of concentration bound to the vesicles in the experimental range employed, and all (except HHC which was not tested) were found to partition into the membrane to an extent greater than 90% at equilibrium. The steady-state anisotropy values listed in Table V suggest that all of the probes examined have rotational mobility that is partially restricted, which is consistent with their integration into a fluid vesicle bilayer (Chen et al., 1977). As illustrated for HAF/HAE in Figure 5, energy transfer for all of the donor–acceptor pairs listed in Table V occurs as a function of acceptor concentration with a relationship that follows the prediction for randomly oriented, noninteracting molecules in an infinite plane (Wolber & Hudson, 1979).

Discussion

Plasma membrane vesicles prepared from RBL cells by a chemically induced cellular vesiculation method (Scott, 1976) as described in this report should complement the membranes prepared by more conventional procedures (Isersky et al., 1982) for investigation of the membrane-associated IgE–receptor complex. The preparation described here has several features that are especially attractive experimentally. Because the vesicles pinch off from adherent cells, they can be isolated easily without the need for long gradient centrifugation steps. A full-scale preparation (from about 3 × 10^8 cells) can be completed in 3–4 h, and the 20% yield of IgE receptor recovered with the membranes is very reproducible (Table I). The virtual absence of contaminating intracellular membranes in this type of preparation has been amply demonstrated by Scott and co-workers for other cell lines (Scott et al., 1979), and our observations of preparations derived from RBL cells are consistent with this. By microscopy the vesicles appear to be uniform in shape, and aggregation or multilamellar membrane stacking is minimal. As revealed in dextran gradient analysis and IgE binding assays, the vesicles are predominantly unilamellar, right side out, and nonpermeable to macromolecules. The preparation appears to be stable with respect to vesicular structure and IgE binding activity for at least several weeks of storage at 4 °C.

From the fluorescence studies described here we conclude that amphiphatic, fluorescent donor and acceptor probes

partition into the vesicle bilayer in a randomly distributed, noninteracting manner at densities sufficiently high for efficient energy transfer to occur between them. Furthermore, the magnitude of the density can be determined directly from the amount of energy transfer that is observed. As shown for HAE in Figure 5, the fluorescence enhancement due to lipid insertion increases linearly with concentration, and when the donor HAF is present in the vesicles and the acceptor HAE is added, the efficiency of energy transfer is a double-exponential function of acceptor concentration as predicted theoretically (Wolber & Hudson, 1979). The energy transfer is independent of donor concentration which rules out a significant contribution from static or dynamic collisional quenching (Fung & Stryer, 1978; Wolber & Hudson, 1979). Similar behavior has been exhibited by all of the amphipathic probes tested (Table V), including those with head groups that are negatively charged (HAF, HAE, and HHC) and positively charged [ORB, $DiOC_6$-(3), and $DiOC_{10}$-(3)]. Previously others have demonstrated the random distribution of fluorescent phospholipid analogues in model lipid vesicles by energy-transfer methods (Fung & Stryer, 1978), but to our knowledge this has not been tested quantitatively before in a biological membrane.

The density of the acceptor probes can be determined from fitting the energy-transfer data to the theoretical equations by numerical methods (Fung & Stryer, 1978; Wolber & Hudson, 1979) or by scaling the double-exponential curves obtained according to the approximation provided by Wolber & Hudson (1979) (e.g., eq 4 and 5). It should be acknowledged that a source of uncertainty in the density determined in this way resides in the dependence of R_0 on the orientation factor κ^2 (eq 3) and our assumption that $\kappa^2 = {}^2/_3$ which is the case for dynamically averaged dipole orientations of donor and acceptor molecules. With the random distribution of the probes in the plane of the bilayer, it is likely that there is at least a static distribution of dipoles, and the steady-state anisotropies that were observed for all of the probes used (Table V) indicate some degree of rotational freedom during the fluorescence lifetime of the donor molecules (Chen et al., 1977). As has been discussed previously for a similar experimental situation, the assumption of $\kappa^2 = {}^2/_3$ is unlikely to result in an error of greater than ±20% for the average density calculated (Stryer, 1978; Fung & Stryer, 1978). Another source of uncertainty is the exact location of the chromophoric groups within the membrane. Although it is very probable that the charges on these groups will confine them to the surface, we have not yet determined whether the probes can partition freely into both leaflets of the sealed vesicle bilayer. The occasional microscopic observation of a small fluorescent vesicle trapped within a larger one does suggest that probes added to one side can penetrate to both halves of the bilayer, but this needs to be examined more quantitatively. It is unlikely that the outcome of this question will significantly affect the qualitative conclusions drawn in this study. However, if penetration does occur, then transbilayer as well as lateral energy transfer would have to be considered. This would result in a decrease of about 20% in the determined density of acceptor probes (using $R_0 \approx 50$ Å) (Fung & Stryer, 1978).

As will be described in detail in the following paper, these vesicles can be used with energy-transfer methods to determine distances between donor probes at sites on receptor-bound IgE and acceptor probes labeling the membrane surface. Interpreting the observed energy transfer in terms of distances requires knowledge of the density of acceptor probes, but as demonstrated here, this can be directly determined fluorometrically which obviates the need to make questionable assumptions about vesicle surface area and lipid content of the membranes. The fact that IgE binds to membrane receptors after vesicle preparation nearly as well as before allows flexibility in preparing samples for the fluorescence measurements and also allows appropriate control samples for nonspecific effects. The absence of contaminating intracellular membranes in the vesicle preparation provides assurance that the measured properties of the amphipathic probes reflect the environment in the IgE–receptor-containing membranes. In general, these vesicles should prove to be very useful as a basic system with which to investigate in detail the structure of the membrane-associated IgE–receptor complex.

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Registry No. HAF, 73024-80-3; HAE, 85735-45-1; HHC, 69377-68-0; ORB, 65603-19-2; $DiOC_6$-(3), 53213-82-4; $DiOC_{10}$-(3), 68006-77-9.

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