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Rotational Dynamics of the Fc Receptor for Immunoglobulin E on Histamine-Releasing Rat Basophilic Leukemia Cells

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ABSTRACT: The rotational diffusion of immunoglobulin E (IgE) bound to its specific Fc receptor on the surface of living rat basophilic leukemia cells was determined from time-resolved phosphorescence emission and anisotropy measurements. The IgE–receptor complexes are mobile throughout the range of temperatures of 5–38 °C. The residual anisotropy does not reach zero, indicating that the rotational diffusion is hindered. The values of rotational correlation times for each temperature are consistent with dispersed receptors rotating freely in the cell membrane and rule out any significant aggregation of occupied receptors before cross-linking by antigen or anti-IgE antibodies. The rotational correlation times decrease with increasing temperature from 65 μs at 5.5 °C to 23 μs at 38 °C. However, the degree of orientational constraint experienced by the probe is unchanged. Thus, the temperature dependence can be attributed primarily to a change in the effective viscosity of the cellular plasma membrane. The phosphorescence depolarization technique is very sensitive (our probe concentrations were 10–100 nM) and thus generally applicable to studies of surface receptors and antigens on living cells.

The cell surface receptor for immunoglobulin E (IgE)[1] of mast cells and basophils is an integral membrane protein with a molecular mass of approximately 100 kDa composed of four subunits arranged as an $\alpha\beta\gamma_2$ oligomer (Perez-Montfort et al., 1983; Metzger et al., 1983). The receptor binds the IgE molecule monovalently (Mendoza & Metzger, 1976; Newman et al., 1977) with a dissociation constant in the range 10^{-9}–10^{-12} M for different species (Metzger et al., 1982; Kulczycki & Metzger, 1974; Conrad et al., 1975). A variety of data suggest that the terminal four domains of the Fc region [$(C_\epsilon3–C_\epsilon4)_2$] interact with the receptor [reviewed in Perez-Montfort & Metzger (1982)] and that a bend may occur at the $C_\epsilon2$–$C_\epsilon3$ interface of the IgE molecule when it binds (Holowka & Baird, 1983; Baird & Holowka, 1985; Holowka et al., 1985). No biological effect is observed after binding of the IgE to the cell surface. However, the cross-linking of the IgE–receptor complexes by such agents as multivalent antigens or anti-IgE antibodies or cross-linking of the receptor by antireceptor antibodies initiates the process of cellular degranulation and receptor internalization [reviewed in Metzger et al. (1986)].

Studies of other cell surface receptors suggest that intramembrane aggregation initiated by binding of a specific effector molecule is one of the initial events required to trigger the biological chain of responses [reviewed in Schlessinger (1979) and Schlessinger et al. (1983)]. The state of association and rigidity of surface receptors can be assessed experimentally by the measurement of rotational and lateral diffusion (Jovin & Vaz, 1986). These properties can also reflect the influence of interactions with other intrinsic membrane-bound proteins, as well as with cytoplasmic and cytoskeletal components. The lateral diffusion of proteins in cellular plasma membranes [reviewed in Edidin (1981) and Schlessinger & Elson (1981)] has been determined by techniques sensitive to macroscopic motion in the micron range. The experimental diffusion constants are generally lower than those estimated for unconstrained mobile proteins in a lipid bilayer and also lower than the values determined for molecules reconstituted into synthetic lipid bilayers (Vaz et al., 1982), indicating an influence of physical barriers within the plasma membrane and/or interactions with other membrane-associated components. The IgE system provides no exception to the general

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[1] Abbreviations: Ig, immunoglobulin; kDa, kilodalton; RBL, rat basophilic leukemia; PBS, phosphate-buffered saline; Er–IgE, rat IgE labeled with erythrosin 5′-isothiocyanate.

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rule. Results from various studies of lateral diffusion lead to the conclusion that both the unliganded and the occupied Fc receptors are monomeric and mobile (Schlessinger et al., 1976; Mendoza & Metzger, 1976; Wolf et al., 1980; McCloskey et al., 1984; Menon et al., 1985).

In the present study, we probed the dynamic properties of the Fc receptor by the technique of time-resolved phosphorescence polarization which measures rotational motion, a process that is much more sensitive than translational diffusion to local molecular environment and to the state of association (Cherry, 1978; Austin et al., 1979; Zidovetzki et al., 1981; Matayoshi et al., 1983; Speirs et al., 1983; Kinosita et al., 1984). The experimentally accessible time scale offered by the excited triplet state (μs–ms) is particularly suitable for assessing the motions of proteins embedded in cell membranes exhibiting high apparent microviscosities (Shinitzky & Inbar, 1974). Our probe, rat IgE labeled with erythrosin (Er–IgE), was bound to its specific receptor on the surface of the 2H3 subclone of rat basophilic leukemia (RBL) cells (Barsumian et al., 1981). These cells respond to appropriate stimuli with an exocytosis of vasoactive amines and other constituents of internal vesicles and have been used extensively in studies of the cell-mediated allergic response. Our results indicate that the Fc receptor can rotate relatively freely in its membrane environment. The fast rotational motion at 37 °C (rotational correlation time, ϕ, $\sim$ 20 μs) rules out the presence of strong associations with cytoskeletal components or membrane-bound enzymes. Finally, the dependence of the rotational correlation time on temperature was measured and attributed largely to changes in membrane viscosity.

Materials and Methods

Cell Culture. Rat basophilic leukemia cells, subclone 2H3 (RBL-2H3; Barsumian et al., 1981), were cultured at 35 °C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Reagents. Rat IgE, purified from the ascitic fluid of rats bearing the tumor IR162 (Kulczycki & Metzger, 1974), was a gift of H. Metzger. Labeling with erythrosin 5′-isothiocyanate (the gift of P. Garland) was performed in borate buffer at pH 9.4. The labeled protein was dialyzed to neutral pH. In the case of other preparations, traces of hydrolyzed dye were removed by chromatography over SM-2 beads (Bio-Rad, Richmond, CA) in phosphate-buffered saline (PBS). The dye to protein ratio of the erythrosin–IgE (Er–IgE) was 2.1:1. The binding of Er–IgE to the RBL-2H3 cells was checked by counterstaining with fluoresceinated mouse anti-rat Ig (Jackson Immuno Research, Avondale, PA) and by its ability to trigger release of radiaoactive serotonin preloaded into the cells. Cross-linking Er–IgE resulted in greater than 85% of the release induced by parallel preparations with unlabeled rat IgE. Maximal release was achieved with 20 nM Er–IgE. Rabbit anti-rat-IgE IgG, the gift of H. Metzger, was prepared as described (Taurog et al., 1977) and used at a concentration of 1.5 nM for cross-linking the IgE in the release assay.

Buffers and Activity Assays. Exocytosis (serotonin release) was assayed according to Taurog et al. (1977) with a buffer of 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 0.1% bovine serum albumin, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.2. Cells were loaded with 1–5 μCi/mL 5-[1,2-^{3}H(N)]hydroxytryptamine binoxalate (New England Nuclear) in the presence of rat IgE at 37 °C for 1 h. Cells were washed, and the degree of degranulation was measured by the release of radioactivity into the supernatant at 30 and 60 min after addition of anti-rat IgE.

The phosphorescence measurements were done in phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.87 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.2.

Preparation of Cellular Samples for Phosphorescence Measurements. Subconfluent cultures were used for labeling with Er–IgE. Cells were released from the culture dishes by a 10-min incubation with 0.8–2 mM EDTA in Tris-buffered saline, washed by centrifugation after restoration of Mg^{2+} and Ca^{2+}, and resuspended to a density of (4–5) $\times$ 10^7 cells/mL either in medium or in PBS with added bovine serum albumin (1 mg/mL). The cells were labeled for about 1 h at 35–37 °C with 10 μg/mL Er–IgE. The cells were then washed twice with PBS and resuspended to a density of ca. 10^7/mL. The binding of the IgE to the cells was confirmed on an aliquot by counterstaining with fluorescent mouse anti-rat Ig. Viability was 90–95% before and after the phosphorescence measurements by vital staining with fluorescein diacetate. Cross-linking of surface-bound IgE by anti-IgE was accomplished by adding rabbit anti-IgE to the sample at a concentration of 20 μg/mL and incubating for 30 min at 37 °C.

Cell suspensions at a density of (1–2) $\times$ 10^7 were purged of dissolved O_2 by a continuous argon flow into the cuvettes and gentle agitation with a Teflon plunger.

Time-Resolved Phosphorescence Measurements. The decay of phosphorescence intensity and polarization was determined according to Zidovetzki et al. (1981). Excitation with a N_2 laser–dye laser combination was at 515 nm, and the phosphorescence emission was collected above 645 nm. Usually, 4096 individual records consisting of both parallel and perpendicular emission components were accumulated and averaged. Analysis of the data was done according to Zidovetzki et al. (1981). Blank corrections were applied by subtracting records generated under identical conditions, but using unlabeled cells. The total phosphorescence emission, $S(t)$ was calculated as $S(t) = I_{\parallel} + 2I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are the parallel and perpendicular polarized emission components, respectively. $I_{\perp}$ was corrected for the effects of gain inequality and the finite aperture of the optical system for detecting the emission (Jovin & Vaz, 1986). The multiexponential analysis of $S(t)$ gives the phosphorescence (triplet) lifetimes and their amplitudes.

The emission anisotropy, $r(t)$, was calculated as $r(t) = (I_{\parallel} - I_{\perp})/S(t)$. The emission anisotropy is a measure of the rotational relaxation of the macromolecule and the attached probe and is in general given by a sum of exponential terms. Our data were adequately described by a fit of $r(t)$ to a monoexponential decay law according to the expression $r(t) = \alpha e^{-t/\phi} + r_\infty$. The time constant, ϕ (rotational correlation time), is an inverse function of the rotational diffusion constant(s) and in general will also reflect any segmental and wobbling motions of the probe and the macromolecule to which it is attached (Zidovetzki et al., 1981; Lipari & Szabo, 1980). The amplitude α is equivalent to $r_{in} - r_\infty$, in which r_{in} is the observed initial anisotropy and r_∞ the limiting anisotropy, a quantity that reflects the anisotropic equilibrium distribution of the Fc receptor in the plasma membrane. For a more complete description of the formalism and its use, see Zidovetzki et al. (1981), Cherry (1978), Lipari and Szabo (1980), Szabo (1984), Jovin and Vaz (1986).

Results and Discussion

Rat basophilic leukemia cells labeled with Er–IgE exhibit a characteristic phosphorescence which decays in the microsecond–millisecond range (Figure 1a). Unlabeled cells at the same concentration show an apparent short-lived component due to scattering and possibly autoluminescence (Figure 1b).

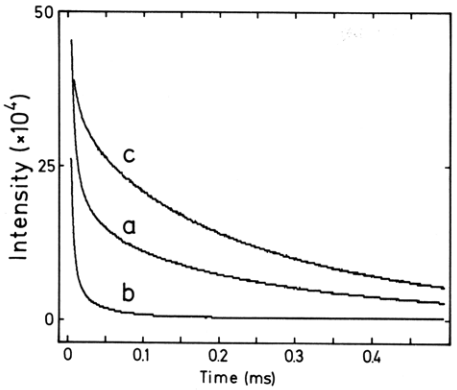

FIGURE 1: Decay of phosphorescence emission of Er–IgE-labeled (a) and unlabeled (b) RBL-2H3 cells. Curve c is twice the difference between curves a and b.

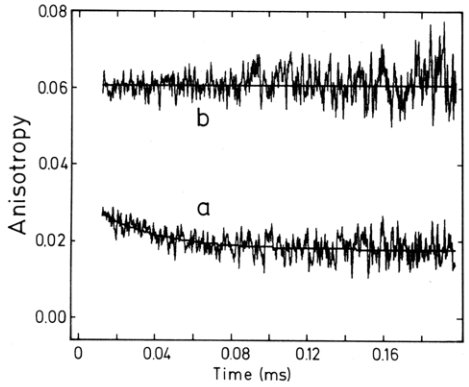

FIGURE 2: Anisotropy decay kinetics of Er–IgE–receptor complexes at 37 °C. (a) Measured before cross-linking by anti-IgE; (b) measured after cross-linking by anti-IgE. The smooth line in (a) shows the fitted curve with the following parameters: $\phi = 31$ μs; $\alpha = 0.013$; $r_\infty = 0.018$. In (b) $r_{in} = r_\infty = 0.060$.

The emission of the labeled cells was corrected by subtraction of the corresponding blank record (Figure 1c). The resultant decay of phosphorescence intensity, $S(t)$, was multiexponential with lifetime components of 4.5, 21, and 93 μs and corresponding fractional amplitudes of 0.33, 0.24, and 0.43, respectively. Such a multiexponential decay is characteristic for the halogenated members of the fluorescein family of probes (eosin, erythrosin) conjugated to proteins (Austin et al., 1979; Garland & Moore, 1979). The heavy atom substituent greatly increases the efficiency of formation of the excited triplet state, which then decays in the microsecond–millisecond range in the absence of O_2.

The phosphorescence emission of Er–IgE labeled cells displays a time-dependent polarization (anisotropy) (Figure 2a). The important features of this anisotropy are the rate of decay (rotational correlation time = 31 μs) and the finite (>0) plateau value (limiting anisotropy, r_∞) of 0.018. Similar properties have been reported for other integral membrane proteins (Zidovetzki et al., 1981; Cherry, 1978, 1985; Matayoshi et al., 1983).

The positive amplitude of the anisotropy decay reflects the inherent randomness in the orientation of the probe relative to the cell membrane, i.e., to the axis of a proposed uniaxial mode of rotation. This is a consequence of the labeling procedure in which the probe is conjugated randomly to sites positioned throughout the several domains of the IgE molecule. In the case of specific labeling, e.g., of the polypeptide hormone epidermal growth factor which has a unique site for the conjugation of erythrosin isothiocyanate, a contrasting behavior is observed in that the anisotropy decays with a negative amplitude (Zidovetzki et al., 1981).

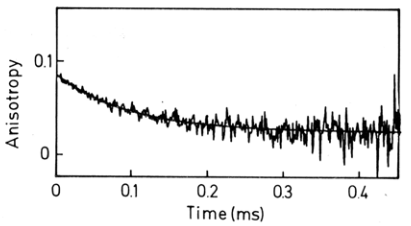

FIGURE 3: Anisotropy decay kinetics of Er–IgE in sucrose solution. The concentration of sucrose, determined with a refractometer, was 71%. The temperature in the cuvette during measurement was 18.6 °C, yielding a solution viscosity of $\eta = 1040$ cP. The Er–IgE concentration was 34 μM. The smooth line represents an exponential decay with the following parameters: $\phi = 96$ μs; $\alpha = 0.060$; $r_\infty = 0.025$.

The initial phosphorescence anisotropy, r_{in}, of 0.03 for Er–IgE-labeled cells is low relative to the value 0.21 obtained for erythrosin immobilized in glasses of DL-arabinose. This finding probably reflects the fact that while the IgE molecule with conjugated erythrosin is bound to the cell surface via its Fc domain, it has enough freedom to undergo segmental motion on a time scale faster (<1 μs) than can be detected in our experiments. These fast motions would significantly depolarize the measured signal. In addition the probe itself may undergo restricted wobbling in a nanosecond–subnanosecond time range (Austin et al., 1979; Kawato & Kinosita, 1981). We sought to distinguish between the contributions to the depolarization made by local probe motion from those due to segmental flexibility of the IgE molecule. Cross-linking IgE bound to the cells by anti-IgE antibodies resulted in the decay curve shown in Figure 2b. The absence of a perceptible decay in the time domain examined (0.2 ms) reflects the immobilization of the cross-linked IgE molecules. The value of $r_{in} = r_\infty = 0.060$ is much greater than the corresponding value (0.031) before cross-linking (Figure 2a). It is reasonable to assume that segmental motions of the IgE molecule would experience a greater degree of restriction by cross-linking than the local excursions of the conjugated erythrosin. Thus, we tentatively conclude that local probe motion on the average accounts for a depolarization from 0.21 to 0.060 and that segmental motion of the IgE itself further reduces r_{in} to 0.03.

In order to demonstrate directly the existence of segmental motions in the IgE molecules, we measured the anisotropy decay of the Er–IgE conjugate in sucrose solutions of high viscosity (Figure 3). The value of the initial anisotropy of the curve in Figure 3, $r_{in} = 0.085$, is significantly lower than that obtained for the immobilized erythrosin in a glass. The viscosity of the sucrose solution in this experiment was 1040 cP, which gives us a scaling factor for the corresponding correlation times in water. In this measurement, the depolarization from $r_{in} = 0.21$ to the observed $r_{in} = 0.085$ took place in the submicrosecond range, which translates into the subnanosecond range for water and agrees with the estimated range of fast probe rotations (Kawato & Kinosita, 1981). The curve in Figure 3 was fit to an exponential decay with a rotational correlation time $\phi = 96$ μs. Normalizing this value to the viscosity of water we obtained $\phi = 92$ ns, a value within the range of the long (82–105 ns) correlation times obtained by Hanson et al. (1981) for various IgG molecules using time-resolved fluorescence anisotropy and attributed by those authors to the motions of the Fab arms about the hinge region. In another recent study using the same technique, a mean value of 124 ns was reported for mouse monoclonal IgE (Oi et al., 1983). Evidence for significant segmental flexibility in mouse IgE has been obtained from steady-state anisotropy measurements which yielded an average ϕ of 54 ns in solution and

Table I: Phosphorescence Anisotropy Decay Parameters of Er–IgE Complexes with the Fc Receptors of RBL-2H3 Cells

T (°C)	$\langle\phi\rangle$	$\langle\alpha\rangle$	$\langle r_\infty\rangle$	$\langle r_{in}\rangle^a$	$\langle r_\infty\rangle/\langle r_{in}\rangle$
5.5	65	0.029	0.030	0.059	0.51
13.8	50	0.028	0.023	0.051	0.45
24.8	26	0.027	0.019	0.046	0.41
38.1	23	0.017	0.016	0.033	0.48

$^a r_{in} = r_\infty + \alpha$.

74–89 ns for IgE bound to membrane vesicles (Slattery et al., 1985). We conclude from these observations and the data reported in this study that the IgE molecule retains its flexibility upon interaction with its cellular receptor. One can speculate as to whether this circumstance arises in spite of or due to the conformational changes (e.g., bending) coupled to the binding reaction.

The curve from Figure 2a can be fit to a single-exponential decay, and the derived value for the rotational correlation time of 31 μs can be attributed to the rotational diffusion of the constituents of the IgE–receptor complex located within the viscous environment of the plasma membrane, i.e., the receptor polypeptide chains. For an estimate of the value expected for ϕ we assume the receptor to be a rigid cylinder undergoing uniaxial rotation about an axis normal to the plane of the membrane. In support of this geometry are the current models of the IgE receptor that depict the bulk of the protein as being embedded in the membrane (Metzger et al., 1982, 1986). In such a situation, two rotational correlation times are expected, ϕ_1 and $\phi_1/4$, where $\phi_1 = 4V\eta/(kT)$ (Cherry, 1978), V is the volume of the receptor molecule experiencing the characteristic membrane microviscosity η, k is Boltzmann's constant, and T is the absolute temperature. We calculate V assuming a 100-kDa protein with partial specific volume 0.72 mL/g. The estimation of the operative membrane viscosity in natural membranes is more problematical. The complex effects of lipid–lipid and lipid–protein interactions and the concentration dependencies cannot be assessed with great confidence (Kleinfeld et al., 1981). It has been questioned recently (Kleinfeld et al., 1981; Clegg & Vaz, 1985) whether the lateral diffusion of membrane proteins is determined primarily by viscous drag according to hydrodynamic models. On the other hand, the apparent values of η can be derived from the diffusion properties of proteins in reconstituted systems, e.g., bacteriorhodopsin in lipid bilayers. They range from 1.1 to 1.8 P for lipid to protein ratios of 210–140 [Peters & Cherry, 1982; see also Vaz et al. (1985)]. Such values are in good agreement with determinations of η from steady-state polarization measurements of lipid probes in membranes of living cells, e.g., 1.8 P for normal lymphocytes at 37 °C (Shinitzky & Inbar, 1974). Therefore, for our estimate we take the value of η to be in the range 1–2 P and calculate the range for the rotational correlation times to be 4–30 μs, at 37 °C. Thus, our experimental value of 31 μs falls into the range expected for a monomeric or a small cluster (two to five) of freely diffusing receptors. The fact that the anisotropy curves were adequately described by a single rotational correlation time is probably due to the finite level of noise and possible nonequivalent amplitudes of two or more components.

We investigated the rotational diffusion of the Fc–receptor–IgE complex as a function of temperature (Figure 4). The experimental curves were fit to exponential decays, and the parameters of the fits are given in Table I and plotted in Figure 5. It is immediately obvious from Table I and Figure 5 that the rotational correlation times decrease with increasing temperature, i.e., from 65 μs at 5 °C to 23 μs at 38 °C. It has been reported that the viscosity of biological membranes decreases 4–5-fold in this temperature range (Shinitzky & Inbar, 1974). Thus, the observed increase in the rotational mobility of the Fc receptors can be accounted for by a change in membrane viscosity. The initial and residual anisotropies, as well as the decay amplitudes, also decrease with increasing temperature (Table I; Figure 5).

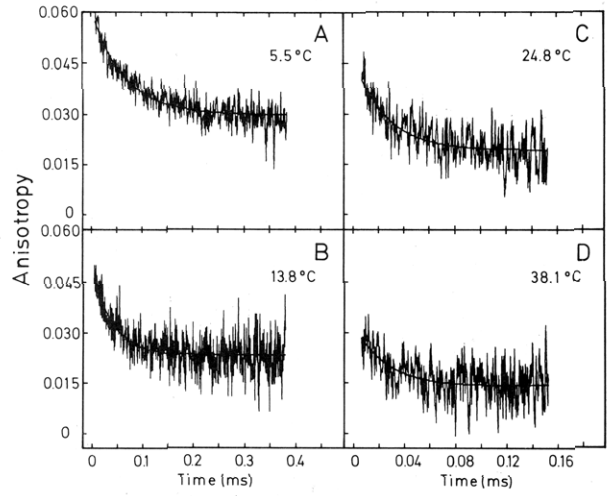

FIGURE 4: Anisotropy decay kinetics of Er–IgE–receptor complexes at different temperatures. The cells were labeled with Er–IgE at 35 °C and then successively measured by phosphorescence at the indicated temperatures in ascending order. In general three to four determinations were made at each temperature. The rotational correlation times and amplitudes, calculated by analysis, varied by less than 25% and 15%, respectively. The smooth lines are decay curves generated with the best-fit parameters given in Table I.

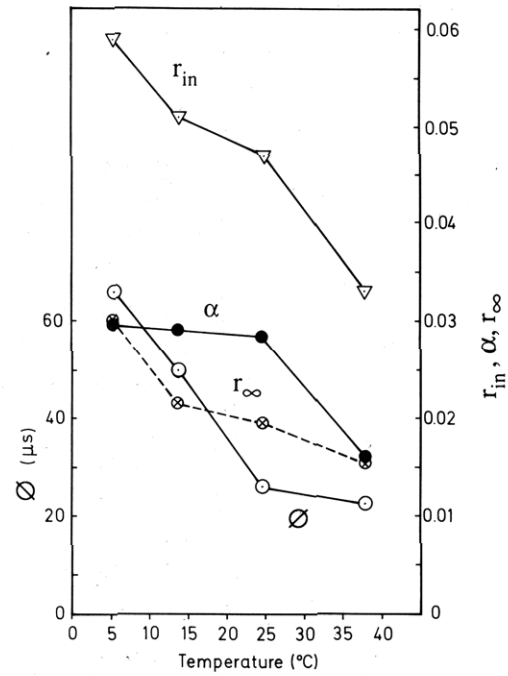

FIGURE 5: Temperature dependence of the rotational diffusion of the Fc receptor–IgE complex. (○) Rotational correlation times, ϕ; (⊗) limiting anisotropies, r_∞; (●) amplitudes of the anisotropy decay, α; (▽) initial anisotropies, r_{in}.

Of further significance is the behavior of the quantity r_∞/r_{in} with temperature. This ratio has been shown to be a measure of the width of the distribution of the probe's transition moments around the bilayer normal and has been designated the "degree of orientational constraint" (Kinosita et al., 1977, 1984; Szabo, 1984). As shown in the present study (Table I) this parameter is invariant with temperature, indicating that the increased depolarization of anisotropy with temperature (Table

I, values of $\langle r_{in} \rangle$, $\langle r_{\infty} \rangle$, and $\langle \alpha \rangle$) is the result of increased local motions of the probe and segmental motions of IgE molecules on a time scale faster than our measurements, with little change in the orientational freedom of the Fc receptor. We conclude that the experimental rotational correlation times and their inverse dependence on membrane fluidity support the view that the receptor molecules are dispersed prior to cross-linking (see introduction), i.e., not associated in a functional sense. Moreover, the fast rotational diffusion of the receptors rules out any strong associations with the cytoskeleton or other peripheral proteins such as enzymes. These properties are entirely consistent with the extreme sensitivity and specificity for the triggering of exocytosis, a process that under physiological conditions is exclusively dependent upon cross-linking through interactions of the externally disposed combining sites of anchored IgE.

The technique of time-resolved phosphorescence depolarization is well suited in general for studies of signaling processes mediated by relatively sparse surface receptors. The RBL-2H3 cell carries (1–3) $\times 10^5$ IgE receptors (Holowka & Baird, 1983). Thus, under our experimental conditions the probe concentration was only about 30 nM.

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