THE EFFECT OF RECEPTOR DENSITY ON THE FORWARD RATE CONSTANT FOR BINDING OF LIGANDS TO CELL SURFACE RECEPTORS

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ABSTRACT For monovalent ligands interacting with cell surface receptors we have directly observed the functional dependence of the forward rate constant on the number of receptors per cell (N). The experimental system we studied consisted of monovalent ligand, 2,4-dinitrophenyl (DNP)-aminocaproyl-L-tyrosine (DCT), binding to bivalent, monoclonal anti-DNP immunoglobulin E (IgE) anchored to its high affinity receptor on rat basophilic leukemia (RBL) cells. To measure the fractional occupation of antibody combining sites by DNP we employed a recently developed fluorescence technique (Erickson, J., Kane, B. Goldstein, D. Holowka, and B. Baird, 1986, *Mol. Immunol.*, 72:769–781). Our results are well fitted by the equation (Berg and Purcell, 1977, *Biophys. J.*, 20:193–219) $k_{on}^{c} = 4\pi DaN\kappa_{on}/[4\pi Da + N\kappa_{on}]$ where k_{on}^{c} is the forward rate constant for binding to the cell, D is the diffusion constant of the ligand, a is the radius of the cell, and κ_{on} is the intrinsic forward rate constant describing a single IgE combining site-DNP interaction. If D is fixed at 10^{-5} cm^2/s, the best fit of accumulated data predicts an average cell radius of ~4 μm and κ_{on} of ~ 1.8×10^{-13} cm^3/s [1.1×10^{8}(M · s)$^{-1}$]; both in excellent agreement with RBL cell size and the single-site forward rate constant for the binding of DCT to IgE in solution, respectively. We believe this is the first report of experimental evidence that directly illustrates the effect of surface density in determining the rates of binding for small molecules to membrane receptors.

INTRODUCTION

Cell surface receptors, because they are restricted to the plasma membrane, are clustered in space. If diffusion can limit the rate of ligand-receptor interaction, theory predicts clustering to have the effect of reducing both the forward and reverse rate constants for ligand-receptor binding. The forward rate constant is reduced because nearby receptors compete for the same ligand and depress its concentration locally. The reverse rate constant is reduced because a ligand that dissociates from one receptor has a finite probability of binding to another before escaping from the vicinity of the cell. This effect of receptor density on ligand binding may have important consequences in biological systems since the rate at which ligands bind to cell surface receptors might serve to regulate the transmission of a chemical signal across the plasma membrane.

The kinetics of the binding of ligands to spherical cells of radius a that have N uniformly distributed monovalent receptors per cell has received considerable theoretical attention (Berg and Purcell, 1977; Berg, 1978; DeLisi, 1980, 1981; DeLisi and Wiegel, 1981; Brunn, 1981; Shoup and Szabo, 1982). For such a cell exposed to a ligand that diffuses in solution with diffusion coefficient D, theory predicts that the ligand-receptor forward rate constant per receptor, k_{on}, will depend on N as follows:

$$k_{on}(\text{cm}^3/\text{receptor} \cdot \text{s}) = \frac{4\pi Da\kappa_{on}}{4\pi Da + N\kappa_{on}}, \qquad (1)$$

where κ_{on} is the intrinsic "reaction-limited" ligand-receptor forward rate constant. The forward rate constant for binding to the entire cell is then k_{on}^{c} (cm^3/cell · s) = Nk_{on}.

The process of bulk ligand being adsorbed by specific receptors confined to a spherical cell surface may be thought of as a two-step process: (*a*) diffusion of ligand to the vicinity of the surface with forward rate constant $4\pi Da$ followed by (*b*) adsorption at the surface by a specific receptor with forward rate constant κ_{on} (Shoup and Szabo, 1982). When the binding to a single receptor is not limited by the former, i.e., when $4\pi Da >> N\kappa_{on}$, then $k_{on} \sim \kappa_{on}$.

Conversely, when the rate of binding is limited by diffusion to the cell, i.e., when $4\pi Da << N\kappa_{on}$, then $Nk_{on} \sim 4\pi Da$. In this case the forward rate constant for binding to the entire cell approaches a maximum value corresponding to that for a fully reactive sphere and increases in receptor density beyond this will have very little effect on the rate of ligand binding. The general form of Eq. 1 should hold for any shaped cell, the only change being the replacement of $4\pi Da$ by the diffusion-limited forward rate constant appropriate for the particular geometry of the cell. Furthermore, if the fraction of surface area occupied by receptors is small and the receptors are modeled as perfectly absorbing disks of radius s, then the rate of binding is entirely controlled by ligand diffusion and the intrinsic reaction-limited forward rate constant characterizing the second step, κ_{on}, may be replaced by the diffusion-limited forward rate constant for ligand reacting with a disk in an infinite plane 4 Ds (Hill, 1975). When this substitution is made, the result of Berg and Purcell (1977) is recovered.

Despite the biologically important implications of this result, direct experimental tests of Eq. 1 have been few. Schwartz (1976) determined the forward-rate constant for phage lambda binding to receptors on *Escherichia coli* K12 at different receptor densities (N = 30–9,500 receptors/bacterium) and was able to show that the forward rate constant for binding to the bacterium was proportional to N for small N and reached a maximum value at large N. Berg and Purcell (1977) and later Brunn (1981) successfully fit Schwartz's data to Eq. 1, although there was some discrepancy between the predicted and observed values of $4\pi Da$ ($= k_{on}^{c}[\max]$). Wank et al. (1983) determined k_{on} for the binding of IgE to its F_c receptor on rat basophilic leukemia (RBL) cells but did not observe any dependence on available receptor density. This was most likely a consequence of the low value for the intrinsic forward rate constant of IgE binding which restricts the system to the regime where $4\pi Da$ is always $> N\kappa_{on}$.

We have been investigating the kinetics of binding of multivalent ligands to cell surface receptor-bound IgE as part of an effort to understand the mechanism by which cross-linking of these receptors triggers a transmembrane signal leading to cellular degranulation (Erickson et al., 1986). As a necessary precedent to these studies we have examined the rate of binding of structurally analogous monovalent ligands. In particular we have used RBL cells with monoclonal anti 2,4-dinitrophenol (DNP) IgE (Liu et al., 1980) anchored to the cell via high affinity $F_c\epsilon$ receptors. With this experimental system we have been able to test Eq. 1 directly by varying the number of IgE molecules bound per cell and determining k_{on} as a function of N ($= F_{ab}$/cell$= 2 \times$ IgE/cell) for binding of monovalent 2,4-dinitrophenylamino caproyl-L-tyrosine (DCT). Our results are in excellent agreement with the theory described by Eq. 1.

METHODS

Sensitization of Cells with ^{125}I-Fluorescein-5-isothiocyanate (FITC)-IgE

The RBL cell subline 2H3 (Barsumian et al., 1981) was maintained in stationary culture as previously described (Taurog et al., 1979) and was used 4–6 d after passage. Cells harvested after a brief incubation at 37°C with 1.5 mM EDTA, 135 mM NaCl, 5 mM KCl, pH 7.4, were incubated with different quantities of ^{125}I-FITC-anti-DNP IgE for 1 h at 37°C in Eagle's minimal essential medium with Earle's salts, 10% (vol/vol) calf serum, and 20 mM HEPES, pH 7.4. The sensitized cells were then washed twice in a buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 20 mM HEPES, pH 7.4) by successive centrifugations at 200 g for 10 min followed by final resuspension at 10^6 to 10^7 cells per milliliter in the same buffer depending on the amount of FITC-IgE bound to each cell. The lowest amounts of ^{125}I-FITC-IgE used (0.1 μg/10^7 cells) resulted in the occupation of $\sim 10^4$ receptors/cell as determined from the specific activity of the IgE ($\sim 10^4$-10^5 cpm/μg bindable IgE). Saturation of all available receptors ($\sim 3 \times 10^5$) was achieved under these conditions using a fivefold bindable excess of ^{125}I-FITC-IgE. These values are consistent with the previously described kinetics for IgE binding to its surface receptor (Wank et al., 1983).

Detection of Ligand-Receptor Binding by Fluorescence Quenching

The proportionate decrease in FITC-IgE fluorescence that accompanies Fab site occupation by DNP ligands, which provides the basis for the quenching method, and the preparation of FITC-IgE derivatives have been described in detail previously (Erickson et al., 1986). All fluorescence recordings were performed on a spectrofluorometer (model 8000; SLM Instruments, Inc., Urbana, IL) operated in ratio mode. The excitation wavelength was 490 nm (bandwidth 8 nm) and the emission was monitored at 526 nm (bandwidth 16 nm). A CS-71 longpass filter (No. 3384; Corning Glass Works, Corning Science Products, Corning, NY) was used in the emission port to reduce scattered light contributions to the signal. In a typical run, the signal was averaged for 0.5 s to provide a single data point. This acquisition time was determined to be optimal for maximizing signal-to-noise while still providing sufficient time resolution for the determination of k_{on}.

For a typical kinetic run, 2 ml of cell suspension were placed in a 10 × 10 × 48 mm acrylic cuvette (Sarstedt, Inc., FDR) and stirred with a Starhead stirbar (Nalge Co., Div. of Sybron Corp. Rochester, NY). Small volumes of a micromolar ligand stock solution were introduced using 3 or 5 μl microcapillary tubes (Drummond Scientific Co., Broomall, PA) fit snugly into the end of a length of PE-90 Intramedic tubing (Clay Adams, Parsippany, NJ). The other end of the tubing was fed through a small port leading from the fluorometer sample chamber to a 50-μl gas-tight Hamilton syringe (Reno, Nevada) which provided the force for injection. Mixing time under our conditions was <2 s. Recording of the fluorescence was continuous before and after injection for adequate periods to observe equilibrium levels of fluorescence as well as the transient decay in signal that immediately followed the introduction of ligand. The maximum quenchable fluorescence averaged ~20–25% of the total signal and was determined at the end of every run by observing the signal following saturation of F_{ab} with excess DNP ligand. Each data set was normalized with respect to this maximum quenching and fit to extract a single time constant (see below).

Treatment of Raw Fluorescence Data and Determination of k_{on}

The data analysis was carried out assuming that the reaction between ligand and surface receptor could be described by a general one step bimolecular reaction scheme. That is, we consider the reaction

$$L + X \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} L\text{-}X,$$

where L and X are the concentrations of free DNP ligand and unbound F_{ab} sites, respectively, and L-X is the concentration of bound complex with all quantities written as moles/liter. Our experimental observable, the decay of fluorescence that accompanies the disappearance of unoccupied F_{ab} may be written as

$$-d[X]/dt = k_{on}[L][X] - k_{off}[L\text{-}X]. \quad (2)$$

The conservation expressions for total DNP and F_{ab} are,

$$[L]_{tot} = [L] + [L\text{-}X], \quad (3a)$$

$$[X]_{tot} = [X] + [L\text{-}X]. \quad (3b)$$

Substitution of Eqs. 3a and 3b into Eq. 2 leads to

$$-d[X]/\{(-k_{off}[X]_{tot}) + \{k_{off} + k_{on}([L]_{tot} - [X]_{tot})\} \cdot [X] + k_{on}[X]^2\} = dt, \quad (4)$$

where the variables have been separated. If we assume k_{on} and k_{off} are constant, integration from $[X] = [X]_{tot}$ at $t = 0$ to $[X] = [X(t)]$ at time t followed by rearrangement yields

$$[X(t)]/[X]_{tot} = [b(\alpha e^{t\beta} - 1) + \beta(\alpha e^{t\beta} + 1)]/\{2c(1 - \alpha e^{t\beta})[X]_{tot}\}, \text{ where}$$

$$a = k_{off}[X]_{tot}$$

$$b = k_{on}([X]_{tot} - [L]_{tot}) - k_{off}$$

$$c = -k_{on}$$

$$\alpha = (2c[X]_{tot} + b - \beta)/(2c[X]_{tot} + b + \beta)$$

$$\beta = (b^2 - 4ac)^{1/2}. \quad (5)$$

Normalized data sets were fit to Eq. 5 to extract k_{on}. Knowledge of $[X]_{tot}$ from the specific activity of the IgE and $[L]_{tot}$ from accurate measurement of DNP in the ligand stock solutions ($\epsilon_{max} = 1.78 \times 10^4$ (M · cm)$^{-1}$ at 365 nm; Kane et al., 1986) allowed these parameters to be entered and held fixed for the computer fitting. Furthermore, since the association constant K_a for DCT and cell bound ^{125}I-FITC-IgE has been previously determined for these experimental conditions (Erickson et al., 1986), k_{on}/K_a could be substituted for k_{off} in Eq. 5, reducing the number of freely varying parameters to one.

Eq. 5 is an approximate result since it ignores the time dependence of the rate constants due to the decreasing free receptor density as binding progresses. Although we did not observe any evidence for this effect, we chose to minimize its possible influence on our results by keeping equilibrium values of fractional receptor occupation below 0.5 in most of our kinetic runs. Alternatively, the raw kinetic data can be analyzed for only the earliest time points with Eq. 2 applied directly yielding $k_{on} \sim(-d[X]/dt)/([L]_{tot}[X]_{tot})$. Following this procedure did not change the values of k_{on} significantly.

RESULTS AND DISCUSSION

Using a well defined experimental system, we have studied the effect of variable anti-DNP IgE surface densities on the rate of DNP ligand binding. Previously we have shown that the fractional occupancy of antibody combining sites belonging to fluorescein-modified IgE was accurately reflected in the fluorescence quenching that accompanies DNP binding (Erickson et al., 1986). The sensitivity of the method allows us to measure binding between nanomolar quantities of F_{ab} and DNP. At these concentrations the binding reactions, typically having characteristic forward rate constants on the order of 10^7 (Ms)$^{-1}$ (Pecht and Lancet, 1977), proceed on a time scale of several seconds. The binding can therefore be followed without resorting to conventional fast kinetic techniques which are difficult to apply to cellular systems.

Fig. 1 illustrates the quality of the raw kinetic data (●) and the best fits of Eq. 5 (—). The top curve is the average of three kinetic runs on identical samples where $N = 1.5 \times 10^5$ F_{ab}/cell and the cell concentration was 1.2×10^7 cells/ml. At very low values of N the averaging greatly improves signal/noise and makes the uncertainty in k_{on} values comparable over the range of IgE receptor occupancies employed ($\sim 10^4 - 5 \times 10^5$ FITC-IgE/cell). The lower kinetic trace shows data from cells possessing the highest receptor density observed in these experiments. It illustrates the other experimental extreme where high F_{ab} density permits the dilution of cells down to $\sim 10^6$/ml

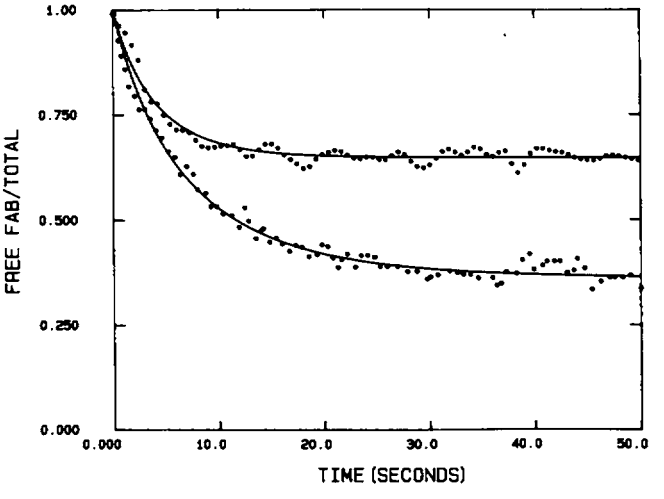

FIGURE 1 Typical kinetic quenching data and best fits (*solid line*) of the equation that follows from simple biomolecular scheme (Eq. 5). The top curve represents a moderately low density of F_{ab} ($\sim 1.5 \times 10^5$/cell) while the bottom curve was obtained with the highest surface density of F_{ab} observed in these experiments (11.5×10^5/cell). *Top curve:* With the following fixed at $K_a = 1.4 \times 10^9$ M^{-1}, $[DCT]_{tot} = 1.5$ nM, $[F_{ab}]_{bulk} = 3.1$ nM, the best fit is obtained when $k_{on} = 6.6 \times 10^7$ (Ms)$^{-1}$. In this experiment cells/ml $= 1.2 \times 10^7$, and F_{ab}/cell $= 1.5 \times 10^5$. *Bottom curve:* Fixing $K_a = 1.4 \times 10^9$ M^{-1}, $[DCT]_{tot} = 4.2$ nM, $[F_{ab}]_{bulk} = 4.5$ nM, gives $k_{on} = 2.6 \times 10^7$ (Ms)$^{-1}$. Cells/ml $= 2.4 \times 10^6$, F_{ab}/cell $= 11.5 \times 10^5$.

without losing the ability to measure the fluorescence decay accurately.

The bimolecular kinetic scheme that results in Eq. 5 does not take into account the localization of F_{ab} combining sites to the surface of cells and in this respect provides a phenomenological constant (k_{on}) to describe the interaction of bulk F_{ab} and DNP ligand. To determine the origin of any observed variation in k_{on}, kinetic experiments were performed on samples where the concentration of bulk F_{ab} was varied systematically in three different ways: (*a*) Fig. 2 shows how k_{on} varies when $[F_{ab}]_{bulk}$ is varied by addition of small aliquots of FITC-IgE to suspensions of cells whose IgE receptors have been previously saturated with rat myeloma IgE (▲). Since the myeloma IgE does not bind DNP and the exchange between solution and cell-bound IgE is slow on the timescale of this experiment (Metzger, 1983), the added DNP ligand binds only to solution IgE. The dashed line is the mean of the four data points and represents a solution $k_{on} = 18.6 \times 10^{-14}$ $cm^3/F_{ab} \cdot s$ ($1.1 \times 10^8 [Ms]^{-1}$). (*b*) At very high values of F_{ab}/cell ($\geq 8 \times 10^5$) there was sufficient signal to allow variance of cell concentration over a fivefold range. Experiments performed with the same surface density of FITC-IgE but a range of cell concentrations showed no apparent change in k_{on} (■). The dashed line at the bottom of Fig. 2 is the mean of the five data points shown and represents the observed k_{on} of 3.6×10^{-14} $cm^3/F_{ab} \cdot s$ for this particular value of N (8×10^5 F_{ab}/cell). (*c*) The results from methods *a* and *b* should be compared with those obtained when cell concentration is fixed at 5×10^6/ml and F_{ab}/cell is varied from ~0.2 to 6×10^5 (●, Fig. 2) by changing incubation ratios of ^{125}I-FITC-IgE/cell described in the Methods section. Under these conditions, k_{on} is apparently a decreasing function of $[F_{ab}]_{bulk}$ and also therefore of N. This trend is in qualitative agreement with the intuitive notion of adjacent receptors competing for available ligand at the cell surface and consequently reducing the observed k_{on} per receptor. At very low receptor densities, k_{on} presumably reaches some limiting value corresponding to the absence of any competing receptors. This idea is embodied in Eq. 1, where in the limit that N goes to zero, $k_{on} = \kappa_{on}$. One might expect this limit to be approximately realized by FITC-IgE in solution (Fig. 2, ▲) where the average distances between IgE molecules are much larger and their competition for the same ligand is correspondingly lower. The fact that the combining sites come in closely spaced pairs due to the antibody being bivalent might be expected by itself to lower the intrinsic forward rate constant by a factor of <ln2 (Goldstein and Wiegel, 1983). No attempt was made to discern this effect by performing similar experiments with F_{ab} fragments.

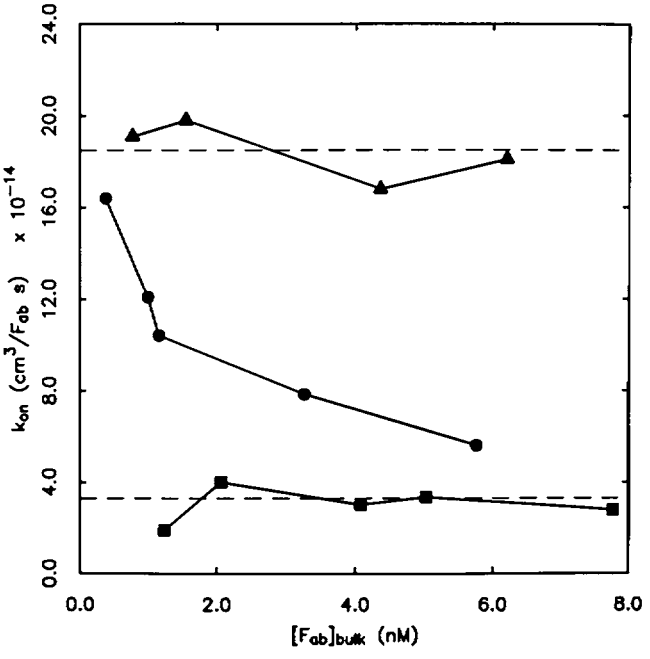

FIGURE 2 The forward rate constant for binding to cells varies only with surface density of F_{ab}. The $[F_{ab}]_{bulk}$ was varied in three different ways: (*a*) Cells were first incubated with unlabeled rat IgE to block the binding of DNP-specific FITC-IgE, which was later added to the cell suspension to give the indicated concentrations (▲) (cells/ml = 6×10^6). (*b*) Cells were incubated with various amounts of FITC-IgE and washed, resulting in only partial saturation of available IgE receptors (●) (cells/ml = 7×10^6). (*c*) Cells were incubated with excess FITC-IgE until all available IgE receptors were saturated. Dilution of these cells gave the indicated concentrations (■) (F_{ab}/cell = 8.2×10^5). Dashed lines represent average values for k_{on} in experiments 1 and 3 (18.5×10^{-14} and 3.3×10^{-14} cm^3/s, respectively). Note: $k_{on}(Ms)^{-1} \times (1{,}000/6.02 \times 10^{23}) = k_{on}(cm^3/F_{ab}\ s)$.

Fig. 3 shows accumulated data from experiments per-

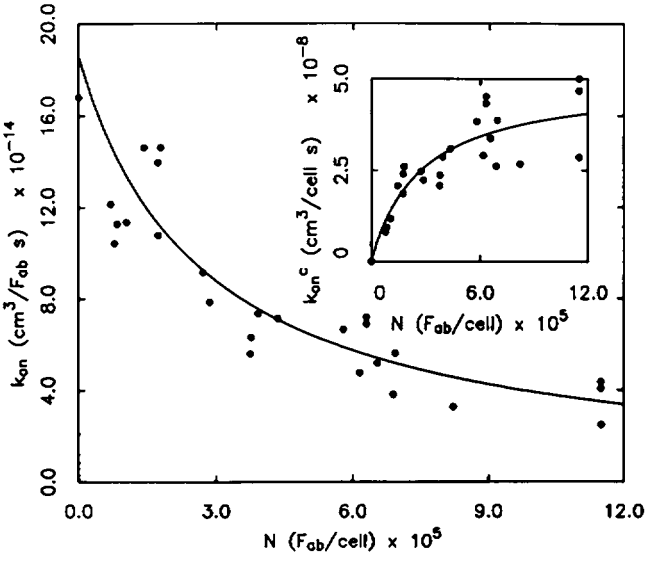

FIGURE 3 Accumulated $k_{on}(cm^3/F_{ab}\ s)$ vs. $N(F_{ab}/cell)$ data and best fit of Eq. 1. The best fit is given by $4\pi Da = 4.96 \times 10^{-8}$ $cm^3/cell \cdot s$ and $\kappa_{on} = 18.6 \times 10^{-14}$ cm^3/F_{ab} s. Note how this latter value coincides with that in Fig. 2 for FITC-IgE in solution (▲). *Inset:* Same data plotted on a per cell basis showing the limiting value at high receptor densities where $k_{on}^c = 4\pi Da = 4.96 \times 10^{-8}$ $cm^3/cell \cdot s$. Solid line represents best fit of Eq. 1 multiplied by N with same parameter values.

formed under a variety of conditions of cell concentration, $[F_{ab}]_{bulk}$, and IgE surface density. Only the last has an effect on the observed value of k_{on} as illustrated in Fig. 2, and the observed k_{on} data is therefore plotted as a function of N. As was the case for the single experiment shown in Fig. 2, the measured rate constant is clearly a decreasing function of the number of F_{ab}/cell. The solid line represents the best fit of Eq. 1 to the data and demonstrates the predicted functional dependence of $k_{on}(cm^3/Fab \cdot s)$ on $N(F_{ab}/cell)$. The best fit was obtained by varying the composite parameter $4\pi Da$ and the intrinsic forward rate constant κ_{on} and yielded values of 4.96×10^{-8} $cm^3/cell \cdot s$ and 1.86×10^{-13} $cm^3/F_{ab} \cdot s$, respectively. Although the exact agreement is fortuitous, it is striking how well the best fit of the accumulated cell data predicts a value for the intrinsic rate constant equal to that observed for DCT and solution FITC-IgE (see above). The plausible quantitative predictions of the theory are further illustrated by choosing an approximate value for the DCT diffusion constant of 10^{-5} cm^2/s and solving for a. This procedure yields a reasonable value for the cell radius of 4 μm.

The upper bound for the rate constant describing binding to the whole cell, $4\pi Da \geq k_{on}^c$, which is approached as N becomes large ($>5 \times 10^6$), is predicted to be 4.96×10^{-8} $cm^3/cell \cdot s$ as noted above. Since the maximum obtainable receptors/cell ($=1/2F_{ab}s$/cell) for RBL cells is generally less than $\sim 1 \times 10^6$, this limit of the model can only be experimentally approximated (the highest value of $N\kappa_{on}/4\pi Da$ obtained was ~4). The inset in Fig. 3 plots the same data points as in Fig. 3 where the k_{on} values have been modified by multiplication by N $[k_{on}(cm^3/F_{ab} \cdot s) \times N = k_{on}^c(cm^3/cell \cdot s)]$ to illustrate graphically that in the limit of large N, k_{on}^c approaches $4\pi Da$. For this system at least, these data indicate that the cell accomplishes half the maximum possible rate of binding DCT to antibody combining sites by having $\sim 10^5$ IgE receptors occupied by antibody specific for DCT. Assuming the radius of an IgE receptor is 50 Å, this represents at most $Ns^2/4a^2 \times 100 \sim$ 4% of the total surface area of the cell with an average inter-receptor spacing of ~450 Å. Given the similar k_{on} observed in solution for a range of antibody specificities (Pecht and Lancet, 1977), our quantitative results concerning the rate of adsorption of small molecules by variable densities of surface immunoglobulin should be directly applicable to other similar ligand-receptor systems. In this regard, we have performed the same experiments with DNP-lysine and DNP-glycine. The equilibrium constants for these ligands are $\sim 1 \times 10^8$ and 1×10^7 M^{-1}, respectively, but the overall k_{on} results are the same as those obtained with DCT (data not shown). Beyond this narrow quantitative interpretation, however, our results demonstrate a general phenomenon with practical consequences for many receptor systems currently under study.

We believe the above data constitute the first experimental evidence revealing the functional dependence of the rate of binding of small molecules on the surface density of a biological receptor. The theory presented by Berg and Purcell in their seminal paper (1977) is in excellent agreement with our findings both qualitatively and quantitatively. Unique values for the composite parameter $4\pi Da$ (4.96×10^{-9} $cm^3/cell \cdot s$) and intrinsic reactivity of the IgE ($\kappa_{on} = 1.86 \times 10^{-13}$ $cm^3/F_{ab} \cdot s$) provided by the best fit of Eq. 1 to the accumulated data (Fig. 3) correspond to a reasonable value for the cell radius of 4 μm if D is assigned a value of 10^{-5} cm^2/s and an intrinsic reactivity equal to that measured for IgE in solution. We are presently using the knowledge gained in this study to characterize the kinetics of receptor/receptor cross-linking by symmetric bivalent ligands of the type $(DCT)_2$-R (R = ethylene diamine or cystine, Kane et al., 1986). Determination of the rate constants describing the two-dimensional cross-linking reactions responsible for signal transduction in this system will provide a much needed complement to the large body of data characterizing the IgE-mediated biological response.

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