Bivalent Ligand Dissociation Kinetics from Receptor-Bound Immunoglobulin E: Evidence for a Time-Dependent Increase in Ligand Rebinding at the Cell Surface†

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ABSTRACT: The bivalent ligand *N*,*N*′-bis[[ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosyl]cystine [$(DCT)_2$-Cys] binds and cross-links anti-dinitrophenyl (DNP) immunoglobulin E (IgE)–receptor complexes on the cell surface of rat basophilic leukemia cells. The rate of dissociation of this bound ligand was monitored by using a fluorescence method under two different conditions. In one case the monovalent ligand DCT was added in large excess to prevent the dissociating ligand from rebinding to unoccupied antibody combining sites. Under these conditions, dissociation of the bivalent ligand from IgE-sensitized cells proceeds to completion with kinetics that are well described by two rate constants that are independent of the time of preincubation of the bivalent ligand with the cells. In the second case, dissociation of $(DCT)_2$-Cys from cell-bound anti-DNP IgE was monitored in the presence of a large excess of anti-DNP IgE in solution that acts as a sink to absorb the dissociated ligand. Under these conditions, the bivalent ligand becomes more resistant to dissociation as the preincubation time of the bivalent ligand with the cells is increased. An increasing fraction of the bound ligand does not dissociate on a measurable time scale in the presence of this sink. The results indicate that cell-associated IgE–receptor complexes undergo a time-dependent change that facilitates the reformation of the cross-linked state when one end of the ligand dissociates to break up the existing cross-link. The possible physical basis and functional implications of these results are discussed.

It is well established that the primary event leading to cellular degranulation in mast cells, basophils, and related cell lines is the cross-linking of high-affinity receptors for IgE[1] on the cell surface. However, the critical features of IgE receptor aggregates that are required for a stimulatory signal remain largely unknown.

On the rat basophilic leukemia (RBL)-2H3 cell line, dimeric IgE receptor complexes are typically ineffective at stimulating degranulation responses (Fewtrell & Metzger, 1980; Menon et al., 1986b), but trimeric and larger complexes formed with covalent oligomers of IgE (Fewtrell & Metzger, 1980), anti-IgE antibodies (Fewtrell & Metzger, 1981; Menon et al., 1986b), or multivalent antigens (Fewtrell, 1985) are capable of eliciting a strong degranulation response. For oligomeric IgE, the distribution of singly bound versus multiply bound species is difficult to assess, and the heterogeneity of cross-linking that is achieved with multivalent antigen or polyclonal anti-IgE antibodies makes it difficult to correlate binding studies of the cross-linking ligand with the functional responses.

In an attempt to overcome these limitations, we have employed a symmetrical bivalent ligand, $(DCT)_2$-Cys, that binds with high affinity to a monoclonal anti-DNP IgE antibody and that triggers transient increases in cytosolic Ca^{2+} (Ryan, 1989) and a weak degranulation response (Kane et al., 1986) in RBL-2H3 cells. A fluorescence method was developed for monitoring the binding of DNP ligands to bivalent anti-DNP IgE as a function of ligand dose or of time. We previously employed this system to determine equilibrium binding behavior for monovalent and bivalent ligand binding to IgE in solution and to IgE that is bound to its receptor on cells (Erickson et al., 1986). This system also allowed the characterization of kinetic behavior of monovalent ligand binding to IgE in solution and on cells (Erickson et al., 1987; Goldstein et al., 1989).

Titrations of cell-bound anti-DNP IgE with increasing concentrations of bivalent $(DCT)_2$-Cys provided evidence for efficient cross-linking of IgE–receptor complexes with this ligand (Erickson et al., 1986). However, it was not possible to determine an unambiguous equilibrium cross-linking constant from these data. One complication is that cross-linking initiates cellular changes that perturb the equilibrium conditions. It became evident that kinetic studies were needed to understand the relationship between cross-linking and the resultant functional response.

In the accompanying paper (Posner et al., 1991), we demonstrated that the dissociation of $(DCT)_2$-Cys from anti-DNP IgE in solution in the presence of monovalent competitor, DCT, is described by a biexponential function and the values for the dissociation rate constants, k_{-1} and k_{-2}, differ by a factor of $\sim$15. The value of k_{-1} is similar to that previously determined for dissociation of DCT (Goldstein et al., 1989), and the smaller value of k_{-2} provides evidence for some further stabilization of the cross-linked complexes, possibly by the formation of small rings.

The present study extends these dissociation measurements to the case of IgE bound to its cell surface receptor on RBL

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[1] Abbreviations: $(DCT)_2$-Cys, *N*,*N*′-bis[[ε-[(2,4-dinitrophenyl)-amino]caproyl]-L-tyrosyl]cystine; DNP, dinitrophenyl; DCT, [ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosine; IgE, immunoglobulin E; RBL, rat basophilic leukemia; FITC, fluorescein 5-isothiocyanate.

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cells. Using the same fluorescence method that was employed in previous studies, we observe a more complex behavior for $(DCT)_2$-Cys that is bound to IgE at the cell surface than that observed for this bivalent ligand bound to IgE in solution or on membrane vesicles derived from RBL cells. Evidence is presented for a time-dependent decrease in ligand dissociability from the cell surface, and this is shown to be the result of increasingly efficient reformation of cross-links by the monovalently bound $(DCT)_2$-Cys. The structural and functional implications of these results for receptor-mediated signal transduction are discussed.

Materials and Methods

Reagents. Fluorescein 5-isothiocyanate (FITC) was obtained from Molecular Probes, Inc. (Eugene, OR), and sodium [^{125}I]iodide was from Amersham Corp. (Arlington Heights, IL). The monovalent ligand [ε-[(2,4-dinitrophenyl)amino]-caproyl]-L-tyrosine (DCT) was obtained from Biosearch, Inc. (San Rafael, CA), as the dicyclohexylamine salt, and the bivalent derivative $(DCT)_2$-Cys was synthesized and chartacterized in this laboratory as described previously (Kane et al., 1986).

IgE and Derivatives. Mouse monoclonal anti-DNP IgE from hybridoma H1 26.82 (Liu et al., 1980) was affinity purified as previously described (Holowka & Baird, 1983). The IgE was then subjected to gel filtration on a Sephacryl 300 column to remove any aggregates or fragments, and peak fractions of monomeric IgE (>4 mg/mL) were either diluted to 2 mg/mL for modification or used unmodified in concentrated form in some dissociation experiments. Preparation and characterization of anti-DNP IgE modified with FITC and Na^{125}I (FITC-IgE) has been described (Erickson et al., 1986). Prior determination of the specific activity of the FITC-^{125}I-IgE (^{125}I cpm/binding site) allowed accurate measurement of the anti-DNP Fab concentration (equal to twice the concentration of intact IgE). For experiments using IgE-sensitized cells, counting the number of cells/per milliliter with a hemocytometer permitted calculation of an average anti-DNP Fab per cell.

Sensitization of Cells with FITC-IgE. The RBL cell subline 2H3 (Barsumian et al., 1981) was maintained in continuous cell culture and harvested as previously described (Taurog et al., 1979). Cells were sensitized with a ≥10-fold molar excess of FITC-IgE over receptors 24 h prior to harvesting. In experiments where less than full occupancy by anti-DNP IgE was desired, rat myeloma IgE (IR162), which has no detectable affinity for DNP, was mixed with mouse anti-DNP IgE in varying ratios and incubated with cells to saturate the IgE receptors. Sensitized cells were washed twice in buffered salt solution [BSS: 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose, 0.05% gelatin (w/v), 20 mM HEPES, pH 7.4] by successive centrifugations at 200*g* for 10 min, followed by final resuspension at ~10^6 cells/mL in the same buffer.

Spectrofluorometric Measurements of DNP Ligand Dissociation. Fluorescence measurements were made with an SLM 8000 fluorescence spectrophotometer operated in ratio mode. In a typical experiment, 2 mL of a solution containing RBL cells sensitized with FITC-IgE in BSS was stirred continuously in an acrylic cuvette (10 × 10 × 48 mm; Walter Sarstedt, Inc., FDR) and thermostatically controlled at either 15 or 25 °C. For membrane vesicle experiments, 125 μL of the FITC-IgE-saturated membrane suspension was placed in a 3 × 3 mm quartz cuvette (NSG Precision, Hicksville, NY). FITC was excited at 490 nm (8-nm bandwidth, fwhm), and emission was monitored at 526 nm (16 nm, fwhm) in all experiments. Small amounts of $(DCT)_2$-Cys solution were delivered in a darkened room with microcapillary tubes (Drummond Scientific, Broomall, PA). The subsequent decay in fluorescence was monitored and, at indicated times thereafter, a small volume of either a concentrated stock solution of unlabeled anti-DNP IgE (≥20 μM) or DCT (2.2 mM) was added to compete for the unbound DNP or for the unoccupied IgE sites, respectively. The restoration of the fluorescence signal that accompanied the decrease in $(DCT)_2$-Cys binding to FITC-IgE sites was monitored continuously, and the data were collected with an AST286 computer for later analysis.

Preparation and FITC-IgE Labeling of Plasma Membrane Vesicles. Preparation and characterization of chemically induced plasma membrane vesicles from RBL cells have been described previously (Holowka & Baird, 1983; Baird & Holowka, 1985). Vesicles were incubated with a 5-fold excess of FITC-IgE over receptors (~100 nM) by rotating the mixture overnight at 4 °C. These were then diluted ~15-fold and pelleted by centrifugation at 25000*g* for 30 min at 4 °C. Following removal of the supernatant containing unbound FITC-IgE, the vesicles were resuspended to approximately 10^7 cell equiv of receptor-bound IgE/mL (~5 nM IgE).

Data Analysis. Interaction of ligand and IgE in solution or on the membrane surface can be considered in terms of the two equivalent Fab binding sites of IgE. A two-step binding and cross-linking reaction sequence for IgE Fab sites and bivalent ligand (C) can be written as

$$Y + C \underset{k_{-1}}{\overset{2k_{+1}}{\rightleftharpoons}} Y_1 \quad (1)$$

$$Y + Y_1 \underset{2k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} Y_2 \quad (2)$$

where Y corresponds to the unliganded IgE binding sites (Fab sites) and Y_1 and Y_2 correspond to IgE binding sites occupied by singly and doubly bound bivalent molecules, respectively. This is essentially the "ligand state" model of Perelson and DeLisi (1980), since the Fab concentration variables are defined in terms of the bound states of the ligand. For the bivalent ligand, $(DCT)_2$-Cys, the fractional quenching of FITC-IgE fluorescence is linearly related to the degree of Fab binding site occupancy at all points on a titration curve (Erickson et al., 1986), and therefore the level of fluorescence gives the proportions of free (=[Y]) and bound (=$[Y_1] + 2[Y_2]$) Fab sites directly. Reducing the number of bound states to two, however, requires the following simplifying assumptions: (1) The ligand cannot bridge two Fab's on the same IgE (i.e., no intramolecular cyclization). (2) Chains of IgE formed when ligand binds bivalently do not include rings that have different binding properties. (3) The forward cross-linking step, i.e., the reaction of the free ligand end in complex Y_1 with a free Fab end, is unaffected by the size of the chain to which these sites may be attached. (4) For the complex Y_2, the intrinsic dissociation rate constant for one end of a ligand is independent of the size of the chain in which it is incorporated.

If experimental conditions are fixed such that the forward reactions in eqs 1 and 2 are prevented from occurring, the model reduces to one describing the sequential release of $(DCT)_2$-Cys from IgE in solution or from the cell surface receptor bound IgE. Such conditions can be achieved if a sufficiently large excess of monovalent ligand is added to completely fill the empty receptor sites and thereby prevent rebinding of the bivalent ligand. The dissociation steps are (1) the breaking of a cross-link when one end of the bivalent ligand in Y_2 dissociates (described by k_{-2} in the reverse of eq 2) and (2) dissociation of the resulting monovalently bound

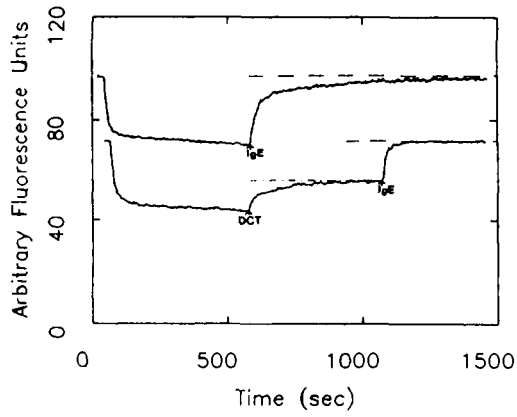

FIGURE 1: Recovery of FITC fluorescence after binding 0.90 nM $(DCT)_2$-Cys to 0.16 nM cell-bound FITC-IgE (3×10^4/cell) for 10 min followed by the addition of unlabeled anti-DNP IgE to a final concentration of 0.50 μM (top curve) or DCT to a final concentration of 60 nM (bottom curve). For comparison the two curves are offset, and the original levels of fluorescence at $t = 0$ for each trace are indicated at the end of the recovery curves (--). Traces are corrected for small dilution effects at each addition. The level of relative fluorescence observed for a separate but identical sample in which all FITC-IgE sites were saturated with DCT (-··-) is also shown for the bottom trace. The temperature for this experiment was 25 °C.

ligand into bulk solution (described by k_{-1} in the reverse of eq 1).

As shown in the accompanying paper (Posner et al., 1991), when a large excess of monovalent ligand, DCT, is used to cause net dissociation of the bivalent ligand, $(DCT)_2$-Cys, from IgE, we obtain the following expression for the relative fluorescence:

$$F = F_{max} - (F_{max} - F_{min})[A \exp(-k_{-1}t) + (1 - A) \exp(-2k_{-2}t)] \quad (3)$$

where F_{max} is the value of the relative fluorescence after dissociation has gone to completion, F_{min} is the relative fluorescence immediately after the addition of the DCT, and A is the normalized preexponential weighting factor. Equation 3 is strictly valid only under conditions where rebinding of the bivalent ligand is prevented.

The data were fit with F_{max}, F_{min}, A, k_{-1}, and k_{-2} taken as free parameters. Values for these parameters were obtained with the International Mathematics and Statistics Library (IMSL) fitting routine "ZXSSQ", which is based on a finite difference, Levenberg-Marquardt algorithm for solving nonlinear least-squares problems. Estimates of the standard deviation of the parameters were obtained by using the bootstrap method described by Efron and Tibshirani (1986). To obtain the estimates each experiment was simulated 100 times.

RESULTS

Dissociation of $(DCT)_2$-Cys from Cell-Bound IgE. Fluorescence intensity changes that accompany binding of DNP ligands to FITC-IgE were used to monitor the kinetics of $(DCT)_2$-Cys dissociation from anti-DNP IgE on the cell surface. The approach is illustrated in Figure 1. Following the addition of $(DCT)_2$-Cys, the fluorescein fluorescence of FITC-IgE is reduced as this bivalent DNP ligand binds. This part of the binding curve reflects both the initial monovalent binding and the cross-linking of two or more IgE–receptor complexes on the cell surface (Erickson et al., 1986). Net dissociation of the bivalent ligand is monitored as an increase in the fluorescence when the binding equilibrium (or steady state) is perturbed. Net dissociation of bound $(DCT)_2$-Cys was initiated in two ways: (1) addition of an excess of unmodified anti-DNP antibody (sink), which absorbs the dissociated ligand (top curve), or (2) addition of an excess of the monovalent DNP ligand, DCT (competitive inhibitor), which fills the unoccupied binding sites but quenches the fluorescence to a lesser extent than does $(DCT)_2$-Cys (bottom curve).

The top curve of Figure 1 shows that if unlabeled anti-IgE is added to the suspension of cells with $(DCT)_2$-Cys having been prebound for a short period of time (<10 min), the fluorescence recovers to a level approaching that measured before $(DCT)_2$-Cys addition. As described below, longer preincubation times with $(DCT)_2$-Cys result in a decrease in the maximum recoverable level of fluorescence under similar conditions. Addition of the monovalent ligand DCT at 200-fold excess over $(DCT)_2$-Cys ~10 min after addition of $(DCT)_2$-Cys (bottom curve, Figure 1) results in a partial recovery of fluorescence to the same intensity observed when the DCT is added without prior addition of $(DCT)_2$-Cys. This result indicates that DCT has fully displaced bound $(DCT)_2$-Cys in this sample. Addition of unlabeled anti-DNP IgE following the DCT-induced dissociation of $(DCT)_2$-Cys fully restores the FITC-IgE fluorescence on a time scale that is consistent with the dissociation kinetics of monovalently bound DCT (Goldstein et al., 1989).

Dissociation of $(DCT)_2$-Cys in the Presence of a Sink of Soluble IgE. We previously showed that the rate of dissociation of DCT from receptor-bound FITC-IgE in the presence of a large excess of soluble unlabeled IgE is sensitive to the density of unoccupied anti-DNP binding sites at the cell surface (Goldstein et al., 1989). These results demonstrated that even for the monovalent ligand, rebinding to cell surface IgE can occur many times before the ligand can dissociate completely from the cell and escape into solution where it is captured by the soluble anti-DNP IgE. However, once steady-state binding is attained with the monovalent ligand, further incubation has no effect on the rate of dissociation that is monitored in the presence of excess unlabeled anti-DNP IgE.

A strikingly different result is obtained with the bivalent ligand $(DCT)_2$-Cys: longer preincubation times with FITC-IgE-sensitized cells lead to a progressive loss in the dissociability of this ligand upon addition of excess unlabeled anti-DNP IgE. Figure 2a illustrates this phenomenon with the dissociation part of the binding curves from three identical samples in which $(DCT)_2$-Cys was added to FITC-IgE-labeled cells at 25 °C and then allowed to incubate for 10 (top), 60 (middle), or 120 (bottom) min before addition of excess anti-DNP IgE. In each case the rate of dissociation is clearly biphasic, and the time-dependent loss in dissociability appears to be approaching a maximal effect after a 60-min preincubation time. In this experiment the fluorescence quenching due to the initial $(DCT)_2$-Cys binding had reached a steady state in these samples within 10 min (similar to the data of Figure 1), before the earliest dissociation measurement was initiated. This indicates that something other than occupancy of binding sites is causing the time-dependent effect. The loss in $(DCT)_2$-Cys dissociability illustrated in Figure 2a is not sensitive to small differences in the amount of anti-DNP IgE that is added; a 2-fold increase in this concentration does not significantly alter the results obtained (data not shown).

Evidence from several experiments (not shown) indicates that the time-dependent decrease in $(DCT)_2$-Cys dissociation from IgE receptors on cells in the presence of soluble anti-DNP IgE (Figure 2a) is not a consequence of IgE receptor endocytosis: (1) Examination of $(DCT)_2$-Cys cross-linked FITC-IgE on RBL cells by fluorescence microscopy shows a uniform distribution of FITC fluorescence at the cell periphery, even

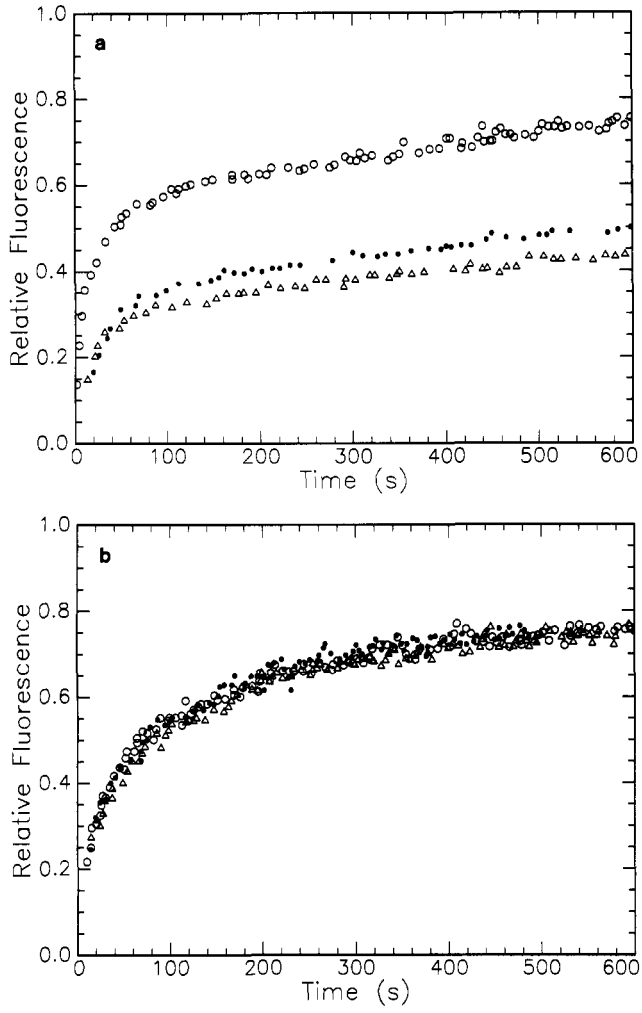

FIGURE 2: Dissociability of $(DCT)_2$-Cys from FITC-IgE–receptor complexes in the presence of excess anti-DNP IgE in solution increases with longer preincubations on cells (a) but not on plasma membrane vesicles (b). (a) FITC-IgE-sensitized cells were suspended at 3.2×10^6/mL to give a concentration of cell-bound FITC-IgE = 0.21 nM, and these were incubated with 0.46 nM $(DCT)_2$-Cys for 10 (○), 60 (●), or 120 (△) min before recovery of fluorescence was initiated by adding soluble anti-DNP IgE to a final concentration of 1.2 μM. (b) FITC-IgE-sensitized vesicles were suspended to give a membrane-bound FITC-IgE concentration = 5.5 nM, and these were incubated with 3.2 nM $(DCT)_2$-Cys for 10 (○), 20 (●), and 135 (△) min before recovery of fluorescence was initiated by adding soluble unlabeled IgE to a final concentration of 0.8 μM. Both experiments were carried out at 25 °C. The surface density of FITC-IgE in the cell samples was adjusted downward to be similar to that in the vesicle samples (as judged by fluorescence intensity) by sensitizing the cells with an appropriate mixture of unlabeled rat IgE and FITC-IgE.

after several hours at 25 °C. Extended incubation of the cells at 4 °C (12–16 h) shows some indications of a large scale redistribution of fluorescence to one pole of the cell without any evidence for internalized fluorescence. (2) Inhibition of any possible cross-link-mediated endocytosis by 10 mM deoxyglucose and 10 mM sodium azide (Menon et al., 1984) does not affect the loss in $(DCT)_2$-Cys dissociability. (3) A large excess of DCT completely displaces $(DCT)_2$-Cys from cell-bound IgE even after long (>1 h) incubation periods with the latter ligand. (4) Experiments performed at 8 °C, a temperature that prevents endocytosis caused by multivalent antigen, display a similar loss in $(DCT)_2$-Cys dissociability.

This time-dependent loss in dissociability for $(DCT)_2$-Cys is not observed when the bivalent ligand is bound to IgE receptors on plasma membrane vesicles derived from RBL cells (Figure 2b). These membrane preparations contain predominantly right-side out, sealed vesicles that lack an intact cytoskeleton but retain the IgE receptor as well as other plasma membrane components (Holowka & Baird, 1983). For the two sets of experiments shown in Figure 2, the density of FITC-IgE on cells and vesicles was similar, as judged by the fluorescence intensities of individual cells and vesicles in the fluorescence microscope. Further evidence that any difference in IgE density in the two preparations is not critical comes from the observation that both give similar dissociation curves after short incubations with $(DCT)_2$-Cys (compare the 10-min curves in Figure 2).

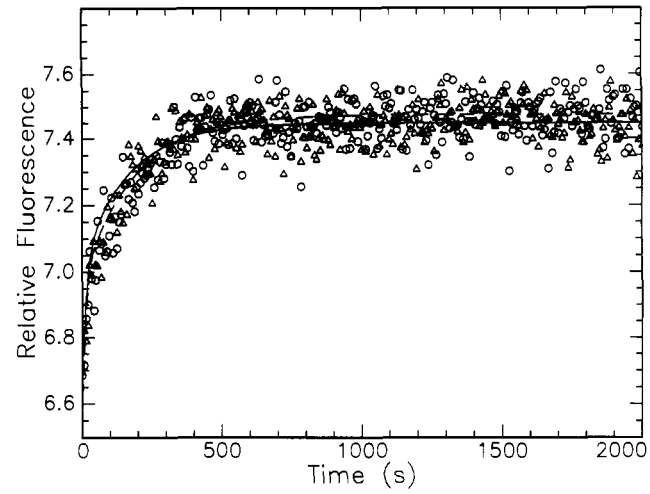

FIGURE 3: DCT-induced dissociation of $(DCT)_2$-Cys from cell-bound FITC-IgE after 3- (○) and 60-min (△) preincubations with the bivalent ligand. Both samples had a FITC-IgE concentration = 3 nM and a $(DCT)_2$-Cys concentration = 3.2 nM. After the indicated preincubation time, the DCT was added to the suspensions to a final concentration of 11 μM. The data are fitted according to eq 3 with, $k_{-1} = 0.089\ s^{-1}$, $k_{-2} = 0.0026\ s^{-1}$, $A = 0.457$, $F_{max} = 74\,770$, and $F_{min} = 74\,500$ (○ and dashed line) and $k_{-1} = 0.063\ s^{-1}$, $k_{-2} = 0.0030\ s^{-1}$, $A = 0.436$, $F_{max} = 74\,500$, and $F_{min} = 67\,000$ (△ and solid line).

Dissociation of $(DCT)_2$-Cys in the Presence of a Monovalent Competitor. A time-dependent decrease in $(DCT)_2$-Cys dissociability from FITC-IgE on cells is not observed when an excess of the monovalent ligand DCT is used to displace the cell-bound bivalent ligand. Because the data do not have this complication, they can be evaluated with simple binding equations. As shown by the representative experiment in Figure 3, the fluorescence recovery curves that reflect the decay of the bound $(DCT)_2$-Cys states in the presence of excess DCT are very similar for preincubation times of 3 and 60 min. In both cases the recovery is biphasic, and the data are well fitted by eq 3, yielding values for k_{-1} and k_{-2}. In both cases $(DCT)_2$-Cys is completely dissociated from FITC-IgE within ~1000 s after DCT is added, which differs markedly from what is observed when soluble unlabeled IgE is used to induce dissociation (Figure 2a).

The data shown in Figure 3 are representative of those obtained under conditions of different FITC-IgE cell surface densities (1.5×10^5 and 4.5×10^5 sites/cell) and different amounts of $(DCT)_2$-Cys preincubated with the cells (2.3 and 3.2 nM). From 10 experiments analyzed with eq 3 (five each with 3- and 60-min preincubation times) we find average values of $k_{-1} = 0.045 \pm 0.006\ s^{-1}$ and $k_{-2} = 0.0024 \pm 0.0001\ s^{-1}$. The value of k_{-1} is similar to the value observed for dissociation of DCT from cell surface FITC-IgE ($0.026 \pm 0.001\ s^{-1}$; Goldstein et al., 1989). The value of k_{-2} in this analysis is ~20 times lower than that of k_{-1}, indicating a larger population of bound DNP sites that are more slowly dissociating than those governed by k_{-1}. These values for the rate

constants are not sensitive to the concentration of DCT used to induce dissociation; very similar values were obtained when 1 μM instead of the standard concentration of 11 μM was used (data not shown). When DCT was used to initiate dissociation of $(DCT)_2$-Cys from FITC-IgE on the membrane vesicles, the values obtained for the two rate constants were very similar to those found on cells (data not shown).

Discussion

The present study was undertaken to characterize the dissociation kinetics of a structurally well-defined bivalent DNP ligand, $(DCT)_2$-Cys, from cell-associated IgE. The results contribute to a more detailed understanding of ligand binding and cross-linking and the relationship of those to the cellular responses they induce. Since IgE is bivalent with respect to its DNP binding sites, $(DCT)_2$-Cys has the potential for forming a complex distribution of linear and cyclic chains of varying sizes (Kane et al., 1986; Posner et al., 1991). Previous equilibrium binding studies indicated that $(DCT)_2$-Cys is capable of cross-linking receptor-bound IgE extensively on the cell surface (Erickson et al., 1986).

Dissociation of $(DCT)_2$-Cys in the Presence of a Monovalent Competitor. These studies showed that dissociation of bivalent $(DCT)_2$-Cys in the presence of a large excess of the monovalent competitor DCT goes to completion. The data are well fitted by a biexponential expression (eq 3) yielding values for k_{-1} [$(4.5 \pm 0.6) \times 10^{-2}$ s^{-1}] and k_{-2} [$(2.4 \pm 0.1) \times 10^{-3}$ s^{-1}] that are similar to the values obtained with these ligands and IgE in solution [$k_{-1} = (2.5 \pm 1.4) \times 10^{-2}$ s^{-1}, $k_{-2} = (1.6 \pm 0.4) \times 10^{-3}$ s^{-1}] (Posner et al., 1991). It is noteworthy that similar values are obtained after variable times of preincubation of $(DCT)_2$-Cys with cell-bound IgE prior to addition of the DCT. The value for k_{-1} probably represents an intrinsic dissociation constant for monovalently bound $(DCT)_2$-Cys because it agrees well with the value obtained for DCT previously [$k_{-1} = (2.6 \pm 0.1) \times 10^{-2}$ s^{-1}; Goldstein et al., 1989]. As discussed in the preceding paper (Posner et al., 1991), it is expected that the intrinsic rate constant for cross-link breakage (k_{-2}) for this symmetric bivalent ligand would be the same as the rate constant for monovalent dissociation both in solution and on cells. The apparent discrepancy between the apparent values of k_{-1} and k_{-2} may reflect some stabilization of two or more IgE molecules in proximity, thereby enhancing rebinding of the untethered end of $(DCT)_2$-Cys before DCT can block the temporarily unoccupied IgE site.

As discussed further below, rebinding of $(DCT)_2$-Cys to IgE on the cell surface is enhanced because the receptors are confined to a surface rather than being dispersed in the three-dimensional solution. However, at the concentration of DCT used in these studies (typically $\sim$10 μM), rebinding of fully dissociated $(DCT)_2$-Cys (total bulk concentration $\sim$2 nM) is negligible, since the forward binding of both ligands is governed by the same k_{+1}. Furthermore, we calculate that the reformation of an intermolecular cross-link by a singly bound $(DCT)_2$-Cys will also be prevented in the presence of excess DCT as long as the receptor-bound IgE molecules are free to diffuse laterally and rotationally in the plane of the membrane at the expected rates for un-cross-linked IgE–receptor complexes of 2×10^{-10} cm^2/s (Menon et al., 1986) and 40 μs (Zidovetski et al., 1986), respectively (B. Goldstein, unpublished results; Erickson, 1988). It is possible, however, that if two or more binding sites are held in proximity, reformation of $(DCT)_2$-Cys cross-links could occur more rapidly than blocking of empty sites by DCT. In this case the product of k_{+2} and the effective concentration of free binding sites would be larger than the intrinsic value of the rate constant for dissociation of cross-links ($=k_{-1}$), and the apparent k_{-2} would be correspondingly reduced and include these rebinding effects.

A reasonable explanation for the apparent discrepancy between k_{-1} and k_{-2} for $(DCT)_2$-Cys dissociating from IgE in solution in the presence of excess DCT is that small cyclic complexes ($[ligand]_2[IgE]_2$) are a major species (Posner et al., 1991). This explanation is also consistent with the results obtained for $(DCT)_2$-Cys dissociating from IgE on cells under similar conditions. Further support for this explanation comes from recent phosphorescence anisotropy measurements, which show evidence for the efficient formation of dimer-sized IgE–receptor complexes on plasma membrane vesicles derived from RBL cells (Myers, 1990). On cells, fluorescence photobleaching recovery measurements indicate that crosslinked IgE–receptor complexes formed by $(DCT)_2$-Cys have laterally mobile fractions and lateral diffusion coefficients that are similar to those for dimeric IgE–receptor complexes formed by monoclonal anti-IgE antibodies (R. Posner and J. Thomas, unpublished results). The apparent absence of large chains of cross-linked IgE–receptor complexes under these conditions suggests that cyclization is an efficient process on vesicles and cells.

Another possible explanation for the biphasic nature of $(DCT)_2$-Cys dissociation from cells is a change in the intrinsic affinity of the IgE binding sites due to a conformational change in the Fab segments that results from the aggregation process (Seagrave et al., 1987). We feel that this is unlikely to account for the observed dissociation results, since other studies indicate that the intrinsic affinity for the monovalent ligand, DCT, remains unchanged when IgE–receptor complexes are aggregated on cells by a variety of means (R. Posner and B. Lee, unpublished results).

Dissociation of $(DCT)_2$-Cys in the Presence of a Sink of Soluble IgE. Dissociation of $(DCT)_2$-Cys from cell surface FITC-IgE in the presence of an excess of unlabeled anti-DNP IgE in solution (sink) is more complex than dissociation in the presence of an excess of the monovalent competitor DCT. The differences are apparently due to the relative abilities of sink versus monovalent competitor to prevent rebinding of the bivalent ligand to IgE on the membrane. Of particular interest is the time-dependent decrease in the ability of the bivalent ligand to dissociate and be absorbed by the sink. This loss of dissociability was shown not to be due to an endocytic process, and it is not observed when the monovalent competitor is used to prevent rebinding (Figure 3). The change apparently reflects the formation of a different state of aggregation on cells that does not occur on the membrane vesicles or in solution.

In order to interpret the observed $(DCT)_2$-Cys dissociation kinetics under these conditions, it is useful to consider factors that may limit the efficiency of the soluble anti-DNP IgE in facilitating unidirectional dissociation of the bivalent ligand. Because of the locally high concentration of FITC-IgE sites confined to the cell surface, ligand rebinding mechanisms are expected to slow the departure of the ligand from the membrane surface. For example, if one end of a bivalently bound $(DCT)_2$-Cys molecule dissociates, this transiently free end may (1) rebind to the same IgE site from which it dissociated or to a different receptor-bound IgE, resulting in no net change in bivalently bound, cell-associated $(DCT)_2$-Cys, (2) bind to an unlabeled IgE molecule in the solution population to form a $(DCT)_2$-Cys-linked dimer consisting of one FITC-labeled cell surface IgE and one unlabeled IgE molecule, or (3) be freed from attachment to the cell surface when the other end of the ligand dissociates before cases 1 or 2 occur. We showed

previously that significant rebinding occurs for monovalent ligand dissociation in the presence of a sink (Goldstein et al., 1989). Rebinding effects will be enhanced in the case of a bivalent ligand since two steps are required for dissociation from the membrane surface.

It appears that soluble IgE does not readily facilitate $(DCT)_2$-Cys dissociation from the cell surface by binding to untethered ends (case 2). When excess ^{125}I-IgE is added to FITC-IgE-sensitized cells in the presence of prebound $(DCT)_2$-Cys, no significant cell-associated radioactivity is detected (less than one ^{125}I-IgE per 10 FITC-IgE; Erickson, 1988). Also, under a broad range of concentrations, soluble anti-DNP IgE causes no enhancement of $(DCT)_2$-Cys-stimulated degranulation, indicating that this antibody fails to bridge cell-bound IgE–receptor complexes via $(DCT)_2$-Cys (Erickson, 1988).

We can further evaluate this situation with the theory formulated in the preceding paper (Posner et al., 1991). We showed that in order for unlabeled IgE to prevent the rebinding of $(DCT)_2$-Cys that is singly-bound to an FITC-labeled IgE molecule to a second FITC-IgE molecule, k_{+2} [unlabeled IgE] $\gg k_{+2}$ [FITC-IgE]. For FITC-IgE in solution the k_{+2} have units of nM^{-1} s^{-1}, and the unlabeled IgE concentration conditions for the inequality can be easily achieved. The same condition holds when the FITC-IgE is cell-bound except that now the right-hand side of the inequality is restricted to two dimensions; i.e., k_{+2} has units of cm^2 s^{-1} $site^{-1}$, and [FITC-IgE] has units of sites cm^{-2}. Since all of the FITC-IgE is restricted to a limited area on the cell surface, the numerical value for its two-dimensional concentration is greatly inceased compared to its bulk (three-dimensional) concentration. Equilibrium binding data with $(DCT)_2$-Cys and FITC-IgE bound to cells can be fit by two affinity constants, K_1 and K_2, where K_1 is the same as for DCT binding and K_2 (in three-dimensional units) is about 10 times larger (Erickson, 1988). This is opposite of the situation in solution where K_2 is $\sim$100 times smaller than K_1. Since the values for k_{-2} appear to be similar for $(DCT)_2$-Cys dissociating from both cell-bound FITC-IgE and solution IgE, it would appear that k_{+2cell} when converted to three-dimensional concentration units is $\sim$1000 times larger than k_{+2soln}.[2] Therefore, to satisfy the inequality and make re-cross-linking of $(DCT)_2$-Cys to 1 nM cell-bound FITC-IgE 100 times less favorable than its binding to soluble IgE, >100 μM (20 mg/mL) soluble IgE would be required. This calculated concentration, which is already impractically high, is probably an underestimate because it does not consider orientational effects. Thus, at the practical concentrations of unlabeled IgE we have used (1–2 μM), rebinding of the monovalently bound $(DCT)_2$-Cys is not prevented, and this method of inducing the dissociation of the bivalent ligand is much less efficient than the monovalent competitor method with the concentrations of DCT we have used.

Although cyclic dimers can explain the bimodal dissociation of bivalent ligand and cell-bound IgE, this is not sufficient to explain the time-dependent loss in the dissociability revealed in the sink experiments. In this regard, the striking difference between cells and membrane vesicles (Figure 2) provides useful insight. Previous studies have shown that these vesicles lack an intact cytoskeleton and that membrane proteins diffuse on them about 10 times faster than on cells (Tank et al., 1982). Also, oligomeric IgE–receptor complexes can coalesce on cells but not on vesicles, suggesting that cross-linked receptors interact with some other cell surface component(s) that are not retained in the vesicles (Menon et al., 1984). Recent phosphorescence anisotropy measurements further indicate that dimeric IgE–receptor complexes can become rotationally immobile on cells but not on vesicles (Myers, 1990). On the basis of these observations, a reasonable explanation for the time-dependent loss of $(DCT)_2$-Cys dissociability on cells is a corresponding increasing probability of reforming the $(DCT)_2$-Cys cross-links. This would go beyond the formation of cyclic dimers and be mediated by interactions between cross-linked IgE–receptor complexes and other cellular components. For example, either time-dependent clustering of dimeric IgE–receptor complexes or restriction in the relative mobility of IgE–receptor complexes within a dimer would increasingly hold them in proximity, facilitating the rebinding mechanisms. The latter possibility is illustrated in Figure 4 in a scheme that shows the cross-linking states identified by our results. Further experiments will be necessary to test the kinetic relationship between the formation of rotationally immobile complexes and the decrease in $(DCT)_2$-Cys dissociability.

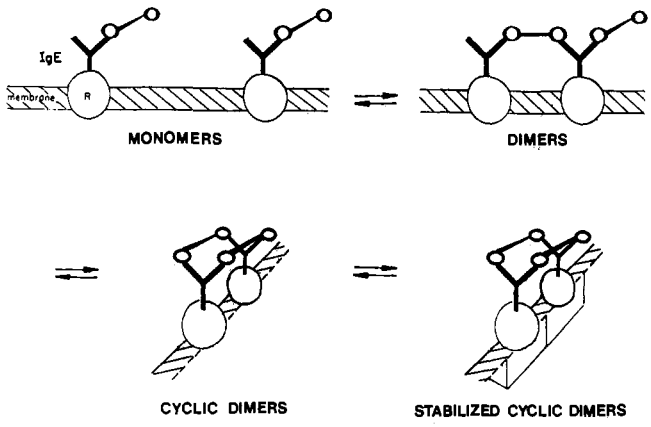

FIGURE 4: Possible sequence of cross-linking events suggested by the dissociation results on living cells. $(DCT)_2$-Cys is represented by O–O, and the pair of rectangular boxes in the last step represents a receptor-associated cellular component. The first two steps in this scheme are relatively rapid and occur in less than 10 min at 25 °C as indicated in Figure 3. The last step is slow ($t_{1/2}$ > 10 min at 25 °C, Figure 2) and gives rise to the observed time-dependent increase in ligand rebinding.

Functional Implications of Dissociation Experiments. The kinetic results presented here provide further support to the previous conclusion based on equilibrium studies (Erickson et al., 1986) that intermolecular cross-linking occurs efficiently between $(DCT)_2$-Cys and anti-DNP IgE bound to its receptor on the cell surface. The observation that this bivalent ligand triggers only small infrequent degranulation responses in RBL cells (Kane et al., 1986; Erickson, 1988) has been rather puzzling given the efficiency with which cross-linking occurs. However, the accumulated data may sugguest that the cross-linked complexes formed are largely confined to the dimer size, perhaps due to the efficient formation of cyclic complexes. Dimeric IgE–receptor complexes have been shown to be relatively ineffective in triggering cellular degranulation in RBL cells (Fewtrell & Metzger, 1980).

The time-dependent increase in $(DCT)_2$-Cys rebinding that is reflected in the loss of $(DCT)_2$-Cys dissociability in the presence of the IgE sink at time ≥10 min at 25 °C may indicate the formation of a state involving other cellular components that is important in the initiation of receptor-mediated signal transduction. Consistent with this possibility is the observation that $(DCT)_2$-Cys triggers transient increases in cytosolic Ca^{2+} (Ryan, 1989). Also, we have recently ob-

[2] For these calculations, $K_2 = k_{+2}/k_{-2}$; $k_{+2,3D} = k_{+2,2D}$ [1/(surface area of cell)][1/(cells/L)](Avogadro's no.).

served that a combination of $(DCT)_2$-Cys and a dimer-forming monoclonal anti-IgE antibody results in a vigorous signal transduction response (manifested by Ca^{2+} fluxes and degranulation) that does not occur with either reagent alone (R. Posner and B. Lee, unpublished results). The formation of these readily rebinding complexes with $(DCT)_2$-Cys could also reflect the formation of a desensitized state that follows the activated state, as suggested by Seagrave et al. (1987), as an explanation for the time-dependent changes in dissociability that they have observed for a multivalent ligand bound to IgE on RBL cells. Further experiments will be necessary to distinguish these alternatives in the present case. An interesting hypothesis to be tested is that efficient reformation of cross-links between the same pair of IgE receptors is much less productive in mediating signal transduction than the continual formation of cross-links between new pairs of IgE receptors.

In summary, these studies demonstrate that the dissociation of bivalent ligands bound to bivalent IgE on cell surface receptors can be a complex process that is determined by a number of different parameters. The results obtained indicate that rebinding of singly bound bivalent $(DCT)_2$-Cys to reform the cross-linked state is a major factor in determining the efficiency with which that ligand either remains bound or dissociates from the cell surface. It is likely that the rebinding process plays an important role in the kinetic behavior of a wide variety of different bivalent or multivalent ligands that bind to different cell surface receptors. The time-dependent increase in rebinding that has been observed with $(DCT)_2$-Cys reveals the formation of a different state, probably involving interactions with other cellular components, that is likely to have important functional consequences for the mechanism of receptor-mediated signal transduction.

Registry No. $(DCT)_2$-Cys, 104077-25-0.

REFERENCES

Baird, B., & Holowka, D. (1985) *Biochemistry 16*, 618–627.

Barsumian E. L., Isersky C., Petrino M. G., & Siraganian R. P. (1981) *Eur. J. Immunol. 11*, 317–323.

Efron, B., & Tibshirani, R. (1986) *Stat. Sci. 1*, 54–77.

Erickson, J. (1988) Ph.D. Thesis, Cornell University, Ithaca, NY.

Erickson, J., Kane, P., Goldstein, B., Holowka, D., & Baird, B. (1986) *Mol. Immunol. 72*, 769–781.

Erickson, J., Goldstein, B., Holowka, D., & Baird, B. (1987) *Biophys. J. 52*, 657–662.

Fewtrell, C., & Metzger, H. (1980) *J. Immunol. 125*, 70–71.

Fewtrell, C., & Metzger, H. (1981) in *Biochemistry of the Acute Allergic Reactions* (Becker, E. L., Simon, A. S., & Austen, K. F., Eds.) Alan R. Liss, Inc., New York.

Goldstein, B., Posner, R., Torney, D. C., Erickson, J., Holowka, D., & Baird, B. (1989) *Biophys. J. 56*, 955–966.

Holowka, D., & Baird, B. (1983) *Biochemistry 22*, 3466–3474.

Kane P., Erickson, J., Fewtrell, C., Baird, B., & Holowka, D. (1986) *Mol. Immunol. 23*, 783–790.

Liu, F. T., Bohn, J. W., Ferry, E. L., Yamamoto, H., Molinaro, C. A., Sherman, L. A., Klinman, N. R., & Katz, D. H. (1980) *J. Immunol. 124*, 2728–2736.

Menon, A. K., Holowka, D., & Baird, B. (1984) *J. Cell Biol. 98*, 577–583.

Menon, A. K., Holowka, D., Webb, W. W., & Baird, B. (1986a) *J. Cell Biol. 102*, 534–540.

Menon, A. K., Holowka, D., Webb, W. W., Baird, B. (1986b) *J. Cell Biol. 102*, 541–550.

Myers, J. N. (1990) Ph.D. Thesis, Cornell University, Ithaca, NY.

Posner, R. G., Erickson, J. W., Holowka, D., Baird, B., & Goldstein, B. (1991) *Biochemistry* (preceding paper in this issue).

Ryan, T. A. (1989) Ph.D. Thesis, Cornell University, Ithaca, NY.

Seagrave, J. C., Deanin, G. G., Martin, J. C., Davis, B. H., & Oliver, J. M. (1987) *Cytometry 8*, 287–295.

Tank, D. W., Wu, E. S., & Webb, W. W. (1982) *J. Cell Biol. 92*, 207–212.

Taurog, J. D., Fewtrell, C., & Becker, E. (1979) *J. Immunol. 122*, 2150–2153.

Zidovetzki, R., Bartholdi, D., Arndt-Jovin, & Jovin, T. (1986) *Biochemistry 25*, 4397–4401.