Immobilization of the Type I Receptor for IgE Initiates Signal Transduction in Mast Cells†

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ABSTRACT: Clustering of the type I receptor for IgE ($Fc_\epsilon RI$) on mast cells initiates a cascade of biochemical processes that results in the secretion of inflammatory mediators. We have studied this clustering process in order to obtain information about receptor density and mobility required for initiating that cascade. Specifically, we examined the role of new cluster formation in sustaining the secretory response and the minimal cluster density required for initiating secretion. The experimental protocol adopted for these studies employed photoactivatable antigens and antigen-carrying solid surfaces which enabled us to control the density and mobility of the $Fc_\epsilon RI$ within the cluster. Our results show that recruitment of new $Fc_\epsilon RI$ into clusters, either by antigen exchange among $Fc_\epsilon RI$-bound IgE molecules or by IgE-bound $Fc_\epsilon RI$ exchange with vacant receptors, is not required for sustaining the cellular secretory response. Furthermore, we find that the cell's secretory response is very sensitive to the density of immobilized $Fc_\epsilon RI$s, increasing steeply above a density of ca. 1000 immobilized molecules/μm^2. Taken together, these findings suggest that immobilization of a fraction of the randomly distributed $Fc_\epsilon RI$s that are in sufficient proximity on the surface of mucosal-type mast cells of the RBL-2H3 line initiates a degranulation signal, and that this is maintained as long as these receptors are kept within this distance. The above conclusions and the experimental protocol presented in this study are expected to have wider applications for the study and understanding of signaling by immuno (as well as other) receptors.

Immunological stimulation of mast cells is initiated by the clustering of their type I receptors for the Fc domains of IgE ($Fc_\epsilon RI$).[1] Physiologically, this process is mediated by polyvalent antigen binding to $Fc_\epsilon RI$-bound, antigen-specific IgE molecules. However, $Fc_\epsilon RI$-specific antibodies were extensively employed as clustering agents for studying the triggering mechanism (Baniyash et al., 1987; Basciano et al., 1986; Ishizaka & Ishizaka, 1969). Mast cells serve as a model system for studying the activation of different cells of hematopoietic origin by clustering of their surface antigen receptors because of their immediate response and the feasibility of its quantitation. A most intensively studied and best characterized permanent mast cell line is the rat mucosal-type mast cell RBL-2H3 that carries ca. 10^3 $Fc_\epsilon RI$ molecules/μm^2 on its surface (ca. 3×10^5 molecules/cell with a diameter of 10 μm (Metzger 1978)). These receptors are randomly distributed (Kubitscheck et al., 1993) and bind IgE with an affinity of 10^8 M^{-1} (Ortega et al., 1988). Most of them diffuse freely on this cell's surface, but ca. 25−30% were found to be immobile (Kubitscheck et al., 1993; Ryan et al., 1988; Schlessinger et al., 1976). Several factors are considered as determining the signaling capacity of $Fc_\epsilon RI$ clusters, namely, their size, mutual receptor orientation (Ortega et al., 1988), and proximity within the cluster (Kane et al., 1988), as well as the length of time which the receptors remain aggregated (DeLisi, 1981; Ortega et al., 1991; Schweitzer-Stenner et al., 1994).

Since the secretory response of RBL-2H3 cells occurs on the time scale of tens of minutes ($\tau_{1/2} \approx 15$ min at 37 °C), it has been argued that maintenance of $Fc_\epsilon RI$ signaling is a dynamic process that requires continuous de novo formation of $Fc_\epsilon RI$ clusters (Oliver et al., 1988). This notion was mainly supported by the observation that the stimulus resulting from clustering IgE-carrying receptors by polyvalent antigens is abrogated by the subsequent addition of a monovalent hapten excess. Signal termination, monitored by both rapid dephosphorylation of tyrosyl-phosphorylated target proteins (Adamczewski et al., 1992) and a halt in the secretory response, was found to be much faster than antigen dissociation from the cell surface (Seagrave et al., 1987). The suggested rationale for this observation was that the excess monovalent hapten occupies free $Fc_\epsilon RI$-bound IgE molecules, thus preventing their recruitment into clusters by exchange of the polyvalent antigen (Oliver et al., 1988). This was however challenged by the recent finding that when $Fc_\epsilon RI$ clustering is caused by chemically cross-linked trimeric IgE, signaling is not inhibited by subsequent addition of excess monomeric IgE. $Fc_\epsilon RI$s clustered in this way were shown to maintain their ability to signal for a prolonged time (Kent et al., 1994). Since the monomeric IgE was expected to inhibit the exchange of trimeric IgE-bound and vacant $Fc_\epsilon RI$s, it was concluded that the capacity of oligomerized $Fc_\epsilon RI$s to initiate a transmembranal signal is maintained and that the formation of new $Fc_\epsilon RI$ clusters is not required.

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[1] Abbreviations: ANPAH, 6-[(4-azido-2-nitrophenyl)amino]hexanoyl; DNP, 2,4-dinitrophenyl; DNPL, N^ϵ-(2,4-dinitrophenyl)-L-lysine; $Fc_\epsilon RI$, type I receptor for IgE; FRAP, fluorescence recovery after photobleaching; SPA, soluble photoactivatable antigen: $(ANPAH)_{27}BSA$; SANPAH, 6-[(4-azido-2-nitrophenyl)amino]hexanoic acid *N*-hydroxysuccinimide ester.

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The minimal number of $Fc_\epsilon RI$s per cluster and the maximal separation distance among them which still allows for signaling were studied in the past by several approaches. Monoclonal antibodies raised against the $Fc_\epsilon RI$ provided evidence that dimers are sufficient for stimulating cells of the RBL-2H3 line and that the secretory response amplitude is probably modulated also by the configuration within the $Fc_\epsilon RI$ dimer (Ortega et al., 1988). The effect of $Fc_\epsilon RI$ separation distance within a cluster was investigated using divalent rigid macromolecules as spacers between the haptens (Kane et al., 1988), suggesting that the maximal separation distance between receptors that still allows signaling may be larger than 20 nm. This separation distance is in the order of the average inter-receptor distance on resting cells, which may be estimated as ca. 40 nm (for a 10 μm diameter cell carrying 3×10^5 $Fc_\epsilon RI$). The probability that a fraction of the randomly distributed receptors are at such separation distance at any point in time may therefore be quite high. However, since the receptors are freely-diffusing, they do not remain within this required distance for a sufficient length of time to initiate a signal (DeLisi, 1981). This led us to investigate whether $Fc_\epsilon RI$ immobilization on the surface of RBL-2H3 cells, even at its resting density, may provide a signal sufficient for triggering secretion.

We have devised protocols aimed at preventing both antigen exchange among $Fc_\epsilon RI$-bound IgE molecules and IgE exchange between occupied and vacant $Fc_\epsilon RI$s. Briefly, these protocols make use of soluble and solid surface-bound antigens carrying the photoactivatable hapten: 6-[(4-azido-2-nitrophenyl)amino]hexanoyl (ANPAH), which specifically binds the 2,4-dinitrophenyl (DNP)-specific monoclonal IgE-A2. Upon ANPAH photoactivation, the produced nitrene reacts covalently with the IgE molecule. We have used these protocols to try and resolve the apparent discrepancy between the results obtained using antigen vs chemically cross-linked IgE in terms of their signaling efficacy in the presence of a monovalent hapten and monomeric IgE, respectively. One of these protocols was further used for immobilizing $Fc_\epsilon RI$s at varying densities on the surface of RBL-2H3 cells, in order to assess the minimal immobilized receptor density required for transmembranal signaling.

MATERIALS AND METHODS

Cell Culture. RBL-2H3 cells (Barsumian et al., 1981), obtained from Dr. R. Siraganian, NIH, Bethesda, MD, were grown in DMEM (Bio-Lab, Israel) supplemented with 10% FCS (Gibco-BRL, Gaithersburg, MD, USA), 2 mM glutamine, and antibiotics (Bio-Lab, Israel) in a humidified atmosphere with 7% CO_2 at 37 °C. Adherent RBL-2H3 cells were harvested by incubation with 10 mM EDTA (in DMEM) for 15 min at room temperature. For routine secretion assays, cells (1×10^5/well) were either grown overnight to confluency in 96-well plates (Nunc, Denmark) in the presence (or absence) of IgE (10 nM) or allowed to settle and adhere for 1 h in the same plates [$(2-3) \times 10^5$ cells/well] at 22 °C in the presence (or absence) of IgE (10 nM).

Antibodies and Soluble Antigens. The DNP-specific IgE clone A2 (Rudolph et al., 1981) and the IgE-specific rat monoclonal antibody (clone 95.3 (Baniyash & Eshhar, 1984)) were a kind gift of Dr. Z. Eshhar (Weizmann Institute of Science, Rehovot, Israel). The dansyl-specific IgE (clone 27−74 (Dangl et al., 1988)) was a kind gift of Dr. V. T. Oi (Becton-Dickinson Immunocytometry Systems). The A2 mAb was purified by affinity chromatography on DNP-Sepharose and centrifuged prior to its use at 100000*g* for 1 h at 4 °C to sediment oligomeric forms. Only the upper two-thirds of the supernatant were collected, kept at 4 °C, and employed within a week following centrifugation. The 95.3 mAb was used as accepted without further purification. BSA and IgE iodination by ^{125}I was performed using the chloramine-T method (Hunter & Greenwood, 1962). Specific activities in the range of 5−10 Ci/g of protein were obtained. The soluble photoactivatable antigen, $(ANPAH)_{27}BSA$, (SPA), was prepared by adding a 20 mM dioxane solution of 6-[(4-azido-2-nitrophenyl)amino]hexanoic acid *N*-hydroxysuccinimide ester (SANPAH, Sigma, St. Louis, MO, USA) to a solution containing 10 mg/mL BSA (fraction V; Boehringer Mannheim GmbH) and 100 mM HEPES, pH 7.5, to a final SANPAH concentration of 7 mM. The mixture was stirred overnight at 4 °C in the dark. The resulting solution was dialyzed overnight against 100 mM HEPES, pH 7.5, to remove hydrolyzed and unreacted SANPAH, and then again against PBS. Next, the dialyzed material was centrifuged at 20000*g* for 10 min at 4 °C to remove aggregates and insoluble material. The supernatant was removed and kept for further use at 4 °C. Complete removal of unreacted and/or hydrolyzed SANPAH was confirmed by carrying out a control reaction under the above conditions in the absence of BSA and using the above protocol for removing hydrolyzed SANPAH.

Conjugation of 5-azido-2-nitrobenzoyl groups to BSA was performed by reacting the *N*-hydroxysuccinimide ester of 5-azido-2-nitrobenzoic acid with BSA essentially as described above for the reaction between SANPAH and BSA. The antigens $(dansyl)_n BSA$ and $DNP_{11}BSA$ were prepared by reacting dansyl chloride and 2,4-dinitrofluorobenzene, respectively, with BSA as described previously (Eisen et al., 1959; Hardy, 1986).

Unless otherwise stated, all other reagents were purchased from Sigma. All organic solvents used were HPLC-grade. All experiments with soluble photoactivatable antigens were performed under darkroom illumination conditions (unless stated otherwise).

Determination of the Number of ANPAH Haptens Bound per BSA Molecule of SPA. The extinction coefficient of ANPAH at 400 nm was calculated from the absorption of its reaction product with glycine (prepared using essentially the same protocol used for the reaction of SANPAH with BSA but with a 10-fold excess of glycine over SANPAH) to be 2.0×10^3 M^{-1} cm^{-1}. Protein concentration in $(ANPAH)_{27}BSA$ (SPA) was determined using the Bradford assay with BSA as a standard. Interference due to the absorption of ANPAH at the respective wavelength was prevented by proper dilution of the sample.

Determination of A2 Affinity for ANPAH. The binding of ANPAH-glycine to IgE-A2 was monitored continuously by measuring the quenching of the intrinsic antibody fluorescence, essentially as described previously (Schweitzer-Stenner et al., 1986). The excitation wavelength was 280.0 ± 0.1 nm, and the emission was monitored at its maximum, i.e., 330.0 ± 0.1 nm. The temperature of the sample was kept constant at 37 °C. The antibody and hapten concentrations and the fluorescence intensity were corrected for dilution as described previously. The measured fluorescence was also corrected for the absorption of ANPAH by

performing the above titration with an immunoglobulin with nonrelated specificity (G63 which is an IgG specific for the MAFA protein).

Preparation of Antigen-Carrying Surfaces. Glass-supported antigen-carrying surfaces were prepared as follows. Soft glass coverslips (12 mm i.d., Assistent, Germany) were washed by sonicating twice for 30 min in dichloromethane and then reacted with 1% (w/w) (3-aminopropyl)triethoxysilane (APTES, Aldrich) in the same solvent with sonication for 30 min at ca. 40 °C in a capped glass cylinder. Next, the coverslips were washed three times by sonication in dichloromethane and once with methanol and air-dried. The resultant (3-aminopropyl)silyl (APS)-carrying coverslips were each placed in 24-well plastic plates (Nunc, Denmark) and further derivatized by glutaraldehyde (10 mM in PBS) for 1 h, followed by two successive washes with PBS and reaction with BSA (1 mg/mL in PBS) overnight at 4 °C. The amount of BSA bound to the glass surfaces was determined by performing the above reaction with ^{125}I-labeled BSA. These BSA-carrying coverslips were washed several times with double-distilled water and dehydrated by three successive washes with absolute ethanol. ANPAH-carrying coverslips were then produced by reaction with 130 μM SANPAH in ethanol for 2 h in the dark. The coverslips were next washed twice with ethanol, once with double-distilled water, and twice with PBS and kept in the latter buffer overnight in the dark.

Alternatively, ANPAH-carrying surfaces were produced on polystyrene supports. Briefly, the latter were prepared by adsorption of BSA (1 mg/mL in PBS) to MaxiSorp 96-well plates (Nunc, Denmark) overnight at 4 °C, followed by several washes with double-distilled water, dehydration by three successive washes with absolute ethanol, and derivatization by SANPAH using the protocol employed for the glass-supported surfaces. The results of experiments performed using these supports were qualitatively identical with those obtained for glass-supported surfaces and therefore are not shown.

All operations involving derivatization by SANPAH and experiments with the ANPAH-derivatized surfaces were performed under darkroom illumination conditions.

Preparation of IgE Complexes with Antigens or Antigen-Carrying Surfaces. IgE (A2) and SPA were diluted in PBS to a final concentration of 1 μg/mL (each) and allowed to form noncovalent complexes at 22 °C for 30 min in the dark. For some experiments covalent complexes were obtained by photoactivation. The samples were further diluted in Tyrode's buffer for dose−response studies. For studies involving IgE binding to antigen-carrying surfaces, the latter coverslips were placed in 24-well plates and binding was performed at 22 °C for 1 h using 0.1−1000 nM IgE in PBS, followed by extensive washings with PBS. For studies involving noncovalently dimerized IgE by the IgE-specific mAb (clone 95.3), the former was first diluted in Tyrode's buffer to the required final concentrations (0.1−100 nM) and then reacted with 10 nM of anti-IgE mAb at 22 °C for 30 min. The IgE dimers formed were next incubated with antigen-carrying surfaces for 1 h at 22 °C, followed by extensive washings with PBS. The covalent binding of A2 to antigen-carrying surfaces was performed by further incubating the monomeric IgE-carrying surfaces with 1 mM BS3 (Pierce) in PBS for 1 h at 22 °C, followed by extensive washes with PBS. In order to determine its surface density, IgE binding according to the above protocols was performed with ^{125}I-labeled IgE.

Secretion Assays. Following incubation with IgE (10 nM overnight at 37 °C) confluent adherent RBL-2H3 cells were washed twice with Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA) and stimulated by the indicated antigen concentrations in this buffer (100 μL/well) at 37 °C for 45 min. Following antigen stimulation, supernatant samples were withdrawn and their content of β-hexosaminidase activity was assayed essentially as described (Ortega Soto & Pecht, 1988). For experiments involving cell stimulation by glass-carried antigens, the cells were preincubated (or not) with 10 nM IgE for 2 h at 22 °C, washed twice with Tyrode's buffer, and allowed to adhere (1×10^6 cells/cm^2) to these surfaces for 1 h at 10 °C in this buffer. Next, the cell-carrying glass surfaces were washed twice by Tyrode's buffer to remove nonadherent cells and further incubated in this buffer at 37 °C for 45 min. Following removal of supernatant samples for β-hexosaminidase assay, the cell-carrying coverslips were transferred to Tryode's buffer containing 2% Triton X-100 to extract remaining β-hexosaminidase. Samples of this extract were taken, and their enzymatic activity was assayed. The absorption was background corrected, and the net percent secretory response was expressed as follows: 100 × (secreted)/(secreted + extracted).

Photoactivation Equipment and Protocols. Photoactivation was performed using a set of 6 lamps (8-W 350 Blacklight, Sylvania, USA) positioned 6 cm apart and 9 cm from the sample for uniform illumination. Photoactivation of SPA and its complexes with IgE was carried out for 30 min (unless stated otherwise) with occasional mixing, whereas photoactivation on cell surfaces or of ANPAH-derivatized surfaces was carried out in 96- or 24-well plates. In the latter cases, the samples were covered by Tyrode's buffer or PBS, respectively, and photoactivated for 15 min (unless stated otherwise). Control samples were placed for similar time periods in the illumination chamber yet covered by aluminum foil. Photoactivation on cell surfaces was performed in a buffer deficient in calcium in order to reduce premature secretion.

In order to exclude possible effect of the photoactivating light on the cell's response to antigen stimulation, the latter was measured in cells which were treated with varying doses of the same light employed in the above experiments. Negligible effect of this radiation on the cell's ability to respond to antigenic stimulus in the period chosen for photoactivation (15 min) was observed. However, longer exposure (>20 min) increased their spontaneous secretion.

RESULTS

Stimulation by Soluble Antigens: (A) The Photoactivatable Hapten ANPAH Cross-Reacts with the DNP-Specific Monoclonal IgE-A2. The cross-reactivity of the 2,4-dinitrophenyl (DNP)-specific monoclonal IgE (A2) with the photoactivatable DNP analog 6-[(4-azido-2-nitrophenyl)amino]hexanoyl (ANPAH) was examined by fluorescence titration. The affinity of A2 toward ANPAH-glycine was determined as 6.5×10^5 M^{-1} at 37 °C. Photoactivation of ANPAH-glycine results with a substantial reduction in its binding to A2 IgE (data not shown).

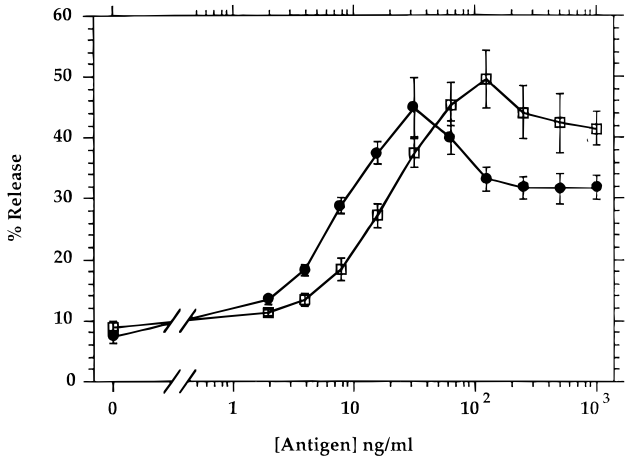

FIGURE 1: Comparison of RBL-2H3 dose−response to stimulation by different antigens. Adherent cells were grown overnight in 96-well plates in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, antibiotics, and 10 nM DNP-specific IgE (A2) at 37 °C. Next, the adherent cells were washed twice with Tyrode's buffer and incubated with the indicated concentrations of DNP_{11}-BSA (□) or $(ANPAH)_{27}$BSA (SPA) (●) for 45 min at 37 °C in this buffer. Supernatant samples were withdrawn, and their β-hexosaminidase activity was assayed. Results are represented as net percent of total (2% Triton X-100 extractable) enzymatic activity. The mean of triplicates is shown, with error bars indicating the standard deviation.

In order to determine whether ANPAH, bound to a macromolecular carrier (termed soluble photoactivatable antigen or SPA), can perform as an antigen, RBL-2H3 cells were saturated with the above IgE, and their secretory dose response to SPA was assayed and compared with that toward DNP_{11}BSA (Figure 1). The results clearly show that SPA is effective in eliciting a secretory response from these cells. The specificity of this process has also been established by using a different photoactivatable analog—5-azido-2-nitrobenzoyl conjugated to BSA—which failed to induce secretion in IgE-carrying RBL-2H3 cells. This was taken as evidence for the sensitivity of the IgE-A2 binding site for the substituent's position on the hapten's aromatic ring.

(B) Photoactivation of SPA Reduces Its Secretion Inducing Capacity of IgE-Carrying RBL-2H3 Cells. The secretory response of IgE-saturated RBL-2H3 cells to increasing doses of photoactivated SPA was studied as a function of its photoactivation time. Thus SPA (1 μg/mL in PBS) was photoactivated (or not) for 3−15 min, diluted to the range of 12.5−100 ng/mL in Tyrode's buffer, and incubated with IgE (A2)-saturated cells for 45 min at 37 °C. Next, supernatant samples were withdrawn, and their content of β-hexosaminidase activity was determined. The results of this experiment are displayed in Figure 2. These results indicate that, for SPA concentrations up to 25 ng/mL, 15 min photoactivation reduced its ability to induce degranulation to background level. Higher SPA concentrations (up to 100 ng/mL) require longer (up to 30 min) photoactivation to achieve this effect (data not shown).

(C) Photoactivation of SPA−IgE Complexes Results in the Formation of Covalent Bonds between the Two That Are Specific for the IgE Binding Site. In order to determine whether SPA photoactivation in the presence of a DNP-specific IgE (A2) would result in the formation of covalent bonds between the two, SPA (1 μg/mL in Tyrode's buffer) was either photoactivated (or not) for 30 min followed by IgE (A2) addition (to a final concentration of 1 μg/mL).

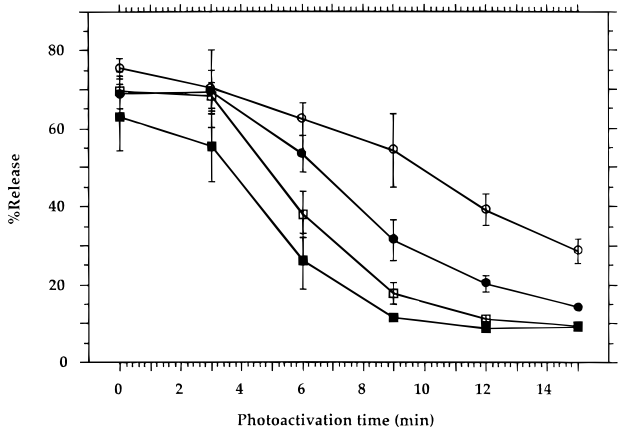

FIGURE 2: Dependence of the secretory response on antigen photoactivation time. Adherent cells were grown overnight in 96-well plates in the presence of IgE. After washing, they were incubated with 12.5 (■), 25 (□), 50 (●), and 100 ng/mL (○) SPA, which has previously been photoactivated for the indicated time periods. All other details are as described in the legend to Figure 1.

Immune complexes were allowed to form for 30 min at 22 °C, followed by photoactivation of IgE complexes with (non-photoactivated) SPA for additional 30 min. Next, both the mixture of IgE and photoactivated SPA and the photoactivated SPA−IgE complexes were each diluted to the range of 1−1000 ng/mL in Tyrode's buffer and incubated with adherent RBL-2H3 cells at 37 °C for 45 min, and the content of β-hexosaminidase activity in their supernatants was determined. The results of this experiment are displayed in Figure 3A. Photoactivated SPA−IgE complexes were found to be more effective in inducing secretion of RBL-2H3 cells than IgE complexed with photoactivated SPA, indicating that photoactivation of the free SPA reduces its ability to bind to the DNP-specific IgE. In order to eliminate the possibility that photoactivation of SPA in the presence of a DNP-specific IgE creates nonspecific covalent bonds between them, a monoclonal IgE of a different specificity (anti-dansyl) was incubated with SPA followed by photolysis under the above-described conditions. Incubation of the product of this reaction with RBL-2H3 cells did not induce a secretory response. Moreover, further incubation of the cells with the appropriate antigen ($(dansyl)_n$BSA) resulted with a significant secretory response (data not shown). This indicates that the photochemically-induced reaction of SPA with the DNP-specific IgE yields specific covalent immune complexes.

The above specificity was further supported by the inhibition of photoactivated (see above) and non-photoactivated SPA−IgE complexes to induce secretion in the presence of 50 μM of the monovalent hapten N^ϵ-(2,4-dinitrophenyl)-L-lysine (DNPL) (Figure 3B). The results show that while 50 μM DNPL fully (>80%) inhibited the secretory response toward non-photoactivated SPA−IgE complexes, it caused only marginal inhibition of the response to photoactivated ones. It is however noteworthy that a 10-fold higher concentration of photoactivated SPA−IgE complexes was required to achieve the cellular response level observed upon stimulation with non-photoactivated ones. Taken together with the results of the previous section, these suggest the following: (1) the SPA−IgE immune complexes dissociate as the consequence of photoactivation, probably due to the photochemically-induced reaction of ANPAH with its macromolecular carrier or solvent. This leads to a

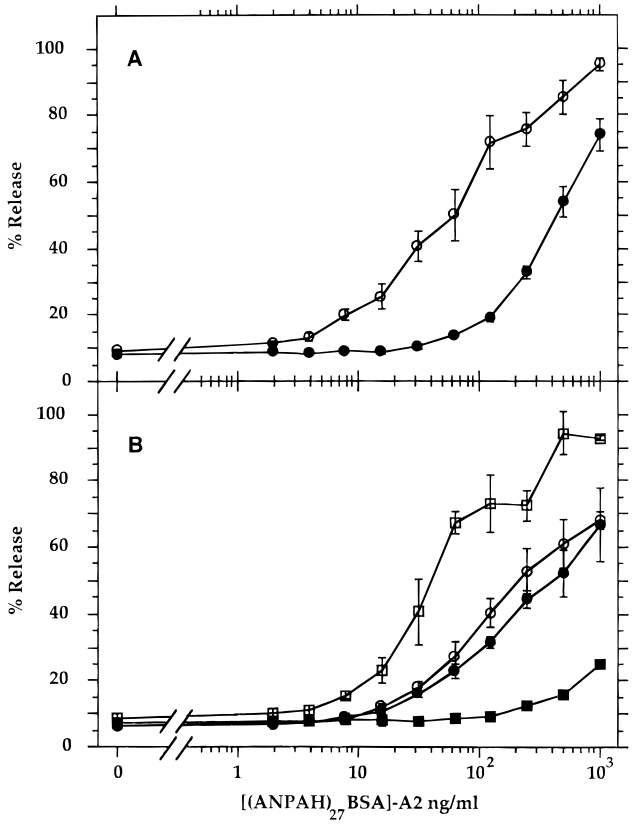

FIGURE 3: Effects of photoactivation and DNPL addition on the antigen−IgE complex-induced RBL-2H3 secretory dose response. Adherent cells were grown overnight in 96-well plates without IgE. After washing, they were incubated with increasing concentrations (based on the antigen) of SPA−IgE complexes. (A) Stimulation by SPA photoactivated before (●) or after (○) IgE (A2) addition. (B) Stimulation in the presence (filled) or absence (empty) of 50 μM DNPL by SPA−IgE complexes which have been photoactivated (circles) or not (squares). All other details are as described in the legend to Figure 1.

reduction in the average number of IgE molecules bound per complex, which accounts for its reduced efficacy in inducing degranulation. (2) A fraction (ca. 10%) of the SPA−IgE complexes undergo covalent binding upon photoactivation, which accounts for their resistance to the inhibitory effect of DNPL. However, the above findings may also be explained by assuming that the photochemically-induced reaction between SPA and the IgE's binding sites is quite efficient while the reduced efficacy of the thus formed covalent complexes in inducing degranulation is due to a requirement for antigen exchange for efficient signaling, which is abolished upon covalent binding of IgE to SPA.

(D) Photochemically-Induced Covalent Binding Reduces the Average Number of SPA-Bound IgE Molecules. In order to establish the reason for the lower dose response to photoactivated SPA−IgE complexes and examine whether photolysis reduces the number of haptenic groups, we added free SPA to these photoactivated complexes. The secretory dose response was measured and compared with that observed to photoactivated and non-photoactivated complexes (Figure 4). We found that this addition causes an increase in the dose response compared with that toward photoactivated SPA−IgE complexes, nearly reaching values observed for non-photoactivated ones. If most of the SPA-bound IgE molecules underwent covalent binding following photoactivation and the reduced efficacy in inducing degranulation results from a requirement for antigen exchange, addition of excess non-photoactivated SPA to photoactivated SPA−IgE complexes should not result with an increase in the secretory response induced by them. Thus we are led to conclude that photoactivation causes covalent binding of only a fraction of the SPA-bound IgE molecules. The above observation also suggests that although the average number of IgE molecules bound per SPA decreases upon photoactivation, the covalent SPA−IgE complexes produced are comparable in terms of their efficacy in inducing secretion with their noncovalent counterparts. This further suggests that there is no requirement for antigen exchange for a maintained stimulus in these cells.

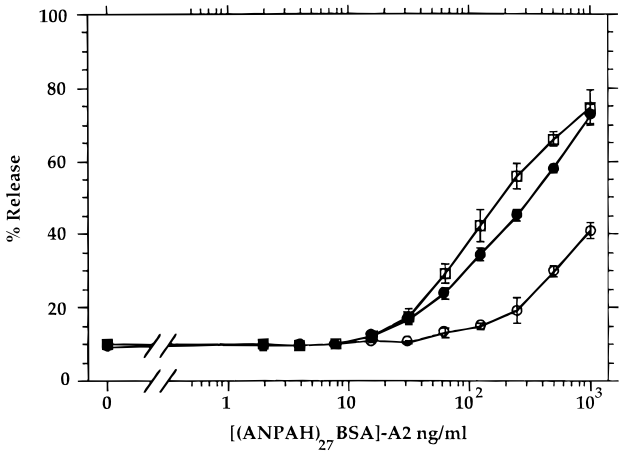

FIGURE 4: Effect of antigen addition to photoactivated antigen−IgE complexes on the elicited RBL-2H3 secretory dose response. Adherent cells were stimulated by the indicated concentrations (based on the antigen) of SPA−IgE complexes which have been photoactivated (○) or not (□) and which were supplemented with the same concentration of free SPA following photoactivation (●). All other details are as described in the legend to Figure 1.

(E) Recruitment into Clusters of New Fc_ϵRIs by Antigen Exchange Is Not Required for Maintenance of Secretion. Based on the above, we should expect a similar difference upon photoactivating SPA−IgE complexes when bound to the Fc_ϵRI on the cell surface. Since the covalent SPA−IgE complexes cannot exchange their antigen with free IgE molecules, they can be used to study the role of the proposed exchange process in inducing the secretory response.

SPA and IgE (A2) were first allowed to react in solution (30 min at room temperature) and then diluted and incubated with the cells under conditions that are nonpermissive to secretion (Tyrode's buffer without added Ca^{2+} at 37 °C) for 60 min under darkroom illumination. The complexes were then photoactivated (or not) on the cell surface for 15 min, either at the beginning or 20 min before the end of the incubation. At the end of incubation, calcium was added to the buffer (to a final concentration of 1.8 mM), and the secretory dose response of the cells was measured after additional 30 min at 37 °C in the presence or absence of 50 μM DNPL (Figure 5). The long incubation under nonpermissive conditions is known to result in desensitization (Warner & MacGlashan, 1988; Weetal et al., 1993) causing a reduction in the secretory response. Indeed, we also observed a reduction in the elicited maximal secretory response to the above stimulation protocol with non-photoactivated SPA−IgE complexes (ca. 51% vs 95%—compare non-photoactivated trace in Figure 5 with the corresponding one in Figure 3B). The background secretory response has

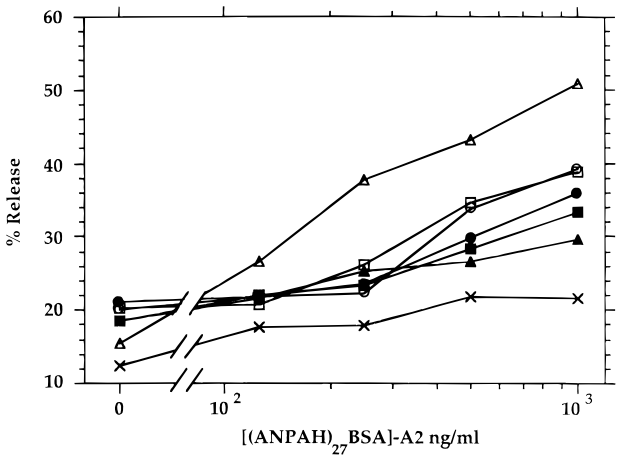

FIGURE 5: Effect of cell-surface antigen−IgE complex photoactivation on the elicited secretory dose response. SPA−IgE complexes were reacted with cells at 37 °C for 60 min in Tyrode's buffer to which no calcium was added. During this incubation period, the cell-carried complexes were either non-photoactivated (△) or photoactivated for 15 min at $t = 0$ (□) and $t = 40$ min (○). At the end of this incubation, calcium was added to the buffer (to a final concentration of 1.8 mM) and the secretory dose response was measured in the presence (filled) or absence (empty) of 50 μM DNPL. As a negative control, the secretory dose response to non-photoactivated antigen−IgE complexes was measured (×) at the end of the incubation period in the calcium-free buffer. Standard deviation of triplicate data points is ≤10%. All other details are as described in the legend to Figure 1.

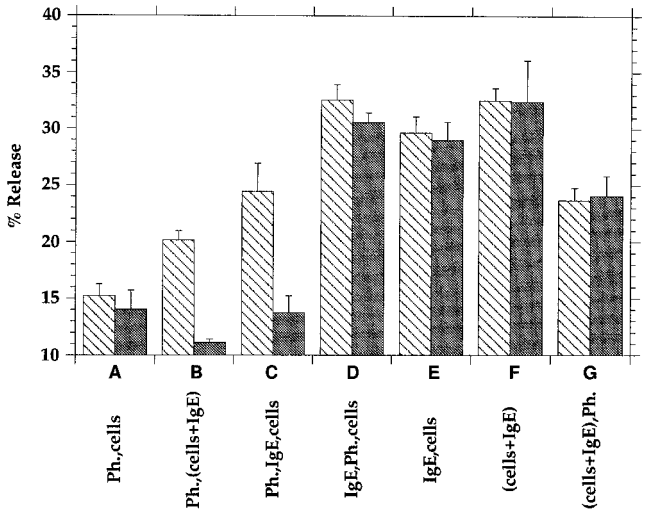

FIGURE 6: Secretory response of RBL-2H3 cells to stimulation by glass surfaces carrying photoactivatable haptens. ANPAH-carrying glass surfaces were prepared as described in the Materials and Methods. Except for controls, either the cells or these surfaces were incubated with 10 nM IgE for 1 h at room temperature followed by three washes with PBS or Tyrode's buffer to obtain IgE-carrying surfaces or cells, respectively. The cells (optionally IgE-carrying) were then incubated on these surfaces for 1 h at 10 °C. The surfaces were optionally exposed to illumination (360 nm) for 15 min at 10 °C either before or after cell adherence. Next, nonadherent cells were removed from the glass surface by washing with Tyrode's buffer, and the glass surface-carried cells were incubated in the latter buffer for 45 min at 37 °C in the presence (dark bars) or absence (hatched bars) of DNPL. Labels on the *x*-axis indicate the sequence of treatments (illumination) separated by a comma. Parentheses indicate preincubation, Ph. = photoactivation. All other details are as described in the legend to Figure 1 and in the text.

also increased from ca. 10% to 15−22%. The latter is probably the result of the combined effects of long incubation under conditions nonpermissive to secretion and the effect of the photoactivating light on the spontaneous secretory response. In accordance with our observations with soluble SPA−IgE complexes, DNPL causes a marked inhibition of the cell's dose response to non-photoactivated complexes under the present protocol (ca. 70% after subtraction of spontaneous degranulation), although the effect is not as dramatic as in the case of the experiments with soluble SPA−IgE complexes because of the above-discussed reduction in the maximal secretory response and the increase in the spontaneous one. As expected from the experiments performed with soluble SPA−IgE complexes, photoactivation of these complexes on the cell surface leads to a reduction in the maximal secretory response. This reduction is actually higher than the one observed for soluble SPA−IgE complexes (ca. 40% vs ca. 30% after subtraction of spontaneous degranulation). Interestingly, the observed secretory response was independent of the preincubation time with the above complexes before photoactivation. Secretion induced by photoactivated antigen−IgE complexes was significantly less inhibited by DNPL (18−25% at 1000 ng/mL SPA) as compared with the non-photoactivated control, clearly showing that photoactivation produces covalently-bound IgE-antigen complexes on the cell surface which induce secretion.

The above results suggest that a sustained secretory response does not require a time-dependent antigen exchange or rearrangement between Fc$_\epsilon$RI-bound IgE molecules on the cell's surface.

Stimulation by Antigens Carried on Solid Supports. (A) Antigen-Carrying Surfaces Induce a Secretory Response That Is Only Partly Inhibited by a Monovalent Hapten. The dose response of IgE-carrying RBL-2H3 cells to simulation by ANPAH-carrying glass supports was studied and found to increase with rising surface hapten density. The latter depends on the derivatizing agent (SANPAH) concentration and/or its reaction time with the surface. However, compared with the dose response to soluble non-photoactivated SPA, the secretory response to antigen-carrying surfaces did not show the local maximum observed at high antigen concentrations, but rather continued to increase up to the maximal hapten density obtainable on these surfaces (data not shown). The maximal secretory response observed on glass supports was found to be ca. 50% of the cell's total β-hexosaminidase activity content. This is much lower than the maximal values observed for identical batches of RBL-2H3 cells stimulated by soluble antigens (ca. 90% for DNP$_{11}$BSA or SPA). However, while excess monovalent hapten (DNPL 50 μM) completely inhibited the secretory response to soluble antigen at antigen concentrations up to 1 μg/mL, it failed to inhibit the response to surface-carried antigens (Figure 6F). This is most probably due to the fact that dissociation of cell-carried IgE molecules from the surface-carried antigen by the competing monovalent hapten does not occur on the time scale of the experiment (see below).

(B) Recruitment of New Fc$_\epsilon$RI into Clusters by IgE Exchange Is Not Required for Maintaining the Secretory Stimulus. The above observed secretory response to ANPAH-carrying glass surfaces was strongly reduced by photoactivating the latter prior to its reaction with IgE-carrying cells (Figure 6B vs -F). However, ANPAH-derivatized glass surfaces, which were first reacted with IgE and then photoactivated, induced a secretory response which was indistinguishable from that induced by non-photoactivated

samples (Figure 6D vs -E). This result contrasts with that observed after photoactivation of SPA−IgE complexes in solution, where the elicited secretory response has been strongly reduced (Figure 3B). These results suggest that the extent of photoactivation-induced IgE dissociation from the antigenic surface is lower than from SPA−IgE complexes. Measurements of IgE dissociation from these surfaces, by its radioactive labeling, show that >75% of the IgE molecules remain bound to the surface following photoactivation at all IgE surface densities studied (1000−10 000 μm^{-2}). Surface photoactivation prior to IgE binding partially prevents the latter, as judged by the reduction in the secretory response (especially in the presence of 50 μM DNPL) to interaction with RBL-2H3 cells (Figure 6C vs -E). This suggests that most of the IgE molecules which remain bound to the ANPAH-carrying surface following photoactivation are covalently attached. It further indicates that the efficacy of the photochemically-induced covalent reaction between the photoactivatable antigen-carrying surface and IgE is higher than that between the latter and SPA.

The above-described properties enabled us to use these photoactivatable hapten-carrying surfaces for studying whether recruitment of new $Fc_\epsilon RI$ into clusters by IgE exchange is required for maintaining secretion for prolonged time periods. Thus IgE-saturated cells were reacted with the above surfaces, followed by photoactivation under nonpermissive conditions for secretion (10 °C) which allow cell adhesion (in a Tyrode's buffer containing 1.8 mM Ca^{2+}). Photoactivation-induced covalent bonds between IgE and surface-carried haptens should inhibit the mobility of these IgEs and thus strongly reduce the probability of exchanging their bound $Fc_\epsilon RI$s. Moreover, under our experimental protocol (cell incubation with 10 nM IgE for 2 h prior to their reaction with the antigenic surface and further incubation of the surface-carried cells with 50 μM DNPL in Tyrode's buffer following photoactivation) both $Fc_\epsilon RI$ and IgE are expected to be respectively saturated. Thus, following photoactivation no new interactions between the cell-carried $Fc_\epsilon RI$ and the surface are expected to occur. The marked secretory response observed upon transition to permissive conditions (37 °C), which is not affected by the presence of 50 μM DNPL (Figure 6G vs -B), indicates that no new $Fc_\epsilon RI$ cluster formation or its recruitment into preexisting clusters is required for maintaining secretion.

(C) Does $Fc_\epsilon RI$ Immobilization on RBL-2H3 Cells Induce Their Secretory Response? Glass surfaces carrying covalently-bound haptens were now employed to assay the relation between cell-surface $Fc_\epsilon RI$ density and the secretory response of RBL-2H3 cells. Since we have observed an almost complete immobilization of DNP-specific IgE molecules on the surface of ANPAH-carrying glass surfaces (see below), these IgE-carrying surfaces could also be used to immobilize the $Fc_\epsilon RI$ at different cell-surface densities. Average IgE densities on these surfaces were monitored by ^{125}I-labeling of the former. The cell's secretory response was measured as a function of IgE density following incubation on these surfaces for 1 h at 10 °C, removal of nonadherent cells by two successive washes with Tyrode's buffer, and transfer to 37 °C for 45 min to allow for secretion.

FRAP measurements established that IgE molecules bound on the above surfaces are practically immobile (I. Tamir, unpublished). Still, an additional protocol was employed in order to eliminate the possible diffusion of IgE into cell-surface contacts, thus increasing its local density. This protocol took advantage of the ability to covalently bind the IgE to the ANPAH-carrying glass surface either by photoactivation or using the covalent cross-linker BS^3. The IgE surface density required for triggering secretion was found to be essentially the same for the covalently-bound surface-carried IgE as that required for the noncovalently bound one (Figure 7). This suggests that no diffusion takes place on the hapten-carrying glass surface towards cell-glass contacts

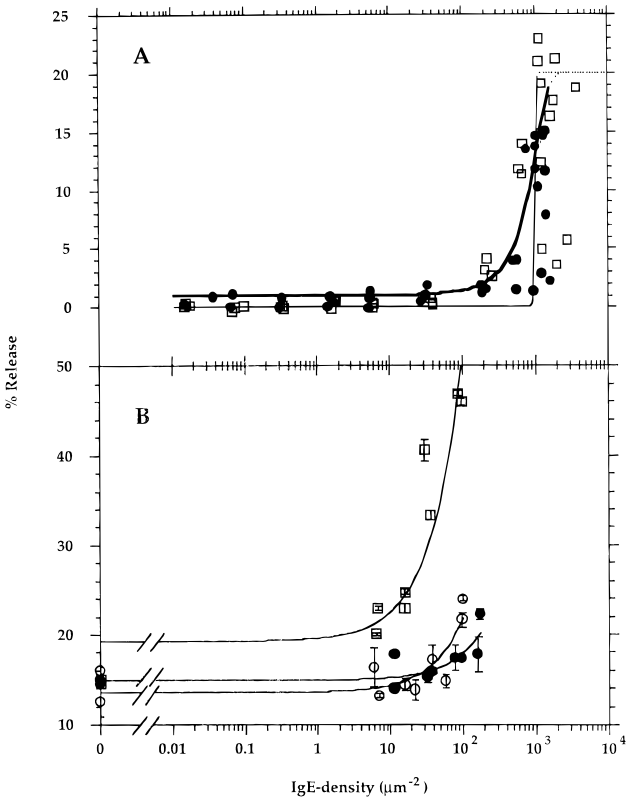

FIGURE 7: Dependence of the secretory response of RBL-2H3 cells on the density of surface-immobilized IgE. ANPAH-carrying glass surfaces were incubated for 1 h with increasing concentrations (0.1−1000 nM) of ^{125}I-labeled IgE (A2) in PBS. Following extensive washing with the latter buffer these surfaces were either photoactivated (15 min) or treated by a covalent cross-linker (1 mM BS^3 (Pierce)) for 1 h at 22 °C followed by consecutive washes with PBS, 100 mM Tris, pH 8.0, and Tyrode's buffers. Next, RBL-2H3 cells were adhered to these surfaces for 1 h at 10 °C followed by removal of nonadherent cells. The glass surface-carried cells were then incubated in Tyrode's buffer for 45 min at 37 °C, and their secretory dose response was measured. (A) Secretory dose response to stimulation by non-photoactivated IgE-carrying surfaces (□) vs photoactivated ones (●). The thin solid line (—) is the result of fitting the data by the first procedure described in the Appendix, the thicker one (bold —) by the second. The dotted lines for both curves represent a plateau which results from the fitting procedure, which however was not observed experimentally in this data set. (B) Effect of chemical linkage to the surface or solution dimerization of IgE on the induced secretory response. Chemical linkage between the surface and its bound IgE was performed by the above-described protocol. IgE dimerization was performed in solution by an anti-IgE antibody (clone 95.3) as described in the Materials and Methods. Secretory dose−response of RBL-2H3 cells to (1) noncovalently bound monomeric IgE (○), (2) covalently-bound IgE (●), and (3) mAb 95.3-dimerized IgE-carrying surfaces (□). Standard deviation of results obtained in triplicate experiments is presented (B) or is ≤10% (A). Curve fits were obtained by a normal regression analysis and introduced to allow for comparison between the different data sets. Other details are as described in the legend to Figure 1 and in Materials and Methods.

during the time course of cell adhesion and secretion, even in the absence of a covalent bond between the IgE and the hapten-carrying glass surface.

The dose−response to surfaces carrying immobilized monomeric IgE indicates the existence of a threshold density below which the release is negligible whereas it steeply increases above. Hence, these dose responses are qualitatively different from that normally obtained with mobile Fc_ϵRI-clustering agents such as antigens (via IgE; cf. Figure 1) or Fc_ϵRI-specific antibodies.

We have further studied the cell's secretory response to stimulation by surfaces which carry a microscopically nonrandom distribution of IgE molecules. IgE was first dimerized in solution by an IgE-specific mAb (clone 95.3) (Baniyash et al., 1986), followed by binding of these IgE dimers to the antigen-carrying glass surface. The observed secretory response to stimulation by these dimeric IgE-carrying surfaces was found to display a considerably lower threshold for the induction of degranulation (ca. 40 molecules/μm^2) as compared with that towards monomeric IgE-carrying ones (Figure 7B). This was taken as further support for the microscopically random distribution of IgE on these surfaces.

DISCUSSION

We have developed and employed experimental protocols designed to determine parameters which govern the capacity of the Fc_ϵRI to trigger the mast cell's secretory response. The parameters considered were Fc_ϵRI mobility, its density within a signaling cluster, and the assumed requirement for new Fc_ϵRI recruitment into clusters in order to sustain a secretory response. Since such a recruitment can take place either by antigen exchange between Fc_ϵRI-carried IgEs and/or by IgE exchange between Fc_ϵRIs, we have prepared reagents that prevent these processes, thus allowing the study of their role in signaling. These reagents carried a photoactivatable DNP analog, namely, ANPAH, shown to bind to the antigen binding site of a DNP-specific monoclonal IgE (clone A2). Photoactivation induced covalent binding to the IgE at the above site, thus preventing dissociation. Conjugation of this hapten to macromolecules or to solid supports produced antigens which could be photochemically induced to covalently bind IgE, thus inhibiting antigen exchange between Fc_ϵRI-carried IgEs. IgE binding to hapten-carrying surfaces was shown by FRAP measurements to result in complete IgE immobilization on these surfaces. Furthermore, photoactivation of these IgE-carrying surfaces leads to covalent binding of IgE to the surface (see below), which inhibits IgE exchange between its bound Fc_ϵRI and free cell-carried Fc_ϵRIs.

In order to resolve between stimuli produced by noncovalent and covalently-stabilized antigen−IgE complexes, soluble macromolecules or solid supports carrying photoactivatable haptens should ideally fulfill the following requirements: (1) The carried haptens should bind specifically and with high affinity to the IgE binding sites. (2) These haptens should undergo a fast and quantitative photoreaction to produce a highly reactive group (e.g., nitrene), which (3) should either react rapidly and covalently with the IgE−antigen binding site or, in the absence of IgE, react with solvent molecules and/or with residues on its macromolecular carrier (such as BSA in SPA). (4) The latter process should transform the haptens so that IgE does not bind them any more and therefore decreases their capacity to cluster it.

We found that the different conjugates of ANPAH fulfill the above criteria. Prior to photoactivation, SPA was shown to be an effective antigen, inducing a secretory dose response of RBL-2H3 cells carrying a DNP-specific IgE, which is comparable in shape to that induced by $DNP_{11}BSA$. Maximal secretory response to SPA occurred however at a 5-fold lower antigen concentration than that towards $DNP_{11}BSA$. This may be due to a difference in the respective affinity (6.5×10^5 M^{-1} for ANPAH-glycine (Tamir, Licht, and Pecht, unpublished) vs 1.0×10^5 M^{-1} for DNP-glycine (Schweitzer-Stenner et al., 1992)) and hence avidity of IgE for the two antigens. Following photoactivation, SPA stimulatory capacity was strongly reduced, probably due to reaction of the photoactivated haptens with their macromolecular carrier or with solvent molecules which renders them unreactive with the DNP-specific IgE. A 10-fold higher concentration of IgE complexes with photoactivated SPA was required to achieve the cellular response level observed upon stimulation with photoactivated SPA-IgE complexes. Furthermore, excess monovalent hapten inhibited the secretory response to non-photoactivated, but not to photoactivated SPA-IgE complexes. Taken together, these results indicate the formation of a covalent bond between IgE and ANPAH upon photoactivation. A further 10-fold increase in the concentration of photoactivated SPA-IgE complexes was required to achieve the cellular response level observed upon stimulation with non-photoactivated ones, suggesting that only a fraction of the antigens undergo covalent binding to IgE while the rest of the ANPAH undergo photolytic inactivation. Thus, the elicited secretory response probably represents that fraction of the photoactivated immune complexes carrying two or more covalently-bound IgE molecule per antigen. This suggests that at least 30% of IgE-bound ANPAH haptens underwent covalent cross-linking with it upon photoactivation, which is higher than the 10% yield reported for the photochemically-induced reaction between the monovalent hapten N^ϵ-(4-azido-2-nitrophenyl)-L-lysine (NAP-lysine) and NAP-specific polyclonal rabbit antibodies (Fleet et al., 1972).

Since a marked secretory response is observed to covalently-linked SPA−IgE complexes (ca. 40% at 1 μg/mL) even after 60 min incubation at 37 °C in a calcium-deficient buffer, we conclude that no recruitment of Fc_ϵRIs into clusters, by antigen exchange among vacant IgE molecules, is required for maintaining transmembranal signaling that leads to secretion. A similar conclusion has recently been drawn drom the results of experiments where the role of IgE exchange between occupied and vacant Fc_ϵRIs in signal transduction was explored (Kent et al., 1994). In that study the Fc_ϵRI was clustered by covalent trimeric IgE, and ca. 50 nM monomeric IgE was added in order to occupy vacant Fc_ϵRIs and inhibit the above exchange process. However, the relatively short time (10 min) allowed for cell−monomeric IgE interaction in the cited study is not sufficient for Fc_ϵRI saturation (Ortega et al., 1991); thus the above exchange process could not be completely prevented.

In the present study we have chosen a different approach to inhibit IgE exchange between occupied and vacant Fc_ϵRIs which made use of surfaces carrying photoactivatable haptens. In analogy with the results obtained with SPA, these

surfaces induced a secretory response of cells carrying the DNP-specific IgE, which was partially reduced upon photoactivation prior to their reaction with the cells. However, in contrast to SPA-IgE complexes, the cell's secretory response to IgE-carrying surfaces was not reduced upon photoactivation of the latter. This probably results from the fact that more than 75% of the antigenic surface-carried IgE molecules remain bound following photoactivation, suggesting their covalent attachment. Furthermore, secretion by cells, stimulated by either photoactivated or non-photoactivated IgE-carrying surfaces, was not inhibited by 50 μM DNPL, while the latter was effective in inhibiting the secretory response of cells stimulated by surfaces which have been photoactivated prior to IgE binding. Taken together, the above results may reflect a slow dissociation rate of IgE (as expected on the basis of the high hapten density (Berg & Purcell, 1977)) from non-photoactivated surfaces, resulting in an increased probability for the corresponding photochemically-driven reaction. However, photoactivation in the absence of IgE strongly reduced the density of available haptens on these surfaces (or the affinity for the DNP-specific IgE toward them), resulting in lower IgE binding to and increased dissociation from them, particularly in the presence of excess monovalent hapten (50 μM DNPL).

It may be argued that surface-carried ANPAH photoactivation within its IgE binding site is inefficient compared with free ANPAH, i.e., that IgE quenches the excited state of ANPAH thus inhibiting the formation of the chemically-active nitrene. This would account for the limited dissociation of IgE from these surfaces following their photoactivation, but it would contrast the results obtained with SPA−IgE complexes where it was clearly shown that, following photoactivation, the immune complexes that did not dissociate have become covalently stabilized. Thus the fact that photoactivation of the above ANPAH-carrying surfaces in the absence of IgE results in their markedly reduced ability to bind it and that photoactivation in the presence of surface-carried IgE molecules does not cause such a reduction in IgE binding clearly indicate that in the latter case IgE becomes covalently attached to the surface.

These interactions of the photoactivatable hapten-carrying surfaces with IgE made them useful reagents for inhibiting IgE exchange between occupied and vacant Fc_ϵRIs. Thus IgE-saturated RBL-2H3 cells were incubated on these surfaces for 1 h, under conditions that are nonpermissive to secretion (10 °C), followed by photoactivation. As discussed above, this protocol results in covalent binding of a fraction of the Fc_ϵRI-carried IgEs to this surface. Next, these surface-attached cells were incubated at 37 °C (in the presence of 50 μM DNPL) to allow for secretion to take place. Under these conditions, formation of new interactions between the photoactivated surface and Fc_ϵRI-carried IgEs is negligible and thus should not contribute significantly to the observed secretion (cf. Figure 6B). Furthermore, exchange of surface-bound IgE with vacant Fc_ϵRIs is inhibited (by the covalent immobilization of the former as well as by saturation of the latter by free IgE). Therefore, only those Fc_ϵRIs that remain bound to the surface via their IgEs following photoactivation can provide a stimulus. A significant secretory response (ca. 24 ± 1%), which was sustained for longer than 30 min following photoactivation, is observed under these conditions. We therefore conclude that neither recruitment of new Fc_ϵRI into preexisting clusters nor the formation of new ones is required for sustaining secretion. The secretory response observed in this case is significantly lower than that observed for the respective non-photoactivated case (Figure 6G vs -F), suggesting that in the latter case additional clusters are formed by further interactions between Fc_ϵRI-carried IgEs and the antigenic surface (since the latter is not photolyzed), which lead to an increase in the observed secretory response. This indicates that new Fc_ϵRI cluster formation increases the secretory response but is clearly not required for sustaining secretion induced by preexisting clusters.

Photoactivation did not cause a significant change in the amplitude of the secretory response induced by IgE-carrying surfaces. This suggests that surface binding of the IgE molecules (1 h at room temperature) immobilizes them already prior to photoactivation. This notion is supported by both FRAP studies showing that the fraction of immobile IgE on these surfaces is close to unity (data not shown) and by the inability of excess monovalent hapten (50 μM DNPL) to inhibit the secretory response they induce. Therefore, the IgE surface distribution and density should not change upon binding to cell-carried Fc_ϵRIs because IgE diffusion into cell−glass contacts is inhibited. Since the secretory dose response to glass surface-carried IgE was not altered by its (photo)chemically-induced covalent binding to the surface (Figure 7), we conclude that no redistribution of IgE took place on the above surfaces following binding to cell-carried Fc_ϵRIs.

A nonrandom distribution of IgE on these surfaces would lead to an increased inhomogeneity of the density of immobilized Fc_ϵRI on the cell surface, which would prevent a statistical treatment of the distribution of immobilized Fc_ϵRIs. Thus it was important to examine both the topography of antigen-carrying surfaces and IgE distribution on them. Therefore, we have first determined the density of BSA molecules covalently bound to the glass surface as 3.6×10^4 μm^{-2}, which suggests practically complete monolayer coverage. This also implies that the actual surface area is not significantly different from the geometrically-calculated one, further suggesting that the surface is essentially molecularly flat. The distribution of ANPAH haptens among these surface-bound BSA molecules can be expected to be random, yielding a similar distribution of surface-bound IgE molecules. The fact that IgE binding to these surfaces was from aggregate-free IgE solutions and found to be noncooperative suggests that it is randomly distributed. This is further supported by the IgE density threshold for the induction of degranulation which has been reproducibly found to be ca. 1000 μm^{-2} (Figure 7A). Since even a relatively small number of Fc_ϵRI clusters per cell are expected to induce a significant secretory response, it is expected to be a function of the probability of finding IgE microaggregates at the glass surface. This is supported by the density-dependent secretory response to stimulation by surfaces carrying IgE dimers, which displays a markedly lower IgE density threshold (ca. 40 μm^{-2}, Figure 7B). In this latter case, the probability of finding two or more IgE molecules (and thus Fc_ϵRIs) in proximity at a given IgE density is expected to be higher than for nondimerized IgE (Figure 7). Thus we are led to conclude that the surface-carried IgE is randomly distributed and not preferentially microclustered.

Since the IgE surface density obtained on the above glass surfaces overlaps with the average Fc_ϵRI concentration on

the surface of RBL-2H3 cells (ca. 1.0×10^3 μm^{-2}), cell attachment to surfaces carrying IgE at the latter density is expected to create at cell−glass contacts an immobile fraction of $Fc_\epsilon RI$s with an average density which matches the average resting cell-surface $Fc_\epsilon RI$ density. The $Fc_\epsilon RI$ density (mobile and immobile ones) is expected to increase at these cell−glass contacts (up to 80% at 1000 immobilized IgE molecules/μm^2) due to the diffusion of mobile $Fc_\epsilon RI$, but this cannot account for the shape of the dose response curve, which exhibits a sharp onset of this IgE density. Hence the results strongly suggest that immobilization of a fraction of the cell-carried $Fc_\epsilon RI$s without significantly altering their density distribution is sufficient for inducing a secretory response.

Triggering this response is now found to require that the density of immobilized $Fc_\epsilon RI$ exceeds a distinct threshold value (ϱ_t). This can be estimated from the data displayed in Figure 7A by virtue of several simple considerations (Appendix). The results of this analysis suggest that the maximal separation distance between immobilized $Fc_\epsilon RI$ that still provides a secretory stimulus is in the range of 30−40 nm. We therefore conclude that immobilization of a fraction of the cells' $Fc_\epsilon RI$s at a density which is close to their average random distribution density on resting cells may be sufficient for initiating a secretory stimulus. It has to be stressed that the calculated ρ_t values should be considered as an upper limit since immobilized glass-carried IgEs are probably not saturated by cell-carried $Fc_\epsilon RI$ at the glass−cell contact regions. Hence our results raise the interesting idea that a secretory stimulus of mast cells solely requires the close proximity of a distinct number of $Fc_\epsilon RI$s over a sufficiently long period of time rather than a distortion of their random distribution. This is in accordance with earlier theoretical predictions (DeLisi, 1981) and corroborates the notion that the corresponding efficacy of $Fc_\epsilon RI$ clusters to trigger release depends on their lifetime (Schweitzer-Stenner et al., 1994). The above is also in line with the earlier observed positive correlation between lateral $Fc_\epsilon RI$ immobilization, caused by its limited clustering, and the secertory response (Menon et al., 1986a,b). This immobilization was suggested to be mediated by interactions of the $Fc_\epsilon RI$ with cytoskeleton-associated components (Menon et al., 1986b), which is distinct from the externally-induced $Fc_\epsilon RI$ immobilization shown in this study. One possible explanation to the above correlation is that interactions of the $Fc_\epsilon RI$ with the cytoskeleton, which constrain $Fc_\epsilon RI$ mobility but do not lead to its association with the detergent-insoluble fraction (Seagrave & Oliver, 1990), may support secretion by prolonging $Fc_\epsilon RI$ encounters. However, it is noteworthy that excessive $Fc_\epsilon RI$ clustering (e.g., by means of an anti-IgE antibody or multivalent antigen) results in a strong reduction in its diffusion coefficient and inhibition of the secretory response (Becker et al., 1973; Lawson et al., 1975; Schlessinger et al., 1976). This phenomenon correlates with the association of the $Fc_\epsilon RI$ with the detergent-insoluble fraction, suggesting that excessive $Fc_\epsilon RI$ clustering leads to desensitization by a yet unknown mechanism which may involve cytoskeleton-associated components (Seagrave & Oliver, 1990; Seagrave et al., 1991). Furthermore, on resting mast cells, displaying only basal secretion, ca. 25% of the $Fc_\epsilon RI$s were found by FRAP studies to be immobilized (Kubitscheck et al., 1993; Schlessinger et al., 1976). Thus interactions with the cytoskeleton may regulate, both positively and negatively, the triggering capacity of $Fc_\epsilon RI$ clusters, depending on the extent of clustering. Our results show that immobilization of $Fc_\epsilon RI$s *per se*, at or below their random distribution density, leads to secretion rather than desensitization, suggesting that the latter is not a direct consequence of the reduction in $Fc_\epsilon RI$ diffusion.

Liposomes carrying DNP-derivatized lipids have been used in earlier studies of the interaction of hapten-carrying surfaces with specific IgE-carrying mast cells (RBL-2H3 line). It was shown that the cells bound specifically to these liposomes surfaces (McCloskey & Poo, 1986), and this induced a secretory response (Balakrishnan et al., 1982). This response was expected to occur only upon cell interaction with "solid" (lateral diffusion coefficient of 10^{-10} cm^2/s or less) hapten-carrying liposomes since the lower mobility of $Fc_\epsilon RI$, bound to these liposomes via IgE, should prolong $Fc_\epsilon RI$ encounters. However, "fluid" (lateral diffusion coefficient of $10^{-7}-10^{-8}$ cm^2/s) hapten-carrying liposomes were also quite effective in inducing degranulation. Since in this case $Fc_\epsilon RI$ mobility should be unaltered, the authors suggested that the unidirectional diffusion of $Fc_\epsilon RI$-bound IgE into cell−liposome contact areas increases their local concentration above a threshold required for signaling. Thus, both $Fc_\epsilon RI$ diffusion capacity and its density within the cluster were suggested to determine the extent of secretory response. Since in the above system the accumulation of both $Fc_\epsilon RI$−IgE and haptens at cell−liposome contacts could not be prevented (and in fact was shown to take place), the distinct effects of $Fc_\epsilon RI$ mobility and density on the induced secretory response could not be resolved. However, marked differences between the secretory response to "fluid" vs "solid" hapten-carrying liposomes were observed, the latter being more effective with respect to (1) the threshold hapten surface density required for inducing significant secretion (0.9 mol % vs 0.45 mol %, respectively), (2) the maximal secretory response induced (ca. 27% vs 55%, respectively), and (3) the nonlinearity (cooperativity) of the secretory response to hapten surface density (Hill coefficients of 2.7 vs 3.8, respectively). Since the amount of "solid" liposomes bound to the cells was ca. 4 times lower than that of "fluid" ones, the above difference in the efficacy of these two liposome types in eliciting secretion is even more striking. Assuming that (1) the interaction of both surface types with the cells results in $Fc_\epsilon RI$-carried IgE accumulation at cell−liposome contact regions and (2) the average density of accumulated $Fc_\epsilon RI$ is the same for both liposome types carrying equal hapten surface densities, the above difference may also suggest that the secretory response of RBL-2H3 cells to stimulation by hapten-carrying surfaces is more sensitive to changes in $Fc_\epsilon RI$ mobility than to its density.

In conclusion, we have provided evidence that $Fc_\epsilon RI$ clustering *per se* is not obligatory for providing a secretory stimulus and that the critical factor is most probably maintaining a fraction of these receptors within a required proximity for a period of time that would allow for a signal to be generated. This time threshold may, for example, be that required for receptor transphosphorylation by receptor-associated protein tyrosine kinases (PTKs, e.g., Lyn) leading to the recruitment and activation of cytosolic PTKs such as Syk (Jouvin et al., 1994) downstream in the coupling cascade. In view of the possible physiological interactions of $Fc_\epsilon RI$-carried IgEs with immobile surface-carried epitopes, results of this study may provide a mechanism for mast cell

activation by organisms and extracellular matrices. Finally, in addition to serving as a model for the latter process, the experimental protocols we have developed may have wider applications for investigating signaling by different immuno (as well as other) receptors.

ACKNOWLEDGMENT

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APPENDIX

Estimation of the Density Threshold of Immobilized $Fc_\epsilon RI$ Required for Stimulating the Secretory Response of Mast Cells. In the following calculations these assumptions are made: (1) Each IgE attached to the glass surface is bound to an $Fc_\epsilon RI$. Hence the cell-surface density of immobilized $Fc_\epsilon RI$ is identical to the IgE density on the glass surface. (2) The two-dimensional distribution of these IgE−$Fc_\epsilon RI$ complexes is considered as random. (3) The secretory response is assumed to be linearly related to the number of cell surface elements having an $Fc_\epsilon RI$ density higher than a distinct threshold value ρ_t. Thus, the response (R) may be estimated by:

$$R(\rho) = (R_0/\delta\rho(2\pi)^{1/2})\int_{\rho_t}^{\infty}\exp\{-{}^1/_2((\rho - \langle\rho\rangle)/\delta\rho)^2\}\,d\rho \quad (1)$$

where R_0 is the cell secretory capacity and $\langle\rho\rangle$ is the average surface density of IgE. The variance $\delta\rho$ of the density can be written as (Davidson 1962):

$$\delta\rho = \rho/N^{1/2} \quad (2)$$

where N is the number of IgE−$Fc_\epsilon RI$ complexes per unit area. The results of fitting eq 1 to both data sets (since no significant difference was found between them) is shown in Figure 7A. It yields the thin solid curve and a value of $\rho_t = 10^3/\mu m^2$. Due to the low density variance $\delta\rho$, the fit according to eq 1 exhibits a rather steep increase at ρ_t, which is in fair agreement with the experimental results. However, some of the data points in Figure 7A seem to indicate that secretion is induced already at lower IgE densities. This is understood if one takes into account that the examined cell population most likely exhibits a distribution $P(\rho_t)$ of threshold values ρ_t which can be assumed to be Gaussian. Hence $R(\rho)$ may in principle be calculated by convoluting $P(\rho_t)$ with eq 1. However, for reasons of simplicity, we solely estimate the influence of $P(\rho_t)$ on the dose response by invoking:

$$\delta\rho^* = (\delta\rho^2 + \Delta^2)^{1/2} \quad (3)$$

as an effective variance in eq 1. $\delta\rho$ is given in eq 2 and Δ reflects the variance in ρ_t. The fit of the thus obtained expression to the dose response is displayed by the thick solid line in Figure 7A. It yields the parameter values $\rho_t = 850\ \mu m^{-2}$ and $\Delta = 500\ \mu m^{-2}$.

Both of the above curve fits yield a plateau in the secretory response at ca. 20% secretion and ≥1000 IgE molecules/μm^2. However, this plateau results from setting $R_0 = 20\%$ as the average maximal secretory response to immobilized IgE observed in this particular experiment. Although we have evidence for the actual existence of such a plateau from other data sets, there is a difficulty in obtaining IgE densities higher than 1000 IgE molecules/μm^2; thus the fitted curve should be considered to be reliable only below this IgE density value (as indicated by the broken line in the curve fits above this value). It should be stressed however that increasing the value of R_0 influences neither the value of ρ_t that is derived from both of the above fitting procedure, nor the shape of the curve fit below 1000 IgE molecules/μm^2; thus the presence of a plateau in the above curve fits is not related to the IgE density threshold for the induction of a secretory response.

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