

THE RECEPTOR WITH HIGH AFFINITY FOR IMMUNOGLOBULIN E^{1,2}

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INTRODUCTION

This review is about one of the physiologically important macromolecules that interact with the Fc domains of immunoglobulins. Such substances (complement, “immunoglobulin binding factors,” Fc receptors) can convert the simple binding of antigen to antibody into a biologically meaningful event.

The protein we review here—the Fc receptor with high affinity for monomeric immunoglobulin E (IgE)—is only one of several proteins that interact with IgE (1–5). In addition to high affinity, its special characteristic is its distribution on the plasma membrane of mast cells and basophils exclusively. Activation of this receptor initiates release of preformed mediators contained in intracellular granules and the biosynthesis of a variety of biologically active lipids such as the leukotrienes. Here we review what is known about the structure of this receptor and the early biochemical events that it initiates. We do not consider the consequences of

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² Abbreviations: IgE, immunoglobulin E; RBL, rat basophilic leukemia; PI, phosphatidyl inositol; PIP, phosphatidyl inositol-4-phosphate; PIP₂, phosphatidyl-4,5-bis phosphate; AdoMet, s-adenosylmethionine; AdoHcy, s-adenosylhomocysteine.

these initial perturbations—an area that deserves separate discussion. Because our subject has been regularly reviewed (6–8), we focus principally on recent data.

MATERIALS AND METHODS

IgE and Cells

Molecular characterization of receptors requires reasonable amounts of material. With respect to the ligand, human IgE myelomas have been available since the late 1960s (9), but the lack of suitable preparations of human cells was a serious drawback. Modest numbers of mast cells can be purified from rats and mice, but preparing sufficient rodent IgE was laborious (10). The discovery by Bazin and his colleagues of rat immunocytomas (11), many of which secreted homogeneous IgE (12), was a major breakthrough. The development of the monoclonal antibody technology (13) now permits the preparation of virtually unlimited numbers and amounts of antigen-specific mouse IgEs. One of these—HI-DNP- ϵ -26.82, which is specific for the dinitrophenyl moiety (14)—has been particularly popular.

The unique development of a *basophilic leukemia* in rats treated with β -chloroethylamine was first reported in 1973 (15). In vitro culture of these RBL cells was initiated in our laboratory that same year (16) and independently by Froese and his colleagues in 1975 (17). These initial isolates are apparently the source of all subsequent work on these widely used cells. By several criteria these cells seem most closely related to so-called mucosal mast cells (18).

The initial cell lines appeared granular and had low but palpable amounts of histamine and substantial numbers of receptors; even so, they failed to undergo receptor-mediated secretion (16). Subsequently secreting cell lines arose spontaneously—an event so unexpected that the cells were originally thought to have arisen from a mouse mastocytoma (19, 20). Cloning of the releasing cells led to a line, 2H3(HR+), (21) which has continued to be highly functional. Recently, other cell lines have been prepared that will be useful for comparative purposes (22).

The IgE-mediated release from RBL cells has many of the characteristics of release from human basophils and rodent mast cells (23). Similarly, no substantial discrepancy between the structure of the receptor on the RBL cells and that on normal cells has been uncovered, although only limited comparative data are available (17, 24–27). The differences that do exist, rather than diminishing the usefulness of the tumor cells, may assist in sorting out the essential from the trivial. Likely, this will be equally true for the reactions initiated by the receptor.

Purification of the Receptor

Once solubilized, the receptor can be absorbed to IgE-adsorbents. After washing, the receptor can then be released by use of a variety of protein-denaturants (7, 28, 29). There are problems with this approach. (a) Since the adsorbents will likely contain aggregates of IgE as well as monomeric IgE, cellular proteins with low affinity for monomeric IgE may coabsorb (1). (b) The aggregated IgE may activate those receptors absorbed to it—if such an event as “activation” indeed occurs (see below). (c) The use of denaturants can dissociate the receptor (30) and may induce functional changes. Since no functional assays as yet exist, such changes are undetectable. (d) Nonspecifically absorbed contaminants may also be released by the denaturant.

An alternative approach involves binding monomeric IgE to the receptor either before or after solubilization and then trapping the IgE either on an antihapten adsorbent utilizing hapten-conjugated IgE (31, 32) or on a hapten-conjugated adsorbent using antigen-specific IgE (33). After washing, the IgE-receptor complexes can be eluted with hapten without employing denaturants. The slow rate of dissociation (34) prevents appreciable losses of receptor throughout purification especially when nmolar concentrations of IgE and receptor are used. The disadvantage of this approach is that IgE-receptor complexes, rather than unliganded receptors, are purified. However, the binding of monomeric IgE to the receptor is not known to lead to any changes in the receptor (see below), so that in principle this is not a serious problem. With this procedure 30% yields of intact receptors can be isolated. Most current lines of RBL cells have approximately 3×10^5 receptors per cell and can be readily grown to a density of 10^6 cells/ml. Thus 10 liter of cells can yield ~ 1.5 nmol receptor.

STRUCTURE OF THE RECEPTOR

Analysis of the receptor was initiated in the mid-1970s in several laboratories, and progress has been regularly reviewed (6, 7). In 1983 we presented a new model for the receptor (35), and we use that model as the basis for discussing recent observations. We focus on several major aspects: The properties of the subunits that constitute the unit receptor; the topology of these subunits in the membrane; the interaction of the receptor with detergents and lipids; and some questions of definition.

Covalent Structure

PROTEIN CHEMICAL APPROACHES The model of the receptor contains four polypeptide chains: a single α chain (36), which contains the binding site for

IgE (17, 24), a single β chain (26, 33); and two identical, disulfide-linked γ chains (27) (Figure 1). Some of the properties of these chains are collected in Table 1.

New findings support specific features of this model. On the basis of the products of proteolytic cleavage of the α chain (46) and, later, on the basis of the kinetics of proteolytic cleavage of receptor bound IgE (47), we proposed that the α chain consists of two similar-sized but nonidentical domains. That the Fab fragments of a particular monoclonal antibody directed to the α chain (antibody BC4) bind in a ratio of ~ 2 mols per mol receptor (48) is consistent with this proposal. We also proposed that the two γ chains are identical, and two-dimensional analysis of intact γ chains subsequently confirmed that conclusion (unpublished observations).

That the γ components are not degradation products of either α or β has been further confirmed by two-dimensional chymotryptic peptide maps of extrinsically iodinated α , β , and γ , which show discrete patterns with essentially no common peptides (G. Alcaraz, J.-P. Kinet, H. Metzger, unpublished observations). This confirms the distinctiveness of the γ chains and implies that lengthy runs of sequence identity between them and β or α are unlikely. We have insufficient data to show that by the same criteria this is also true for the relationship between α and β .

GENETIC APPROACHES To explore the sequences and sequence-relationships further, cloning of the genes that code for them is the most reasonable strategy. We plan to prepare cDNA probes synthesized on the basis of limited sequence analysis. Because the amino terminus of each of the chains appears to be blocked (36; J.-P. Kinet, G. Alcaraz, H. Metzger, unpublished observations, in collaboration with M. Bond), isolation of one

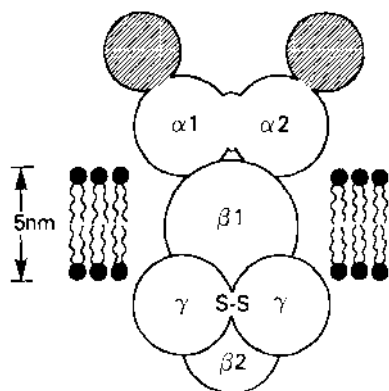


Figure 1 Schematic diagram of receptor for immunoglobulin E. The circles represent spheres whose volumes are proportional to the mass of the peptide and carbohydrate (shaded areas) they define. From Ref. 35 by permission.

or more peptides is necessary to obtain such data; isolation of tryptic peptides has been initiated.

An alternative approach is to prepare cDNA from mRNA with or without prior fractionation of the latter. Using RBL cells, Pure et al (49) as well as Liu & Orida (50) obtained 13-14S fractions of mRNA from sucrose gradients that, when injected into frog oocytes, induced binding activity for IgE. Analysis of this IgE-binding material on polyacrylamide gels showed principally a 31 kd component of uncertain relationship to either the α , β , or γ chains (50). Liu et al (51) have cloned the double-stranded cDNA prepared

Table 1 Properties of the subunits of the receptor for immunoglobulin E^a

Properties	Subunit		
	α	β	γ
Mol/mol receptor (35) ^b	1	1	2
M _r (kd) (27, 33, 37)	45	33	9
Labeling properties			
Extrinsic			
Surface			
Intact cells, vesicles (17, 24, 38)	+	---	-
Lysed vesicles (38) ^c	-	+	+
Intramembrane (27, 39)	-	+	+
Intrinsic			
Glucosamine (24, 27, 40)	+	-	-
Phosphorus (41, 42) ^d	-	+	+
Fatty acids (45)	+	+	+
Composition			
Carbohydrate (wt%) (36)	30%	-	-
Amino-acids (mol%) (36) ^e			
Basic (Lys, Arg, His)	14.6	11.2	15.7
Acidic (Glx, Asx)	20.7	20.9	20.7
Hydroxy (Ser, Thr)	16.1	19.4	12.3
Hydrophobic (Val, Leu, Ile)	22.7	23.6	26.1
Aromatic (Tyr, Phe, Trp)	11.3	8.3	8.0
Other			
Cys	2.36	2.05	1.92
Met	1.04	1.3	0
Gly, Ala, Pro	14.0	18.6	17.1

^a Data from RBL cells exclusively.

^b Reference.

^c Refers to labeling in addition to that seen on intact vesicles.

^d Refers to intact cells only. Hempstead et al (43, 44) report labeling of α chains on rat peritoneal mast cells.

^e The data for the β and γ chains are from G. Alcaraz, T. Y. Liu, J.-P. Kinet, H. Metzger (unpublished studies).

from such mRNA fractions, and this cDNA in turn hybridizes with the mRNA coding for the 31 kd protein. In the amino acid sequence published so far (encompassing approximately one half of the peptide), there appear to be five methionines—more than our own analysis suggests for either the α , β , or γ chains (Table 1). Liu et al suggest that possibly their protein represents the similar-sized material described by Kulczycki & Parker (29) and Holowka & Baird (38).

The relationship of the protein described by Liu and his colleagues to the α , β , and γ chains could be clarified with suitable antibodies. Reports exist of anti- α -chain antibodies (7, 48, 52, 53), but no rigorous analysis of their specificity or of their cross-reactivity with the cloned protein has been published. We (and perhaps others) have tried to prepare antibodies specific for β and γ but as yet we have not succeeded.

Topology

New evidence supports one or another aspect of the topology of the receptor, as depicted in Figure 1. Holowka & Baird (38) used lactoperoxidase to label regions of the receptor exposed on plasma membrane vesicles prepared by treating RBL cells with formaldehyde and dithiothreitol (54). The intact vesicles, which appeared to be primarily right side out, revealed labeling of the α chain almost exclusively. Labeling after lysis of these vesicles produced no increase in the modification of α but now revealed labeling of β and γ . These results are consistent with the model. The results of Lee & Conrad (55) are also confirmatory. As we did also (26, 56, 57), they observed covalent cross-linking of α , β , and γ when bifunctional reagents were reacted with the solubilized receptors. However, when membrane-impermeable cross-linking reagents were reacted with intact cells, no cross-linking was evident (55). [With membrane-permeant reagents such as the bis-imidates, cross-linking of α , β , and γ on intact cells is obtained (26).] These observations are consistent with minimal or no exposure of the β and γ chains on the surface (Figure 1). Although some additional analyses are needed to improve the accuracy of our amino acid analyses, the current data (Table 1) show that the general distribution of hydrophobic and hydrophilic amino acids in α , β , and γ is similar. Though enriched in hydrophobic residues compared to soluble proteins in general (58), each chain has numerous hydrophilic and charged residues; this indicates that each is partially exposed to an aqueous environment.

We emphasize that the current topological scheme is based in part on negative data and in part on no data whatsoever. For example, there are no data that allow one to speculate whether α interacts with β or with γ . Only a hint exists that α does not independently interact with both (see below).

Interaction with Detergents and Lipids

DETERGENTS An unusual feature of the $\alpha\beta\gamma_2$ complex is its dissociation in so-called mild detergents (30, 56), and it was this property that delayed detection of the β and γ chains. Two observations have allowed us to examine this dissociation in detail. The first was that the instability could be largely obviated by maintaining the solubilized receptor in submicellar detergent (30, 59). The second was that by briefly exposing the receptor to micellar detergent in the absence of lipid and then maintaining it in submicellular detergent, even the α chain in the IgE- $\alpha\beta\gamma_2$ complex could be labeled (see also below) (30, 45, 59).

Our principal findings on the dissociation of β and γ_2 from α were as follows (30): (a) The β chain and dimer of γ chains always dissociated in unison. This is a hint that β and γ don't interact independently with α (Figure 1). (b) The dissociation is due to detergent micelles and, under appropriate conditions, the extent of dissociation correlates well with the concentration of micells regardless of which of six different commonly used mild detergents is employed. (c) Under otherwise mild conditions, the molar ratio of detergent monomers (in micellar form) to receptor that is required for dissociation is about 10^5 . This ratio is strikingly higher than the ratio of detergent to lipid that is required to dissolve membranes, where a ratio of ~ 2 suffices (60, 61). (d) Detergents that are most effective for dissolving biomembranes—those with an HLB number (62) of about 13 (63)—disrupt the receptor most efficiently. (e) At low levels of micellar detergents, the kinetics of dissociation implicate a component other than the receptor and the detergent, that influences the process. We believe this component is lipid that is tightly associated with the receptor (below).

The dissociability in mild detergents of the β and γ chains from the α chains is useful for preparatory isolation of the subunits, especially when Triton X-114 is employed (59, 64). With this detergent we can separate the α subunits of the receptor from the β and γ chains under conditions that maintain high concentrations of all the subunits and that are otherwise extremely gentle (59). Nevertheless, we have not so far been successful in recombining the β and γ chains with α , once they have been dissociated.

LIPIDS Recent studies indicate that lipids bind to the receptor in several ways (45). Evidence for *loosely* bound lipid is derived from observations on the conditions necessary to iodinate extrinsically the α chain of the receptor in the IgE- $\alpha\beta\gamma_2$ complex (see above). Previous data had indicated that α could not be oxidatively iodinated once bound to IgE (17, 25). We found that if the complex was briefly exposed to lipid-free micellar detergent and then iodinated in submicellar concentrations of detergent, substantial

iodination of the α chain is obtained. If micellar detergent, with or without lipids, is added back to the complex prior to iodination, the labeling of α is again suppressed. These observations suggest that there are sites on or near the α chain to which lipid binds weakly and that these are rather nonspecific.

Evidence for *tightly* bound lipid comes from the observation that phospholipids prevent the disruptive action of detergent (45, 56), and from the dissociation studies cited above that implicated a third component. Cell-derived mixed lipids are the only ones that have so far been found to protect fully against the disruption induced by detergent (45, 56); this suggests that the tightly-bound lipid may be rather specific. A direct analysis to test this proposal has been initiated (65).

The receptor also contains *covalently* bound lipid (45). Because only small amounts of radioactive fatty acids are incorporated, it is impractical to assess the molar ratio of fatty acid to polypeptide. In the absence of knowledge of whether a substantial proportion of the receptors are modified, it is futile to speculate about the potential function of such lipid.

Defining the Receptor

β and γ_2 dissociate from α under conditions in which most multisubunit membrane proteins remain intact, and the β and γ chains associated with α chains already inserted in the plasma membrane incorporate radioactive phosphorus (42) and fatty acids (45). These facts raise the question of whether it is appropriate, therefore, to consider the β and γ chains as true subunits of the receptor rather than as receptor-associated components. Once the molar ratio has been defined as reasonable (a small integral number), we consider the most decisive criteria to be biosynthetic ones. An examination of the biosynthesis more detailed than had been previously performed (27) has been published (66). These new experiments showed that α , β , and γ are coordinately synthesized and degraded, and cannot exchange independently of each other once inserted into the plasma membrane. It, therefore, seems reasonable to consider the $\alpha\beta\gamma_2$ complex as the unit receptor.

BINDING OF IgE TO THE RECEPTOR

Reaction Mechanism, Kinetics

The principal findings have been reviewed (e.g. 67) and can be summarized easily. All the evidence favors a simple bimolecular, nondiffusion-controlled interaction between a monomer of IgE and a single univalent receptor to which it binds (34, 68, 69). The forward reaction can be described by a single-valued rate constant of about $10^5 \text{ M}^{-1} \text{ s}^{-1}$ for intact cells and

about 30-fold greater for solubilized receptors. The reason for this discrepancy is uncertain. Possibly the slower rate is due to a "gating" effect of other glycoproteins on the cell surface (69). Alternatively, the bending of IgE, which may occur when it binds to cell-bound receptors (see below), may not happen when IgE binds to solubilized receptors (70). The former proposal can be studied by using receptors reincorporated into liposomes free of other proteins (57); the latter, with comparative studies using energy transfer techniques (70, 71). The forward reaction is unremarkable either in its rate constant or in its sensitivity to pH, temperature, salts, etc (34, 72). The dissociation is slow and has a concentration dependence appropriate for a unimolecular reaction. When studied over long time periods, the kinetics suggest that the receptors are heterogeneous since the dissociation data show a concave-upward shape on a semilog plot (73). The shape of this plot is not influenced by the fractional occupancy of the receptors (69), so there is no reason to invoke any (negative) cooperative effect. These data likewise preclude a diffusion-controlled reaction (69). Thus, the slow rate of dissociation ($k_{-1} < 10^{-5} \text{ s}^{-1}$) accounts for the high affinity of the receptor for IgE.

Specificity

The specificity for the epsilon isotype is virtually perfect—no homologous monomeric isotype can compete effectively against the binding of IgE. The species specificity is imperfect. For example, rodent IgE can bind to human cells (74), and recent evidence was presented that human IgE could bind to rodent cells (75). The former observation is not surprising; it is only surprising that it had not been adequately tested previously. The latter finding is unexpected because it had earlier been found that human IgE cannot block the binding of rodent IgE (16, 76). In those instances the human IgE may have become largely inactivated, but because very large concentrations of the IgE were used, this explanation seems improbable. Another unusual aspect of these recent findings is that RBL cells failed to bind human IgE, whereas peritoneal mast cells did (75). Previous studies had suggested very similar binding properties for these cells (76, 77), and peptide maps failed to pick up reproducible differences in the α subunit which binds IgE (25). Some older studies had suggested that human reaginic sera could sensitize rodent mast cells (reviewed in 67); this could have resulted from minimal binding of IgE or perhaps from binding to other receptors. It was in any case difficult to reproduce (78).

Binding Sites on IgE

The binding of IgE to the receptor is not known to cause any significant perturbations by itself. Therefore, definition of the sites on IgE that interact

with the receptor is unlikely to clarify the mechanism of action of the receptor. The interest in this area of research is prompted, rather, by the therapeutic implications of success. That is, if one could prepare suitable peptides based on knowledge of the interactive sites, one might have a useful way of preventing sensitization of mast cells and basophils with endogenous IgE.

OLDER DATA A problem in this line of investigation has been the failure to obtain small fragments of IgE that are active. Human IgE can be split by proteases to produce active Fc fragments $[(C_{\epsilon}2-C_{\epsilon}3-C_{\epsilon}4)_2]$ (79–81), but no smaller fragment has been found to be reproducibly active (reviewed in 47).

Dorrington & Bennich (82) correlated the inactivation of binding of human IgE with the irreversible changes induced by denaturants and concluded that both the $C_{\epsilon}3$ and $C_{\epsilon}4$ domains—particularly the former—were involved.

Perez-Montfort & Metzger (47) attempted to generate active fragments by carefully controlled proteolytic digestion of rat and mouse IgE in the presence and absence of receptor. Although no active fragments (not even an Fc fragment) could be generated, the kinetics of cleavage at several sites yielded some useful information. The presence of receptor slowed (but did not totally inhibit) digestion at sites presumed to be between $C_{\epsilon}3:C_{\epsilon}4$ and $C_{\epsilon}3:C_{\epsilon}2$ (mouse IgE) or within $C_{\epsilon}3$ and between $C_{\epsilon}3:C_{\epsilon}2$ (rat IgE). The latter cleavage was most profoundly affected, the rate being sufficiently slow that the breakdown might actually have been of the small amount of IgE that had dissociated during the course of the experiment. These results clearly implicate a region close to the $C_{\epsilon}2:C_{\epsilon}3$ juncture on each epsilon chain as one that interacts with the receptor.

NEW RESULTS

Genetic Approaches Two groups have infected bacteria with plasmids bearing an insert of the cDNA that codes for all or most of the Fc region or the human epsilon chain (83, 84). The bacteria produced unglycosylated Fc fragments that bound to human mast cells or basophils with the requisite specificity. These results confirm previous data (with rodent IgE) that carbohydrate does not importantly contribute to the binding of IgE (85). The fragment isolated by Liu et al (84) is missing the NH_2 -terminal one third of the $C_{\epsilon}2$ region, so this region is apparently also not required.

Both Kenten et al and Liu et al had to use rather harsh conditions to isolate and purify the epsilon fragment dimers. This is not a trivial matter for the following reason. Both groups indicate that it will now be possible to perform site-specific mutations and to study their effect on the binding to the receptor. Such mutations may, however, change the sensitivity to, or reversibility of, the denaturing conditions used to prepare the material. It

will be difficult to disentangle losses in activity due to such effects from those due to alterations at the binding sites themselves.

Even if conditions could be found whereby the use of denaturants could be avoided, the underlying assumption of all such studies, i.e. that an exchange of a specific amino acid will have only local effects, is not universally true (86). It is likely that many mutants will have to be screened before an interpretable pattern emerges.

Physicochemical approaches Holowka & Baird and their colleagues have employed energy transfer techniques to assess the interaction of IgE with receptors on cells or on membrane vesicles (54, 70, 71, 87). They inserted acceptor probes into the bilayer via hydrophobic tails and measured the quenching of fluorescent donor probes chemically attached to various sites on IgE, or on antibodies to IgE, and of a fluorescent hapten in the combining sites. Energy transfer between probes on antibodies that bind at or near C_ε1 and somewhere in C_ε3 or C_ε4 (88) was also measured. The quenching data can, with certain assumptions, be interpreted to yield an average distance between the donor and acceptor with an estimated accuracy of $\pm 20\%$.

Such studies yield only indirect information and require too lengthy an interpretive analysis to review here. The authors conclude that the IgE binds via only one face of the Fc region and bends at the C_ε2: C_ε3 interface. Bending at the analogous interface in IgM has been proposed, and this is also the principal locus of the limited segmental flexibility of IgM and IgE. It is, therefore, surprising that no substantial changes in the flexibility of IgE occur when it binds to the cell-bound receptor (89). More direct alternative approaches will be necessary to confirm the model of Holowka et al. It is, however, consistent with the observation that interactions occur well removed from the distal end of the Fc and that the terminal portions are not totally prevented from interacting with enzymes (47) or antibodies (88).

Studies with monoclonal antibodies Another way to identify the regions on IgE critical for binding to the receptor is to employ monoclonal antibodies. Baniyash and coworkers (90, 91) have described a monoclonal antibody "51.3," which binds to the Fc portion of mouse IgE and has some interesting properties. The antibody blocks the binding of IgE to RBL cells but only partially—55–65% (90). Nevertheless, this antibody reacts with cell-bound IgE, albeit to a lesser extent than to unbound IgE. When Fab fragments of the anti-IgE were prepared and the stoichiometry of their binding assessed, Baniyash et al (91) estimated that 3 mols Fab bound per mol IgE in solution and only 2 mols per mol with cell-bound IgE. These observations could be related. We suggest there may be two pairs of similar epitopes that this antibody recognizes. If the antibody binds to sites of one set (A) first, it

inhibits binding of antibody to the other set (B) but does not inhibit binding of IgE to the cell. Contrariwise, those molecules of IgE to which antibody binds at site B first are inhibited from binding to the receptor and have a reduced capacity to interact with the antibodies at site A. This could explain the partial inhibition of binding, a phenomenon based on a stochastic event, rather than on the heterogeneity of receptors, as the authors suggest (90). Our proposal could also explain the decreased binding of the antibodies to cell-bound IgE. The B sites (above) are unavailable on cell-bound IgE, whereas the A sites would still react with antibody.

Differences in the reactivity of free and cell-bound IgE and the apparent discrepancy in the observation that antibody can inhibit binding but still react with cell-bound IgE, could also be explained by changes in the conformation of IgE when it binds, as noted above.

Other strategies Each of the approaches described so far yields results that are difficult to interpret unambiguously. It is appropriate therefore to consider other stratagems. One is to compare the sequences of IgE from different species with each other and with sequences of other isotypes of immunoglobulin. Given the partial cross-reactivity of mouse and human IgE with the corresponding heterologous receptors (above), it is difficult to know what to look for! Notably, the least divergent portion of the mouse and human epsilon chain is in C_ε4 (92)—a region that is implicated to a lesser extent than C_ε3 (above). It seems likely that comparative sequence analysis can at best provide a basis for deciding on which site-specific mutations it would be most profitable to focus (see above). We previously (6) suggested alternative approaches that still seem worth exploring by those interested in this area: (a) Use of cross-linking reagents and (b) modification of IgE in the presence and absence of the receptor. Although the work in analyzing the appropriate peptides will be tedious, the results are likely to be less ambiguous than those produced by most other methods.

AGGREGATION AS THE INITIATING SIGNAL

We shall not recount once more the oft-reviewed extensive evidence that aggregation of the receptors is the initiating signal in IgE-mediated release (6). Rather, we shall focus on newer studies that relate to several specific aspects of such aggregates.

Size of the Aggregates

Earlier studies had suggested that aggregates of IgE as small as a dimer might be sufficient to initiate release (93, 94) but the possibility that minor quantities of larger aggregates were in fact the critical stimuli could not be

excluded. The quantitative analysis by Segal et al (95) appeared to settle the issue, since this showed that highly purified, covalently cross-linked dimers could indeed initiate release from rodent mast cells. Similarly, human basophils were shown to be stimulated by dimeric IgE (96). The water was only a little muddied when Fewtrell & Metzger (97) observed that with RBL cells, dimers were virtually inactive in the absence of D₂O (below), and even in its presence were considerably less effective than trimers, and the latter less than higher oligomers. Analogous studies have been performed by Menon et al (98) using a monoclonal anti-IgE (A2(88)) that at low concentrations forms dimers of IgE almost exclusively but which at higher concentrations can form larger aggregates (71). With this reagent, peritoneal mast cells show the same poor response to dimers as is shown by RBL cells, whereas with preformed dimers of IgE, Segal et al studying cutaneous mast cells (95) and Fewtrell & Metzger using peritoneal mast cells (97) found excellent responses. Because putatively similar cells may show substantial differences in their responsiveness to dimers (below), experiments with both types of reagents need to be performed with a single batch of cells in order to determine the basis of this variability.

The initial studies on human basophils yielded quantitatively less dramatic differences between dimers, trimers, and higher oligomers but the results were qualitatively similar to those reported on RBL cells (96). More recent studies by MacGlashan & Lichtenstein show a correlation between the "releasability" of cells and their ability to discriminate between dimers and higher oligomers, i.e. their ability to respond to dimers (99, 100). It is known that the peripheral blood basophils of 10–20% of donors fail to release when challenged with antigen or anti-IgE. Other donors respond to different degrees even when care is taken to compare cells that have equivalent quantities of bound IgE. MacGlashan & Lichtenstein find that cells that are more responsive in general also respond well to dimers, whereas those that respond sluggishly in general respond poorly to dimers but quite adequately to trimers (100).

A fundamental question about the interpretability of the studies with small oligomers of IgE is raised by a recent observation of Holowka & Baird's group. They showed that cell-bound small oligomers of IgE with a multiplicity < 6 slowly coalesce and form larger clusters containing 100s to 1000s of molecules (101). This raises the question of whether the concept of a "unit signal" being as small as a dimer of receptors must now be discarded. The implicit assumption of the original proposal (95) was that oligomers of IgE whose *maximum* size was well defined would induce aggregates of receptors, the multiplicity of which had a similarly well-defined upper limit. If this is not the case, then although it might still be true that a dimer could initiate the chain of events, the size of the mechanistically active aggregate

of receptors would remain uncertain. Holowka & Baird and their colleagues have provided data that suggest the original proposal is still likely to be correct. Allowing receptors occupied by small oligomers to coalesce into larger aggregates prior to initiating release by a temperature jump had no effect on either the magnitude or the kinetics of the subsequent degranulation (98). Thus, although it is difficult to rule it out completely, the coalescence of aggregated receptors into larger clusters does not seem to play an essential role, and with responsive cells, dimers of receptors appear to be capable of generating at least a modest or, in some cases, an optimal signal. Whether this signal is adequate to induce substantial release appears to be related to other factors, still undefined. Where the question has been examined, all the perturbations that appear to reflect early events (see below) exhibit the difference between stimulation by dimers and trimers, etc.

Number of Aggregates

Localized aggregation of receptors by reagents bound to beads induces only localized degranulation (102). Furthermore, all the quantitative analyses suggest that more than a single aggregate of receptors is necessary to get maximum degranulation. But these studies also indicate that only a few per cent of the total receptors need to be aggregated to observe substantial release (23, 97). Nevertheless, the dose response curves are relatively shallow, and per unit of release, progressively larger numbers of receptors must be engaged as maximal release is approached (97). There are insufficient data to assess the stage at which the relative refractoriness occurs, but it is likely to be an early one. For example, the breakdown of phosphatidyl inositols (PIs) can be studied on RBL cells (see below) using trimers of IgE, and the maximum number of receptors that could have been aggregated can be determined from the number of trimers that were bound. Detectable breakdown is observed when no more than ~ 100 receptors per cell could have been aggregated (103), but even though more and more receptors become aggregated, a proportional increase in breakdown of PI does not occur. It is likely that the progressive disproportionality is amplified by the process of desensitization (see below). In any case, until some of the fundamental biochemical phenomena in both activation and inactivation can be identified positively and studied independently, the relationship between the numbers of receptors aggregated and the amount of release probably cannot be studied rigorously. On the other hand, it is already clear that the maximum release of mediators attained is not limited by either the number of receptors aggregated nor, for example, by the amount of PI broken down. Thus, maximal (but incomplete) release is

obtained before either of the latter two phenomena have reached a maximum (103).

Mechanistic Role of Aggregates

In later sections, we review a variety of phenomena each of which has been reported as an early consequence of aggregation of the receptors. The role of the receptor has not been defined for any of these events. In this section we, therefore, consider some general aspects of this problem and review what progress has been made in its solution.

INTRINSIC ACTIVITIES We previously indicated that it is useful to consider two classes of possible mechanisms (6). Upon aggregation, the receptor itself may assume one or more functions, such as forming a channel for ions or carrying out an enzymatic activity. The first steps in testing for such *intrinsic* activities have been accomplished: Methods of purification have been devised that obviate the need for using ordinary denaturants (32), and the deleterious effects of the mild detergents necessary to solubilize the receptors can be minimized either by maintaining a low micellar detergent:lipid ratio or only submicellar concentrations of detergents throughout purification (30, 56). Uncovering such intrinsic activities presents several problems. First, one must decide for which activities to test. Such experiments may require a considerable effort, because in such analyses *negative results mean nothing*, and because the activity one is searching for may be rather specific. For example, it has been proposed that a serine protease is activated as an early consequence of aggregation (see below). This could be analogous to the mechanism of activation of C1 where C1r becomes a protease. However, C1s is virtually the only known substrate for this enzyme! Therefore, the protease activity of the receptor for IgE, if it exists, could be missed entirely without the appropriate substrate. Furthermore, the intrinsic activity sought for may require the receptor (and its substrate, if there is one) to be incorporated in a bilayer structure—possibly in a bilayer whose total concentration of proteins is similar to the native one. Some general aspects of this latter problem are discussed in Grasberger et al (104). So far no evidence for an intrinsic function of the receptor has been presented.

EXTRINSIC ACTIVITIES The receptor, like IgE, may have no intrinsic function, and a distal component may be the one activated by interaction with the aggregated receptor. We have termed this an *extrinsic function* of the receptor (6). There are a variety of ways to find evidence for such components. Methods such as radiation inactivation and measurement of rotational or translational diffusion have the advantage of being applicable

to intact systems—even living cells—but can give only very indirect information. Direct methods involve trying to isolate a complex composed of the receptor and the relevant molecule, by maintaining the interactions with the use of gentle procedures for purification or covalent cross-linking reagents. Currently two candidates exist for molecules that interact with the receptor (see below), though it is not known whether this interaction is due to some intrinsic action of the receptor upon them or simply to aggregation of the receptor per se.

INITIATION AND PERSISTENCE OF PERTURBATIONS We comment on one additional general aspect of the role of aggregation: that is, whether a particular perturbation, once initiated, can persist in the absence of aggregation of the receptor. Only the very late stages of internalization of the receptor show this property. Thus, whatever the mechanistic role of the aggregation may be, it, rather than some later step, appears to be the time-dependent one for virtually all receptor-mediated events. However, there are the perturbations that, although initiated by and dependent upon aggregation throughout their own lifetime, appear not to persist throughout the lifetime of other aggregation-dependent steps (Table 2). The role of such phenomena is unclear. Whereas results with various inhibitors suggest that at least some of these perturbations play an essential role, they clearly

Table 2 Early perturbations stimulated by aggregating the receptor for IgE

Perturbation	Initiated	Lifetime	Dependent on extracellular Ca^{2+}
Morphological changes in plasma membrane	Very early	Throughout secretion	No
Immobilization of receptors	Very early	Throughout secretion	No
Internalization of receptors	Delayed	Throughout secretion	No
Activation of protease	Very early	Uncertain	No
Activation of methyl transferase	Very early	Brief ^a	Partial ^a
Rise in cAMP	Very early	Brief	No
Hydrolysis of PIs	Early	Throughout secretion	Yes ^b
Opening of channels for Ca^{2+}	Early	Throughout secretion	Yes

^a Refers to incorporation of counts into phospholipids.

^b See text. There are some data which suggest otherwise.

cannot simply be the initial step in a single linear sequence of events. They could, however, represent an essential, parallel stimulus.

EARLY EVENTS INITIATED BY THE RECEPTOR

Changes in Plasma Membrane and in Mobility and Distribution of Receptor

CHANGES IN THE PLASMA MEMBRANE Morphological changes in the plasma membrane of cells that were reacted with a variety of ligands have been documented in several systems (105–108). Similar events have recently been documented by Pfeiffer et al on RBL cells, after aggregation of receptor-bound IgE (109). Prior to stimulation the surface of these cells shows numerous microvilli (109, 110), but within 30 sec after aggregating the cell-bound monomeric IgE, the surface becomes highly lamellar. If the aggregation is induced by a multivalent hapten-protein conjugate the original morphology of the membrane is restored within minutes after the addition of hapten. As little as 5 ng of antigen—a dose that produced maximal secretion but only after 30 min—produced maximal morphological changes in seconds (109). Other changes that occur equally rapidly include a dramatic spreading of the cells and increased areas of adhesion to the substrate. A generalized increase in fluid pinocytosis occurs somewhat more slowly. All these events can proceed in the absence of added Ca^{2+} in the medium—a medium in which exocytosis does not occur. Even in the presence of Ca^{2+} , neither the morphological changes nor exocytosis occur if the cells are stimulated while in mitosis. This is true despite the fact that the internalization of the aggregated receptors (111) as well as the receptor mediated influx of Ca^{2+} (112), occurs normally.

CHANGES IN MOBILITY OF RECEPTORS Earlier studies using fluorescent labeling techniques and light microscopy, or ferritin-labeling techniques and electron microscopy (68, 113–119), showed that the diffusely distributed receptors for IgE, like many other membrane proteins, can be clustered with a suitable multivalent reagent. At least in those analyses that involved minimal perturbation of the cells (68), the involved receptors for IgE appeared to be independently mobile. Photobleaching techniques confirmed these observations and showed that the translational diffusion coefficient— $2 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ (117)—was similar to that found for other membrane proteins when these were studied with probes that did not aggregate them (120). Examination of the rotational dynamics and electrokinetic mobility of unliganded receptors and of receptors liganded with monomeric IgE has led to similar conclusions (120a, 120b).

Menon et al (98, 121) have extended such photobleaching studies on RBL

cells, using small preformed oligomers of IgE, as well as the "A₂" monoclonal anti-IgE antibody (88) which, as noted above, primarily forms dimers at low concentrations and larger aggregates only at high ratios of antibody to IgE. On a time-scale similar to that observed for the morphological changes in the plasma membrane, substantial decreases occurred in the recovery of fluorescence in the bleached areas—most likely due to immobilization of the receptors. This immobilization occurs in the absence of added Ca²⁺ in the medium and even at sharply reduced levels of cellular ATP. Dimers of IgE, which induce virtually no exocytosis in these cells, also do not induce immobilization. If D₂O is added, the cells do show a modest amount of release and a corresponding immobilization (121). Thus, a close correlation exists between the degree of immobilization of receptors, biochemical perturbations such as the influx of Ca²⁺, and the breakdown of phosphoinositides (see below), and secretion. Because all of the latter processes require added Ca²⁺ and normal levels of ATP, it is apparent that they cannot account for the immobilization. It is more difficult to prove the opposite, i.e. that the immobilization is a *necessary* antecedent for the other changes.

Suggestive data implicate an interaction of the aggregated receptors with cytoskeletal elements during immobilization. First, immobilization is not seen on the blebs of plasma membranes with which these investigators have recently worked (98). If anything, the mobility of unaggregated receptors on these blebs is increased, so that aggregation of receptors, which should proceed normally on them, appears, in and of itself, insufficient to explain the immobilization. Such blebs are, however, depleted of filamentous F-actin (122). In normal plasma membranes, a reversible attachment of aggregated receptors to actin has been implicated because of partial colocalization of fluorescently labeled clusters of IgE and phalloidin (see below). More direct evidence for such an interaction has also been obtained. If cells are reacted with a covalent cross-linking reagent prior to solubilization with detergent, the aggregated receptors remain in the detergent-insoluble pellet (26, 123)—a pellet which contains cytoskeletal elements.

The process by which aggregated receptors could become attached to cytoskeletal structures is unclear. Indirect evidence for an active process comes from the observation that phorbol esters can induce internalization of membrane proteins (124–28) including the receptor for IgE (K. Furuichi, C. Isersky, personal communication). The phorbol esters probably do aggregate the receptors directly. It is more plausible that they induce covalent changes in one or more cellular components involved in the interaction between the membrane protein and cytoskeletal components, either by activating protein kinase C (128) or by some other mechanism. Nevertheless, because the immobilization of the receptors for IgE (as well as

their coalescence and internalization; see below) is readily reversed by disaggregating them, it is likely that the normal (nondrug-induced) process is passive and based upon multivalent interactions.

Interaction with cytoskeletal components is also implicated in the clustering of aggregated receptors. As noted previously, Holowka & Baird and their colleagues (101) noted that when receptors are aggregated even with relatively small oligomers of IgE, patches containing thousands of receptors gradually form. Such clustering is not observed on the actin-depleted blebs, even when the latter are still attached to cells on which unblistered portions of membrane exhibit large-scale aggregation. These latter clusters appear to be enriched in actin, as assessed by staining with phalloidin (98).

INTERNALIZATION AND REEXPRESSION OF RECEPTORS

Effect of Monomeric IgE When the surface proteins of RBL cells are oxidatively iodinated, e.g. with ^{125}I , and the cells reacted with ^{131}I -labeled IgE at various times thereafter, a fall in the ratio of ^{125}I to ^{131}I is observed in the IgE-receptor complexes subsequently isolated (66, 129). Since any increase in the total number of IgE-binding sites is too small to account for the change in the ratio, the simplest interpretation is that labeled receptors are progressively degraded and replaced by new unlabeled receptors. Assuming such a mechanism, the half-life of the unliganded receptors has been calculated to be 8–12 hr (66, 129). If a similar experiment is conducted, but the cells are exposed to the IgE immediately after labeling and at the start of the incubation, the receptors liganded with IgE and the bound IgE are degraded more slowly. The latter finding agrees with the observation that in cells reacted with radiolabeled IgE and examined by electron-microscopic autoradiography, the retained radioactivity is observed on the surface of the cell only (73). One explanation for all of these results is that whereas unliganded receptors are internalized and degraded, receptors liganded with IgE are not internalized. If so, then the binding of monomeric IgE has some significant effect on the receptor and on its interaction with other cellular constituents. An alternative explanation should be considered. This posits that the binding of IgE produces no significant change in the receptor; the liganded and vacant receptors alike are internalized and exposed to lysosomes. However, just as the receptor can protect bound IgE from being hydrolyzed by exogenous proteases (47), so the bound IgE can protect the receptor from being degraded (28). Such undegraded IgE-receptor complexes would then recycle to the plasma membrane. That receptor-IgE complexes once internalized can be reexpressed (see below) shows that such a pathway exists. The failure to observe internalized monomeric IgE-receptor complexes in the electronmicroscopic studies (73) could be explained quantitatively. At any instant in time only a small

fraction of the total IgE bound to a normally cycling receptor might be in the interior, and thus it could escape detection.

That RBL cells incubated with IgE show an increase in total receptors (66, 129) could be related to the observation that basophils from individuals with high levels of serum IgE have increased numbers of receptors (130). Possibly, the normal mechanism by which the receptors/cell is controlled is simply a balance between degradation and biosynthesis, rather than a more elaborate counting mechanism.

Effect of oligomeric IgE If cells are reacted with preaggregated IgE, or the monomeric IgE on their surface is aggregated with an appropriate multivalent antigen, the receptors, the IgE, and, if present, the antigen are fairly rapidly internalized. This phenomenon has been examined in detail by Isersky and her collaborators (119, 131–135). Their principal findings are: (a) Dimers of IgE provide a sufficient stimulus for internalization. Even on RBL cells, where dimers induce little secretion (especially in the absence of D₂O) and exhibit minimal immobilization (see above), the process proceeds expeditiously although not as rapidly as when trimers or higher oligomers are tested (119). (b) The internalized covalent oligomers and the receptors to which they are bound are not reexpressed (119). (c) The kinetics of internalization are similar on rat peritoneal mast cells and the RBL cells ($t_{1/2}$ 3–5 min), even though the rate of secretion by the mast cells is ~20-fold greater than that by RBL cells (131). (d) As is true of the morphological changes in the membrane and the immobilization of receptors described above, internalization proceeds as rapidly in the absence of added Ca²⁺ as in its presence (131). (e) The process is incomplete, with 40% or more of the IgE remaining on the surface (119, 131, 132). (f) A fraction of vacant receptors, or receptors liganded with monomeric IgE, transiently “coendocytose” when receptors aggregated with IgE are internalized (119). The cointernalization is not totally independent of added Ca²⁺, and several inhibitors of exocytosis ablate cointernalization of monomeric receptors without affecting internalization of aggregated ones (133). Peculiarly, the fractional coendocytosis is relatively insensitive to the ratio of monomeric to aggregated receptors. The process appears to be specific in that internationalization of monomeric IgE-receptor complexes is not observed even when the receptors for IgG on the same cells are extensively aggregated. These results suggest that at least a proportion of the monomeric receptors may interact with aggregated receptors of the same type (134). A similar conclusion was reached by the Ishizakas on the basis of quite different methods (118). The failure to observe such interactions when copatching or immobilization were examined (68, 98, 117) may have resulted from methods that were less sensitive and that therefore failed to detect

small amounts of such interactions. (g) Even at fairly late stages of internalization induced by a multivalent hapten-conjugate, the process can be reversed by addition of hapten (135). Thus, internalization, like so many other phenomena we shall review, is in large part maintained only so long as the receptors remain aggregated.

Relationship to secretion Since the morphological changes in the plasma membrane and the changes in mobility and distribution of the receptors are manifested in the absence of added Ca^{2+} , they must be initiated independently of other early events, such as the influx of Ca^{2+} and the hydrolysis of the phosphatidyl inositides, which require added Ca^{2+} (see below). It is unclear, however, whether the former are parallel phenomena or early steps in the sequence leading to secretion. This question is difficult to explore because most methods provide average data for the entire population of receptors, whereas it is known that aggregation of only a tiny fraction of receptors is sufficient to obtain a near-maximal response of the cells (see above). One experimental finding—but only one—suggests that at least the internalization of the aggregated receptors is not required: When the surface IgE is aggregated by concanavalin A attached to large beads, localized degranulation is observed (102). A test of whether aggregated, internalized receptors are competent to induce secretion could be performed with an impermeant hapten.

Activation of Proteases

Over 20 years ago Austen & Brocklehurst reported that inhibitors and substrates of proteolytic enzymes inhibited secretion by lung mast cells from guinea pigs (136). This basic observation has been reproduced many times with a variety of mast cells, with basophils, and with RBL cells, and with many different inhibitors and substrates that interact with serine proteases (reviewed in 8). Some confusion arose because there appear to be two stages of secretion sensitive to such inhibitors, but it now appears clear that one of these is at an early step. Thus, all of the biochemical changes we describe in subsequent sections appear to be blocked by these inhibitors. Whether the inhibitors also block the morphological changes of the plasma membrane and the changes in mobility and distribution of the receptor that occur upon aggregation of the latter has not been reported. However, preliminary data on RBL cells (K. Furuichi, C. Isersky, unpublished results) suggest that internalization is not inhibited by doses of phenylmethylsulfonyl fluoride that would be expected to block secretion.

The inhibitors of proteases are only effective if they are present during the aggregation of the receptors. If the cells are reacted with the inhibitors and washed prior to stimulation, the receptor-mediated changes occur nor-

mally. This implies that aggregation of the receptors is required to activate a protease which then becomes sensitive to the inhibitor. This protease has been dubbed E^r (137). Evidence exists for an analogous entity (E^d) the receptor-mediated activation of which is required for receptor-induced desensitization (see below) (137, 138). The latter enzyme presumably has a lower K_i for the inhibitors than E^r , since *low* doses of inhibitors (10–40 nM in mouse mast cells) can block desensitization (induced by aggregating receptors in the absence of Ca^{2+}) without blocking the release that occurs when Ca^{2+} is subsequently restored (137, 138). This also indicates that E^d can be activated in the absence of Ca^{2+} . Similarly, activation of E^r appears to be Ca^{2+} independent. Thus, if the receptors are aggregated in the absence of Ca^{2+} and in the presence of *high* doses (10^{-4} – 10^{-3} M) of inhibitor and the cells then washed prior to addition of Ca^{2+} , the expected secretion is not observed. Presumably, the receptor-activated E^d and E^r were both inactivated by the inhibitors during the incubation in the Ca^{2+} -free medium. These results are consistent with receptor-mediated activation of two discrete proteases as an early event.

That exogenously added proteases can induce Ca^{2+} -dependent release (139 and references therein) is consistent with such a model, but of course this result can also be explained otherwise. Although it would be interesting to know if exogenous proteases also initiate some of the biochemical changes induced by aggregation of the receptors for IgE, it is difficult to conceive how their use can provide insight into the nature of the putative endogenous enzyme(s). The substantial concentrations of inhibitors that are required to inhibit E^r (see above) make it impractical to use affinity-labeling techniques with radioactive inhibitors to try to identify the enzyme, although this approach might work for E^d . Identifying the molecular basis for the phenomenological observations is a major experimental challenge.

Activation of Methyltransferases

Activation of methyltransferases as an essential step in IgE-mediated secretion by mast cells was first reported by Hirata & Axelrod (140). We discuss these and related studies in some detail, first because it has been proposed that methylation may be one of the earliest steps in secretion, and second because of the controversy that surrounds these findings. To evaluate the experimental data, the metabolic pathways must be understood and some of the methodological problems in exploring them appreciated. We divide our discussion into two sections. We first consider the evidence that methyl transferases are activated by the receptor. We then analyze the evidence that the relevant substrates are phospholipids.

GENERAL ASPECTS Methylation reactions control many biochemical pathways. Discrete methyltransferases catalyze the transfer of a methyl group to

either phospho- or neutral lipids, to proteins, or to DNA, or RNA (t, r, and m) (141, 142). These modifications are thought to be important in cellular functions such as chemotaxis, transport, and gene expression. Despite the wide range of substrates, all methyl transfer enzymes use s-adenosylmethionine (AdoMet) as the methyl donor and all are competitively inhibited by s-adenosylhomocysteine (AdoHcy), the demethylated product of AdoMet (Figure 2). It should be noted, however, that AdoMet is also a substrate for AdoMet decarboxylase (143) and interacts with a variety of other proteins (reviewed in 141).

Since the K_i for AdoHcy of various transmethyases vary over a 1000-fold range (144), the activity of a particular enzyme will depend on the relative concentrations of AdoHcy and AdoMet, and on the ratio of its K_m for AdoMet to its K_i for AdoHcy. Certain of these enzymes may thus be regulated by the intracellular concentration of AdoHcy via AdoHcy hydrolase.

The latter enzyme is the only one that metabolizes AdoHcy in eukaryotic cells. It cleaves AdoHcy into adenosine and homocysteine (Figure 2)—a hydrolysis that is readily reversible. In fact, the equilibrium constant probably favors synthesis, but under normal conditions adenosine is rapidly metabolized by adenosine deaminase and the AdoHcy hydrolase reaction is pulled towards hydrolysis.

Many investigators use pharmacological agents to raise the intracellular levels of AdoHcy, in order to establish that a methylation reaction is involved in a particular cellular function. Addition of adenosine plus homocysteine increases AdoHcy by driving AdoHcy hydrolase in the synthetic direction. This effect can be potentiated and prolonged by

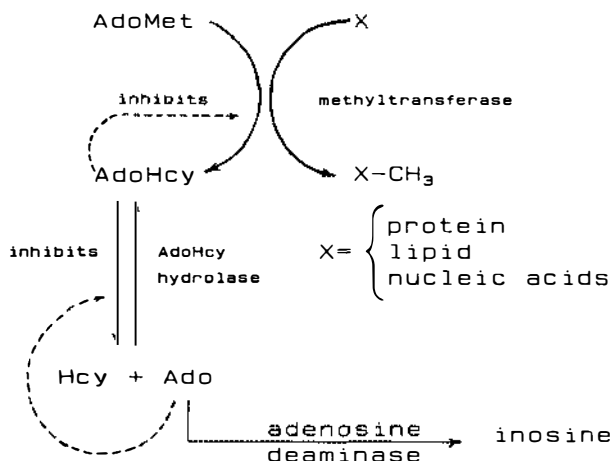


Figure 2 Enzymes that control methyl transferase reactions.

inhibitors of adenosine deaminase. In addition, there are analogues of adenosine and AdoHcy that inhibit AdoHcy hydrolase and cause an elevation of AdoHcy. Several adenosine analogues are substrates for AdoHcy hydrolase. Administering these compounds, along with homocysteine, or an analogue thereof, results in the buildup of analogues of AdoHcy that also inhibit transmethyloses (141, 142).

EFFECTS OF INHIBITORS Inhibitors of methyltransferases block IgE-mediated secretion by a variety of mast cells as well as by RBL cells (8, 145). Evidence that early receptor-mediated events are affected is based on the concomitant inhibition of (a) stimulated methylation of phospholipids, (b) the early rise in cAMP, (c) the influx of ^{45}Ca , and (d) an absence of an effect on histamine release stimulated by Ca^{2+} ionophores (146). Numerous studies of the effects of inhibitors of methylation have been reported. For example, human lung mast cells were incubated with 3-deaza-adenosine and homocysteine (thiolactone (147). These should induce AdoHcy hydrolase to synthesize 3-deaza-adenosine-homocysteine, an inhibitor of methyltransferases. That action on the latter was responsible for the observed inhibition of the cellular activities was strongly supported by the counter-inhibitory action of AdoMet (148, 149). Although higher doses of the inhibitors were required to diminish histamine release than to diminish the rise in cAMP and the influx of ^{45}Ca (Figure 6 in 147), this can be easily rationalized. Only a fraction of the receptors must be aggregated to produce maximal secretion (see above), whereas at least some of the biochemical perturbations that are stimulated by the receptors are directly proportional to the number of aggregates formed (see below). Thus, under conditions where most of the receptors are aggregated, it is likely that a receptor-mediated early event would have to be substantially inhibited before a decrease in secretion was evident. Studies on RBL cells have similarly shown that inhibitors of methyltransferases cause concomitant inhibition of influx of ^{45}Ca and of secretion (150).

Further evidence that the action of the inhibitors of methyltransferase is specific comes from studies of their effect on desensitization, which we discuss more fully below. That the inhibitors can actually prevent inactivation of the cells under conditions where secretion by untreated cells is inhibited (137) is reassuring.

If the aggregation of receptors leads to activation of methyltransferases, one might be able to observe this indirectly by finding some decrease in their substrate, AdoMet. The published data on such experiments are contradictory. Moore et al failed to observe any decrease in RBL cells (151), whereas Ishizaka et al (152) saw a 12-fold drop in incorporated radioactive AdoMet with mast cells. The latter is rather surprising since it implies that

upon triggering of the receptors for IgE, the cells metabolized over 90% of their pool of AdoMet in ~ 15 sec!

METHYLATION OF PHOSPHOLIPIDS Hirata et al first reported that bridging the cell-bound IgE on mast cells with concanavalin A promoted methylation of phospholipids (153). This group investigated the phospholipid methyltransferases in several systems. From studies on red blood cells, they proposed that methyltransferase I, in the inner leaflet of the membrane, monomethylated phosphatidylethanolamine which, upon flipping to the outer leaflet, was further methylated by a methyltransferase II located there (154). Although this pathway is a physiologically minor one for generating phosphatidylcholine, they proposed that activation of these enzymes is a critical event in the stimulation of cells by several different receptors (140).

Before reviewing the findings on mast cells and RBL cells, two methodological points should be stressed. Virtually all the studies where changes in methylation of phospholipids have been claimed involve simply the measurement of chloroform extractable counts from cells whose AdoMet pool has been labeled by incubation in [methyl³H] methionine. Although it has been stated that the phospholipids in the chloroform phase showed "a marked" incorporation of radioactive methyl groups (152), the fraction of the radioactivity in such extracts is not regularly assessed. Moore et al recently showed that as little as 30% of such counts may be associated with phospholipid, the remainder being in undefined moieties (151). It should also be noted that in experiments with inhibitors, only a single time point is routinely reported so that one cannot determine whether incorporation of methyl groups into chloroform-soluble moieties was reduced or simply slowed.

Ishizaka and her colleagues regularly observe incorporation in mast cells whose receptors for IgE have been aggregated in a variety of ways (8). Recently they showed that interrupting the aggregation diminished the incorporation (149), but a dose response curve relating the number of receptors aggregated to the extent of incorporation has never been reported.

The time course of incorporation is curious. It begins promptly, peaks at 15 sec after addition of stimulant and returns virtually to baseline in 30 sec to 2 min. Since their earlier data showed rapid marked depletion of radioactive AdoMet (above), one can rationalize a failure to see a further rise in radioactivity, but the decline suggests virtually immediate breakdown of the chloroform-soluble moieties. There is nothing in the current models that suggests a mechanism or role for this rapid change. If the radioactively labeled AdoMet is rapidly metabolized, this does not of course mean that there is a net change in total cellular AdoMet since this

might be continuously replenished. If so, then similarly any methylated products derived from the newly synthesized AdoMet would be unlabeled. Therefore, these experiments are not informative about any net changes in phospholipids as a consequence of aggregation of the receptors. In analogous experiments on RBL cells, Crews et al presented similar results, although the kinetics of incorporation, of ^{45}Ca uptake and of secretion were all substantially slower (150). Using nominally the same line of cells, Moore et al (151) failed to observe stimulated incorporation of methyl groups, although IgE-mediated influx of $^{45}\text{Ca}^{2+}$, release of arachidonic acid, breakdown of PIs (see below), and secretion were substantial. They used conditions that gave optimal incorporation of [methyl- ^3H] methionine into AdoMet but observed no enhanced incorporation of counts into individual phospholipids as assessed by high pressure liquid chromatography. As already noted, they also failed to see any enhanced metabolism of AdoMet.

The basis for this discrepancy is unclear. Clearly, it is more reliable to quantitate changes in the specific phospholipids directly than to rely simply on counts extracted in the chloroform phase. It would also be useful to see if the differences in the specific methods used were important (cf 150, 151). Finally, it is regrettable that in the study by Moore et al, the effects of inhibitors were not tested since, as we emphasize here, the question of whether methyltransferases are involved and if so, which substrates they modify, are separate issues.

Ishizaka tried to define more precisely which of the phospholipid methyltransferases were involved (148) by exploiting the finding of Hirata & Axelrod that transferase I requires Mg^{2+} but not Ca^{2+} (155). Ishizaka reported that when mast cells were incubated in the presence of Mg^{2+} and EGTA, methyl groups were incorporated only into phosphatidylethanolamine as expected. She also observed that receptor-mediated incorporation of ^{32}P into phosphatidic acid, phosphatidylcholine, and phosphatidylinositol was unabated. It should be noted that this is the first report to claim that inhibitors of methyltransferases such as 3-deaza-adenosine inhibit receptor-mediated breakdown of PIs.

If an early consequence of aggregating the receptor for IgE is activation of phospholipid methyltransferases, it might be expected that this could be observed with preparations of plasma membranes. Positive results have been reported for membrane preparations from rat mast cells (156) but are difficult to evaluate. First, the results are reported in dpm, whereas comparable experiments on mast cells are reported in pmol, and there is insufficient information to convert one unit into the other. Second, the incorporation by the intact cells was measured at 15 sec as usual, whereas that by the membranes was determined at 60 min. Thus, it remains to be

proven that such preparations show a significant amount of activity relative to intact cells.

McGivney et al reported an innovative approach to this subject (157). They treated RBL cells with a powerful mutagen and selected clones grown from the small number of surviving cells which (a) bound IgE, (b) failed to secrete in response to aggregation of cell-bound IgE, (c) did secrete in response to ionophore, and (d) were deficient in either phospholipid methyltransferase I or II. When a clone missing enzyme I was fused with one missing enzyme II, IgE-mediated secretion was partially restored. Their strategy is modeled on one used to select specific bacterial mutants. However, with bacteria, specific metabolic intermediates can be used to growth-select appropriate auxotrophs. This could not be done with the mutagenized RBL cells, so the number of genes mutated in the chosen variants is indeterminate. Many complementation studies would have to be performed to demonstrate that it was loss of the phospholipid methyl transferases that was the only relevant variable.

APPRAISAL After reviewing the published data we conclude that the evidence for involvement of methyltransferases—evidence derived principally from studies with inhibitors—is more solid than the evidence that phospholipids are the relevant substrates. That no increase in methylation of bulk cellular proteins (and polynucleotides) has been observed (150, 152) could simply reflect inadequate sensitivity or unrecognized degradation. As noted above, even the incorporation into chloroform-soluble moieties is relatively transient. It is also worth noting that whereas the results with inhibitors suggest that methyltransferases are involved in early steps that precede the rise in intracellular Ca^{2+} , the methylation of phospholipids could involve much later steps, unless it can be proven that lipids are the only substrates on which the stimulated methyltransferases are acting. In the end, proof for the role of methyltransferases will have to await information about what their activation accomplishes in molecular terms. There are no data whatsoever on this matter.

Activation of Adenylate Cyclase

PRINCIPAL FINDINGS Aggregation of the receptor on mast cells initiates a rapid rise in cAMP (8). The nucleotide increases to a maximum in less than 30 sec and returns to basal levels in ~ 2 min. The rise in cAMP begins a little later than the stimulated incorporation of methyl groups (see above), and despite earlier reports to the contrary (158), recent studies show that it can be blocked by inhibitors of methyl transferases (8). Since the rise in cAMP occurs in the absence of extracellular Ca^{2+} (137) and prior to the stimulated uptake of $^{45}\text{Ca}^{2+}$ (seen in the presence of Ca^{2+}) (148), it appears to be an

early event initiated by aggregation of the receptors. As with other receptor-mediated events, the apparent stimulation of adenylate cyclase is blocked by protease inhibitors (8). Indirect evidence for a receptor-mediated increase in cellular cAMP has also been obtained by demonstrating increased levels of cAMP-activated protein kinases (159, 160).

Since adenylate cyclase is a plasma membrane protein, one anticipates that the receptor-mediated stimulation of this enzyme should also be observable on isolated membranes. Although positive results have been claimed (156), the stimulation appeared to be substantially less than that observed on intact cells. For the membranes, the rise in cAMP took ~ 60 min to reach a maximum (cf above), and we calculate that, per cell equivalent, the stimulation of the cyclase in the membranes was only $\sim 2 \times 10^{-5}$ of that observed in intact cells! Even if we correct for the fact that binding of IgE to the membranes was only 0.3% of that expected for intact mast cells, the recovery of activity was only $\sim 0.6\%$.

SIGNIFICANCE The significance of the apparent stimulation of adenylate cyclase is unknown. That no rise in cAMP is seen in receptor-mediated secretion from RBL cells (145) suggests that the rise observed in mast cells may be incidental rather than essential. Possibly, it is related to receptor-mediated inactivation (desensitization) (below), since mast cells are much more readily desensitized than RBL cells. Furthermore, when intracellular cAMP is increased artificially, secretion of mediators is frequently inhibited rather than enhanced, although discrepant results have been reported (161). Perhaps the rise in cAMP represents simply a side effect of the interaction between the receptor and GTP-binding regulatory proteins—an interaction that has been proposed to mediate receptor-initiated activation of phospholipase C (see below). Studies analogous to those with inhibitors of methyl transferases that have strengthened the case for the importance of those enzymes (see above) would be useful for evaluating the significance of the stimulation of adenylate cyclase.

Breakdown of Phosphatidyl Inositides (PIs)

The breakdown of PIs in calcium-dependent activation of cellular events by membrane receptors is receiving increasing attention (162–164). After outlining the elements of this system, we will briefly describe older data on mast cells and then focus on newer studies on RBL cells.

METABOLISM OF PIs Phosphatidyl inositol (PI) and its phosphorylated derivatives phosphatidyl inositol-4-phosphate (PIP) and phosphatidyl inositol-4,5-bisphosphate (PIP₂) are ubiquitous components of eukaryotic membranes; in mammalian cells they constitute 5–7% (PI), 1% (PIP), and 0.4% (PIP₂) of the total phospholipids (164). PI is synthesized from the

glycerol backbone in the endoplasmic reticulum, from which it is transferred to the plasma membrane. Because the PI kinases as well as other relevant enzymes appear in the cytoplasm (as well as on membranes), it is assumed that the PIs are chiefly localized in the inner leaflet of the plasma membrane (165, 166).

In some instances (167) the receptor-mediated release of the inositide moiety can be catalyzed by a phospholipase D (which, therefore, also generates phosphatidic acid), but in most systems it is phospholipase C (which generates diacyl glycerol and inositol phosphates) whose action is enhanced. Soluble cytoplasmic forms of the latter enzyme have been isolated from liver and from sheep seminal vesicles (168, 169). The enzymes from the latter show greater activity towards PIP and PIP₂, when these lipids are presented in vesicles; and the vesicles hydrolyze them at submicromolar concentrations of Ca²⁺ (170). Whether membrane-bound phospholipase C activity (171–173) represents a distinct entity is uncertain. It is also unclear whether the role of receptors in initiating breakdown of the PIs is to activate a membrane-bound enzyme, to recruit cytoplasmic enzyme to the membrane, or simply to mobilize membrane PIs making them more susceptible to hydrolysis.

Despite the ability of variations in Ca²⁺ concentrations to modulate the activity of phospholipase C (170, 174, 175), current models of receptor action postulate that this pathway is activated directly. Receptors are thought to stimulate the enzyme—possibly through an intermediary GTP-binding regulatory protein (below)—releasing inositol phosphates that in turn release Ca²⁺ from intracellular stores (163).

OLDER REPORTS ON MAST CELLS Early studies reported turnover of PI as a consequence of aggregating receptors for IgE on mast cells on the basis of an enhanced incorporation of ³²P and/or ³H-inositol into PI, phosphatidyl choline, and phosphatidic acid (176–178). The dose response curve of release of histamine and incorporation of ³²P into these lipids correlated well. However, when stimulation was performed in the absence of Ca²⁺, incorporation of ³²P and ³H-inositol into PI persisted whereas secretion did not occur. These studies have methodological drawbacks that were common to a number of early reports in this field: (a) The techniques for either extracting or separating the lipids did not permit one to visualize the inositol polyphosphates (179) and (b) brief preincubation with the radioactive label (³²P or ³H-inositol) and measurements of the lipids after rather lengthy stimulation with the agonist prevented direct observation of the initial stages in the enhanced breakdown. In mast cells the breakdown of the PIs is initiated 5 to 30 sec after activation, and 1 to 2 min later the resynthesized lipids return to their baseline values, increasing further after

longer incubations (180, 181). Short incubation times are particularly important for observing the rapid hydrolysis of the inositol polyphosphates, especially when this is assessed by quantitating the lipids rather than the water-soluble breakdown products. Thus, an enhanced phosphorylation of diacyl glycerol (to phosphatidic acid) is observed in mast cells within 8 sec after activation, whereas labeling of PI and phosphatidyl choline starts only after two min (177).

STUDIES ON RBL CELLS

Correlation with aggregation of the receptors Activation of RBL cells by aggregating the bound IgE with an appropriate antigen increases the level of the inositol phosphates within 10 to 40 sec (182). Experiments with preformed oligomers of IgE showed an excellent correlation between the amount of oligomer bound (and, therefore, of receptors aggregated) and the rise in the inositol phosphates (103). Larger oligomers were more effective than trimers, and trimers much more than dimers—exactly what was observed when secretion and rises in $[Ca^{2+}]_i$ were measured (see below). Similarly, the effectiveness of all of the oligomers was enhanced by D_2O . As was true for the rise in intracellular Ca^{2+} , doses of oligomers above those necessary to achieve maximal secretion further enhanced the breakdown of PIs. Thus, the failure to obtain 100% release of mediators cannot be ascribed to limitations of the metabolism of PIs. Finally, it is noteworthy that—again as was true for the rise in $[Ca^{2+}]_i$ —doses of trimer and larger oligomers, necessary to achieve maximal secretion, led to equivalent breakdown of the PIs. Interrupting the aggregation of the receptors promptly halts several receptor-initiated phenomena including secretion, but such protocols have not yet been applied with respect to turnover of PIs.

Characteristics of breakdown products After stimulation, RBL cells exhibit an initial rapid rise in IP_2 , followed by a smaller increase in IP_3 . Both of the latter reach a plateau value at about 10 to 15 min, whereas the rise in IP, which begins at about the same time as the rise in IP_3 , continues virtually linearly for over 20 min (Figure 1 in 182). Since the polyphosphoinositides are present in much smaller amounts than PI (the ratio is about 1:1:20 in RBL cells for PIP_2 , PIP, and PI, respectively), it is apparent that there is a substantial preferential breakdown of PIP_2 and PIP. However, it is not clear whether the IP is generated exclusively from the action of phosphatases on IP_3 and IP_2 (as is the case in some systems (183)), or whether it also results from direct breakdown of PI. If the latter is not the case, then only continuous synthesis of PIP and PIP_2 can account for the amount of IP that is generated. Possibly the inhibition observed when the cells are depleted of ATP (184) is due to reduced synthesis of PIP and PIP_2 .

In mast cells PIP also decreases, reaching a minimum 5 sec after stimulation and thereafter increasing (apparently at the expense of PI) back to baseline values at about 30 sec (181). In those studies both PIP and PI reached levels higher than those prior to stimulation at 1 and 2 min respectively. No substantial changes in PIP_2 were noted.

Dependence on Extracellular Ca^{2+} The breakdown of PIs initiated by aggregating the receptors for IgE on RBL cells is dependent upon adequate extracellular Ca^{2+} , in apparent contradiction to the situation observed in mast cells (see above). It is possible that the results on mast cells were amplified by a failure to reach isotopic equilibrium during the short incubations employed. The apparent synthesis of PIs may have been relatively minor and cannot be assumed to reflect accurately the extent of breakdown of the PIs. In RBL cells the variation in breakdown as a function of external Ca^{2+} parallels exactly the variation in the rise of intracellular Ca^{2+} and secretion (182). However, ions such as La^{3+} and Zn^{2+} that inhibit the rise in $[\text{Ca}^{2+}]_i$ and secretion [presumably by blocking the passage of Ca^{2+} through receptor-induced channels (see below)] do not block receptor-mediated stimulation or breakdown of PI. Since under these circumstances the IP_3 generated should release Ca^{2+} from internal stores, it is curious that no change in quin-2 fluorescence was detected (182). Raising the intracellular Ca^{2+} directly with ionophores does not in itself substantially stimulate hydrolysis of PIs.

ROLE OF GTP-BINDING PROTEINS We have already noted that in some systems a GTP-binding regulatory protein has been postulated to serve as a go-between from the activated receptor to the phospholipase C, in analogy to the role such proteins play in receptor-mediated activation of adenylate cyclase (185–188). The evidence is based partly on the effect of GTP or its nonhydrolyzable analogues and partly on the use of toxins such as pertussis, at least one of those actions is on the inhibitory GTP-binding protein N_i . Although effects of both types of reagents have been noted on mast cells, their site of action is by no means certain (189–191).

Pertussis toxin has been reported to inhibit passive cutaneous anaphylaxis in the rat and to inhibit partially IgE-mediated release of histamine from peritoneal rat mast cells in vitro (192). We have seen no effect of the toxin on IgE-mediated turnover of PIs or on secretion from RBL cells, even though ADP-ribosylation of a 41 kd protein was documented (unpublished observations in collaboration with J. Moss). The effect of the toxin on breakdown of PIs mediated by the receptor for IgE has not been reported for mast cells. The GTP analogue, Gpp(NH)p, introduced into transiently permeabilized mast cells, causes secretion in the presence of Ca^{2+} , and this reaction is inhibited by neomycin (thought to act on PIs) (179). However, these findings are insufficient to prove the involvement of a GTP-binding

regulatory protein in the breakdown of PIs induced by the receptor for IgE.

We have explored the possibility that the receptor itself might be a member of the family of the GTP-binding regulatory proteins, since there are some obvious similarities between the subunit structure of the receptor and these proteins (185). Comparative peptide-mapping data and our initial analyses of the amino acid composition of the β subunit suggest no such relationship. Nor have we seen consistently positive results on Western blots using antibodies (193) that react with the β chain common to all GTP-binding proteins (J.-P. Kinet, H. Metzger, unpublished observations).

SUMMARY AND FUTURE DIRECTIONS The special feature of the receptor-induced breakdown of PI in RBL cells (but less clearly on mast cells) is its dependence on extracellular Ca^{2+} —a dependence not observed in several other systems. Since the evidence is against the stimulation of breakdown being initiated (or even requiring) a rise in *intracellular* Ca^{2+} (see below), the high concentration of extracellular Ca^{2+} that is required [$K_m \sim 0.4$ mM (192)] must be for an earlier step. The receptor-induced *formation* of ion channels occurs even in the absence of Ca^{2+} , but the *specificity* of such channels for Ca^{2+} requires concentrations of extracellular Ca^{2+} similar to those required to initiate breakdown of PIs (see below). If these observations are related, then either these latter phenomena share a common Ca^{2+} -dependent antecedent step or one of them precedes the other. If the breakdown of PIs is a necessary antecedent for the initiation of channels specific for Ca^{2+} , this would provide a ready explanation for the existence of the PI pathway in this system.

It should be emphasized that there is still no evidence that hydrolysis of PIs plays a significant role in the cellular functions stimulated by the receptor for IgE. The lack of specific inhibitors of this system forecloses one obvious approach to this question. One substance that does inhibit receptor-induced breakdown of PIs and the rise in cytosolic Ca^{2+} completely (but inhibits secretion only partially) is phorbol myristic acetate (194; M. Beaven, personal communication). This is interesting because this substance stimulates protein kinase C, an enzyme that is also activated by a metabolite of PI (and other phospholipids): diacylglyceride. Whether the receptor-mediated breakdown of PI secondarily stimulates protein kinase C, and how this may relate to other receptor-mediated secretory events, are under investigation.

It will ultimately be important to reconstitute the receptor-mediated hydrolysis of PIs using purified components. As a step in that direction, the demonstration of receptor-mediated breakdown on isolated plasma membranes is important. Although a variety of preparations of such membranes from RBL cells contain phospholipase C activity, we have not observed

stimulation of the enzyme when the receptors are aggregated, even after readdition of cytoplasmic components. Indeed, cells simply permeabilized (with saponin), as gently as possible, lose their responsiveness even though the cytoplasmic contents have not been aspirated from the sample. In experiments on cytoplasmic phospholipase C, the hydrolysis of the PIs is markedly affected by the other phospholipids present in the mixture and by deoxycholate (174, 175). Similarly we and others (195) have found that hydrolysis of PIs in intact cells is exquisitely sensitive to low concentrations of organic solvents. It appears that the topological relationships between the proteins and lipids are critical in these reactions.

Increase in Intracellular Ca^{2+}

OLDER STUDIES (196) Since the observations of Mongar & Schild (197), it has been repeatedly shown that mM concentrations of extracellular calcium are required for optimal receptor-mediated secretion. Because maneuvers that directly raise the intracellular Ca^{2+} induce secretion (198–202), it is reasonable to postulate that the receptors for IgE initiate a transfer of calcium ions from outside the cell to the inside. Aggregation of receptors induces an enhanced uptake of $^{45}\text{Ca}^{2+}$ (150, 203), which supports this proposal. Although this apparently enhanced uptake could be due to a stimulated decrease in efflux, direct assessment of this with RBL cells showed that efflux actually was stimulated, not diminished (204). The enhanced uptake of $^{45}\text{Ca}^{2+}$ could also result simply from an enhanced exchange across the membrane without a net increase in cytoplasmic Ca^{2+} , and this possibility cannot be excluded by such studies. Recent investigations have more directly demonstrated a net rise in intracellular Ca^{2+} and provided evidence that this occurs owing to the receptor-induced opening of ion channels in the plasma membrane.

MEASUREMENTS OF $[\text{Ca}^{2+}]_i$ The development of the fluorescent tetracarboxylate probe quin-2 by Tsien (205) has permitted rather simple quantitation of intracellular Ca^{2+} , and a detailed examination using this approach with RBL cells has been performed by a group in Cambridge, United Kingdom (151, 182, 206). Measurements on unstimulated cells showed the intracellular concentration of Ca^{2+} to be 104 ± 5 nM, and over brief periods this was essentially independent of extracellular Ca^{2+} in the range 0.05 to 1.0 mM. At very low concentrations of extracellular Ca^{2+} (< 5 μM) obtained by EDTA, the $[\text{Ca}^{2+}]_i$ progressively decreased, the rate of efflux being ~ 60 amol ions/cell/min; adding back Ca^{2+} to the medium caused a corresponding rate of uptake. This dynamic balance of $> 3.6 \times 10^7$ ions/cell/min, which maintains an intracellular concentration of ~ 100 nM, was resistant to marked decreases in intracellular ATP.

Upon aggregation of the cell-bound IgE with antigen (ovalbumin), or with concanavalin A, a prompt increase in $[Ca^{2+}]_i$ was observed at rates greater than 1 fmol/cell/min under maximal stimulation. This is greater by a factor of two than the rate that can be calculated from the ^{45}Ca measurements of Crews et al on RBL cells (150). Intracellular concentrations up to 12-fold higher than the resting concentrations were observed by Beaven et al (206). The essential features of receptor-mediated increases in $[Ca^{2+}]_i$ were these: (a) The increase was only observed at $[Ca^{2+}]_o > 50 \mu M$. Whereas the normal influx pathway appeared to saturate at $[Ca^{2+}]_o > 0.1 mM$, the pathway stimulated by the receptors was saturated only at 10-fold higher concentrations, with an estimated $K_m \sim 0.4 mM$. In studies on rat mast cells, White et al (207) observed an increase in $[Ca^{2+}]_i$ upon aggregation of receptors even in the presence of EGTA. However, in those studies the cells were exposed to 100 μM quin-2 (cf 206), a concentration at which there is evidence of cell cytotoxicity (151). This seriously complicates the interpretability of that study. (b) The rise in $[Ca^{2+}]_i$ was directly related to $[Ca^{2+}]_o$ (206). (c) After an initial rapid rise, the $[Ca^{2+}]_i$ showed only a slow decrease as long as the cross-linking of the receptors was maintained. When aggregation of the receptors was disrupted, there was a rapid decrease similar to the decrease observed by addition of lanthanide ions or EDTA. (d) The enhanced transport of Ca^{2+} into the cell required cellular ATP levels well above those required for the transport of Ca^{2+} into unstimulated cells (see above).

These studies were recently extended in experiments employing covalent oligomers of IgE (103). Such oligomers allow one to vary both the total number of receptors aggregated and the multiplicity of the individual aggregates that form more rigorously than can be accomplished either with antigen or with anti-IgE antibodies. The new studies by Maeyama et al (103) document the relationship between the aggregation of receptors and rises in $[Ca^{2+}]_i$. They also show that the enhanced effectiveness of larger oligomers as inducers of secretion is mirrored by the enhanced increases in the intracellular Ca that they induce. At the higher doses of the larger oligomers, more receptors become aggregated than appear to be necessary to generate the maximal amount of secretion. It is noteworthy that in the studies of Maeyama et al the maximal $[Ca^{2+}]_i$ continues to increase until doses of IgE are reached that saturate the receptors. This differs from the release of histamine but is similar to the hydrolysis of PIs (see above). These findings suggest that the maximal amount of secretion is not limited as a consequence of the mechanism that generates the rise in cellular Ca^{2+} . Similar to all other receptor-mediated events that have been examined so far, D_2O substantially enhanced the uptake of Ca^{2+} (103).

The relatively low extinction coefficient (< 5000) and quantum yield (0.03

to 0.14) of quin2 require that loadings of several tenths of millimolar or more (internal concentration) are necessary to overcome cellular auto-fluorescence. Such concentrations may perturb cells [e.g. mast cells (207)], buffer transient changes in $[Ca^{2+}]_i$, and may have other undesirable side effects (208). A new generation of indicators (208) have 30-fold brighter fluorescence, exhibit a shift in wavelength (not just an increase in intensity) when they bind Ca^{2+} , and have an increased selectivity for Ca^{2+} . Their development should substantially improve the usefulness of this approach.

DEPOLARIZATION OF PLASMA MEMBRANE POTENTIAL Evidence for a receptor-mediated opening of a channel for Ca^{2+} through the plasma membrane has been sought, using the tetraphenylphosphonium cation as a probe. Equilibration of this ion between the medium and the interior of cells is thought to reflect a combination of the potential difference ($\Delta\psi$) across the plasma and mitochondrial membranes (209, 210). The formation of channels through the plasma membrane that allows the entrance of positive ions diminishes $\Delta\psi$, and phosphonium ions are released into the medium to an extent governed by the Nernst equation: $\Delta\psi = -(RT/F)\ln[(\text{probe}_{in})/(\text{probe}_{out})]$. Here R is the gas constant, T , the absolute temperature, and F is Faraday's constant. Experimentally, the change in potential can be indirectly assessed by measuring the decrease in cell-associated tritiated phosphonium ions.

Two groups used this approach on RBL cells stimulated with either anti-IgE (211–213) or an appropriate multivalent hapten-conjugate (211). Both groups observed slow (~ 30 min) equilibration of the probe, which makes kinetic experiments (212) difficult to interpret. Upon aggregation of the receptor-bound IgE, partial release of the phosphonium ions was observed. Addition of hapten, which did not affect release induced by anti-IgE, led to prompt reuptake of the ions (211).

The results of these studies differed in two important respects. In order to assess the role of the plasma membrane in the receptor-induced effects, both groups sought to eliminate the influence of the mitochondria. Sagi-Eisenberg & Pecht (213) used the protonophore carbonyl-cyanide-p-trifluoromethoxy phenyl hydrazone; they observed a 50% drop in the potential of unstimulated cells and little change after aggregation of the receptors. They, therefore, concluded that the changes observed on unpoisoned cells did *not* reflect changes in the plasma membrane, but rather reflected uptake of Ca^{2+} by the mitochondria, secondary to an increase of that ion in the cytoplasm. Kanner & Metzger (211) blocked the mitochondrial respiratory chain with rotenone and the proton-dislocating adenosine triphosphatase with oligomycin. They observed an $\sim 20\%$ drop in the baseline potential. Notably, the *relative* decrease initiated by aggregation

of the receptors was still observed. High concentrations (100 mM) of the probe—which preferentially collapse the mitochondrial potential difference—gave similar results. They, therefore, concluded that the receptor-induced changes were *principally* the result of a change in the plasma membrane potential.

A further difference was in the influence of external Ca^{2+} . Eisenberg & Pecht observed a parallel decrease in receptor-mediated depolarization and secretion (212), whereas Kanner & Metzger observed comparable receptor-induced depolarization in the presence or absence of mM Ca^{2+} , even though in the latter medium, secretion was totally blocked (211).

The independence of depolarization from the concentration of external Ca^{2+} led the latter workers to suggest that the putative plasma membrane channel could pass Na^+ at low concentrations of Ca^{2+} . This was experimentally verified by studying the uptake of $^{22}\text{Na}^+$ (214). The authors concluded that the plasma membrane channels are induced by aggregation of the receptors, regardless of the concentration of external Ca^{2+} , but become specific for Ca^{2+} only at mM concentrations of the latter (214). Their conclusions are consistent with the “direct” measurements of cytoplasmic Ca^{2+} using a fluorescent probe (206), as well as with the characteristics of the putative channel protein (see below). The latter approaches can yield more easily interpretable data. It seems, therefore, more productive to focus future efforts on these more direct measurements, rather than to try to unravel the cause of the contradictory findings from studies on the membrane potential.

MOLECULAR BASIS OF CHANNEL ACTIVITY In a series of provocative papers (215–220) Mazurek, Pecht, and their collaborators characterize a plasma membrane protein from RBL cells that they propose is the Ca^{2+} -channel-forming protein whose activity is initiated by aggregation of the receptors for IgE. The basis of their work is the drug cromolyn—a drug known to inhibit IgE-mediated secretion from mast cells. One of the mechanisms that has been proposed for the action of this drug is that it interferes with the receptor-mediated gating of Ca^{2+} through the plasma membrane. The correctness of this proposal forms the conceptual basis for interpreting the results of Mazurek et al. It should be noted, however, that the mechanism(s) of action of cromolyn is by no means certain. Cromolyn can have a variety of actions and, indeed, after a thorough review of the subject (196), Pearce concluded that “the calcium gating mechanism must now be rejected and alternative explanations for . . . [its] . . . action should be sought.”

The principal findings of Mazurek et al are as follows: (a) Rat peritoneal mast cells and RBL cells bind beads conjugated with cromolyn, in a Ca^{2+} -dependent manner (215). (b) A surface membrane protein (M_r 60,000 in

unreduced gels) can be isolated from detergent lysates of RBL cells using the ligand-binding properties of the protein and either immunoprecipitation or affinity chromatography (216). Substantial amounts of the protein can be isolated. If we assume an overall yield of $\sim 30\%$, their data (219) suggest $\sim 6 \times 10^5$ copies/cell, i.e. an amount at least equal to the number of receptors on these cells. (c) Using a cell sorter, variants of RBL cells can be isolated that are deficient in binding cromolyn and in IgE-mediated influx of Ca^{2+} and secretion (217). These variants appear to have a normal capacity to bind IgE and to respond normally to ionophore. (d) If such variants are fused with envelopes of Sendai virus implanted with isolated cromolyn-binding protein, virtually full IgE-mediated influx of Ca^{2+} and secretion are restored. These activities can be blocked by ascitic fluid said to contain monoclonal antibodies to the cromolyn-binding protein (218). From the sigmoidal-dose-response data the authors calculate that two molecules of the protein are required to generate a unit response (218). (e) If isolated cromolyn-binding protein is reconstituted into planar bilayers and reacted with a monoclonal antibody said to initiate degranulation of RBL cells, conductances can be measured in the presence of Ca^{2+} and Na^+ (219). The conductances are similar to those observed when receptor-bound IgE in planar bilayers prepared from the plasma membranes of RBL cells is aggregated. In either case, cromolyn blocks the conductances. The flux of either Na^+ (at low concentrations of Ca^{2+}) or Ca^{2+} (at mM concentrations of Ca^{2+}) is consistent with the observations of Kanner & Metzger referred to above (214). The findings of Mazurek et al are obviously of considerable interest; they report the first direct evidence for a cellular component that may interact with the receptor and that performs one of the critical receptor-mediated events.

There are, however, several aspects of the published work that require clarification: (a) Most, though apparently not all, workers have failed to observe any reproducible inhibition of degranulation by RBL cells with cromolyn, and indeed, Mazurek et al have published only minimal data in this regard. (b) The published method for preparing the conjugates of cromolyn—an essential procedure without which none of their studies can be reproduced—does not work in our laboratory or apparently in the hands of others. An analysis of the derivative of cromolyn from which the conjugates were made has not been given. (c) The evidence that the cromolyn-binding protein used in the various experiments is pure is, as yet, inadequate. Indeed, close inspection of their gels shows a disturbing similarity between their principal component and serum albumin, and they themselves appear to be uncertain about the significance of a variably copurifying 24 kd protein (216, 219). It is possible that this, rather than the larger component, contains the activity they are measuring. (d) The

characteristics of the monoclonal antibodies that are essential reagents in their recent studies have not been published. For example, there has been no description of the cellular proteins with which these antibodies react.

The work of Mazurek et al raises the question of the role of ATP in the opening of the calcium channel (see above), since reconstitution of active channels with purified preparations of receptors and cromolyn-binding protein has been reported by these workers (220). Similarly, it is at present difficult to integrate their findings with those of Ishizaka and her coworkers on the essential role of a serine protease, methyltransferases, and activation of adenylate cyclase in receptor-mediated influx of Ca^{2+} (see above).

Protein kinase activity Phosphorylation and dephosphorylation of proteins are widely used mechanisms by which cells regulate their functions, and several instances in which these mechanisms may control the function of plasma membrane receptors are currently under intensive investigation (221–223). In some of these, the membrane protein is itself a kinase, as well as one of the substrates for that activity (221, 222). Because no biochemical function can yet be assigned to the receptor for IgE, the possible role of phosphorylation in regulating its function can only be explored indirectly. Here we consider three questions: (a) Can one or more of the subunits of the receptor serve as a substrate for protein kinases? (b) If so, are there changes in the extent (or type) of phosphorylation upon aggregating the receptors, and is this a primary or secondary event? (c) Is the receptor itself a kinase?

THE RECEPTOR AS A SUBSTRATE FOR KINASES Hempstead et al (43, 44) observed incorporation of $^{32}\text{P}_i$ into phosphothreonine and phosphoserine on the α chain of receptors for IgE on rat mast cells. This finding on the intact mast cells is intriguing because it implies that the α chain may be a transmembrane protein (cf Figure 1). So far, the evidence that α does not penetrate the membrane has been based exclusively on negative results (above) which by their nature are much less convincing. Intact RBL cells incorporate ^{32}P into phosphoserine in the β chains and into undetermined sites in the γ chains, but not into the α chains (41, 42). Using membranes from RBL cells incubated with ^{32}P -ATP, we have observed labeled phosphoserine and phosphothreonine in α chains, and phosphoserine and phosphotyrosine in the β and γ chains (R. Quarto, H. Metzger, unpublished observations). Teshima et al (224) reported incorporation of counts into phosphoserine in a 36 kd protein that coprecipitated with the receptor for IgE when membranes of RBL cells were incubated with ^{32}P -ATP. This kinase activity had the properties of protein kinase C (224, 225). There are insufficient data in that report to determine whether the 36 kd component represents the β chain of the receptor. Finally, we have observed Mn^{2+} -dependent kinase activity in preparations of solubilized receptors that

actively incorporate counts into phosphotyrosine on both the β and γ chains (but not into the α chain or IgE) of solubilized IgE- $\alpha\beta\gamma_2$ complexes (below). Thus, each of the subunits of the receptor for IgE can serve as a substrate for one or more protein kinases.

CHANGES IN AGGREGATED RECEPTORS

Mast cells Hempstead et al reported enhanced phosphorylation of the α chains of the receptor for IgE on mast cells when the latter were reacted with antigen (44). No changes were observed in the regions of the gel where the β and γ chains would have been located.

There are several aspects of this study that make interpretation difficult: (a) The purification procedure used in their study (44, 52) would reduce the yields of just those receptors in which one is most interested, i.e. those that had bound IgE on the intact cells and that had been aggregated with antigen. We are not convinced by the evidence that such losses were minimal (44). Furthermore, the purification procedure employed leads to virtually complete loss of the β and γ chains in our hands (30). (b) No estimate of the extent of modification was possible so that the fraction of the receptors that were modified is unknown. (c) The changes were similar to those observed when the cells were triggered with the ionophore A23187 (43). Since it is unlikely that this drug has any direct effect on the receptors, it suggests that the changes observed in the α chains in both studies (43, 44) may have been secondary ones, e.g. induced by the rise in intracellular Ca^{2+} (226). That RBL cells show no change in the phosphorylation of α chains upon triggering (see below) even though their receptor-mediated secretion in many ways resembles that of normal mast cells, also suggests that the modification of α is not a critical event for activation.

RBL cells Changes in the phosphorylation of the β and γ chains of the receptors from stimulated RBL cells were described by Perez-Montfort et al (42). They used cells that had biosynthetically incorporated ^3H -leucine so that the yields of triggered and untriggered receptors could be assessed (they were equivalent) and the change in phosphorylation per unit peptide could be determined. The principal findings were: (a) Receptors from antigen-triggered cells showed an average increase in phosphorylation in β of about 45%, whereas the γ chains showed an average decrease of about 35%. In these experiments, the changes in the unaggregated and aggregated receptors from the antigen-treated cells were equivalent. (b) The fraction of the receptors that incorporated ^{32}P was small—less than 10% and perhaps much less. This could be assessed because the phosphorylated chains had a slightly reduced mobility on polyacrylamide gels. Neither that study nor a previous one (41) showed evidence of phosphorylation of α chains.

Thus, neither the results of Hempstead et al nor ours provide evidence

that phosphorylation or dephosphorylation of the receptor is a primary event in the early steps initiated by aggregation of the receptors.

INTRINSIC KINASE ACTIVITY OF THE RECEPTOR We recently observed that when receptors that had been purified by the technique we regularly employ (see above) were incubated with ^{32}P -ATP, substantial kinase activity was present (R. Quarto, H. Metzger, unpublished observations). This activity has the properties of a tyrosine kinase and substantial incorporation of phosphorous into tyrosine is observed on both the β and γ chains. When casein is present also, it too is modified but, on a molar basis, much less efficiently than the β and γ chains. If prior to elution of the receptors from the affinity column the latter is briefly "stripped" with micellar detergent (using the same protocol we employ to make the α chain of the receptor susceptible to iodination—see above), activity is found in the detergent wash. These findings suggest that the observed activity may not be due to the receptor itself. To what extent it is specifically associated with the receptor is currently being examined.

Receptor-Induced Inactivation

PRINCIPAL OBSERVATIONS When receptors on mast cells or basophils are aggregated under nonpermissive conditions, such as in the absence of extracellular Ca^{2+} , the cells not only fail to secrete but may become inactivated. That is, restoring permissive conditions—in this case adding back Ca^{2+} —does not lead to degranulation. This inactivation is referred to as desensitization. In some cases the inactivation is specific. For example, using the protocol just cited, if relatively few receptors were initially aggregated in the absence of Ca^{2+} , only these will be incapable of initiating release, whereas others subsequently aggregated after the restoration of mM Ca^{2+} can induce release from the same cells (227, 228). If larger numbers of receptors are aggregated under the nonpermissive conditions, desensitization becomes nonspecific, and it is then no longer possible to initiate any receptor-mediated secretion (227, 228). Studies on RBL cells have given somewhat different results. In this case desensitization only becomes prominent if minimal aggregation of receptors is employed (204, 229), although here too it is likely that the inactivation only involved those receptors aggregated in the absence of Ca^{2+} . The process of inactivation has the following properties: (a) It is initiated by aggregation of the receptors, and as with the activation process, dimers of IgE are sufficient but larger oligomers are more effective (96). (b) It appears to involve steps prior to the rise in intracellular Ca^{2+} . Thus, the receptor-mediated increase in cellular uptake of $^{45}\text{Ca}^{2+}$ is ablated in desensitized cells (230), but if the intracellular Ca^{2+} is raised directly with ionophores, normal secretion is

observed (231). (c) The process is an active one and shows interesting parallels to the process that initiates secretion. Thus, inactivation is not observed if the desensitization is conducted at low temperature (232). Furthermore, Kazimierczak et al (138) observed that within a narrow range of doses, DFP when added during aggregation of receptors under nonpermissive conditions could interfere with desensitization as assessed by following secretion from human basophils. Ishizaka et al (137) reported analogous studies on mouse mast cells and showed that the inhibition of desensitization was observable by examining uptake of $^{45}\text{Ca}^{2+}$ as well as secretion. The desensitization was not only blocked by very low doses of DFP but also by inhibitors of methyltransferases (137). (d) The process of desensitization appears to be a normal accompaniment of the activation process. For example, it occurs even under permissive conditions if the aggregating stimulus is minimal (233 and references therein). Furthermore, in the study by Kazimierczak et al noted above, DFP actually enhanced secretion under permissive conditions if added at appropriate doses (138).

MECHANISM The mechanism by which aggregation of receptors initiates desensitization is uncertain. One particularly simple explanation does not seem adequate. Thus, it might be supposed that during the desensitization phase of the experimental protocols described above, the receptors were shed, degraded, or internalized (234) and were, therefore, not available to be aggregated upon subsequent exposure to the appropriate cross-linking reagents. The observation of MacGlashan et al (235) that *no* internalization occurred with human basophils in the absence of Ca^{2+} could not be substantiated at least with RBL cells (109, 133). However, all studies on this aspect suggest that more than enough IgE-receptor complexes remain on the surface of the desensitized cells to stimulate release.

Thus, the data support the proposal that secretion results from a (positive) balance between activation and inactivation (137, 138, 236). They further suggest that the inactivation occurs as an early event involving reactions similar to those that initiate secretion.

CONCLUDING REMARKS

In our introductory comments, we noted that the receptor for IgE is one of a class of proteins whose interaction with the Fc domains of immunoglobulins transforms the reaction of antibody with antigen into a physiologically important event. In common with other such systems the initiating signal is dependent upon aggregation of the antibody (237, 238).

The proximal component that "senses" the aggregation of the IgE antibody—the receptor—is understood only in broad outline. It is clear

that the α chain binds the IgE, but we know nothing yet about what the β and γ subunits do. Information about the more distal components is based largely on phenomenological data. The molecular nature of these components and how they interact with the receptor and with each other must now be defined. The literature is replete with elaborate flow diagrams—sometimes several in a single paper—that illustrate putative events taking place in the plasma membrane. What is missing is even a single report of a preparation of membranes that demonstrates a meaningful level of any activity initiated by aggregation of the receptor. We are convinced that the goal of developing such preparations deserves highest priority by those wishing to understand the early events mediated by the receptor for IgE.

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