Changes in the Receptor for Immunoglobulin E Coincident with Receptor-Mediated Stimulation of Basophilic Leukemia Cells†

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ABSTRACT: Aggregation of the receptor for immunoglobulin E on mast cells and related tumor cells initiates exocytosis. We examined tumor cells that had incorporated [^{3}H]leucine and ^{32}P to see if stimulating them produced modifications in the receptors themselves. No changes were observed in the yield of receptors or in the relative proportion and the molecular weights of their α, β, and γ subunits. In addition, no new "receptor-associated" components were observed. However, after the cells were stimulated, the γ chains of the receptors showed an average 35% decrease in their associated ^{32}P. Changes in the β subunits were more variable but on the average showed a similar-sized increase in ^{32}P. Using a novel protocol that permitted examination of aggregated and unaggregated receptors from the same cell, we found that changes in the unaggregated receptors were quantitatively indistinguishable from those exhibited by the aggregated receptors. These findings raise the possibility that the changes are related to one of the inactivation reactions thought to accompany the activation sequence.

Immunoglobulin E (IgE)[1] binds with high affinity to a receptor on the surface of mast cells, basophils, and a tumor analogue, rat basophilic leukemia (RBL) cells. Aggregation of this receptor is the initial event in the sequence of reactions leading to degranulation of such cells. However, the immediate molecular consequences of the aggregation are completely unknown.

It is not obvious how best to search for such undefined biochemical events experimentally, with some reasonable hope for success. One potentially useful approach is to look for changes in the receptor itself, since there are numerous examples of systems in which the successive reactants are covalently modified (Reid & Porter, 1981; Jackson & Nermerson, 1980). Our group previously attempted this approach (Holowka et al., 1980; Fewtrell et al., 1982), but our efforts were seriously hampered by the instability of the receptor during purification (Holowka & Metzger, 1982). Recently we developed procedures with which the intact receptor could be reproducibily purified (Rivnay et al., 1982) and as a consequence found that the receptor contained an additional pair of subunits (γ) that had been overlooked (Perez-Montfort et al., 1983a). It seemed opportune, therefore, to reexplore the question of aggregation-mediated changes in the receptor. This paper describes studies in which we examined receptors from stimulated cells that had been biosynthetically labeled with [^{3}H]leucine and [^{32}P]orthophosphate.

Materials and Methods

The procedures and materials used in this study have virtually all been described previously (Perez-Montfort et al., 1983a; Fewtrell et al., 1982). Only the protocol for stimulating the cells and subsequently isolating the receptors was modified and requires detailed presentation here.

RBL cells of the secreting subline 2H3 (Barsumian et al., 1981) were harvested from stationary flasks on the fourth or fifth day, washed with complete culture medium, reacted with monomeric, radioiodinated, and benzenearsonylated mouse anti-dinitrophenyl (or rat nonspecific) IgE, and then washed twice with a phosphate-free salt solution (Fewtrell et al., 1982). In certain experiments, the cells had been grown in [^{3}H]leucine prior to addition of the IgE (Rivnay et al., 1982). The cells in the phosphate-free salt solution were then incubated with 10 mCi of carrier-free [^{32}P]orthophosphoric acid for 1 h at 37 °C. When the cells were stimulated, bovine immunoglobulin G conjugated with an average of 15 dinitrophenyl groups was added to a final concentration of 1 μg/mL. This concentration of antigen was more than adequate to give maximal release when the cells were incubated for 1 h. However, after 15 min (the time used in these experiments), release would be expected to be approximately 50% maximal. Immediately after stimulation, the cells were washed with ice-cold phosphate-buffered 0.155 M NaCl (pH 7.4) containing 20% fetal calf serum and then once with ice-cold buffer alone.

The cells were solubilized at 5×10^7 cells/mL with a 10 mM concentration of the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Hjelmeland, 1980) in the presence of the protease, phosphatase, and phosphokinase inhibitors we regularly use (Chaplin et al., 1980; Fewtrell et al., 1982). After 15 min at 4 °C, dinitrophenyllysine was added to 0.1 mM and the incubation continued for an additional 15 min prior to centrifugation. Addition of the hapten disrupts the IgE–antigen interaction and is required to obtain complete recovery of the triggered receptors after solubilization.

The receptor–IgE complexes were purified on anti-benzenearsonate columns as described previously (Kanellopoulos et al., 1979; Rivnay et al., 1982) using 10 mM detergent and 2 mM tumor-derived phospholipids in the solvent. The receptor–IgE complexes were subjected to a clearing precipitation using added human IgE plus anti-human IgE, and the

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[1] Abbreviations: IgE, immunoglobulin E; RBL, rat basophilic leukemia; kDa, kilodalton.

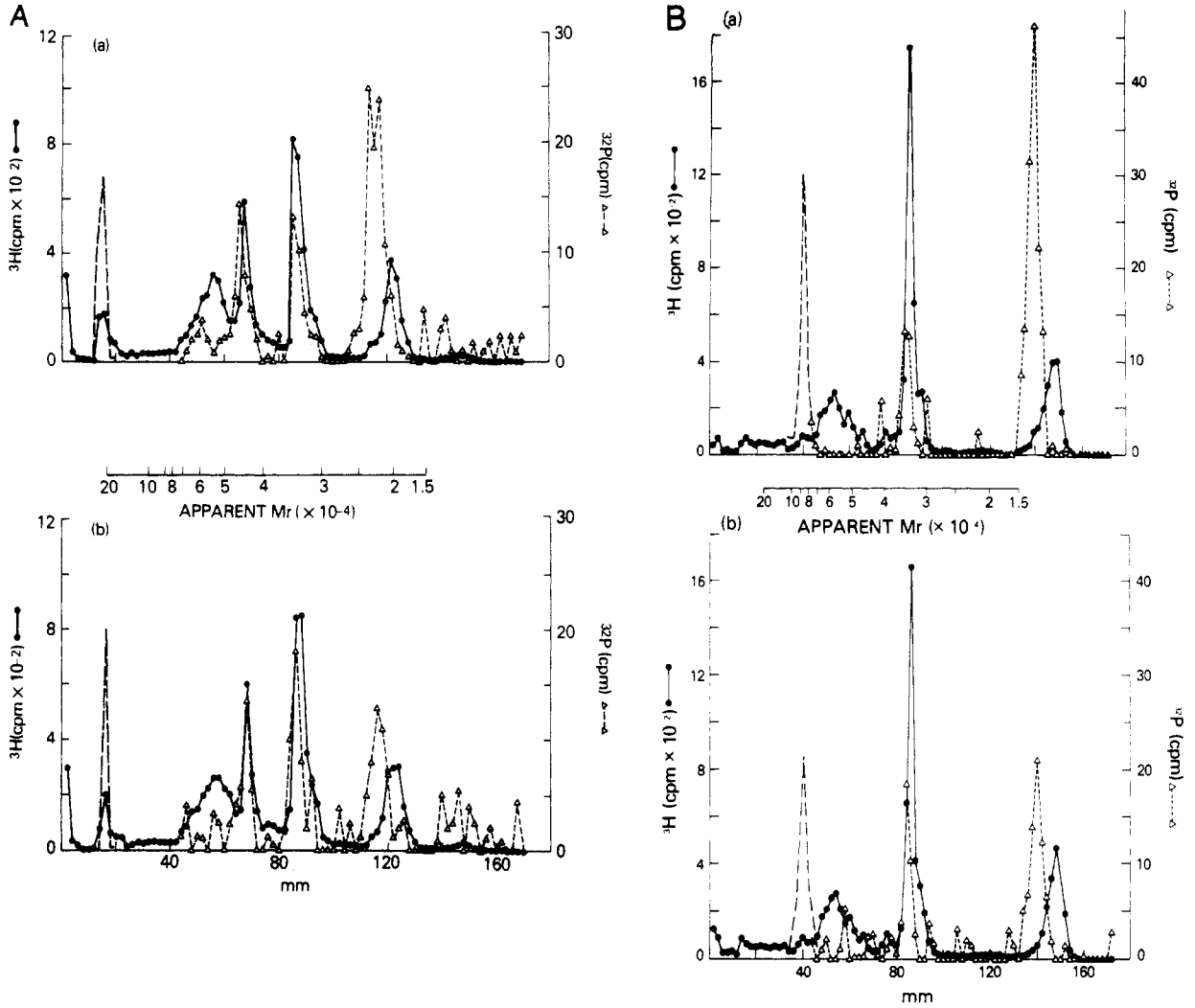

FIGURE 1: (A) Analysis of IgE–receptor complexes isolated from unstimulated (a) and stimulated (b) cells. Electrophoresis was performed on a 12.5% polyacrylamide gel in the absence of reducing agent in the sodium dodecyl sulfate buffer. The gel was dried, exposed for autoradiography, and then cut into 2-mm slices and counted for ^{125}I. The slices were then processed further for the determination of ^{3}H and ^{32}P. The peak drawn with long dashes represents the ^{125}I associated with the ϵ chains of IgE and is on the same scale as that used for the tritium counts. No ^{125}I was detected elsewhere on the gel. The molecular weight scale is based on eight standards which covered the range 200–14 kDa (high and low standards; Bio-Rad, Richmond, CA). (B) Same as (A) except in the presence of reducing agent.

specific precipitations were performed with appropriately absorbed anti-rat IgE or anti-mouse IgE.

Results

Cells were biosynthetically labeled with both [^{3}H]leucine and [^{32}P]phosphate and the receptors reacted with IgE. In the experiment described here, duplicate aliquots were left untreated, and two further aliquots were stimulated to degranulate by adding antigen. The four specimens were individually solubilized and the receptors isolated from them. Comparable yields of the receptor-bound ^{125}I-IgE were recovered in the column eluates from each aliquot: unstimulated cells, 15.4 and 19.2%; stimulated cells, 15.7 and 18.4%. Thus, the samples accurately reflected the aggregated and unaggregated receptors from the stimulated and unstimulated cells, respectively.

The receptor–IgE complexes were precipitated with anti-IgE and the precipitates analyzed on polyacrylamide gels with or without prior reduction. Figure 1 shows an analysis of two of the samples, each of which was examined prior to reduction (Figure 1A) and after reduction (Figure 1B). In each case, the top panel (a) is an analysis of receptors from unstimulated cells and the lower panel (b) an analysis of the receptors from cells reacted with antigen.

The peaks drawn with long dashes at 200 and 85 kDa represent the iodinated IgE and ϵ chains in the unreduced and reduced samples, respectively. In the latter samples, there are virtually no iodine counts corresponding to the light chains of the mouse IgE because these chains are poorly iodinated (Fewtrell et al., 1982).

Components Labeled with Leucine. If the simpler patterns generated by the reduced samples (Figure 1B) are considered first, the [^{3}H]leucine (solid circles) is distributed in three principal components in the specimen from the unstimulated cells (Figure 1B, panel a): at 58, 33, and 10 kDa. These represent the α, β, and two γ polypeptide subunits that constitute the receptor as we currently envision it (Perez-Montfort et al., 1983a). Figure 1B, panel b, shows the corresponding pattern for the specimen from the stimulated cells. The pattern of [^{3}H]leucine is indistinguishable from that obtained with the receptors from the unstimulated cells. The apparent molecular weights of the three components are the same, and they are present in the same proportions (Table I). Furthermore, no additional components are observed.

The corresponding pattern generated by the unreduced samples (Figure 1A, panels a and b) shows the 45-kDa component previously identified as a disulfide-linked complex of β and γ chains (Kinet et al., 1983). Otherwise, the patterns

Table I: [^{3}H] Leucine and ^{32}P Recovered in the Subunits of Receptors Isolated from Stimulated and Unstimulated Cells

specimen	[^{3}H] leucine[a]			^{32}P/^{3}H[b]		
	α	β	γ	α	β	γ
stimulated cells	0.24	0.43	0.24		1.07	3.65
unstimulated cells	0.23	0.45	0.24		1.20	7.36
ratio	1.04	0.96	1.00		0.89	0.50

[a] The tritium counts associated with the individual component were divided by the total counts in the gel. [b] The ^{32}P counts were divided by the corresponding tritium counts and multiplied by 100.

are similar to the patterns seen after reduction except that the γ chains are present as the 20-kDa dimer. Again there is no apparent difference in the pattern of leucine counts in the material from the unstimulated and stimulated cells (cf. panels a and b).

Nature of Components Labeled with ^{32}P. The open triangles and the lines represented with short dashes show the distribution of ^{32}P in the precipitated material. in the unreduced gels (Figure 1A, panels a and b), three phosphorylated components were observed: at 45, 33, and 23 kDa. The 45-kDa phosphorylated component is only minimally displaced from the peak of [^{3}H]leucine representing the complex of one β and two γ chains (Kinet et al., 1983). The 33-kDa component corresponds closely to the position of the β subunit. However, the 23-kDa band is shifted by about 4 kDa from the peak of tritium representing the dimer of γ chains. Nevertheless, the following evidence confirms that the 23-kDa component represents phosphorylated γ chains: (a) Upon reduction (Figure 1B, panels a and b), the 23-kDa component disappears, and a new 14-kDa peak is observed. The displacement of the latter from the peak of tritium counts representing the monomers of γ chains is similar to the displacement of the 23-kDa peak of ^{32}P from the tritium counts representing the dimers of γ chains. Notably, no additional components other than the one at 14 kDa are observed after reduction. (b) From a quantitative analysis of the tritium counts, we calculated that ~30% of the β and γ chains were complexed in the 45-kDa component in the specimens derived from the stimulated and unstimulated cells alike. Upon reduction, there was a coordinate increase in the ^{3}H and ^{32}P in the γ chains and 14-kDa component, respectively. If we define R as the ^{32}P (14 or 23 kDa) to ^{3}H (γ chain) ratio, then the ratio R(reduced)/R(unreduced) was 1.00 and 1.02 for the specimens from the stimulated and unstimulated cells, respectively. These results indicate that the 14-kDa phosphorylated component becomes incorporated into the 45-kDa complex to the same extent as the γ chains do.[2] (c) The 23- and 14-kDa phosphorylated components from unreduced and reduced specimens were resistant to digestion with protease V8, exactly as is the case for the γ chains (Perez-Montfort et al., 1983a).

Neither the quantitative analysis of the specimens shown in Figure 1 nor the radioautographs of all four specimens (Figure 2) showed phosphorylation of the α chains, which is in agreement with our previous findings (Fewtrell et al., 1982).

Changes in Phosphorylated Components after Stimulation of Cells. When the patterns of ^{32}P counts in the specimens from stimulated cells were analyzed, the most reproducible difference was a decrease in the phosphorylation of the γ chains (Figure 1 and Table I). In this experiment, there was little or no change in the phosphorylation of the β chains. However, as described below, this was variable, and on the average, there was an increase in the ^{32}P associated with the β chains. Figure 2 shows the radioautographs of all the specimens before (A) and after (B) reduction. These radioautographs were developed under conditions such that only the iodinated and phosphorylated components would be visualized. The principal feature to be noted is the fine structure of the bands corresponding to the β chains, seen particularly clearly in the reduced samples. Lanes B and D are specimens from the duplicate samples of the unstimulated cells. They show a doublet at 33 kDa with the band at the lower apparent molecular weight being more intense. In the duplicates from the stimulated cells (lanes C and E), this pattern is clearly reversed.

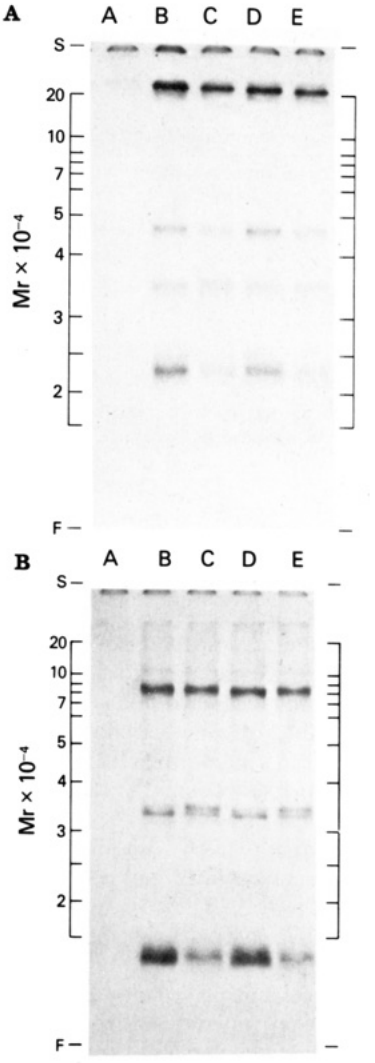

FIGURE 2: (A) Autoradiograph of the gel on which unreduced control and specific immune precipitates of IgE–receptor complexes were analyzed. The gel is the same one from which the data in Figure 1 were derived. S is the end of the stacking gel; F is the dye front. (Lane A) Control precipitate of complexes isolated from stimulated cells (lane C). The control precipitates from the three other specimens were similarly negative. (Lanes B and D) Specific precipitates of IgE–receptor complexes isolated from unstimulated cells. (Lanes C and E) Specific precipitates of IgE–receptor complexes isolated from stimulated cells. The data in Figure 1 are derived from lanes D (panel A) and C (panel B), respectively. (B) Same as (A) except in the presence of reducing agent.

[2] In this experiment, we also observed that upon reduction, the ratio of ^{32}P to ^{3}H in the 33-kDa β subunit fell sharply for the specimens derived from the unstimulated cells (−49%) and stimulated cells (−39%) alike (Figure 1). This was due to a decrease in the ^{32}P associated with the β chains since there was no discrepant increase in the tritium counts. We have no explanation for this finding, and in general it was not observed.

Table II: Recovery of ^{32}P in the β and γ Subunits of Aggregated and Unaggregated Receptors Isolated from the Same Cells

	^{32}P ratio in subunits from stimulated/unstimulated cells[a]			
	aggregated receptors		unaggregated receptors	
expt	β	γ	β	γ
1	1.32	0.64	1.40	0.64
2	1.36	0.57	1.45	0.74
3	1.10	0.59	1.01	0.52
4	2.07	0.72	1.86	0.74
mean	1.46	0.63	1.43	0.66
SD	0.42	0.067	0.35	0.10

[a] The ^{32}P counts associated with the subunits of the receptor were normalized on the basis of the amount of radioiodinated IgE recovered.

Phosphorylation of Aggregated and Unaggregated Receptors from the Same Cells. The receptors for IgE on RBL cells are monovalent and bind rat and mouse IgE with equivalent high affinity (Mendoza & Metzger, 1976a,b). These characteristics allowed us to design a unique experimental protocol with which aggregated and unaggregated IgE–receptor complexes from the same cells could be analyzed individually. This enabled us to determine whether changes in the phosphorylation of the receptors were restricted to those receptors that had been aggregated or whether they occurred in all receptors from stimulated cells.

Briefly, cells were reacted with an equimolar mixture of iodinated, benzenearsonylated mouse anti-dinitrophenyl-IgE and rat nonspecific IgE. The cells were incubated with ^{32}P, stimulated with dinitrophenylated antigen, and solubilized, and all the IgE–receptor complexes were purified on an anti-benzenearsonate column. The mouse IgE–receptor complexes which had been aggregated by antigen were specifically isolated from the eluate by precipitating them with anti-mouse IgE. The unaggregated rat IgE–receptor complexes were correspondingly recovered by precipitating them with anti-rat IgE. A specimen of cells that had not been stimulated with antigen was similarly processed. The precipitates were examined on polyacrylamide gels and the counts of ^{32}P in the β and γ chains quantitated.

Table II presents the results of four separate experiments in which this protocol was used. It is apparent that the aggregated and unaggregated receptors from stimulated cells undergo equivalent changes when compared to the receptors from unstimulated cells. There is an approximately 35% decrease in ^{32}P associated with the γ chains. The number of ^{32}P counts associated with the β chains was more variable, but on the average, the β subunits from stimulated cells showed a 45% increase.

Discussion

The results obtained with cells intrinsically labeled with [^{3}H]leucine demonstrate that aggregation of the receptor for IgE—the initial event in IgE-mediated exocytosis—leads to no substantial changes either in the relative proportions of the three subunits or in their size. We could not, of course, have detected slight changes such as the removal of a small peptide. To rule out such modifications, peptide mapping will be required.

The labeling studies also failed to reveal components that became newly associated with the receptors after stimulation. It is unlikely that our procedures for purification can be made much more gentle than they already are (Rivnay et al., 1982), so that if this line of investigation is to be pursued, chemical cross-linking will have to be employed. Our initial attempts in this regard were not encouraging (Holowka et al., 1980).

With respect to the incorporation of ^{32}P, two aspects of the results deserve discussion. One deals with the nature of the phosphorylated components. The phosphorylated component at 33–35 kDa is the β component. As shown previously (Fewtrell et al., 1982), it becomes covalently attached to the α chain when the receptors are exposed to cross-linking reagents and dissociates from the α chain in the absence of an appropriate stabilizing solvent just as the β subunit does (Rivnay et al., 1982). In our previous study, the peak of ^{32}P had an apparent molecular weight indistinguishable from that for the β subunit labeled with the intramembrane probe iodonaphthyl azide (Fewtrell et al., 1982). On other gels (e.g., Figure 1), there appears to be a slight difference between the components labeled with ^{32}P and tritium. This is explainable by the aberrant mobility of some phosphorylated polypeptides in polyacrylamide gels (below). Hempstead et al. (1983) have also observed a phosphorylated component in this molecular weight range. They propose that whereas the component they observe is the phosphorylated β chain, the phosphorylated component that we have described in the same region is likely to represent unglycosylated α chains. We find this a curious claim for the following reasons: (1) The purification procedure they employ permits only traces of the β chain to be recovered—so little in fact that when tritium counts in the 30-kDa range were first noted by them, they referred to these as "small amounts of contamination" (Kulczycki & Parker, 1979). Indeed, they have never provided any data to show that the material they are observing is *not* a contaminant. (2) On the other hand, the component that we have characterized as the β subunit is isolated in substantial amounts [cf. Figure 5c in Kulczycki & Parker (1979) with Figure 7 in Holowka et al. (1980), Figure 4 (bottom panel) in Rivnay et al. (1982), Table I in Perez-Montfort et al. (1983a), and Figure 1 in this paper], and we have provided evidence that there is 1 mol of β per mol of α. We have elsewhere detailed the evidence that the phosphorylated component at 33–35 kDa is not a fragment of α chains or unglycosylated α chains as Hempstead et al. (1983) propose (Fewtrell et al., 1982). Furthermore, any α chains in the isolated preparations must have been bound to IgE, and there is no evidence from all previous studies that such unglycosylated α chains exist on the surface of unmodified cells.

The apparent masses of the phosphorylated 23- and 14-kDa components seen in the unreduced and reduced specimens, respectively, are substantially greater than those observed for the peak of γ-chain dimers and monomers as determined from the incorporated [^{3}H]leucine. Nevertheless, by all available criteria (Results), these phosphorylated components represent γ chains. The increase in the apparent molecular weight, 4000 to 5000 in this instance, is not without precedence. Such apparent increases for phosphorylated vs. unphosphorylated polypeptides have been noted several times by others (Axelrod, 1978; Julien & Mushynski, 1982; Loube et al., 1983). The shift coincidently provides information on the fraction of the γ chains that is phosphorylated. As can be seen from Figure 1B, the 10-kDa peak of counts due to [^{3}H]leucine is asymmetric. We have repeatedly seen such asymmetry [cf. Figure 4 in Rivnay et al. (1982); Figures 2 and 6 in Perez-Montfort et al. (1983a), and Figure 14 in Kinet et al. (1983)], and if this is entirely due to the phosphorylation of the γ chains, the aggregate data suggest that 10–20% of these subunits are phosphorylated in the unstimulated cell.

The peak of counts due to [^{3}H]leucine associated with the β chain also shows either some asymmetry or a frank doublet band. That the *phosphorylated* β chains also appear as doublets (Figure 2B) suggests that there are β chains which contain more than one phosphorus. During the course of this study, we made two attempts to determine whether the phosphorus was on serine, threonine, or tyrosine but could not come up with an interpretable result. We had previously found evidence for *O*-phosphoserine (Fewtrell et al., 1983), and we are continuing our efforts in this respect.

In that earlier study (performed before the γ subunits had been identified), a phosphorylated 14-kDa component was also observed. It was conjectured that this might represent the β2 domain of the β chain—a domain whose existence was postulated on the basis of experiments in which the β was proteolytically cleaved (Holowka & Metzger, 1982). It now appears that what was observed was in fact phosphorylated (monomeric) γ chains. Nevertheless, in the previous study, we did not observe a phosphorylated β1 fragment in the face of apparent breakdown of the β chain. This suggests that our conclusion that the β chain is phosphorylated in the β2 domain was correct.

A second aspect about which we wish to comment concerns the changes induced by stimulating the cells. Such stimulation resulted in reproducible decreases in the phosphorylation of the γ chains and somewhat more variable increases in the phosphorus associated with the β chains. That the changes in the latter were not observed in our previous study (Fewtrell et al., 1982) is, therefore, not surprising, particularly since at that time we had not yet developed procedures for obtaining consistent yields of the subunits.

Hempstead et al. (1983) propose that our failure to see phosphorylation of the α chains on RBL cells, whereas they do see phosphorylation of the α chains upon stimulation of rat peritoneal mast cells, means that the tumor cells are unsuitable for functional studies. We reject this argument. We have analyzed elsewhere the secretory properties of RBL cells (Fewtrell & Metzger, 1982) and have demonstrated that these properties closely resemble the fundamental properties of basophils and, to a lesser extent, mast cells. There are, of course, likely to be a variety of physiological differences between discrete cell types—as there are between mast cells and basophils (Fewtrell & Metzger, 1982; MacGlashan et al., 1983)—whether tumor cells or not. That there should be fundamental differences between the mechanisms by which a particular membrane receptor induces signal transduction on different cell types is much less probable. Indeed, since the RBL cells undergo relatively normal antigen-induced, IgE-mediated release in the absence of phosphorylation of the α chains, it seems more likely to us that the latter process is not on the main pathway that initiates secretion and is a coincidental (though not necessarily uninteresting) event that occurs when mast cells are stimulated to secrete. In agreement with this interpretation, it was found by Hempstead et al. (1982) that the α chain was phosphorylated when cells were stimulated with the calcium ionophore A23187 which bypasses the receptor altogether.

The significance of the changes we observed cannot be assessed yet either. Because equivalent changes occurred on both the aggregated and unaggregated receptors from stimulated cells (Table II), it is more likely that these alterations also reflect a secondary, rather than a primary, event. We emphasize that observation of this phenomenon was only possible because we were able to isolate and separate both the aggregated (activated) and unaggregated receptors from stimulated cells. We are not aware that such internal controls have been applied in similar studies.

It is thought that aggregation of the receptors of IgE initiates three events: (1) an activation sequence leading to exocytosis; (2) an inactivation reaction involving only those receptors that were aggregated; and (3) an inactivation reaction that can prevent subsequent stimulation even by receptors not previously aggregated (MacGlashan & Lichtenstein, 1981). It is possible that the changes we have observed are related to this third phenomenon.

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