Association of the crosslinked IgE receptor with the membrane skeleton is independent of the known signaling mechanisms in rat basophilic leukemia cells

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Crosslinking of the IgE receptor on the surface of rat basophilic leukemia (RBL) cells by multivalent antigen induces an association of these receptors with the detergent-insoluble membrane skeleton. Detergent insolubility of the receptor can also be induced on purified plasma membranes isolated from RBL cells by the use of either IgE oligomers or IgE monomers plus multivalent antigen. The critical event in initiating this interaction between the receptor and the membrane skeleton is crosslinking of the receptor. This association is rapid, and, when triggered by multivalent antigen, it is quickly reversed by the addition of excess monovalent antigen. The fact that this association occurs with the use of purified plasma membranes indicates that all of the components necessary for this interaction are present in the plasma membrane and that intracellular components are not required. Although crosslinking of the receptor activates phospholipase C and phospholipase A2 leading to the generation of several second messengers, none of these signaling mechanisms appears to be involved in IgE receptor interaction with the membrane skeleton. This interaction cannot be induced by phorbol 12-myristate 13-acetate (PMA), ionomycin, or a combination of these two reagents, although this will result in degranulation. Furthermore, receptor detergent insolubility is temperature independent when triggered by multivalent antigen, thus indicating that enzyme-catalyzed reactions are not important. This was verified by the fact that a variety of inhibitors that block phosphatidylinositol metabolism, arachidonic acid metabolism, Ca^{2+} influx, and protein kinase C (PKC) activation had no effect on antigen-induced association of the receptor with the membrane skeleton. These results indicate that the signaling mechanisms leading to the degranulation response are not involved in the association of the crosslinked receptor with the membrane skeleton.

Introduction

The plasma membrane skeleton and the cytoskeleton are believed to regulate many cellular events by controlling the distribution and movement of cell surface receptors. It has been observed in many cell types such as platelets (Wheeler *et al.*, 1985), pheochromocytoma cells (Vale and Shooter, 1983), granulocytes (Jesaitis *et al.*, 1984), rat mammary adenocarcinoma cells (Jung *et al.*, 1984), RBL cells (Robertson *et al.*, 1986; Apgar, 1990; Seagrave and Oliver, 1990), and B lymphocytes (Braun *et al.*, 1982; Woda and McFadden, 1983; Woda and Woodin, 1984) that binding of ligand and crosslinking of cell surface proteins induces detergent insolubility of those receptors. Often this immobility of crosslinked receptors is a prelude to patching, capping, and internalization of the receptors, which in turn may be important in the down-regulation of responses. In other cases, some cell surface receptors appear always to be associated with a detergent-insoluble structure (Galvin *et al.*, 1984; Ranscht *et al.*, 1984; Landreth *et al.*, 1985).

In rat basophilic leukemia (RBL) cells it has been demonstrated that crosslinking of the IgE receptor leads to immobility of the complexes in the plane of the membrane (Menon *et al.*, 1985a,b) and to detergent-insolubility (Robertson *et al.*, 1986; Apgar, 1990; Seagrave and Oliver, 1990). It has also been shown that this is probably due to an interaction of the crosslinked receptors with the underlying, detergent-insoluble membrane skeleton (Apgar, 1990). The membrane skeleton, which is biochemically and structurally distinct from the cytoskeleton, is composed of a discrete set of proteins that interact to form a matrix that is continuous over the entire cytoplasmic face of the plasma membrane (Mescher *et al.*, 1981; Apgar *et al.*, 1985; Apgar and Mescher, 1986). This structure is also believed to be able to interact with microfilaments and may therefore be acting as an intermediate between cell surface proteins and the cytoskeleton.

Crosslinking of the IgE receptors on RBL cells leads to several other physiological responses,

including an increase in filamentous actin (F-actin), changes in cell shape, aggregation and internalization of the crosslinked receptors, and degranulation with the release of inflammatory mediators (Ishizaka and Ishizaka, 1984; Metzger *et al.*, 1986; Oliver *et al.*, 1988). Crosslinking of the IgE receptors also leads to the activation of phospholipase A2 and phospholipase C, which are both believed to be associated with the receptor through G proteins (Narasimhan *et al.*, 1988, 1990; Wilson *et al.*, 1989). Phospholipase C hydrolyzes the phosphoinositides to produce inositol phosphates and diacylglycerol, which lead to the increase in intracellular Ca^{2+} and activation of protein kinase C (PKC), respectively (Beaven *et al.*, 1984a,b; Cunha-Melo *et al.*, 1987). The increase in cytoplasmic Ca^{2+} is due mainly to the influx of extracellular Ca^{2+} through an ion channel, although there is also probably release from intracellular stores (Beaven *et al.*, 1984b; Fewtrell and Sherman, 1987; Mohr and Fewtrell, 1987). Phospholipase A2 activation is responsible for the production of lysophospholipids and arachidonic acid, which is further metabolized into the prostaglandins and leukotrienes (Crews *et al.*, 1981). In addition, there is membrane depolarization (Kanner and Metzger, 1983; Sagi-Eisenberg and Pecht, 1983), activation of a $\text{Na}^+/\text{Ca}^{2+}$ antiport (Stump *et al.*, 1987), and changes in intracellular pH (Ali *et al.*, 1989). Exactly which of these signals is responsible for degranulation is not known, but several of them—such as activation of PKC, increase in intracellular Ca^{2+} , arachidonic acid production, and changes in pH—have been implicated. In addition, the increase in F-actin which may be involved in the down regulation of the response is believed to be activated by PKC (Oliver *et al.*, 1988; Apgar, 1991).

Previous studies have shown that association of the crosslinked IgE receptors with the membrane skeleton is very rapid and may be one of the earliest events to occur (Menon *et al.*, 1985a,b; Robertson *et al.*, 1986; Apgar, 1990). The purpose of this study was to determine whether the known second messenger pathways that are activated on crosslinking of the receptor are involved in signaling for the association of the receptors with the membrane skeleton. Using purified plasma membranes, we found that crosslinking of the receptors is the critical event leading to this association and that all of the components necessary are found in the plasma membrane. Furthermore, although crosslinking leads to transmembrane signaling in RBL cells, none of these signals appears to have any effect on the level of detergent-insolubility of the receptor.

Results

Requirements for and kinetics of receptor detergent insolubility

Other investigators have shown that the critical event leading to detergent insolubility and degranulation is crosslinking of the receptors (reviewed in Metzger, 1983). However, these studies were performed using whole cells, where other cellular events could be taking place. It was therefore important to determine whether the same was true in purified plasma membranes. Monomers and dimers of IgE bind to purified plasma membranes but are detergent soluble when the membranes are extracted with Triton. Using IgE trimers, a small but consistent increase in detergent insolubility is seen, whereas higher-order oligomers are >80% detergent insoluble when tested on plasma membranes (Table 1). Control experiments in which IgE oligomers were added to tubes that did not contain any plasma membranes showed that the oligomers do not pellet themselves under the centrifugation conditions that were used. Thus, only IgE oligomers that have bound to the receptor and induced an association with the membrane skeleton are insoluble. Furthermore,

Table 1. Detergent insolubility of ^{125}I -IgE oligomers bound to purified plasma membranes

Oligomer fraction ^a	First addition ^b	Second addition	% detergent insoluble
Monomer	—	—	9.8 ± 0.8
Dimer	—	—	10.0 ± 0.9
Trimer	—	—	20.3 ± 1.4
Higher Oligomers	—	—	80.8 ± 5.2
Monomer	DNP-lysine	—	9.8 ± 0.2
Monomer	DNP-BSA	—	37.9 ± 2.3
Monomer	DNP-BSA	DNP-lysine	9.1 ± 1.1
Higher Oligomer	DNP-lysine	—	84.4 ± 3.9
Higher Oligomer	DNP-BSA	—	86.5 ± 6.7
Higher Oligomer	DNP-BSA	DNP-lysine	85.4 ± 4.0

^a ^{125}I -IgE was chromatographed on a Sephacryl S-300 gel filtration column; and monomers, dimers, trimers, and higher-order oligomers were isolated. The IgE oligomers were bound to purified plasma membranes, and detergent insolubility induced by these fractions was determined by solubilizing the membranes in ice-cold buffer containing 1% Triton X-100.

^b Purified plasma membranes were sensitized with either monomers or higher-order oligomers. The plasma membranes were then triggered with a first addition of either 100 ng of DNP-BSA or 100 μg of DNP-lysine at room temperature. After 15 min, a second addition of either buffer or DNP-lysine was made. The reaction proceeded for another 5 min before the membranes were solubilized and receptor detergent insolubility was determined.

Figure 1. Kinetics of receptor detergent insolubility. IgE anti-DNP primed RBL cells (○) and purified plasma membranes (■) were triggered with 500 ng/ml of DNP-BSA for various lengths of time at 23°C. The reaction was stopped by the addition of ice-cold solubilizing buffer. The chemical crosslinker DTBP was not used in this experiment. Background values of 9.8% for detergent-insolubility using plasma membranes, and 3.1% using whole cells, were subtracted from each time point value. The error bars represent the SE.

IgE monomers only become detergent insoluble when aggregated by multivalent antigen such as dinitrophenyl-bovine serum albumin (DNP-BSA). Monovalent antigen (DNP-lysine) not only fails to induce detergent-insolubility but it rapidly reverses insolubility induced by DNP-BSA. Oligomeric IgE remains detergent insoluble regardless of whether monovalent or multivalent antigen is present, and DNP-lysine is incapable of reversing the detergent-insolubility. These results indicate that association of the IgE receptor with the detergent-insoluble membrane skeleton in purified plasma membranes is induced by aggregation of the receptors and that this association can be reversed on disaggregation of the receptors. This is consistent with the results seen by other investigators using whole cells.

Previous experiments using whole cells have shown that association of the crosslinked receptor with the membrane skeleton is a relatively rapid event as measured by fluorescence recovery after photobleaching (Menon *et al.*, 1985a,b) and the detergent-insolubility assay (Robertson *et al.*, 1986). As can be seen in Figure 1, the time course of the reaction is similar whether using whole cells or purified plasma membranes. Because of the nature of the experiment, the chemical crosslinker dimethyl 3,3'-dithiobispropionimidate (DTBP) could not be used to stabilize the association, thus accounting for the relatively low levels of receptor detergent insolubility. The half-maximal response is reached in 30–60 s, whereas a plateau level

is achieved in 5–10 min. A response can be observed in as little as 10 s, which is the earliest time point that could be measured. On the basis of the kinetics of phosphatidylinositol metabolism, arachidonic acid metabolism, Ca^{2+} influx, and degranulation (Beaven *et al.*, 1984a,b; Cunha-Melo *et al.*, 1987; Maeyama *et al.*, 1988; Apgar, unpublished results), detergent insolubility appears to be one of the first events to occur after receptor crosslinking.

Failure to induce receptor detergent insolubility using PMA and ionomycin

Antigenic stimulation of RBL cells leads to phosphatidylinositol metabolism with the subsequent generation of inositol phosphates and diacylglycerol. These metabolites then lead to a rise in intracellular Ca^{2+} and activation of PKC, both of which are important second messengers in the degranulation response (Metzger *et al.*, 1986). To determine whether Ca^{2+} and PKC activation are important mediators of the association of the receptor with the matrix, we used phorbol 12-myristate 13-acetate (PMA) and ionomycin to mimic these two responses. In the experiments shown in Figure 2, RBL cells were stimulated with different concentrations of antigen in the presence of different concentrations of either PMA or ionomycin. With no antigen present, a background level of ~8% detergent-insolubility is seen regardless of whether iono-

Figure 2. Effect of ionomycin and PMA on DNP-BSA-stimulated receptor detergent insolubility. RBL cells were sensitized with IgE anti-DNP and stimulated with different concentrations of DNP-BSA in the presence of different concentrations of ionomycin (upper panel) or PMA (lower panel). Association of the crosslinked receptor with the membrane skeleton was stabilized with the addition of DTBP. The error bars represent the SE.

mycin or PMA are present. This indicates that ionomycin concentrations as high as 800 nM and PMA concentrations as high as 50 nM do not induce receptor association with the membrane skeleton. Furthermore, the level of detergent insolubility induced by suboptimal and optimal concentrations of antigen are not affected by any concentration of either PMA or ionomycin. Thus the degree of detergent-insolubility of the crosslinked receptor is not influenced by altering either the intracellular Ca^{2+} level or the level of PKC activity.

To determine whether these two signals together can induce association of the receptor with the membrane skeleton, we incubated RBL cells with 100 nM ionomycin and varying concentrations of PMA. Neither 100 nM ionomycin nor any concentration of PMA alone causes degranulation, although the two reagents together can synergize to lead to degranulation (Figure 3). However, under the same conditions, there is no increase in receptor detergent-insolubility. Thus, the combination of ionomycin and PMA cannot induce the receptors to associate with the membrane skeleton even though they can cause degranulation.

Temperature dependence

The previous experiments indicated that the detergent-insoluble response could not be mimicked either by ionomycin, PMA, or a combination of the two reagents. In an attempt to further dissociate the signaling events from the association of the receptor with the membrane skeleton, we sensitized RBL cells with IgE anti-DNP; triggered them with DNP-BSA at different temperatures; and measured phosphatidylinositol metabolism, arachidonic acid metabolism, Ca^{2+} influx, degranulation, and detergent insolubility. Metabolic events are very temperature dependent and therefore no increase in inositol phosphates or release of either arachidonic acid or its metabolites can be detected at 4°C or 15°C (Figure 4). At 23°C, phosphatidylinositol metabolism and arachidonic acid metabolism proceed at ~50% of the level reached at 37°C. Ca^{2+} influx and degranulation are both known to be dependent on metabolic signals and are therefore also temperature dependent. As can be seen in Figure 4, there is a very strong correlation between these two events and the metabolism of phosphatidylinositol and arachidonic acid. However, measurements of receptor detergent-insolubility show that although there is a 30–40% decrease at 4°C, detergent-insolubility of the crosslinked receptor is relatively temperature independent using either whole

Figure 3. Effect of PMA and ionomycin on detergent-insolubility and ^3H -5HT release. RBL cells were incubated with 100 nM ionomycin and different concentrations of PMA. The ^3H -5HT assay was performed at 37°C for 45 min. The detergent insolubility assay was allowed to proceed for 30 min at 23°C before the addition of DTBP. The assay was terminated with the addition of ice-cold solubilizing buffer. The detergent-insolubility assay was also performed at 37°C with similar results. The error bars represent the SE.

cells or purified plasma membranes. This experiment shows that association of the crosslinked receptors with the membrane skeleton can occur in the absence of detectable phosphatidylinositol and arachidonic acid metabolism.

Effect of inhibitors on antigen-induced receptor detergent insolubility

An alternate means of dissociating detergent-insolubility of the crosslinked receptor from known signaling mechanisms is through the use of inhibitors. Using whole cells, ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) effectively inhibits the influx of extracellular Ca^{2+} , arachidonic acid metabolism, and phosphatidylinositol metabolism (Table 2). However, crosslinking of the IgE receptor in the presence of 5 mM EGTA has no effect on the degree of detergent-insolubility. In addition, quinacrine, nordihydroguaiaretic acid (NDGA), and quercetin—which inhibit arachidonic acid metabolism and phosphatidylinositol metabolism to varying degrees—also have no effect on the association between the crosslinked receptor and the membrane skeleton. These results agree with the temperature studies and argue that Ca^{2+} influx as well as arachidonic acid and phosphatidylinositol metabolism are not required for receptor detergent-insolubility. Association of the crosslinked receptors with the underlying detergent-insoluble structures is independent of these signaling mechanisms.

Inhibitors of PKC have been shown to inhibit degranulation as well as the F-actin response (Cunha-Melo *et al.*, 1989; Apgar, 1991). How-

Figure 4. Temperature dependence of receptor detergent-insolubility, ^3H -5HT release, Ca^{2+} influx, phosphatidylinositol metabolism, and arachidonic acid metabolism. RBL cells were sensitized with IgE anti-DNP, equilibrated to 4°C, 15°C, 23°C, or 37°C. Detergent-insolubility of the receptor was also measured on purified plasma membranes at different temperatures. The cells and plasma membranes were triggered with 200 ng/ml of DNP-BSA and measured for detergent-insolubility of the receptor, ^3H -5HT release, $^{45}\text{Ca}^{2+}$ influx, production of inositol phosphates, and arachidonic acid release. The results are presented as the percentage of the response at 37°C. The control value for specific detergent insolubility using whole cells was 27.7 and 23.6 using purified plasma membranes. The control value for specific degranulation was 33.0%, 8519 cpm for Ca^{2+} influx, 5841 cpm for arachidonic acid release, and 4957 cpm for inositol phosphate production. Error bars represent the SE.

ever, as Table 3 indicates, the addition of EGTA and EDTA to remove Ca^{2+} and Mg^{2+} , which are necessary requirements for most kinases, has no effect on receptor detergent-insolubility using purified plasma membranes. This also shows that association of the crosslinked receptors with the membrane skeleton does not require these divalent cations. Furthermore, the addition of ATP as a phosphate donor does not have any effect on detergent-insolubility, even in the presence of Ca^{2+} and Mg^{2+} . This result is in agreement with previous work that showed that detergent insolubility of the receptor is not energy dependent. Finally, PKC inhibitors such

as sphingosine, staurosporine, and K252a have no effect on detergent-insolubility resulting from crosslinking the receptor either on whole cells or on purified plasma membranes.

Recent evidence has shown that phospholipase C and phospholipase A2 are activated through G proteins in RBL cells (Ali *et al.*, 1989; Wilson *et al.*, 1989; Narasimhan *et al.*, 1990). Therefore, another possibility was that association of the crosslinked receptor with the membrane skeleton was also mediated either directly or indirectly through a G protein. Purified plasma membranes sensitized with ^{125}I -IgE were therefore incubated in the presence of GTP or the

Table 2. Effect of inhibitors of arachidonic acid and phosphatidylinositol metabolism on RBL cell activation^a

Inhibitor	Detergent insolubility	Ca^{2+} influx	Arachidonic acid release	Inositol phosphates
Control	100.0	100.0	100.0	100.0
EGTA (5 mM)	97.1	3.8	9.1	1.9
Quinacrine (50 μM)	100.1	96.0	11.0	92.3
NDGA (50 μM)	91.1	0.0	10.7	36.9
Quercetin (25 μM)	112.0	32.8	10.4	88.4

^a RBL cells were sensitized with IgE anti-DNP and preincubated with the appropriate inhibitor for 15 min. The cells were triggered by the addition of 500 ng/ml DNP-BSA. The detergent-insolubility assay was performed at 23°C, whereas all other assays were performed at 37°C. The results are expressed as percentage of the control response. The control value of specific detergent insolubility was 29.14, 3886 cpm for Ca^{2+} influx, 7445 cpm for arachidonic acid, and 9696 cpm for inositol phosphates. In all cases the error is <5%.

Table 3. Effect of protein kinase C inhibitors on receptor detergent insolubility^a

Inhibitor	Whole cell % control	Plasma membrane % control
Control	100.0 ± 4.7	100.0 ± 10.8
EGTA, EDTA (5 mM)	ND	114.2 ± 5.7
ATP (5 mM)	ND	98.7 ± 2.8
Sphingosine (50 mM)	97.0 ± 1.7	82.0 ± 0.5
Staurosporine (1 mM)	93.0 ± 2.8	94.0 ± 1.9
K252a (1 mM)	99.0 ± 1.4	101.0 ± 2.8

^a Either whole cells or purified plasma membranes were sensitized with ¹²⁵I-IgE anti-DNP. The cells or membranes were then preincubated with the inhibitor for 15 min before the addition of 500 ng/ml DNP-BSA. The reaction was terminated by the addition of ice-cold solubilizing buffer. The results are expressed as percentage of the control response. For these experiments, the control specific detergent insolubility using whole cells was 33.1% and 28.0% using purified plasma membranes.

GTP analogues, GTP γ S or GDP β S, and the receptors were crosslinked with DNP-BSA. As can be seen in Table 4, none of these reagents had any effect on the level of receptor detergent-insolubility.

It has also been shown in RBL cells that pH changes may synergize with the Ca²⁺ influx during the signaling process (Ali *et al.*, 1989). To test the effects of pH, we sensitized purified plasma membranes with ¹²⁵I-IgE and triggered them with DNP-BSA in buffered media with pH values ranging from 5.9 to 7.7. The results shown in Figure 5 indicate that neither the background level nor the antigen-triggered level of receptor detergent-insolubility is affected by pH. These data demonstrate that the association of the crosslinked receptor with the membrane skeleton is independent of pH changes.

Discussion

The experiments presented here using purified plasma membranes and IgE oligomers indicate that crosslinking of the IgE receptor is the critical event in inducing an association between the receptor and the detergent-insoluble membrane skeleton. Monomers and dimers of IgE bind to the receptor but remain detergent soluble, whereas trimers and higher-order oligomers lead to receptor association with the membrane skeleton. Monomeric IgE receptor complexes can be induced to associate with the membrane skeleton by crosslinking with DNP-BSA. These results are in complete agreement with those seen using whole cells. Numerous investigators have shown that the key event leading to degranulation is crosslinking of the

Table 4. Effect of GTP analogues on receptor detergent insolubility using purified plasma membranes^a

Analogue	% Control
Control	100.0 ± 0.9
GTP (5 mM)	89.6 ± 4.0
GTP γ S (1 mM)	85.4 ± 6.4
GDP β S (5 mM)	84.1 ± 6.5

^a Purified plasma membranes, sensitized with ¹²⁵I-IgE, were preincubated with GTP, GTP γ S, or GDP β S for 15 min before the addition of DNP-BSA. The reaction was stopped after 30 min by the addition of ice-cold solubilizing buffer. The degree of detergent insolubility was determined, and the results are expressed as the percentage of the control response. The control level of specific detergent insolubility was 25.6%.

IgE receptor and that the minimum triggering unit is a trimer (Fewtrell and Metzger, 1980; Menon *et al.*, 1985b; Robertson *et al.*, 1986), although under special circumstances a dimer will work (Kane *et al.*, 1986, 1988). Studies by Menon *et al.* (1985a,b) using RBL cells and fluorescence recovery after photobleaching found that trimers were the minimum unit necessary to induce immobility of the receptor in the plane of the membrane and that this correlated with the requirements for degranulation. Robertson *et al.* (1986) also found that trimers became detergent insoluble on whole cells, whereas dimers and monomers did not. All of these results indicate that simple binding of IgE to the receptor does not trigger association with the membrane skeleton nor does it result in transmembrane signaling. The fact that higher-order oligomers are more detergent insoluble than trimers is probably due to the greater degree

Figure 5. Effect of pH on receptor detergent-insolubility. Purified plasma membranes were sensitized with IgE anti-DNP and triggered with either buffer (○) or buffer containing 500 ng/ml of DNP-BSA (■) at pH ranging from 5.9 to 7.7. The assay was performed at 23°C for 30 min before the addition of ice-cold solubilizing buffer. Error bars represent the SE.

of multivalent interaction with the membrane skeleton. The studies using purified plasma membranes extend the previous work using whole cells and show that all of the components necessary for association of the receptor with the membrane skeleton are present in the plasma membrane and that cytoplasmic components are not required.

The fact that crosslinking of the receptor is important for inducing detergent insolubility of the receptor is also shown by the fact that it can be reversed by the use of monovalent antigen. This has been seen using purified plasma membranes in the present study and in whole cells (Apgar, 1990; Seagrave and Oliver, 1990). The fact that monovalent antigen reverses the association with plasma membranes indicates that DNP-lysine is causing its effect by deaggregating the receptors and not through an indirect effect on the cell. These findings also correlate well with other studies that have shown that monovalent antigen rapidly shuts down any further signaling and degranulation (Maeyama *et al.*, 1988).

Although crosslinking of the receptor induces an association with the membrane skeleton and activates several second-messenger systems, five lines of evidence indicate that these signaling mechanisms are not necessary for detergent insolubility of the receptor. 1) The addition of PMA, ionomycin, or a combination of the two failed to induce detergent-insolubility or have any effect on the level of detergent insolubility because of suboptimal or optimal concentrations of DNP-BSA. These reagents are known to trigger degranulation and the F-actin response (Oliver *et al.*, 1988; Apgar, 1991). 2) Association of crosslinked receptors with the membrane skeleton using either whole cells or plasma membranes was independent of temperature. This is consistent with the findings of Menon *et al.* (1985a,b), who found that immobility of the crosslinked receptor complexes, as measured by fluorescence photobleaching techniques, occurred at 4°C. 3) Inhibitors of phosphatidylinositol metabolism, arachidonic acid metabolism, PKC, and Ca²⁺ influx had no effect on the level of detergent insolubility induced by multivalent antigen. 4) The fact that detergent-insolubility of the receptor can occur using plasma membranes shows that changes in pH, other ions, and membrane potential are not involved. 5) Neither GTP nor the GTP analogs, GTP γ S and GDP β S, had any effect on the level of receptor detergent-insolubility using purified plasma membranes. Thus, either the association between the crosslinked receptors and the membrane skeleton is not actively

signaled for or the pathways in operation are unknown and unaffected by low temperatures and these drugs. It is not currently known which membrane skeletal proteins are involved. However, because of the relative simplicity of purified plasma membranes and the detergent-insoluble membrane skeleton, it is probable that the crosslinked IgE receptors are associating with the same membrane skeletal component under all of the conditions that have been tested.

It is also not known whether detergent insolubility of other crosslinked receptors on other cells is independent of known signaling mechanisms. However, crosslinking of cell surface receptors by antibody on B cells (Braun *et al.*, 1982; Woda and McFadden, 1983; Woda and Woodin, 1984), T leukemic cells (Marano *et al.*, 1989), and neutrophils (Jack *et al.*, 1986), as well as lectin-induced receptor crosslinking on neutrophils (Suchard and Boxer, 1989) and PC12 pheochromocytoma cells (Vale and Shooter, 1982), does lead to receptor detergent-insolubility even if the assays are performed at 4°C. Although not measured in any of these cases, it is unlikely that any enzyme-based signaling mechanisms would be active at 4°C. Thus, it is quite possible that transmembrane signal-independent association of crosslinked receptors with the membrane skeleton is a general phenomenon.

In RBL cells it is known that crosslinking of the IgE receptor leads to the activation of phospholipase C and phospholipase A2 and the metabolism of phosphatidylinositol and arachidonic acid. This in turn leads to the generation of many second messengers. A rise in cytoplasmic Ca²⁺, activation of PKC, changes in intracellular pH, and arachidonic acid production are all believed to be important signals for degranulation (Ishizaka and Ishizaka, 1984; Metzger *et al.*, 1986; Oliver *et al.*, 1988), whereas PKC activity appears to be critical in the antigen-induced rise in F-actin (Apgar, 1991). The kinetics of the detergent-insolubility response using either whole cells or purified plasma membranes is relatively rapid, indicating that association of the crosslinked receptor with the membrane skeleton is one of the initial events to take place. Using IgE oligomers, there is very good correlation between detergent insolubility of the receptor, signaling, and degranulation. For these reasons, it has been proposed that this association may in fact be necessary for transmembrane signaling to occur (Robertson *et al.*, 1986). The findings reported here are consistent with this proposal in that detergent-insolubility of the receptor could be neither induced nor inhibited

by reagents that affect the known signaling mechanisms. It is therefore possible that detergent-insolubility may be occurring before transmembrane signaling and may be necessary for this signaling to occur.

The results reported here are consistent with a model proposed by Oliver *et al.* (1988), with some modifications. RBL cells contain receptors that bind IgE in a 1:1 ratio. In this state the receptors are detergent-soluble, uniformly distributed on the cell surface, and freely mobile (Menon *et al.*, 1985a,b). At this stage, receptors probably interact with the membrane skeleton in a very transient fashion, which may be responsible for their decreased mobility relative to lipid probes. Addition of multivalent antigen leads to the crosslinking of the receptors into very small clusters. This is presumed to lead to multivalent interaction with the membrane skeleton, which is stable, and therefore to lead to immobility and detergent-insolubility of the receptor complexes. This association does not require any of the known signaling pathways, and only plasma membrane components are necessary. Stable interaction of the receptor complexes with the membrane skeleton may then lead to an active state and the generation of transmembrane signals. A variety of second messengers are subsequently generated, including the activation of PKC, which leads to the rise in F-actin and microfilament interaction with the receptors. Microfilaments may then be important for the directed movement of the small active clusters into larger clusters, which are believed to be inactive or even inhibitory. Thus the membrane skeleton may be involved in positive signaling whereas the cytoskeleton may be important in down-regulation.

Material and methods

Cells and plasma membrane preparation

RBL-2H3 cells were routinely grown at 37°C in flasks containing RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 5% fetal calf serum, 5% newborn calf serum, 2 mM glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, and penicillin-streptomycin. Cells were removed from the flasks by incubating them with phosphate-buffered saline (PBS) containing 5 mM EDTA for 10 min at 37°C.

Purified plasma membranes were isolated from RBL cells on the basis of previously published methods (Apgar *et al.*, 1985). The cells were grown in 850-cm² roller bottles in complete media until they reached confluence. They were then removed from the bottles using 5 mM EDTA in PBS, washed three times in Dulbecco's-PBS (D-PBS), resuspended in D-PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and frozen at -70°C. The frozen cells were thawed, further disrupted by nitrogen cavitation (600 psi for 5 min), and centrifuged at 3600 × *g* for 15 min. The supernatant was saved, and the pellet was resuspended in D-

PBS and centrifuged again. The supernatant from the second centrifugation was combined with the first supernatant, and they were spun at 22 000 × *g* for 30 min to pellet the membranes. Plasma membranes were purified by sucrose gradient ultracentrifugation.

Reagents

The mouse monoclonal IgE, which is specific for DNP, was a generous gift from Dr. Fu-Tong Liu of the Medical Biology Institute (Liu *et al.*, 1980). The IgE was labeled with ¹²⁵I and Iodobeads (Pierce Chemical, Rockford, IL) according to the instructions of the manufacturer. Unreacted ¹²⁵I was separated from ¹²⁵I-IgE using a PD-10 gel filtration column (Pharmacia, Piscataway, NJ). The iodination process itself causes some aggregation of the IgE. ¹²⁵I-IgE monomers and oligomers were therefore separated and purified by gel filtration using Sephacryl S-300 (Pharmacia) equilibrated in D-PBS (Menon *et al.*, 1985a).

³H-myo-inositol (60–100 Ci/mmol) was from Amersham (Arlington Heights, IL), whereas ³H-arachidonic acid (60–100 Ci/mmol), ¹²⁵I, and 5-(1,2-³H)-hydroxytryptamine binoxalate (15–30 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Ionomycin, staurosporine, and K252a were purchased from Calbiochem (San Diego, CA), whereas DTBP was from Pierce. Quin-2/AM was from Molecular Probes (Junction City, OR). All other reagents were obtained from Sigma (St. Louis, MO). DNP-BSA containing ~50 mol of DNP per mole of BSA was prepared according to published methods (Mishell and Shiigi, 1980). PMA, ionomycin, staurosporine, K252a, Quin-2/AM, quercetin, and NDGA were prepared as stock solutions in dimethyl sulfoxide, whereas sphingosine was dissolved in ethanol. In all cases, the maximum concentration of organic solvent in the experiments was 0.05%.

Receptor detergent insolubility assay

The method of Robertson *et al.* (1986) was used with some modifications (Apgar, 1990). Briefly, RBL cells, at a concentration of 3 × 10⁶ cells/ml, were incubated with 1 μg of ¹²⁵I-IgE per ml for 1 h at 37°C. The cells were then washed three times in D-PBS, resuspended in complete RPMI 1640 media, and allowed to adhere for 1 h at 37°C to 24-well plates at a concentration of 1 × 10⁶ cells/well. The supernatant was then removed and the cells were stimulated by the addition of 300 μl of Hank's balanced salt solution containing 0.5% BSA and DNP-BSA. Detergent-insolubility assays were usually performed at 24°C because internalization of the receptors does not seem to occur at this temperature (Robertson *et al.*, 1986). In some experiments the interaction was stabilized by the use of DTBP, at a final concentration of 10 mM. Unreacted DTBP was blocked with lysine, the supernatant was removed, and the cells were solubilized with 1 ml of ice-cold solubilizing buffer (50 mM NaCl, 2.5 mM MgCl₂, 300 mM sucrose, 1 mM PMSF, 0.5% Triton X-100, 10 mM HEPES, pH 7.2) for 15 min at 4°C. The supernatant was then removed and the Triton insoluble shells were solubilized with 4% sodium dodecyl sulfate (SDS) in D-PBS. The ¹²⁵I content of the Triton soluble and insoluble material was determined using a Micromedic gamma counter.

Detergent-insolubility of the IgE receptors on purified plasma membranes was measured as previously described (Apgar, 1990). Monomeric ¹²⁵I-IgE, purified by Sephacryl S-300 gel filtration, was centrifuged in a Beckman airfuge for 30 min at 149 000 × *g* before being added to plasma membranes at a concentration of 1 μg IgE per 10 μg of plasma membrane. After a 2-h incubation at 4°C, the plasma membranes were washed three times using D-PBS. The membranes were then pelleted in an airfuge for 7 min at

149 000 $\times g$ and resuspended in D-PBS containing 1 mM PMSF at a concentration of 200 $\mu\text{g/ml}$; 50- μl aliquots were placed in tubes. The IgE-sensitized plasma membranes were then incubated with 50 ng of DNP-BSA in 50 μl for 30 min at room temperature. The reaction was stopped by the addition of ice-cold solubilizing buffer containing 1% Triton X-100. After a 30-min incubation at 4°C, the membrane skeleton and any associated proteins were isolated by centrifugation for 7 min in the airfuge. The pellets were then washed once in D-PBS containing 0.5% Triton X-100. The results are expressed as a percentage (cpm in pellet/total cpm bound to plasma membranes) $\pm$ SE.

³H-5HT release assay

RBL cells were incubated with IgE and 3 $\mu\text{g/ml}$ of ³H-5HT for 2 h at 37°C. The cells were then washed three times, allowed to adhere to 24-well plates, and stimulated with DNP-BSA for 45 min. Degranulation was measured by removing the supernatant and centrifuging to remove any detached cells. Total incorporation of ³H-5HT into the cells was determined by lysing the cell monolayers with 3% SDS. Percent specific release of ³H-5HT was calculated as (cpm in supernatant)/(cpm in supernatant + cpm in cell monolayers) $\times$ 100%.

Measurement of arachidonic acid metabolism

RBL cells were grown for 24 h in complete RPMI 1640 containing 0.5 $\mu\text{Ci/ml}$ of ³H-arachidonic acid. The cells were then washed three times in Hank's balanced salt solution containing 0.5% BSA and sensitized with IgE as described above. After sensitization, the cells were washed three times, allowed to adhere to 24-well plates, and stimulated with DNP-BSA in Hank's balanced salt solution containing 0.1% BSA. After 30 min, the supernatants were removed, the wells washed with Hank's balanced salt solution, the combined supernatants centrifuged at 1000 rpm for 5 min, and an aliquot removed for measurement in a liquid scintillation counter.

³H-inositol phosphate production assay

RBL cells were grown in Eagle's basal medium and labeled with 1 μCi of ³H-myo-inositol for 24 h. The cells were harvested, washed three times in Hanks balanced salt solution containing 0.5% BSA, and sensitized with IgE for 2 h. The cells were then washed three times and finally resuspended in Hank's balanced salt solution containing 0.1% BSA and 10 mM LiCl at a concentration of 3.3×10^6 cells/ml. Aliquots (300 μl ; 1×10^6 cells total) were placed in glass tubes and preincubated with the appropriate concentration of inhibitor for 15 min at 37°C. The cells were then stimulated with 25 ng/ml DNP-BSA for 30 min at 37°C. The reactions were stopped by placing the tubes on ice and adding 500 μl of ice-cold PBS, 100 μl 0.22 M HCl, and 2 ml of chloroform/methanol (1:2 v/v). The tubes were then vortexed thoroughly and incubated on ice for 30 min. The phases were separated by centrifugation at 1000 rpm for 5 min. The upper aqueous phase was removed and saved. One milliliter of water was added to the lower phase, vortexed, and incubated on ice for 15 min. The tubes were then centrifuged and the upper phases removed and combined. The inositol phosphates were isolated using Dowex 1-X8 in the formate form according to published procedures (Berridge *et al.*, 1982).

⁴⁵Ca²⁺ influx assay

This assay was based on the method of Fewtrell and Sherman (1987). IgE-sensitized RBL cells were placed in 24-well

plates as described above in RPMI containing 0.1% BSA, 20 mM HEPES, and 100 μM Quin-2/AM and incubated for 1 h at 37°C. The supernatant was then removed and replaced with the same media without Quin-2/AM. The cells were incubated for 20 min at 37°C to allow intracellular hydrolysis of the Quin-2/AM to take place. After the removal of this supernatant, the cells were preincubated for 15 min with the appropriate inhibitor. The media was removed, and the cells were stimulated in media containing 10 $\mu\text{Ci/ml}$ of ⁴⁵Ca²⁺ and the appropriate inhibitor for 5 min. The media was removed, and the cells were washed twice with 2 ml/well of ice-cold quenching buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM LaCl₃, and 10 mM HEPES, pH 7.4). The cells were lysed with 500 μl of 3% SDS, and the ⁴⁵Ca²⁺ content was determined by liquid scintillation counting.

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