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IgE-MEDIATED DESENSITIZATION IN HUMAN BASOPHILS AND MAST CELLS

Donald MacGlashan, Jr., Sandra Lavens-Phillips, and Katsushi Miura

Johns Hopkins University, Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224

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1. ABSTRACT

Secretion from mast cells and basophils, two cells central to immediate hypersensitivity reactions, has characteristics that suggest the existence of intrinsic signal transduction processes that limit the extent of the cell's response. This process(es) has been termed desensitization. One goal of current research efforts is to determine the mechanisms used by mast cells and basophils to down-regulate an ongoing secretory reaction. Recent studies have indicated that, like secretion itself, the mechanisms of down-regulation or desensitization differ according to the mediator being studied. Thus, for human basophils, there appear to be distinct signaling pathways leading to the secretion of the three major classes of mediators -- granules contents, lipids, and cytokines -- and each pathway appears to have distinct down-regulatory processes. For an ongoing secretory reaction, the secretion of histamine and LTC4 are limited by a process that does not involve the earliest steps in activation, activation of the early tyrosine kinases, lyn and syk. These early events persist for long periods which more appropriately correspond to the regulation of cytokine secretion. Recent studies have also indicated that the process of desensitization is altered during stimulation in the absence of extracellular calcium, the traditional method of examining this process. These studies indicate that down-regulation studied in this manner is not dependent on any of the signaling events currently defined as being necessary for secretion. A variety of processes are discussed and potential mechanisms based on most recent studies using cell lines are explored.

2. INTRODUCTION

Allergic disease is characterized by a variety of symptoms that reflect the activation of mast cells in the tissues and basophils which have migrated into the tissues. The canonical characteristics of an acute allergic reaction -- swelling, erythema, and itching-- result from the release of histamine from either mast cells or basophils. The reaction is triggered by the binding of antigenic proteins to IgE antibody bound to high affinity receptors (FcεRI) on these two cell types. Because of the multivalent nature of both the cell bound IgE antibody and the antigenic protein, these reactions are initiated by the aggregation (crosslinking) of the cell surface IgE. Indeed, recent efforts to understand the very earliest stages of the IgE-mediated signal transduction cascade have focused on why aggregation is required. Like most signal transduction cascades, there appear to be pathways which lead to activation and secretion as well as pathways which serve to limit the extent of the reaction. In this context, it is useful to examine the characteristics of a typical histamine release kinetic curve (the most commonly studied mediator). Figure 1 demonstrates that histamine release is complete within 15-30 minutes of the addition of stimulus. It is notable that release reaches a plateau that is generally much less than 100% of total cellular histamine (histamine is a preformed mediator stored in cell granules so that the extent of the reaction is often expressed as the amount of histamine secreted into the extracellular medium relative to what is available in the cell). This plateau in release suggests a self-limiting reaction and this review will describe what is known about this reaction in human basophils and mast cells. It should be noted that there are several choices for cell models of IgE-mediated secretion. Rodent mast cells, either rat or mouse, are the most commonly used model of this type of secretion with studies of the rat basophilic leukemia cell (RBL-2H3) contributing the vast bulk of information on this reaction. However, RBL cells don't appear to demonstrate the same kind of down-regulation that is observed in human cells. So while studies of human cells are complicated by the

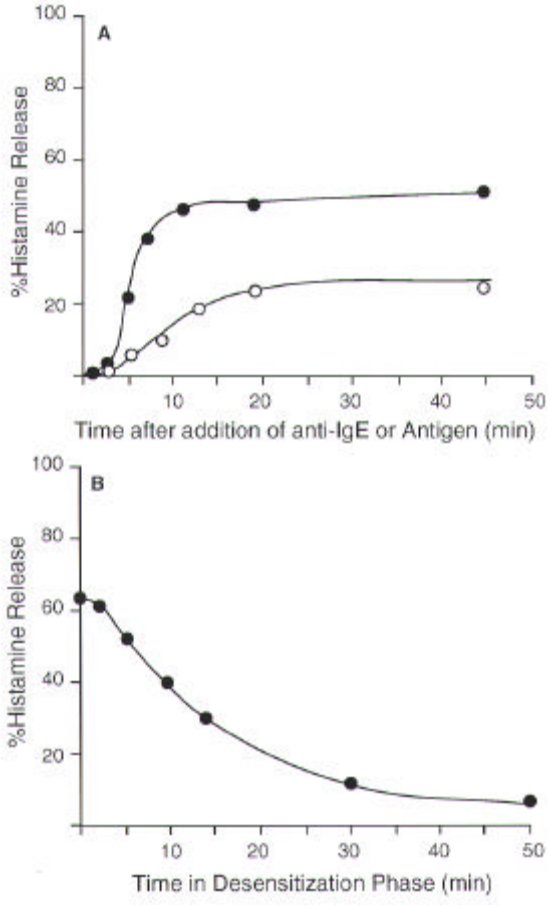

Figure 1. Kinetics of release and desensitization for human basophils. Panel A shows examples of two typical kinetic curves of histamine release for human peripheral blood basophils stimulated with an optimal (•) and suboptimal (○) concentrations of anti-IgE antibody. The ordinate is percent of the total cellular histamine content, obtained by lysing an aliquot of cells with perchloric acid, released into the supernatant. The points represent times, after the addition of stimulus, at which samples are harvested to determine the amount of histamine release. Panel B shows a typical kinetic curve of desensitization. The ordinate is the same as in panel A. The abscissa is the time that cells are exposed to an optimal concentration of anti-IgE antibody in the absence of extracellular calcium. Following a specific period of time, extracellular calcium is returned to the buffer and the reaction allowed to proceed to completion (usually another 45 minutes) before harvesting to measure the amount of histamine in the supernatants.

difficulties of purifying sufficient numbers for biochemical studies, they possibly represent the best available model of this particular reaction. Furthermore, the direct relevance of information obtained from studies of normal human basophils or mast cells also makes this an appealing model. Therefore, this review will focus on the human model but draw many inferences from the vast array of studies performed with RBL cells or other rodent mast cells.

3. IS SECRETION SELF-LIMITING?

Before proceeding with a description of the self-limiting reaction in these cells, it is useful to make a few observations about mediator release from basophils and mast cells. Historically, the most often studied mediator is histamine. Histamine has been known to be a feature of the allergic reaction for many decades but it was only in the 1960s that the precise cellular source of the histamine was identified as the mast cell or basophil. As noted above, the fact that it is preformed and therefore measurable in resting cells that have been lysed, led to the method of characterizing its release as a percentage of what is available in the cell. This particular measure of the extent of the reaction effectively normalizes the results for different numbers of basophils or mast cells present in the reaction and it reveals the self-limiting nature of the response. However, basophils and mast cells secrete other mediators, the most well studied being newly synthesized after stimulation. For example, both basophils and mast cells synthesize and secrete leukotriene C4. Since this mediator is not preformed, its secretion is generally characterized in units of picomoles/10^6 cells. But this gives no sense of relative strength of signal and it is therefore difficult to judge whether an IgE-mediated stimulus is inducing the maximum response or something less than maximum. The plateau of release has less meaning if it represents the maximum release possible (i.e., the exhaustion of stored mediators or newly synthesized mediator precursors) so it is useful to note that other stimuli, physiological or non-physiological, generally induce far more LTC4 release than the maximum obtainable with an IgE-mediated stimulus. With this consideration, it can be concluded that like histamine release, IgE-mediated LTC4 release also appears self-limited. Likewise, IL-4 secreted from human basophils is newly synthesized and while IgE-mediated stimulation induces substantial release of this mediator, it is clear that much more synthesis is possible (1, 2).Indeed, for both LTC4 and IL-4 release, the average donor's basophils secrete approximately 4 fold more of these mediators when ionomycin is the stimulus than when an optimal concentration of IgE-mediated stimulus is used. Therefore, IgE-mediated secretion of both IL-4 and LTC4 is probably self-limited in a manner consistent with histamine secretion. However, as will be elaborated on below, the precise mechanism for down-regulation of the secretion of each of the mediators may be different.

Because the IgE-mediated reaction is dependent on aggregation, the reaction between antigen and IgE (or anti-IgE and IgE) has characteristics entirely analogous to the classical immunoprecipitation reaction in solution. In other words, a maximum number of crosslinks forms on the cell surface at some concentration of antigen or anti-IgE antibody related to the affinity of the interaction (3,4) while higher concentrations lead to fewer crosslinks and at extreme concentrations all IgE binding sites are saturated with antigen but no crosslinks exist. In practice, the crosslinking curves can be quite broad but experiments to test for maximal histamine release are performed with concentrations that are first shown to be optimal. Since normal cell surface IgE bound to FcεRI is a mixture of different antigen specificities, the most commonly used stimulus is anti-IgE antibody which crosslinks all cell surface IgE and should presumably lead to the maximum

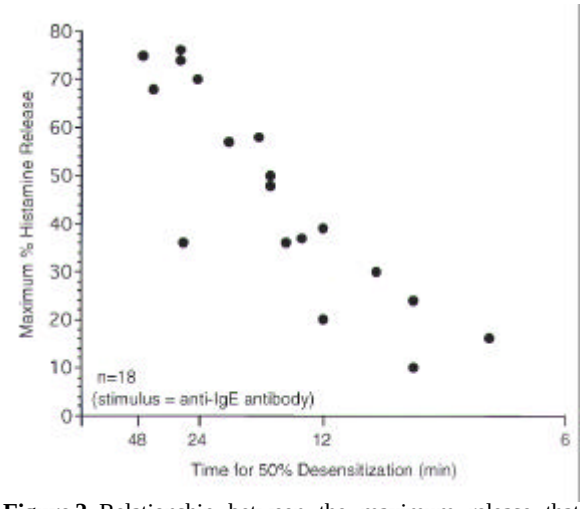

Figure 2. Relationship between the maximum release that can be obtained from a donor's basophils and the rate that the same cells can be desensitized. For this type of study, the optimum concentration for anti-IgE antibody-mediated histamine release is first determined. Using this concentration of anti-IgE antibody, a desensitization kinetic curve is generated similar to the one shown in figure 1B. The point at which 50% of the non-desensitized response occurs is extrapolated from the desensitization curve and plotted against the maximum release obtained with the same concentration of anti-IgE antibody. Each point in the figure represents the results of these two measurements for 18 different individuals.

level of signal transduction. Thus, when mediator release is limited to values less than what is obtainable by other means, one can be assured that maximal crosslinking has occurred despite the limited response. It should also be noted that basophils usually express far many more receptors than are required for histamine release. A typical donor's basophils are capable of a half-maximal response with only 2000-3000 IgE molecules per cell while the cell normally expresses greater than 100,000 receptors and often >95% of these are occupied with IgE antibody (5). Therefore, stimulation with an optimal concentration of anti-IgE antibody should generate a signal that is more than needed for secretion.

Expressing histamine release as a percentage of total cellular histamine tends to promote the question (although the question should arise whether or not the data is expressed this way) of whether less than 100% release results from a subpopulation of cells releasing 100% and the remainder not releasing at all (or some closely similar bimodal distribution of responses). This question is not entirely settled, but a variety of recent studies have indicated that for the most part, the response of single cells is graded according to the magnitude of the stimulus and that response distributions in cell populations are unimodal. One might also consider the possibility that some granules in all basophils or mast cells are simply not releasable. For example, 50% histamine release might actually represent 100% release of the releasable granules. However, combining anti-IgE antibody with other non-IgE dependent physiological stimuli can lead to 100% release, indicating that all granules are secretable. In the same context, enhancing agents such as IL-3 or deuterium oxide (to partially replace H_2O in the buffers) can enable the cells to release 100% in response to IgE-mediated stimulation. Finally, by definition, suboptimal concentrations of antigen or anti-IgE antibody leads to release which is less than optimal concentrations but with the important point that this is not a kinetic argument, a plateau of release is also reached at these less than optimal concentrations (see figure 1A). It should also be noted that secretion does not lead to cell death, i.e., this reaction is not limited because the cells die. Indeed, it can be shown that given enough time, human basophils and mast cells can reconstitute secreted granules (6, 7). Taken together, these data suggest that all cells participate, all granules can participate and that less than 100% release is an indication that down-regulatory events limit the extent of the reaction.

4. RELATIONSHIP BETWEEN DESENSITIZATION AND RELEASE

If down-regulatory events occur in parallel with activating events, the final response should represent the balance of the two opposing processes. Years ago it was shown that the self-limiting aspects of the reaction could be separated from the activating events by dividing the experimental protocol into two phases (8).In the first phase, cells are incubated with anti-IgE antibody or antigen in a buffer that doesn't contain calcium. Often a small amount of calcium chelator, like EGTA (5-10 μM), is included to insure the free calcium level is below the intracellular resting concentration of ≈100 nM. The extracellular calcium is necessary for secretion to occur, so this stimulation presumably results in some aspects of the activation cascade minus the sustained elevation of intracellular calcium that normally accompanies secretion (9, 10). Under these conditions, the cells begin to lose their responsiveness. This is demonstrated by returning calcium to the extracellular medium, allowing secretion to occur. One finds that the longer the period of incubation in the absence of extracellular calcium, the more poorly the cell secretes when calcium is returned to the medium (see figure 1B). On average, a 15 minute pretreatment (what is termed desensitization) in the absence of extracellular calcium leads to a 50% loss of the response of cells not pretreated with stimulus. An important technical point is that this decay in the response does not occur if the cells are simply incubated without calcium. For at least 3 hours, the human basophil can be kept in medium without extracellular calcium without altering its response when calcium and then antigen are added back. The kinetics of this antigen-induced decay of response resemble a first order decay process although the resemblance is only superficial. Nevertheless, it is convenient to speak of the desensitization kinetics as a rate, i.e. the time it takes for the response to decay by 50%. The typical 'rate' of desensitization is 15 minutes at concentrations of anti-IgE antibody that are optimal for histamine release in human basophils. In human mast cells, the rate is faster, typically 5 minutes. For different donors, there is a characteristic 'rate' of desensitization and a characteristic maximum release. A plot of 'rate' of desensitization versus maximum release for a variety of donors is shown in figure 2. A good inverse correlation is observed (if 'rate' of desensitization is plotted so that desensitization is measured as the inverse of the time required

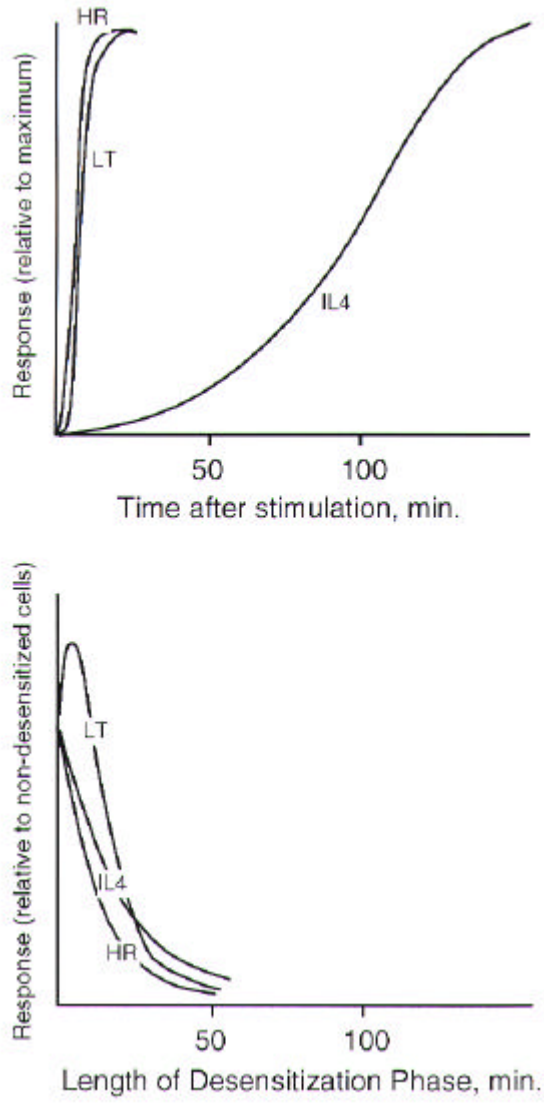

Figure 3: A stylized representation of the kinetics of histamine release and desensitization. The left panel shows the relative kinetics of histamine, LTC4 and IL-4 release from human basophils stimulated with an optimal concentration of anti-IgE antibody. Similar results are obtained when antigens are used. The right panel shows the kinetics of desensitization as measured by loss of ability to secrete either histamine, LTC4, or IL-4.

for 50% decay in the response). This analysis suggests that the faster the desensitization process, the more poorly the basophils release. Because the points in this plot represent different donors, the results suggest that desensitization may be an important determinant of basophil responsiveness.

An underlying hypothesis, that has only occasionally been tested, is that the nature of desensitization, both its rate and mechanism, is the same whether basophils or mast cells are stimulated in the presence or absence of extracellular calcium. In other words, the nature of the process studied in the absence of extracellular calcium is thought to be an accurate reflection of the process that limits ongoing secretion. There are a variety of experiments that indicate that this is only partially true. Early mathematical modeling very successfully predicted a number of release characteristics of basophils by explicitly formulating this hypothesis into a model of secretion. While this provided theoretical support for the hypothesis, experiments using the serine protease inhibitor, DFP (diisopropylfluorophosphate), provided experimental support. DFP treatment of basophils has two interesting characteristics. First, treating the cells with DFP and then washing the cells free of DFP prior to stimulation results in no change in secretion (11).However, if DFP is added with the stimulus, or even after stimulation, high concentrations effectively inhibit secretion. This irreversible inhibitor presumably alters the function of an enzyme that is only present following stimulation. The second characteristic is that DFP inhibits secretion at concentrations greater than 1 mM but markedly enhances release between 100 and 500 µM. Like inhibition, enhancement did not occur if the cell was incubated with DFP and then washed to remove the drug. It was then shown that DFP effectively inhibited desensitization and that the enhanced release probably resulted from the inhibition of desensitization. Indeed, the kinetic curves of histamine release, in the presence of DFP, showed the a characteristic one might expect under these conditions -- linear release without the plateau of release described above for normal secretion. These data supported the idea that desensitization represented the self-limiting process that regulates ongoing secretion.

In contrast, more recent experiments indicate that while some aspects of the desensitization process may be similar in the presence or absence of extracellular calcium, the kinetics of the process appear markedly altered for some of the newer endpoints examined, like IL-4 release (12). Figure 3 summarizes recent experiments which provide this newer perspective. When desensitization was first described as a process limiting histamine release, histamine was the only mediator measured from secreting basophils. In the early 1980s leukotriene release was added to the list. The release of this mediator had some qualitatively distinct characteristics from histamine release but the kinetics was otherwise similar and any working models of the overall reaction did not require substantial changes. In the early 1990s it was discovered that mast cells and basophils secrete cytokines. For human basophils, IL-4 secretion is prominent. Although mast cells may also secrete cytokines, the exact profile of released proteins is not yet clear. In basophils, secretion of IL-4 requires several hours rather than the minutes required for histamine or leukotriene release (13).This fact added an interesting wrinkle in the model of regulated secretion. If some self-limiting process serves to shut off histamine and leukotriene release within minutes, why does IL-4 secretion continue for hours? It was shown that this is not simply the result of a reaction which is started and then continues running without further input. If basophils are sensitized with an IgE specific for penicillin and stimulated with a multivalent penicillin-albumin (HSA) conjugate, histamine release is complete by 30 minutes but IL-4 secretion is just beginning. If the crosslinks are dissociated with monovalent penicillin, added after 30 minutes of stimulation, no further secretion of IL-4 occurs (12). This result indicates that the

primary signal must be maintained throughout secretion and that the reaction hasn't become dissociated from the original signal. Together, these data lead to the conclusion that if desensitization events serve to limit histamine release, they do not operate on signaling that leads to IL-4 secretion. Since IL-4 secretion is also self-limiting, another signaling pathway controls secretion of this mediator. Thus far, these results only suggest that there is more than one regulatory pathway. However, a further complication is that the process of down-regulation or desensitization that occurs following stimulation in the absence of extracellular calcium operates on all studied endpoints. The bottom panel in figure 3 summarizes these results. The primary result is that when cells are stimulated without extracellular calcium, the decay in the responsiveness occurs on a time scale similar for each type of mediator. In particular, IL-4 release desensitizes as quickly as histamine release. This would not be expected from the kinetics of release; the desensitization of IL-4 release should have occurred more slowly, possibly requiring hours. Therefore, the kinetics of down-regulation is altered by the absence of extracellular calcium. In particular, the down-regulation event(s) controlling IL-4 release appear to occur more slowly when extracellular calcium is present. Alternatively, the absence of extracellular calcium reveals a process which is not normally operative during secretion, one which serves to alter all subsequent signaling events. Weighing-in against this later interpretation are the DFP studies which show that inhibition of desensitization results in enhancement of histamine release.

5. SPECIFICITY OF DESENSITIZATION

Human basophils and mast cells can be stimulated with a variety of secretagogues although the list for basophils is currently much longer than the list for mast cells. In addition, because circulating IgE binds to FcεRI on either cell type, the binding of IgE to basophils and mast cells confers on these cells the range of antigen specificities found for the circulating IgE. One prominent distinction between the various stimuli is the immediacy of the response. Basophils respond to FMLP, C5a, PAF, chemokines like MCP-1 (and other chemokines that bind to either CCR2, 3 or 4) and the response is very rapid. Calcium elevations occur within 1-2 seconds of the addition of the stimulus (at concentrations that induce mediator release). In contrast, IgE-mediated responses are relatively slow to develop. The most obvious distinguishing characteristic is that non-IgE mediated secretagogues are 7 transmembrane receptors that couple through pertussis toxin sensitive GTP binding proteins. If basophils are desensitized through the FcεRI, there is no apparent change in the responsiveness to these non-IgE dependent secretagogues. Results like these suggest that IgE-mediated down-regulation operates on pathways specific to FcεRI stimulation and not on the myriad of shared pathways that probably are needed for secretion.

If basophils are desensitized with a specific antigen, the effects on release induced by non-crossreacting antigens are dependent on the densities of cell surface IgE specific for both the desensitizing antigen and the rechallenge antigen. In other words, desensitization may be specific for the antigen used to desensitize the cell or cross over to effect other antigen specificities and there is a continuum lying between these extremes. If the density of the antigen specific IgE is less than 2000-3000 molecules per basophil, desensitization is largely specific for the desensitizing antigen. The transition to progressively more desensitization of the non-crossreacting rechallenge antigen appears to occur between 3000 and 25000 IgE molecules per basophil (specific for the desensitizing antigen) (14, 15).For example, if a cell possesses 20,000 IgE molecules specific for ragweed antigen and 10,000 molecules of IgE specific for rye grass antigen, desensitizing the cell with an optimal concentration of ragweed antigen will likely ablate the response of the cell to rye grass antigen. To a modest extent, high densities of IgE specific for an antigen not used to desensitize the cell may allow the noncrossreacting antigen to overcome the nonspecific desensitization. It also appears that specific desensitization is relatively permanent state while the response to crossreacting antigens, if initially inhibited, slowly recovers over 24 hours (16).In these recovery experiments, the desensitizing antigen was dissociated from the cells with monovalent hapten prior to culturing the cells to determine whether recovery from nonspecific desensitization occurred. It is not known whether this dissociation step is critical for recovery.

As noted previously, studies of basophil and mast cell function are generally carried out with anti-IgE antibody as the IgE-dependent stimulus. Because the anti-IgE antibody has the potential of crosslinking all cell surface IgE, it must be assumed that desensitization experiments using anti-IgE antibody induce both specific and nonspecific desensitization. The degree of nonspecific desensitization cannot be assessed with normal reagents because the anti-IgE has aggregated all cell surface IgE. However, if the cells are first sensitized with mouse anti-DNP (dinitrophenyl) IgE (which binds with reasonable affinity to human FcεRI but doesn't crossreact with anti-human IgE), it can be shown that anti-human IgE antibody does indeed desensitize the cell nonspecifically. Since most studies of signal transduction use anti-IgE antibody as the stimulus, both the mechanisms of specific and nonspecific desensitization are presumably being studied. It is also interesting to note that concentrations of antigen that are too low to induce measurable mediator release may still desensitize the cell, albeit very slowly. Since mediator release does not occur, the desensitization can take place in the presence of extracellular calcium.

6. UNDERLYING MECHANISMS OF DESENSITIZATION

6.1. Extracellular Negative Feedback?

Thus far, the above discussion has only considered the general characteristics of release and desensitization and their potential relationship. The signaling pathways that lead to desensitization remain unknown. However, there are a variety of studies that suggest what aspects of signal transduction are not responsible for desensitization. The cartoon in figure 4 summarizes some general ways that cells use to down-regulate their response. The first process depicted in figure 4 would lead to experiments to test for feedback inhibition by mediators released from basophils. This possibility cannot be entirely excluded since not all granule contents or newly synthesized mediators (or other metabolites)

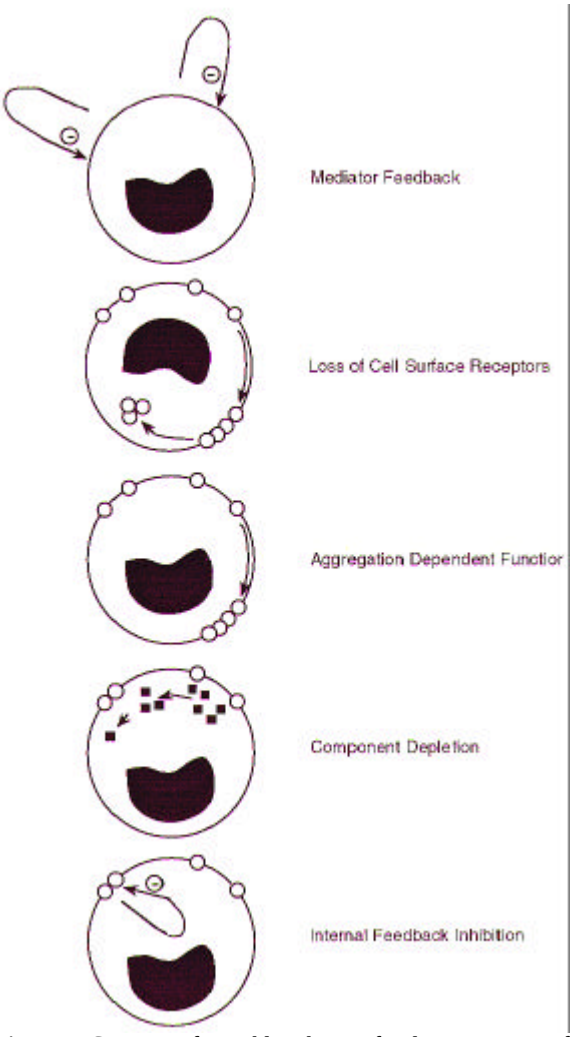

Figure 4: Cartoons of possible schemes for desensitization of IgE-mediated secretion in human basophils and mast cells. Refer to the text for a description of the underlying processes represented.

are known and even if they were known, the existence of receptors on basophils for these substances would also need to be known. However, the three major mediators have been examined. Basophils, but not mast cells, have a type 2 histamine receptor which will mediate inhibition of histamine release through an elevation in intracellular cAMP (17, 18).Histamine could, therefore, feedback on the cell response to down-regulate further secretion. However, H2 antagonists like cimetidine do not enhance histamine release. Nor could this process, or any released mediator account for desensitization that takes place during stimulation in the absence of extracellular calcium and therefore the absence of secretion. LTC4 and IL-4 do not appear to have any effects on basophil secretion. Similar studies have not been done for mast cells.

6.2. Receptor loss from the cell surface?

Many receptors are down-regulated by removal of the receptor from the cell surface. In many cases, the receptor undergoes recycling through endocytotic vesicle compartments to ultimately return to the cell surface. In some cases the receptor is degraded rather than recycled. IgE-mediated stimulation, within the time frame of histamine release desensitization (<1 hour) does not result in loss of cell surface IgE or its receptor. In light of the time course for IL-4 release discussed above, this makes sense. If enough receptor were removed to down-regulate histamine release, it seems likely that IL-4 secretion would also be ablated (for basophils stimulated with anti-IgE antibody, there is a vast excess of receptors such that a profound loss of receptors would be required to markedly reduce the signals leading to histamine release).

A subset of this scheme is depicted in the third process, the formation of large aggregates that are not removed from the cell surface but only poorly stimulate signaling. It seems likely that such a process can account for a specialized form of down-regulation in basophils. High concentrations of polyclonal anti-IgE antibody can be shown to induce extremely large aggregates that are visible as patches and eventually caps of IgE on the cell surface. The appearance of visible patching coincides with a marked decrease in histamine release (19) and if monovalent anti-IgE antibody (Fab' fragments) is added to this reaction, histamine release is enhanced (20).These results indicate that under some circumstances too large an aggregate is actually a poorer stimulus for release. It should be noted that this remains a special case because antigens and monoclonal anti-IgE antibodies do not lead to this result: antigen excess results in fewer crosslinks rather than larger aggregates and for all points on the dose response curve, the co-addition of monovalent antigen (usually monovalent haptens rather than single epitope antigens) causes fewer crosslinks to form and therefore, less histamine release. With respect to the large aggregates formed by polyclonal anti-IgE antibody, one speculation is that after a certain size aggregate is formed, further recruitment of more FcεRI into such aggregates does not lead to better signaling. As the reaction "matures", progressively more of the FcεRI is involved in these aggregates while no increase in signaling results, leading to less mediator release. We speculate that such a process may explain the different optimums of histamine and IL-4 release when basophils are stimulated with polyclonal anti-IgE antibody but not antigens (1, 13).The longer reaction time for IL-4 secretion and its requirement for a maintained signal mean that even a slow progression of aggregate size to larger aggregates would be detrimental to IL-4 release. It is also interesting to note that carrying out desensitization at 4^{o}C inhibits the process when antigens are used. If polyclonal anti-IgE antibody is used at supraoptimal concentrations to desensitize the basophil at 4^{o}C, desensitization is only modestly inhibited. This result further supports the hypothesis that the loss of response under conditions where large aggregates form may result from a process which is not dependent on the normal signaling apparatus. However, since this mode of down-regulation doesn't appear to operate for antigens, it does not explain the many other characteristics of desensitization. Indeed, since simple bivalent haptens can be demonstrated to induce the formation of only very small aggregates, dimers and trimers, and these haptens can still cause desensitization, it is apparent that other events occur.

Determining which signal transduction events participate in desensitization is made more difficult by the relatively difficult task of obtaining large numbers of purified basophils or mast cells. While there are hundreds of signaling pathways that have been studied in RBL cells, less than 10 signal pathways have been examined in any depth in human basophils or mast cells. Mechanisms that involve internal feedback have received the most attention. The implication of the DFP studies noted above is that passive component depletion (the fourth scheme in figure 4) doesn't explain desensitization. These DFP studies suggest that an active enzymatic process is involved (the fifth scheme in figure 4) and that desensitization is an active process rather than a passive depletion. Several pathways have been partially ruled out.

6.3. Negative Regulation by Protein Kinase C?

The PKC family of isozymes have been considered a central component of signal transduction for a large variety of intracellular signaling reactions. Early studies in human basophils noted that the phorbol esters alone were strong stimulants of histamine release (which is curiously not true for human or rodent mast cells) (21). Shortly after these observations were made, it was found that the phorbol esters were relatively selective activators of PKC, so this result suggested that PKC may play a pro-degranulatory role in basophil secretion. Interestingly, PMA does not induce LTC4 or IL-4 secretion. Another interesting characteristic is that PMA induces histamine release from basophils even if the cells are heavily loaded with the calcium chelator BAPTA and stimulated in the presence of extracellular EGTA. By the mid 1980s, it was also appreciated that PKC could act in a down-regulatory manner. Pre-incubation of RBL cells with PMA would inhibit an IgE-mediated elevation of cytosolic calcium. Later studies found a similar result for human basophils. Somewhat later, IgE-mediated stimulation of human basophils was found to induce a kinase activity with the characteristics of PKC, Ca^{++}/phosphatidylserine sensitive phosphorylation of histone III proteins (22).By this time, it had been found that all cells expressed multiple PKC isozymes. RBL cells expressed PKC isozymes that appeared to mediate activation, PKC β and PKC δ, as well as PKC isozymes that mediated down-regulation, PKC α and ε (23-26).Only recently were human basophils examined for the expressed isozymes of PKC (27).One surprise in the basophil studies was the absence of PKC α. Preliminary studies suggest that human lung mast cells do express this isozyme. In basophils, PKC βI, βII, δ and ε are present and there are pilot experiments which suggest that PKC ζ and τ are not present, although the commercial antibodies for these isozymes may not be good enough to draw a strong conclusion. While PKC translocation (cytosolic to membrane fractions) could be observed following stimulation with PMA or FMLP, no translocation could be observed following stimulation with anti-IgE antibody. This is not necessarily a concern since PKC δ and ε are distributed evenly between cytosolic and membrane fractions in resting basophils and translocation is not required for PKC to have a role.

To ascertain a role for PKC in the IgE-mediated reaction in human basophils or mast cells, pharmacological agents must be used although caution must be used in interpreting pharmacological experiments. Early PKC inhibitors are now known to be too nonselective. For example, staurosporine is an excellent inhibitor of both tyrosine kinases and PKC and since tyrosine kinases are required for IgE-mediated signaling, the use of staurosporine to study PKC is excluded. We have recently found that the second generation PKC inhibitor, Go-6976, is also an excellent tyrosine kinase inhibitor and is therefore not useful in these studies (27).Calphostin C has been used in a variety of studies but its use in basophil studies is also problematic. It inhibits ionomycin-induced release and also induces marked spontaneous release from basophils. However, the indolylmaleimides, bis-indolylmaleimide I and II and Ro-31-8220 have been found to be reasonably selective inhibitors of PKC isozymes. These inhibitors effectively reverse a wide variety of PMA-induced events in basophils. For example, PMA induces histamine release directly, synergizes with ionomycin to induce secretion, induces phosphorylation of cPLA2, inhibits ionomycin-induced IL-4 secretion, inhibits the cytosolic calcium elevation that follows IgE-mediated stimulation and induces marked morphological changes. For each of these endpoints, the indolylmaleimides (200 - 1000 nM) completely reverse the effects of PMA. Therefore, these compounds effectively inhibit the activity of some, if not all, PKC isozymes. However, they do not inhibit IgE-mediated histamine release (27). Nor do they inhibit desensitization. This was, naturally, a surprising result and to conclude that PKC isozymes do not play a role in active secretion or desensitization may be premature. However, the negative results are strengthened considerably by the clear ability of these compounds to inhibit a wide variety of PMA-induced events in basophils.

It should be noted that while the indolylmaleimides do not inhibit histamine release, they do cause modest enhancement of antigen-induced histamine release. Enhancement of release induced by anti-IgE antibody is sporadic and modest so that it is not statistically different from controls. The enhancement of antigen-induced release suggests that during active secretion, PKC may play a down-regulatory role. This seems contradictory to the experiments where the indolylmaleimides inhibited neither antigen or anti-IgE antibody induced desensitization. But, as noted previously, there is evidence that down-regulation may change in the presence or absence of extracellular calcium. Provided the modest enhancement observed with antigen-induced release is not a result of nonselective effects of these inhibitors, the data could indicate that a calcium-dependent PKC has a modest role in down-regulating secretion or that the interaction of a PKC with the signaling complex is dependent on calcium. Interestingly, these inhibitors cause a more marked enhancement of IL-4 release. Recent studies have noted that PMA inhibits the IL-4 release induced by ionomycin (a calcium ionophore), in stark contrast to the synergy between PMA and ionomycin observed when lymphocytes are stimulated for IL-4 secretion (2).While this result may be a red-herring, as the PMA-induced histamine release seems to be, the enhancement of IgE-mediated IL-4 release by indolylmaleimides may indicate a down-regulatory role for PKC in this signaling pathway. If the distinction between stimulation in the presence or absence of extracellular calcium indicates the involvement of a calcium-

dependent PKC, the only two available in the human basophil are PKC βI and βII. In RBL cells, PKC β appears to play a role in activation rather than de-activation but the role may be different in human cells.

As noted above, the observation that down-regulation differs if the cells are stimulated in the presence or absence of extracellular calcium was unexpected. It leads to experiments to determine what is different about the two conditions. One obvious difference is the nature of the cytosolic calcium response. If basophils or mast cells are stimulated in the presence of extracellular calcium there is a biphasic character to the cytosolic calcium elevation. As in most cells, there is an initial release of calcium from internal stores that is followed by an influx of calcium from the extracellular buffers. If cells are incubated in a calcium containing buffer and a calcium chelator like EGTA is added just prior to the addition of stimulus, the release from internal stores persists and the initial phase of the (Ca^{++})i response looks similar to that observed without the pre-addition of EGTA. However, the sustained (Ca^{++})i response is ablated. It should be noted that a sustained (Ca^{++})i response does not always follow receptor stimulation of basophils or mast cells. For example, stimulation of basophils with C5a (28) or mast cells with substance P (29) results in a strong initial release of calcium from internal stores and a rapid return to resting calcium levels, with little or no obvious influx component. So the sustained response following stimulation through FcεRI represents an aspect of the calcium response which is clearly dependent on the nature of the signal transduction events. The absence of the late component of the (Ca^{++})i response therefore distinguishes stimulation in the presence and absence of extracellular calcium. However, the presence of the initial response indicates that early signaling events are intact and that there is a transient opportunity for signaling components that are dependent on an elevation in (Ca^{++})i to be active.

More recent experiments provided a somewhat different perspective on the desensitization that is observed in the absence of extracellular calcium. Different cell types are variably sensitive to incubation in the absence of extracellular calcium. For example, it is known that treatment of RBL cells with EGTA leads to a rapid loss of internal stores of calcium. Human basophils also appear to lose these internals stores, but over a longer time frame. Operationally, a typical desensitization experiment is carried out on cells which have been handled in buffers not containing calcium (with little or no EGTA used). Recent studies indicate that stimulation with anti-IgE antibody results in a markedly blunted release of internal stores of calcium in cells which have not been suspended in a calcium containing buffer for some time before challenge.

Because the release of calcium from internal stores has a highly nonlinear dependence on the signal strength, a blunted calcium response could either indicate a severely blunted early signal transduction cascade or simply the marked reduction of internal stores of calcium. It appears that the latter situation explains the blunted calcium response so that this observation cannot be used to infer what it happening to earlier signaling steps. However, the fact that no calcium response occurs in cells which clearly desensitize reinforces the conclusion that desensitization doesn't even require the transient release of internal stores of calcium discussed above.

Studies in RBL cells indicate that the earliest steps in FcεRI-mediated signaling involve the phosphorylation of the receptor and two associated tyrosine kinases, lyn and syk. While the exact progression of events remains unclear, it appears that there is a low level of association between membrane bound lyn and FcεRI (30).When the receptors are crosslinked, lyn phosphorylates the beta subunit of FcεRI to which it is not directly associated, so-called cross-phosphorylation (31-33).This in turn leads to the further recruitment of lyn to the phosphorylated beta subunit and cross-phosphorylation of other beta subunits by the newly recruited lyn occurs. Phosphorylation of FcεRI γ follows and syk is recruited to bind to FcεRIγ and becomes enzymatically active. Downstream elements are then phosphorylated by syk. At this point the signal cascade is somewhat less clear. A hallmark of these early reactions is the apparent absence of any requirement for increases in (Ca^{++})i., i.e., lyn and syk phosphorylation occur equally well in RBL cells stimulated in the presence or absence of extracellular calcium. For human basophils, this is not true (34).Unlike RBL cells, a short treatment with EGTA prior to stimulation leads to a blunted and transient phosphorylation of both lyn and syk (receptor phosphorylation has yet to be studied). If cells are stimulated under conditions more often associated with a desensitization experiment (prolonged handling in calcium free buffers, but not necessarily containing EDTA or EGTA), phosphorylation of lyn and syk is markedly blunted. Recent unpublished experiments indicate that lyn and syk activity may not be required for normal desensitization kinetics. Treatment of the cells with Go-6976, which inhibits lyn and syk phosphorylation, has no effect on desensitization. The evidence thus far indicates that cells which have not been in calcium containing buffers, display blunted lyn and syk phosphorylation, blunted release of calcium from internal stores and in data not discussed, blunted production of diacylglycerols. Taken together, the data suggests that desensitization, studied this way, does not need the components of signal transduction most often associated with mediator release.

6.4. Down-regulation during active secretion

Down-regulation of signaling in the presence of extracellular calcium has a different set of characteristics. As might be expected from the long, crosslink-dependent, time course of IL-4 secretion, the phosphorylation of lyn and syk appears equally sustained. This observation is interesting for the fact that the early signaling components remain active when the release of histamine and LTC4 has altogether stopped (34).In contrast, the phosphorylation states of ERK1/2, whose phosphorylation states seem associated with the generation of arachidonic acid by cytosolic PLA2 and the release of LTC4, are transient on a time scale fully compatible with the start and stop of LTC4 release (35).The phosphorylation of ERK1/2 is thought to result from a signaling cascade that starts with the activation of Ras which is in turn, indirectly activated by syk activities. The observation that the activity of this cascade is transient while syk phosphorylation is sustained is intriguing and the data

suggests that down-regulation occurs proximal to MEK activation and distal to syk activation.

6.5. Alternative Mechanisms

If the changes that occur to basophils and mast cells during stimulation in the absence of extracellular calcium do not require the activation of signaling components normally associated with secretion, the next step is to find events which don't depend on these steps. An intriguing recent observation made in RBL cells is that FcεRI must associate with specific microdomains of the plasma membrane in order to express activity (36).These domains are associated with detergent insoluble fractions of RBL cells and phosphorylation of FcεRI β and γ are dependent on association with these domains. The microdomains appear enriched in lyn kinase and phosphorylation of the receptor subunits is not required for association of aggregated receptors to the domains. Cytoskeletal elements also appear to regulate persistence of receptor in the microdomains. If the cytoskeleton is disrupted with cytochalasin D, the reaction persists for longer periods (36, 37).It appears that the cytoskeleton regulates association with the domains and that slowly, receptors are eliminated from the domains by cytoskeletal activity. In this description of events, down-regulation can result from the removal of aggregates from the functional microdomains. Similar studies in human basophils or mast cells have not been done. However, it is useful to note that cytochalasin B markedly enhances histamine release from basophils (38).Its effects on the secretion of other mediators has not been studied. To study its effects on desensitization requires some care be taken to wash out the drug prior to testing for the extent of desensitization by challenge in the presence of extracellular calcium. While this technique has worked for a study of the effects of indolylmaleimides and Go-6976 on desensitization, it has not yet been effective for cytochalasin D.

Another potential mechanism relates to recent studies showing the recruitment of phosphatases to the receptor complex following aggregation. Phosphatases such as SHP-1, SHP-2 and SHIP, once recruited, could regulate or blunt the subsequent reaction. It will be some time before this possibility can be examined in detail in human basophils or mast cells but this reaction may also be dependent on some phosphorylation of the receptor to effect the recruitment process, so more immediate studies will need to focus on the phosphorylation of the receptor during stimulation in the absence of extracellular calcium.

One theme not yet mentioned relates to recent observations that Fc receptors or T cell receptors can be modulated by the co-aggregation with other cell receptors that recruit phosphatases to the receptor complexes (39).For example, co-aggregation of IgG receptors with IgE receptors down-regulates the antigen driven response. This appears to result from the recruitment of SHIP or SHP-1 to the receptor complex where the phosphatases de-phosphorylate the relevant receptor or associated kinases required for activation (40, 41).At this point in time, there have been no indications that IgE-mediated desensitization results from co-aggregation with other cell surface receptors. However, the case of antigen initiating a co-aggregation of the IgE and IgG receptors is a very real possibility and may ultimately prove to be an important part of basophil and mast cell biology.

7. SUMMARY

It is evident that mediator release from basophils and mast cells is a self-limited reaction. One way of studying this down-regulatory aspect of signaling is to challenge these cells in the absence of extracellular calcium. While this does reveal an aspect of down-regulation, it is now clear that this process is not exactly the same during stimulation in the presence of extracellular calcium. It now also seems likely that this process of desensitization doesn't require some of the early signaling events normally associated with active secretion. Some down-regulatory processes occurring during active secretion have a different time course from those occurring during a desensitization-style experiment and appear to be selective for a particular mediator release process. Basophils and mast cells appear to have multiple points of auto-regulation and may be able to selectively regulate different classes of mediators by altering specific desensitization processes. It is now becoming clear to investigators studying activation of hematapoietic cells, T and B cells in addition to mast cells and basophils, that this process of fine-tuning the responsiveness of the cells by altering the down-regulatory elements is an important theme in regulating the characteristics of the immune response.

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Send correspondence to: Dr Donald MacGlashan, Jr., Johns Hopkins University, Asthma and Allergy Center, 5501 Hopkins Bayview Circle Baltimore, MD 21224, Tel: (410)-550-2145, Fax:410-550-2130,E-mail: dmacglas@welchlink.welch.jhu.edu