Desensitization of Mast Cells' Delayed Response to the Type 1 Fcε Receptor

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The regulatory mechanism leading to desensitization of cellular response has been widely investigated in the case of several non-immune membrane receptors (e.g., for growth factors, hormones, or neurotransmitters). However, knowledge of this process in the case of response to the multichain immunorecognition receptors, notably the type I receptor for immunoglobulin E, is rather limited. We first investigated whether the FcεRI-mediated secretory responses of mast cells can be subjected to the desensitization protocol already found to be effective for non-immune receptors (i.e., by excessive, prolonged or repetitive exposure to the stimulus), under physiological conditions (i.e., in the presence of extracellular source of calcium). The rat mucosal-type mast cells of the RBL-2H3 line that enable a rigorous examination of the response to the FcεRI stimulus were previously found to undergo such a process when subjected to prolonged FcεRI-IgE clustering by specific antigen (DNP_{11}-BSA) or by the IgE-specific monoclonal antibody 95.3 at concentrations close to the threshold of inducing secretion. The induced desensitization was then resolved in a reduced secretory response upon optimal FcεRI-IgE clustering. The extent of the observed desensitization was further found to depend on the concentrations of cell-bound IgE and of the clustering agents, as well as on the length of the desensitization period.

The question whether the prolonged FcεRI-IgE sub-threshold clustering by antigen would also desensitize the secretion of *de novo* synthesized mediators (i.e., arachidonic acid-derived products and cytokines) has now been addressed and was found to effectively suppress the secretion of newly synthesized leukotrienes and cytokines (as monitored by secretion of leukotriene B4 or tumor necrosis factor alpha and interleukin-4 respectively). Moreover, our findings also indicate that the *de novo* synthesis of late-phase mediators is regulated by desensitization at the pretranslational level.

Prolonged sub-threshold FcεRI-IgE clustering by antigen or by an IgE-specific monoclonal antibody causes desensitization of the secretion of *de novo* synthesized leukotrienes by RBL-2H3 cells

The conditions of desensitization of FcεRI-induced cellular response under defined physiological conditions (i.e., in the presence of an extracellular source of calcium) were investigated in the rat mucosal-type mast cells of the RBL-2H3. FcεRI-IgE clustering on these cells induces the *de novo* synthesis and secretion of several arachidonic acid-derived products (i.e., leukotrienes and prostaglandins) [1]. We first identified the conditions leading to desensitization of these newly synthesized mediators' secretory response to the FcεRI stimulus, as monitored by the secreted LTB4. Control cells were kept for equal periods of time without antigen. Desensitized (●) and non-desensitized (O) cells were next optimally stimulated for 30 minutes with 10 ng/ml antigen. The decrease in secretion due to desensitization (right scale, □) was calculated by subtracting the desensitized response from the non-desensitized one and expressed as a percentage of maximal response. The value of cells' basal LTB4 secretory response is (◊). Points represent average secretion of triplicates ± SD. Data are taken from one experiment out of two.

To this end, the threshold antigen concentration, defined as the lowest concentration of the clustering agent yielding minimal, yet significant, secretion of LTB4, was first determined. Then, cells were subjected to prolonged incubation (12 hours) with sub-threshold concentrations of the FcεRI clustering agent, i.e., the antigen DNP_{11}-BSA. The threshold and optimal concentrations of the antigen at both IgE concentrations (0.5 and 5 nM) were also determined by measuring the dose dependence of the cells' LTB4 secretion.

As previously found for the immediate secretory response, i.e., the degranulation [2], a clear inverse relation was established between the IgE concentration and the extent of desensitization, i.e., the lower the IgE concentration, the larger the desensitization effect. Thus, cells reacted with 0.5 nM IgE exhibited an up to 94% decrease of the LTB4 secretion [Figure 1A], while cells incubated with 5 nM IgE exhibited a 80% decrease of the secretory response [Figure 1B]. Still, as observed in the case of degranulation, the sub-threshold antigen concentration (1.5 ng/ml) used for inducing desensitization was found to cause the largest decrease of LTB4 secretion, regardless of the IgE concentration used [Figure 1]. These results established that the protocol we identified as causing desensitization of the immediate secretory response also effectively desensitizes secretion of the examined *de novo* synthesized leukotrienes.

Prolonged sub-threshold FcεRI-IgE clustering also causes desensitization of another late-phase cellular response: the secretion of *de novo* synthesized cytokines

We further investigated the feasibility of desensitizing, by the same protocol, the secretion of *de novo* synthesized cytokines by

FcεRI = type I receptor for immunoglobulin E
Ig = immunoglobulin
LTB4 = leokotriene B4

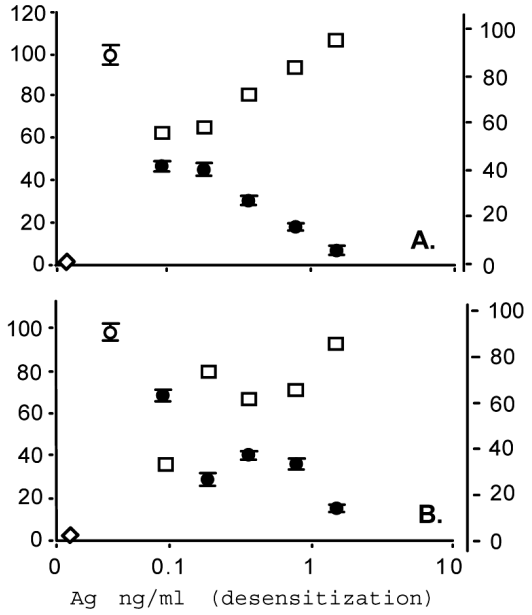

Figure 1. Prolonged sub-threshold FcεRI-IgE clustering by antigen causes desensitization of leukotriene B4 secretion. Adherent RBL-2H3 cells (1 x 10^5 cells/100 μl/well) were reacted for 2 hours with 0.5 nM **[A]** or 5 nM **[B]** of IgE. Cells were then incubated for 12 hours with a sub-threshold range of antigen content followed by optimal stimulation.

monitoring IL-4 and TNFα secretion [2]. It is noteworthy that in this case we observed that both the threshold and optimal concentrations of antigen were found to be three to sixfold lower than those previously required for inducing desensitization of degranulation or leukotriene secretion respectively. Evidence that untreated RBL-2H3 cells already store limited amounts of TNFα was provided by an earlier report [3]. Hence, in order to ascertain that changes in secretion of the newly synthesized mediator were monitored, desensitization and stimulation phases were always carried out for a period of least 4 hours.

The desensitization protocol and its results have been presented previously [2]. Briefly, RBL-2H3 cells were preloaded (1 hour) with IgE (0.05 or 0.5 nM) and then subjected to prolonged sub-threshold clustering (14 hours) with the relevant antigen concentrations (0.045–1.5 ng/ml). The desensitized cells and their non-treated control were then stimulated for an equal period of time with the appropriate optimal antigen concentrations. The dependence on the IgE and antigen concentrations of the extent of desensitization was determined. As found for the desensitization of the immediate response (degranulation) and that of LTB4 secretion, that of TNFα secretion depended on concentrations of both IgE and of the desensitizing antigen. Thus, cells treated with 0.05 nM IgE exhibited up to 90% of desensitization, while those treated with 0.5 nM IgE exhibited a noticeably smaller decrease in secretion (~53%). The desensitization of the TNFα secretion was found to develop in a time-dependent manner: At low IgE (0.05 nM) a decrease of 70% of the secretory response was observed after 8 hours and 90% following 14 hours of sub-threshold treatment. In cells reacted with 0.5 nM, the time dependence of the process was noted only when treatment was carried out with lower sub-threshold level antigen concentrations (i.e., 0.75 ng/ml or less), reaching the maximal desensitization (40%) after 14 hours. Moreover, desensitization of secretion of the other cytokine (IL-4) was also found to develop under analogous conditions and to reach the same magnitude as observed for TNFα.

Taken together, these results confirm the effectiveness of the physiological protocol of sub-threshold FcεRI-IgE clustering by antigen for desensitization of secretion of *de novo* synthesized mediators in the RBL-2H3 cells. It should be stressed that the extent of desensitization was found to crucially depend on the concentrations of the IgE and of the FcεRI aggregating agent and optimally develops at low levels of the FcεRI clustering. Furthermore, these findings suggest that the extent of the FcεRI-IgE clustering might function as a common regulatory mechanism under non-pathological conditions (i.e., at low levels of specific IgE molecules). However, the consistently observed inverse relation between the IgE concentration and extent of desensitization restricts the conditions of optimal desensitization to very low concentrations of the receptor-bound IgE.

We also identified a protocol that successfully desensitizes a late-phase secretory response (i.e., TNFα secretion) from cells incubated with high IgE concentrations (5 or 50 nM). Briefly, cells reacted with high concentrations of IgE (5 or 50 nM) were incubated for 4 hours with an antigen concentration previously determined to be sub-threshold and which desensitized the secretion of this cytokine. At the end of this short period, the cells were washed and re-incubated for an additional 4 hours with the same sub-threshold antigen concentration. These desensitized cells and their control (maintained in antigen-free medium for equal periods of time) were then subjected to a final 4 hour stimulation with a range of antigen concentrations. This protocol was found to cause a decrease of up to 90% of the TNFα secretion at both IgE concentrations used. Hence, the late-phase response of secretion of the *de novo* synthesized mediators can be effectively desensitized also at high concentrations of IgE by exposing the cells to repetitive sub-threshold clustering by antigen.

These findings strongly suggest that the limited extent of the FcεRI-IgE clustering could be relevant in terms of a general regulatory intracellular mechanism induced by the desensitization process.

Prolonged sub-threshold FcεRI-IgE clustering inhibits the production of mRNA of late-phase cytokines

In an effort to identify the molecular mechanisms of desensitization we investigated whether the transcription of cytokine genes was affected by the desensitization protocol used. To this end, the RBL-2H3 cells were desensitized (see legend of Figure 2), their total mRNA was isolated, and the expression levels of specific mRNAs for several cytokines was then determined. As shown, antigen-mediated optimal stimulation generally induced robust transcription of the investigated genes. In turn, treating

TNFα = tumor necrosis factor alpha

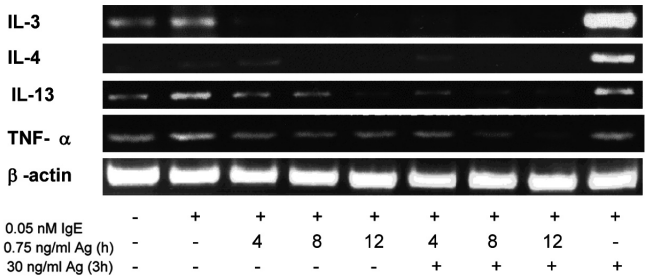

Figure 2. FcεRI-IgE clustering by sub-threshold concentrations of antigen markedly reduces the amount of the expressed cytokines' mRNA. Adherent RBL-2H3 cells grown in Petri dishes (1 x 10^7 cells/plate) were treated for 2 hours with 0.05 nM IgE and then incubated for the shown time periods with 0.75 ng/ml antigen. The control cells were maintained without antigen for equal time periods. Desensitized cells and their control were then stimulated for 3 hours with 30 ng/ml of antigen. At the end of the treatment all the cells were lysed and their total mRNA was isolated using a RNeasy kit (Qiagen, CA, USA). Its c-DNA was obtained by using a Reverse iT™ 1st Strand Synthesis kit (ABgene, UK), further amplified in a polymerase chain reaction with the appropriate primers for IL-3, IL-4, IL-13, TNFα and β-actin, resolved on 1% agarose gel and visualized under ultraviolet light. Data are derived from one out of four independent experiments.

the cells with the sub-threshold antigen concentration (0.75 ng/ml) for 1–12 hours did not generate detectable amounts of their specific mRNA [Figure.2]. However, when the cells were subjected to desensitization followed by optimal stimulation, a significant decrease in the amounts of all investigated transcripts was lower than the basal level observed in untreated cells (i.e., not treated with antigen). These results indicate that *de novo* synthesis of late-phase mediators is regulated by the desensitization at the pretranslational level, either by reducing the activity and/or expression of the transcription factors (AP-1, NFAT) or by processing the primary transcript of the encoding gene(s).

The considerable efficacy of this inhibition suggests that the control process operates upstream of the gene transcription, i.e., it affects the performance of the transcription factors. Several reports [4-6] propose that transcription of cytokine genes in mast cells is induced by distinct transcription factors (e.g., the production of IL-13 is under control of NFAT, while TNFα is regulated by AP-1). These findings, together with the present ones, suggest that discrete transcription factors may similarly be regulated by the desensitization, most likely by interference with specific elements of the FcεRI coupling network.

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