ORIGINAL ARTICLE

Allergen-Sensitization Increases Mast-Cell Expression of the Exocytotic Proteins SNAP-23 and Syntaxin 4, Which Are Involved In Histamine Secretion

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Abstract

Background: Activation of mast cells (MCs) via aggregation of immunoglobulin E (IgE) bound to its high affinity receptor (FcεRI) results in release of inflammatory mediators from secretory granules. Histamine is one of the critical biological mediators released in the allergic response. Synaptosomal-associated protein of 23 kDa (SNAP-23) and syntaxin 4 are plasma membrane proteins that have been associated with exocytosis in MCs. Studies with monoclonal IgEs indicate that binding of IgE to FcεRI induces molecular and biological changes in MCs.
Objectives: The aim of this study was to investigate changes in the expression of SNAP-23 and syntaxin 4 by MCs following rat sensitization with ovalbumin (OVA). In addition, we assessed whether these proteins were involved in histamine secretion.
Methods: SNAP-23 and syntaxin 4 expression was analyzed by Western blot using MCs from control and sensitized animals. Successful sensitization was confirmed based on the passive cutaneous anaphylaxis reaction. To test the role of these exocytotic proteins in histamine secretion, permeabilized MCs were incubated with SNAP-23 and syntaxin 4 antibodies.
Results: Expression of SNAP-23 and syntaxin 4 was significantly higher in MCs from OVA-sensitized rats than in cells from control animals. In addition, incubation of permabilized cells with antibodies to SNAP-23 and syntaxin 4 led to a marked reduction of histamine secretion in stimulated cells.
Conclusions: Sensitization with OVA increases the expression of SNAP-23 and syntaxin 4 in MCs. Furthermore, our data suggest that these exocytotic proteins participate in histamine secretion.

Key words: Histamine. Mast Cells. Sensitization. SNAP-23. Syntaxin 4.

Resumen

Antecedentes: La activación de los mastocitos (MCs) mediante la agregación de inmunoglobulina E (IgE) ligada a su receptor de alta afinidad (FcεRI) produce una liberación de mediadores inflamatorios de los gránulos.
La histamina es uno de los mediadores biológicos críticos liberados en la respuesta alérgica. La proteína asociada al sinaptosoma de 23kDa (SNAP-23) y la sintaxina 4 son proteínas de la membrana plasmática que se han relacionado con la exocitosis en los MCs.
Los estudios con IgE monoclonal indican que la unión de IgE a FcεRI induce cambios moleculares y biológicos en los MCs.
Objetivos: El objetivo de este estudio fue investigar los cambios en la expresión de SNAP-23 y sintaxina 4 MCs, después de una sensibilización de las ratas con ovoalbúmina (OVO). Además, evaluamos si estas proteínas estaban implicadas en la secreción de histamina.
Métodos: Se analizó la expresión de SNAP-23 y de sintaxina 4 mediante transferencia Western, utilizando MCs de animales sensibilizados y controles. A través de la reacción anafiláctica cutánea pasiva se pudo confirmar que la sensibilización se había realizado de modo correcto. Para examinar el papel de estas proteínas de la exocitosis, en la secreción de histamina se incubaron MCs permeabilizados con anticuerpos anti-SNAP-23 y anti-sintaxina 4.
Resultados: La expresión de la SNAP-23 y de la sintaxina 4 fue significativamente más elevada en los MCs de ratas sensibilizadas a OVO que en las células de los animales control. Además, la incubación de células permeabilizadas con anticuerpos anti SNAP-23 y anti sintaxina provocó una marcada reducción de la secreción de la histamina en las células estimuladas.
Conclusiones: La sensibilización con OVO aumenta la expresión de SNAP-23 y sintaxina 4 en los MCs. Asimismo, nuestros datos sugieren que estas proteínas de exocitosis participan en la secreción de histamina.

Palabras clave: Histamina. Mastocitos. Sensibilización. SNAP-23. Sintaxina 4.

Introduction

Mast cells (MCs) are immune cells that play a crucial role in allergic and inflammatory reactions. In allergy, the binding of immunoglobulin (Ig) E to its high-affinity receptor (FcεRI) sensitizes MCs. Subsequent cross-linking of IgE-FcεRI by multivalent antigens results in activation of the MCs and the release of mediators responsible for allergic inflammatory reactions from their secretory granules [1]. This process, known as degranulation, takes place by compound exocytosis [2]. Histamine is one of the critical mediators during allergic responses and affects smooth muscle, endothelial cells, nerve endings, and mucous secretion [1]. Synaptosomal-associated protein of 23 kDa (SNAP-23) and syntaxin 4 are exocytotic proteins in the MC plasma membrane, and they have been linked to the degranulation process [2,3]. Incubation of antibodies to SNAP-23 in permeabilized MCs impairs ß-hexosaminidase secretion [2], and overexpression of syntaxin 4 in the rat MC line RBL-2H3 (rat basophilic leukemia) inhibits expression of the intragranular protein p80 [3].

Recent studies indicate that at high concentrations some monoclonal IgEs bound to FcεRI have effects on MCs [4], such as upregulation of FcεRI expression and increased histamine release [5,6]. These experiments suggest that sensitization leads to functional changes in MCs.

The aim of our study was to investigate the expression level of SNAP-23 and syntaxin 4 in MCs from allergen-sensitized rats to evaluate the possible regulation of these proteins during allergen sensitization. The role of SNAP-23 and syntaxin 4 in histamine release from MCs was also analyzed.

Material and Methods

Ovalbumin Sensitization

Thirty male Wistar rats weighing 180 g were sensitized with an intramuscular injection of 1 mg of ovalbumin (OVA) (Grade V, Sigma, St. Louis, USA) precipitated in 7.8 mg of aluminum hydroxide gel in 1 mL of saline solution. Simultaneously and as adjuvant, 0.5 mL of *Bordetella pertussis* vaccine (kindly donated by ISEA, Aguascalientes, Mexico) containing $10\text{-}15 \times 10^9$ heat-killed bacilli/mL was injected subcutaneously. A booster sensitization was injected 7 days later. Thirty control rats were injected with aluminum hydroxide gel and the vaccine, but without OVA. Serum was collected from each rat on day 14 and stored at –20°C until used.

Passive Cutaneous Anaphylaxis Reaction

Sera from the 30 sensitized and control rats were individually analyzed for passive cutaneous anaphylaxis (PCA). Twenty naive male Wistar rats weighing 500 g were anesthetized with ether and the dorsal skin shaved. Fifty microliters of each serum diluted 1:64, 1:32, 1:16, 1:8, 1:4, and 1:2 (to titer determination) or undiluted serum (to analyze the response magnitude) were injected intradermally in the dorsal skin. Twenty-four hours later the rats were anesthetized and injected intradermally with 50 µL of saline and histamine (2 µg in 50 µL of saline) as negative and positive controls, respectively. The rats were then injected in the jugular vein with 2 mg of OVA and Evans blue (34 mg/kg) in 3% saline. After 30 minutes, animals were sacrificed by anesthetic overdose and the diameter of each spot measured. For MC experiments, the titer of the anti-OVA IgE antibody was expressed as the highest dilution causing a lesion more than 5 mm in diameter [7]. To measure the magnitude of the anaphylactic reaction, equal areas from the injected sites (blue spots) were removed with a hollow punch and the amount of dye in the tissue sample determined colorimetrically after dye extraction with 1 mL of 1.0 N KOH and 9 mL of a mixture of acetone and 0.6 N phosphoric acid (5:13). The sample absorbances were measured at 620 nm using a spectrophotometer, and the amount of dye in the tissues determined using a standard curve (0.1 to 5 µg/mL Evans blue).

Isolation and Purification of Peritoneal Mast Cells

Rats were sacrificed by ether anesthesia. Peritoneal exudate cells (PECs) were obtained by peritoneal lavage with 20 mL of HEPES-buffered Tyrode solution (HBT) at pH 7.4, supplemented with 1% bovine serum albumin (BSA). PECs were washed in ice-cold BSA-HBT by centrifugation (180 × *g*, 4°C, 6 minutes) and resuspended in 1 mL of ice-cold HBT. MCs were purified using a 30%-80% discontinuous Percoll gradient [8]. Cells were centrifuged at 220 x *g*, 4°C for 20 minutes and pelleted at the bottom of the centrifuge tube. Residual Percoll was eliminated with 2 washes in BSA-HBT. MCs were resuspended in 1 mL of HBT. Cells were examined for purity using a light microscope and the trypan blue exclusion test was used to assay viability. Only those cell suspensions with an MC purity of 95% to 100% were used.

Electrophoresis and Immunoblot for SNAP-23 and Syntaxin 4

MCs (2.5×10^6) from 10 control or sensitized rats were homogenized in a manual tissue grinder in ice-cold lysis buffer. Proteins, from 20 µL of each sample, were separated in 13.5% sodium dodecyl sulfate polyacrylamide gels [9]. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Sigma, St. Louis, USA) by electroblotting [10]. Blots were treated with Tris buffer containing 3% BSA for 1 hour at room temperature and incubated at 4°C overnight with rabbit polyclonal antibodies against SNAP-23 (1:1000, Synaptic Systems, Göttingen, Germany), syntaxin 4 (1:1000, Synaptic Systems), or ß-actin (1:2000, Sigma). The membranes were incubated for 2 hours with alkaline-phosphatase-conjugated secondary antibody (1:20000, Zymed, South San Francisco, USA). Proteins were detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). ß-Actin signal was used as an internal standard against which to assess changes in SNAP-23 and syntaxin 4 expression levels. Quantification of protein bands was carried out by densitometry using a Kodak Digital Science imaging system (Eastman Kodak Company, Rochester, USA).

Inhibition of Histamine Secretion With SNAP-23 and Syntaxin 4 Antibodies in Permeabilized MCs

MCs from 10 untreated rats were permeabilized using a modification of an established method [2,11]. Briefly, cells

(50 000) in 1 mL ice-cold PIPES buffer (PB) were washed by centrifugation (250×*g*, 4°C, 3 minutes) and resuspended in 500 µL of PB containing 2 mM ethylene glycol tetraacetic acid (EGTA) and 2 µM digitonin (Sigma) [2]. Following gentle mixing, cells were incubated for 3 minutes at 37°C for permeabilization. To evaluate histamine secretion, 500 µL of ice-cold PB containing 2 mM EGTA (basal secretion) or 10 µM buffered Ca^{2+} concentration plus 40 µM guanosine 5'-O-3-thiotriphosphate (GTPγS) (stimulated secretion), was added in the presence or absence of the antibodies (anti-SNAP-23 or anti-syntaxin 4, diluted 1:200) [8]. As a control for specificity, permeabilized MCs were incubated with nonspecific rabbit immunoglobulins diluted 1:200. Cells were kept on ice for 30 minutes to allow antibody uptake and then incubated at 37°C for 10 minutes to stimulate histamine secretion. Supernatant and pellet samples were diluted 1:3.5 (volume by volume) in PB and placed in a water bath at 100°C for 5 minutes to inactivate histaminase. Proteins were precipitated with trichloroacetic acid (2.5%, final concentration) and the histamine content of the supernatant and the cell pellet was determined by spectrofluorometry using the o-phthaldialdehyde reaction with a Perkin Elmer LS 50B luminescence spectrometer (Perkin Elmer Co., Beaconsfield, UK) [12]. The percentage histamine release was calculated as follows: histamine in supernatant/(histamine in supernatant + histamine in pellet) × 100.

Statistical Analysis

Data were presented as mean (SEM) and compared using the Tukey-Kramer multiple comparison test. Differences were considered statistically significant when $P < .05$.

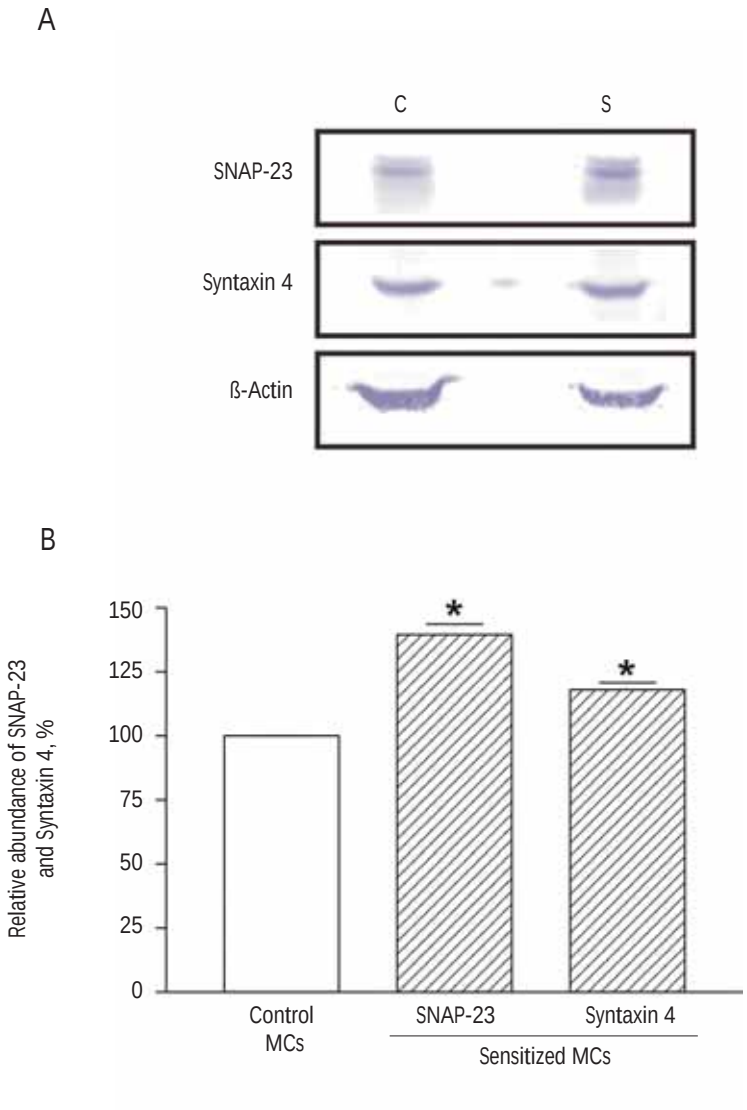

Figure 1. Western blot analysis of SNAP-23 and syntaxin 4 in control and sensitized mast cells (MCs). A, Immunoblot of peritoneal MCs from control (C) and sensitized (S) rats incubated with SNAP-23, syntaxin 4, and ß-actin antibodies. Data were analyzed by densitometry (B) and relative abundance of SNAP-23 and syntaxin 4 was calculated as the ratio of the mean intensity of each protein to that of ß-actin and was normalized to the ratio obtained in control MCs. Bars show the mean of 3 different experiments and whiskers show SEM. Samples for each experiment were obtained by pooling MCs from 10 control or 10 sensitized rats to obtain 2.5×10^6 MCs. Asterisks (*) indicate $P < .05$ compared with control MCs.

Results

Sensitization Increases Expression of SNAP-23 and Syntaxin 4 in MCs

Western blot analysis was performed to explore the level of SNAP-23 and syntaxin 4 expression in sensitized peritoneal MCs. Bands of 29 and 36 kDa corresponding to SNAP-23 and syntaxin 4, respectively, were detected. Data were normalized with respect to the 42 kDa ß actin band (Figure 1A). Densitometric quantification showed that expression of both proteins was significantly increased in MCs from OVA-sensitized rats: SNAP-23 expression was increased by 39.5% and expression of syntaxin 4 by 18.1% as compared with control rats (Figure 1B). MCs from sensitized rats with a titer of anti-OVA IgE antibody ≥ 1:8 and from control rats with negative PCA reaction were used. As shown in Figure 2, serum from sensitized rats caused a significant increase in vascular permeability (Evans blue dye concentration) compared with the effect of serum from control rats. The amount of dye extravasated in response to immune serum was 4-fold greater than that extravasated in response to histamine injection.

Inhibitory Effect of Anti-SNAP-23 and Anti-Syntaxin 4 on Histamine Secretion in Permeabilized MCs

Digitonin-permeabilized MCs were used to enable internalization of anti-SNAP-23 and anti-syntaxin 4 antibodies. In basal conditions (EGTA) the permeabilized MCs released 8% of the total cell content of histamine, whereas with the

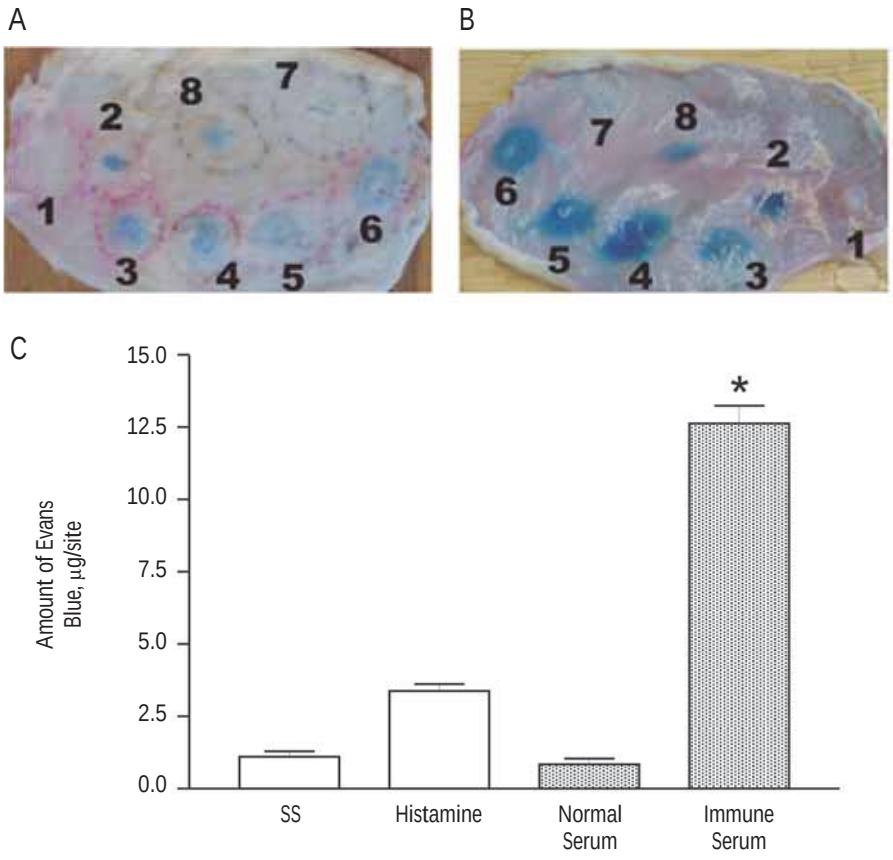

Figure 2. Effect of normal and immune serum on vascular permeability in passive cutaneous anaphylaxis (PCA). Photographs show dorsal outer (A) and inner (B) skin reactions in PCA generated as follows: 1, serum from control rat (normal serum); 2-6, sera from sensitized rats (immune serum); 7, saline; 8, histamine (2 μg). C, Vascular extravasation was evaluated by measurement of the amount of Evans blue dye extravasated in the skin. Bars show mean of 8 different experiments; whiskers show SEM. Asterisks (*) indicate $P < .001$ compared with normal serum.

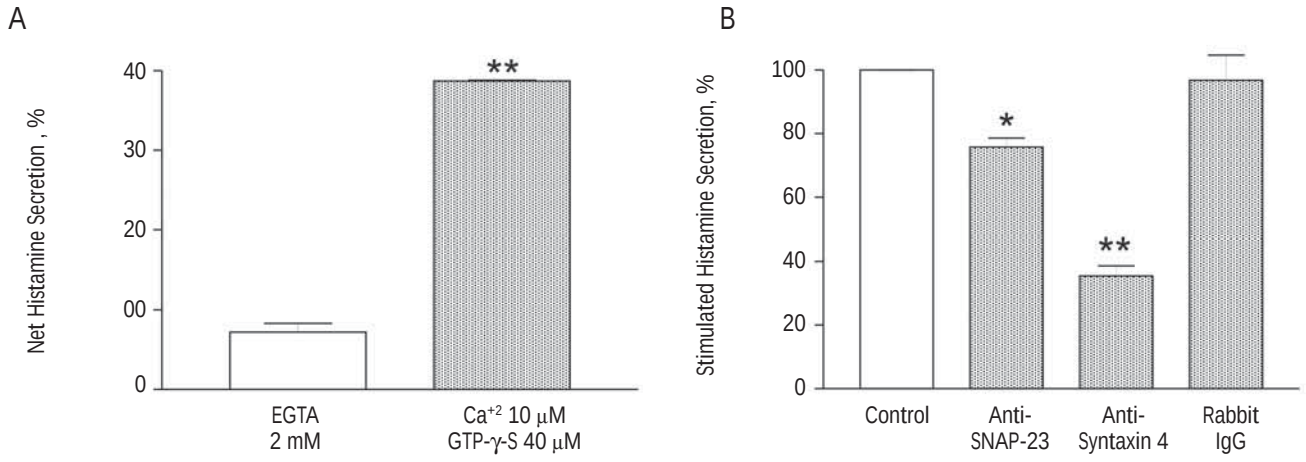

Figure 3.Histamine secretion in permeabilized mast cells (MCs). A, Net histamine secretion in digitonin-permeabilized MCs. Cells were permeabilized and then incubated with control buffer (ethylene glycol tetraacetic acid [EGTA] 2 mM) or stimulation buffer (Ca^{2+} 10 μM, guanosine 5'-O-3-thiotriphosphate[GTPγS] 40 μM). B, Effect of antibodies against SNAP-23 and syntaxin 4 on stimulated histamine secretion. Cells were permeabilized and incubated with stimulation buffer, with (1:200 dilution) or without (control) antibodies and the stimulated histamine secretion was evaluated. Results were expressed as the percentage of total secretion obtained in control assays without antibodies. Bars show the mean of at least 3 different experiments; whiskers show SEM. IgG indicates immunoglobulin G; *, $P < .05$ compared with control; **, $P < .001$ compared with control.

stimulation buffer about 40% was released (Figure 3A).To determine whether SNAP-23 and syntaxin 4 participate in the release of histamine, the exocytotic proteins were blocked with anti-SNAP-23 or anti-syntaxin 4. Histamine secretion in the presence of stimulating buffer was significantly reduced by the blockade of SNAP-23 (25%) or syntaxin 4 (65%) (Figure 3B). To demonstrate the specificity of the antibody inhibition, nonspecific rabbit immunoglobulins were used as controls, with no effect on histamine secretion (Figure 3B).

Discussion

Monoclonal IgEs bound to FcεRI have been reported recently to induce molecular and biological changes in MCs [4]. Monoclonal IgEs can substantially upregulate FcεRI expression on mouse MCs in vitro and in vivo. This enhanced FcεRI expression may increase the ability of these cells to release histamine in response to IgE-dependent activation [6]. Our results provide evidence that MCs from sensitized rats show significantly increased expression of SNAP-23 and syntaxin 4. This effect is correlated with high titers of allergen-specific IgEs in the sera of these animals. In macrophages, the levels of SNAP-23 and syntaxin 4 are increased by lipopolysaccharide exposure in a temporal pattern that coincides with the peak of tumor necrosis factor-α secretion [13]. A study in the interleukin-3-dependent MC line MC9 demonstrated that stimulation with stem cell factor, interleukin-3, and interleukin-10 induced high levels of SNAP-23 mRNA expression [14]. Nevertheless, data are unavailable in relation to SNAP-23 regulation by these cytokines. Our results represent the first demonstration that allergen sensitization causes molecular changes in MCs. Whether upregulation of SNAP-23 and syntaxin 4 in MCs from sensitized rats is directly caused by IgE-FcεRI binding or by other factors such as cytokines remains to be elucidated.

We also used anti-SNAP-23 and anti-syntaxin 4 to evaluate the participation of the exocytotic proteins in the stimulated histamine secretion. Our results showed that in MCs syntaxin 4 was more important than SNAP-23 for histamine secretion. At the same time, SNAP-23 has a lesser role in histamine secretion than in the secretion of ß-hexosaminidase described by other authors [2]. Synaptotagmin II, another protein involved in degranulation, had a less important role in the regulation of serotonin secretion than ß-hexosaminidase secretion [15]. Taken together, these results suggest that secretion of different substances within MCs may be differentially regulated by the same proteins.

In conclusion, our study showed that SNAP-23 and syntaxin 4 expression is modified during sensitization of MCs and that these 2 exocytotic proteins are involved in histamine secretion. The higher expression of exocytotic proteins in sensitized MCs may be related to the upregulation of FcεRI and the increased capacity for histamine release by MCs treated with monoclonal IgEs. It has traditionally been considered that IgE-FcεRI binding is a passive action of "sensitization" before receptor aggregation by antigens, and thus, that sensitized MCs remain in a "resting" state. Our findings suggest that during allergen sensitization MCs undergo molecular changes, such as increased expression of SNAP-23 and syntaxin 4, and that these might induce more intense inflammatory responses.

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