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Life Sciences, Vol. 66, No. 21, pp. PL 283-290, 2000

0024-3205/00/$–see front matter

PII S0024-3205(00)00538-5

PHARMACOLOGY LETTERS
Accelerated Communication

ACTIVATION AND INHIBITION OF MAST CELLS DEGRANULATION AFFECT THEIR MORPHOMETRIC PARAMETERS

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(Submitted June 29, 1999; accepted September 16, 1999;
received in final form December 14, 1999)

Abstract. Activation of mast cells, the key cells of allergic inflammation, causes typical morphological changes associated with an increase in volume, that is a function of area and perimeter. The purpose of this study was to evaluate the effect of mast cell activation to degranulate, carried out by the secretagogue Compound 48/80, and of inhibition of this activation carried out by Nedocromil sodium, a mast cell stabilizing drug, on mast cell area, perimeter and shape factor by a computerized image analyzer. Mast cells were isolated and purified by peritoneal lavage of rats (purity >98%) and co-cultured with mouse 3T3 fibroblasts to which they adhere. Cultures were incubated for 10 min at 37°C with culture medium alone (Enriched Medium) or Enriched Medium containing either Nedocromil (10^{-4} M) or Compound 48/80 (0.3 μg/ml) or Compound 48/80 and Nedocromil (0.3 μg/ml and 10^{-4} M respectively). Supernatants were then assessed for histamine release, as a marker of mast cell activation and the cell monolayers were fixed and stained with an alcoholic-acidic toluidine blue solution and examined with a computerized image analyzer connected with a light microscope. Mast cells incubated in Enriched Medium or Nedocromil possessed similar morphometric parameters. Mast cells activated with Compound 48/80 (70% histamine release) had a significant increase in area and perimeter and a decrease in shape factor in comparison to mast cells in Enriched Medium alone. Simultaneous incubation of mast cells with Compound 48/80 and Nedocromil significantly inhibited their histamine release (36% histamine release) and the increase in area and perimeter, but did not affect significantly their shape factor, in comparison with mast cells incubated with Compound 48/80 alone. These data clearly show that there is a relationship between mast cell activation, consequent histamine release and changes in cell area, perimeter and shape factor and that Nedocromil not only inhibits mast cell histamine release but also the activation induced morphometric changes in mast cells.

Key Words: mast cell activation, Nedocromil sodium, morphometric parameters

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Introduction

Mast cells, the key cells of allergic inflammatory reactions, are high-affinity IgE-receptor bearing cells that contain prominent cytoplasmic granules staining metachromatically with cationic dyes. Activation of mast cells in allergy occurs in response to challenge with a specific antigen against which the surface IgE is directed or by other IgE-directed ligands (1). Alternatively to IgE mediated activation, a variety of biological substances (products of complement activation, neuropeptides and certain cytokines), chemical agents (Compound 48/80, calcium ionophore A23187), drugs (morphine, codeine, desferroxamine) and physical stimuli can elicit release of mast cell mediators (2).

Mast cell activation both by IgE-dependent and IgE-independent stimuli, brings about the process of degranulation that results in the fusion of the cytoplasmic granule membranes with the plasma membrane. This is accompanied by both the fast external release of granule-associated stored mediators (histamine, neutral proteases, acid hydrolyses, proteoglycans, chemotactic factors, cytokines, etc.) as well as by the generation and release of newly generated mediators, such as products of arachidonic acid metabolism (3) and, at later times, by the production and release of an array of cytokines (2).

The mast cell signal transduction events resulting from activation, has been extensively studied both in human and in rat (4). Some studies have also been carried out to investigate the fate of activated mast cells. From both "in vivo" (5,6) and "in vitro" (7) studies it is evident that mast cells regenerate their morphology, mediator content and susceptibility to be stimulated.

Rat peritoneal mast cells co-cultured with 3T3 fibroblasts adhere to the monolayer and keep their viability and functional activity for more than a month (8). In this system we observed that the mast cells following activation with Compound 48/80 gradually regenerate within 14 days, as demonstrated by morphological, functional and biochemical criteria (9). Studies aimed to evaluate morphometric properties of activated mast cells are still scattered. In this study we wanted to assess area, perimeter, shape factor, in cultured rat peritoneal mast cells after activation with Compound 48/80 or upon their simultaneous incubation with Compound 48/80 and Nedocromil Sodium, a mast cell stabilizing drug that is known to inhibit IgE-dependent and independent histamine release.

Methods

Mast cells obtention and co-culture establishment

Mast cells were obtained from Sabra male rats (an outbred strain of the Hebrew University) weighing 250-300 g and co-cultured with 3T3 fibroblasts as previously described (10). Briefly, mast cells isolated and purified from the peritoneal cavity by a sterile procedure were cultured at a density of 5 X 10^5/2 ml in enriched medium (EM: RPMI 1640 containing 10% heat-inactivated fetal calf serum (v/v), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutammine, 0.1 mM nonessential amino acids, pH 7.2. Biological Industries, Israel) on a confluent layer of the contact-inhibited Swiss albino mouse derived 3T3 fibroblast cell line (ATCC, Rockville, MD). After seeding, the mast cells adhered to the fibroblast monolayer in about 10 min. Activation experiments were performed 48 hours after mast cells seeding.

Mast cells/3T3 fibroblast activation and inhibition of activation

The culture medium was aspirated and mast cells/3T3 cultures were washed twice by adding 1 ml of Enriched Medium and removing it by aspiration. Duplicate cultures were incubated with either

1 ml of Enriched Medium, or 1 ml of Enriched Medium containing 0.3 μg/ml Compound 48/80 (Sigma, St. Louis, MO), or 1 ml of Enriched Medium containing Nedocromil sodium 10^{-4} M (a kind gift of Dr Alan Norris, RPR France), or 1 ml of Enriched Medium containing both Compound 48/80 and Nedocromil for 10 min at 37°C in a humidified atmosphere of 5% CO_2. In the fourth group Nedocromil was added immediately before Compound 48/80. Reagents were prepared as fresh stock solutions (10 times concentrated) each time before the experiments. After 10 min part of the cultures were sacrificed for histamine release determination (see below). Supernatants were removed from other replicate cultures and the monolayers were rinsed twice with Tyrode's buffer (TG, Tyrode's buffer containing 0.1% gelatin). Mast cells/3T3 were then incubated for 20 min with an acidic solution of 0.5% toluidine blue (pH 3.5) (Sigma Chemicals, St. Louis, MO) in methanol for 1 min to fix and stain the cells.

Determination of mast cell histamine content and release

To determine mast cell-associated and -released histamine in treated and control cultures, both in triplicates, supernatants were collected and the cell monolayers were scraped from the plate with a Teflon policeman in 1 ml of TG. The cell suspension was disrupted by continuous sonication for 30 sec (output 5, 50% duty cycle, Heat Systems Ultrasonics W380). Histamine content was then determined in the supernatants and in the cells by a radioenzymatic assay using ^{14}C-labeled methyl-S-adenosyl-L-methionine and a crude preparation of rat kidney methyl transferase (11). The percentage of histamine released under the different conditions was calculated by dividing the amount of histamine the supernatant by the sum of that in the supernatant and the respective sonicated cells.

Morphometric analysis of mast cells

The morphometric changes of mast cells were measured by computerized image analysis using the Galay Cue-2 program modified for our specific research topics (12,13). The system consists of a videocamera (Model CUE, Scan Array 2) connected by C-mount to Axioscope (Zeiss, Germany), IBM-PC-AT computer with screen (Sony) and printer Epson 80. This system was set to work both automatically and manually. The intensity range of the staining color of mast cell was set between 0 and 30. The mast cell size was set at 20 μm^2, according to the information in the literature. Experiments were carried out in triplicates. In each plate 40 mast cell were analyzed according to their area, perimeter and shape. The shape factor was calculated based on the following formula: $sf = 4\pi \times area/perimeter^2$. The results of five different biological experiments performed in duplicate showed that the shape ranges from 0 to 1 (the rounder the cell the closer its shape value to the unit) and the calculation is based on this. The computer then calculated the size of the cell by combining the perimeter, the area and the shape data. Since the size of the cells displays a normal distribution, the Student's t test was suitable for statistical analysis.

Results

The activation of the mast cells with Compound 48/80 led to a high level of released histamine that was significantly different from the medium incubated mast cells and mast cells incubated with Nedocromil Sodium (70 ± 7%, 5 ± 2% and 7 ± 4% respectively, mean ± SEM, n=4, $p<0.001$). The mast cells incubated with both Compound 48/80 and Nedocromil released a significantly lower level of histamine in comparison to those treated with compound 48/80 (36 ± 10%, $p<0.04$), indicating that Nedocromil effectively inhibits mast cell degranulation.

As shown in Fig. 1 mast cells incubated in Enriched Medium or Nedocromil sodium kept their typical highly granulated and resting morphology as assessed by light microscopy after staining the monolayers with toluidine blue. Compound 48/80-activated mast cells immediately after

activation, assumed a classical raspberry wrinkled appearance and many mast cell granules were detected on the monolayer.

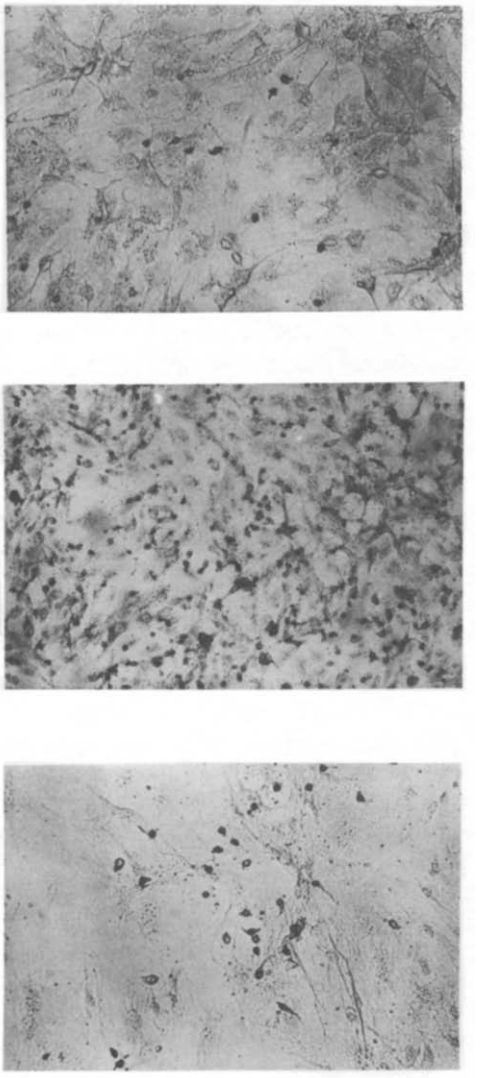

Fig. 1

Morphology of mast cells in the co-cultures.

Morphology of mast cells/3T3 co-cultures was evaluated under the light microscope after staining with toluidine blue. Mast cells/3T3 incubated in Enriched Medium or Nedocrimil sodium (not shown) maintained a structurally intact appearance and looked highly granulated (a). Mast cells/3T3 challenged with Compound 48/80 (0.3 μg/ml) assumed the classical raspberry wrinkled appearance (b). Mast cells/3T3 incubated with Compound 48/80 (0.3 μg/ml) and Nedocromil sodium (10^{-4} M) displayed a granulated resting appearance as did those in control co-cultures (c).

Nedocromil by itself did not influence the area, the perimeter or the shape factor of the mast cells (Figs. 2,3,4 n=3). This is consistent with the data that show that Nedocromil does not influence the histamine release of resting mast cells. Compound 48/80 activated mast cells displayed a larger size and less round shape in comparison to the control mast cells (Fig. 1). Mast cells activated with Compound 48/80 had a significant increase in area ($p<0.01$; Fig. 2) and perimeter ($p<0.025$; Fig. 3) and a significant decrease in shape factor ($p<0.025$; Fig. 4) in comparison to mast cells in Enriched Medium alone.

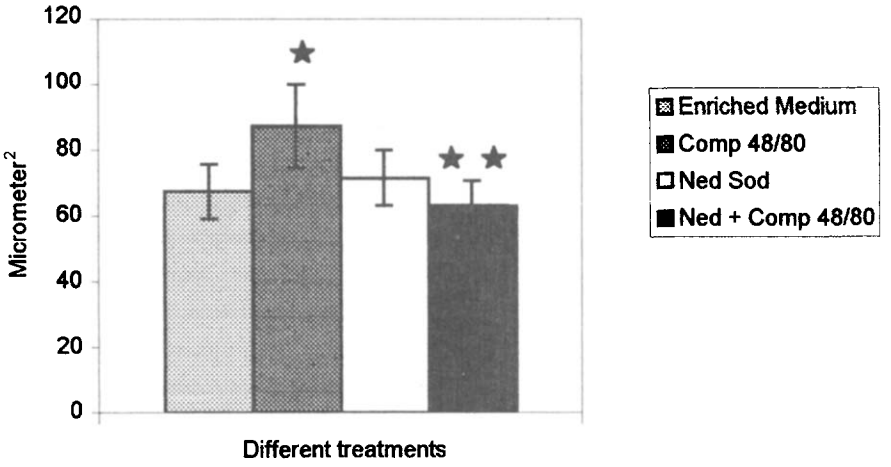

Fig. 2

Effect of activation and inhibition of activation on mast cell area.

Mast cells co-cultured with 3T3 fibroblasts were incubated for 10 minutes at 37°C either with medium alone (Enriched Medium) or with Compound 48/80 (Comp 48/80), or Nedocromil sodium (Ned Sod) or with Compound 48/80 in the presence of Nedocromil sodium (Ned + Comp 48/80). At the end of incubation cultures were fixed and stained and analyzed by computerized image analyzer as described in Methods.

Data are the mean ± SEM of 3 experiments performed in duplicates.

★ $p<0.01$

★★ $p<0.005$

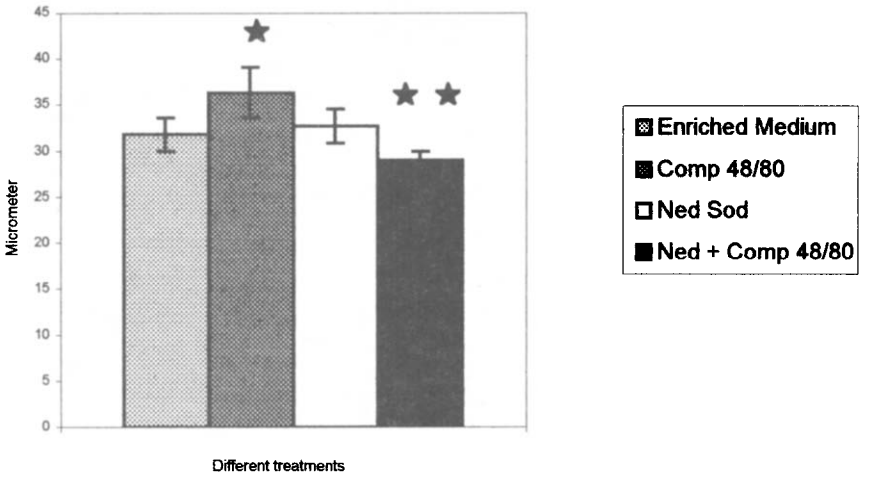

Fig. 3.
Effects of activation and inhibition of activation on mast cell perimeter.
Experiments were performed as described in legend to Fig. 2.
★ $p<0.025$
★★ $p<0.05$

Simultaneous incubation of mast cells with Compound 48/80 and Nedocromil significantly inhibited the increase in area and perimeter ($p<0.05$) but did not influence significantly their shape factor, in comparison with mast cells incubated with Compound 48/80 in the absence of the drug.

Discussion

Cromolyn sodium and the more recently developed Nedocromil sodium, both belonging to the same family of chromones, are considered to be mast cell stabilizers based on several "in vitro" studies, and they also have been used successfully for the prevention of allergic diseases. For example, in asthma, Nedocromil prevents the bronchial obstruction induced by a variety of stimuli, including antigen. When given before antigen inhalation, Nedocromil prevents the early and the late reaction and the increase of bronchial hyperresponsiveness (14). These effects may result from the activity of Nedocromil on inflammatory cells observed "in vitro", particularly on mast cells (14) where it inhibits both IgE-dependent and IgE-independent histamine release (15). Despite intense efforts, the mechanism by which Nedocromil and Cromolyn inhibit histamine release from mast cells is still poorly understood. Historically it has been suggested that chromones may inhibit histamine release by suppressing both calcium uptake from the external milieu (16) and the release of the cation from sequestered internal stores (17). Cromolyn was found to phosphorilate a 78kDa mast cell membrane protein involved in the termination of histamine release (18). More recently, studies have suggested the importance of chloride channels that provide the driving force for calcium influx during mast cell activation. Nedocromil was shown to block intermediate

conductance chloride channels in cultured mucosal-like mast cells (19) and Chloride channels may play a functional role in mediator release from mast cells.

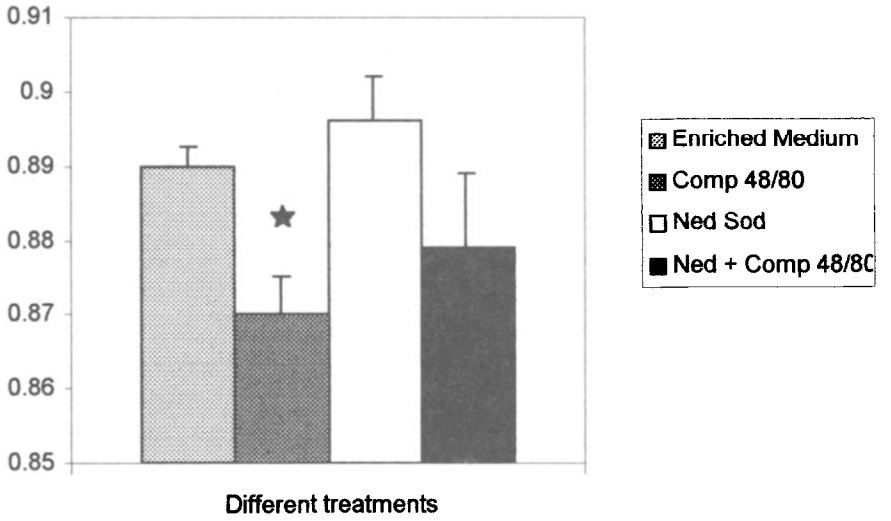

Fig. 4
Effect of activation and inhibition of activation on mast cell shape factor.
Experiments were performed as described in legend to Fig. 2.
★$p<0.025$

The data presented in this study clearly show that there is a relationship between mast cells activation, consequent histamine release and changes in cell area, perimeter and shape factor. We found that Compound 48/80 activation of mast cells caused significant increases in cell area and perimeter and decrease in shape factor. The degranulation event indeed causes dramatic morphologic changes in the mast cells (20). Nedocromil, on the other hand significantly inhibited both histamine release and the mast cell increase in area and perimeter. However it did not affect significantly their shape factor. This would indicate that Nedocromil does not affect the changes in cell shape induced by activation, but rather it affects the process of granule fusion and mediator release. Nedocromil can inhibit chloride channels (19) that in epithelial cells are linked to cell volume regeneration (21). It is therefore possible to postulate that the inhibition of increase in mast cell area and perimeter with Nedocromil is related to the inhibition of chloride channels. In addition these effects of Nedocromil on mast cell morphometric parameters appear to be in accordance with reported findings on the interaction of chromones with actin-binding proteins in mast cells which, in addition to regulating exocytosis, may be involved in volume regulation (22).

In summary we have demonstrated that there is a relationship between mast cell activation, consequent histamine release and changes in cell area, perimeter and shape factor. Furthermore we have shown that Nedocromil sodium not only inhibits mast cell histamine release but also the activation induced morphometric changes.

Acknowledgments

This study was partially supported by a grant from the Aimwell Charitable Trust, UK. Dr. L. Armetti was partially supported by a grant from the UCB Institute of Allergy, Belgium.

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