The Effect of Ouabain on Histamine Release from Human Skin Mast Cells

Mustafa Şenol, MD[1], İ. Halil Özerol, MD[2], Asha V. Patel, MS[1], David P. Skoner, MD[1]

There are controversial reports on the effect of sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) inhibition on mast cell mediator release. Some of them have indicated that ouabain (strophanthin G), a specific Na^+-K^+ ATPase inhibitor, inhibited the release, whereas the others have shown that ouabain had no effect or even had a stimulatory effect on the mediator secretion. Most of these studies utilized animal-derived cells. The aim of this study was to determine the effect of Na^+-K^+ ATPase inhibition on human skin mast cells. Unpurified and purified mast cells were obtained from newborn foreskins and stimulated by calcium ionophore A23187 (1 μM) for 30 min following a 1 hr incubation with various concentrations (10^{-4} to 10^{-8} M) of ouabain. Histamine release was assayed by enzyme immunoassay. The results indicated that ouabain, a Na^+-K^+ ATPase inhibitor, had no significant effect on the non-immunologic histamine release from human skin mast cells in vitro and suggested differences between human and animal mast cells. [Journal of Turgut Özal Medical Center 1996;3(4):277-283]

Key Words: *Calcium, calcium ionophore, histamine, Na^+-K^+ ATPase, ouabain*

Kutanöz mast hücrelerinden histamin salınımı üzerine ovabain'in etkisi

Sodyum-potasyum adenozin trifosfataz (Na^+-K^+ ATPaz) inhibisyonunun, mast hücrelerinden mediatör salınımı üzerine etkileri konusunda çelişkili bilgiler mevcuttur. Bazı çalışmalar, spesifik bir Na^+-K^+ ATPaz inhibitörü olan ovabain (strofantin G)'in, mediatör deşarjını bloke ettiğini, bazıları ise herhangi bir etkisinin olmadığını, hatta mediatör sekresyonunu artırdığını ileri sürmektedir. Bu çalışmaların çoğu hayvanlardan elde edilmiş mast hücreleri kullanılarak yapılmıştır. Bu çalışmanın amacı, Na^+-K^+ ATPaz inhibisyonunun, insan kutanöz mast hücrelerinden mediatör salınımı üzerine etkilerini incelemektir. Sağlıklı yenidoğanların sünnet derilerinden elde edilen mast hücreleri, değişik ovabain konsantrasyonlarıyla inkübe edildikten sonra, kalsiyum iyonoforla stimüle edildi. Histamin salınımı ELISA ile değerlendirildi. Sonuçlar, spesifik bir Na^+-K^+ ATPaz inhibitörü olan ovabain'in, insan kutanöz mast hücrelerinden in vitro nonimmünolojik histamin salınımı üzerine belirgin bir etkisi olmadığını gösterdi. İnsan ve hayvan mast hücreleri arasında bu açıdan da farklılıklar olduğu düşünüldü. [Turgut Özal Tıp Merkezi Dergisi 1996;3(4):277-283]

Anahtar Kelimeler: *Kalsiyum, kalsiyum iyonofor, histamin, Na^+-K^+ ATPaz, ovabain*

The release of inflammatory mediators, especially histamine, from tissue mast cells plays an important role in the pathogenesis of a variety of allergic and inflammatory conditions (1-3). The

[1] University of Pittsburgh, Department of Pediatrics Section of Allergy/Immunology, Children`s Hospital of Pittsburgh Pittsburgh, PA 15213, USA

[2] University of Nebraska, Department of Pathology/Microbiology Omaha, NE 68198, USA

release is caused by immunologic and non-immunologic stimuli and is modulated by many humoral factors (4,5). In allergy, the high affinity Fc receptor (FcεRI) cross-linking activates a signal transduction cascade, which is associated with influx of extracellular calcium and mobilization of intracellular calcium, activation of phospholipases and proteases, increased phospholipid metabolism, release of arachidonic acid metabolites, tyrosine phosphorylation, cyclic AMP production, and activation of protein kinases (3,6).

Na^+-K^+ ATPase, and the pump related to this enzyme, are essential in many physiologic events such as maintenance of cell osmotic balance and cation gradients, resting and action potentials, water-electric balance, and transport of sugars and amino acids (7). It has been proposed that Na^+-K^+ ATPase is involved in the regulation of mediator release from mast cells together with cyclic nucleotides and phospholipid metabolism, in a way very similar to that of most secretory processes (8). Significant ATPase activity has been demonstrated in human mast cells and basophils (9). Ouabain and other digitalis glycosides are generally considered as specific inhibitors of Na^+-K^+ ATPase (10). It has been suggested that Na^+-K^+ ATPase inhibition causes an increase in intracellular sodium levels. This increase in intracellular sodium may affect the intracellular concentration of calcium (cytosolic calcium) via the Na^+-Ca^{2+} exchange system that can exchange intracellular sodium for extracellular calcium (11,12). Additionaly, Na^+-K^+ ATPase blockade can lead to an increase in the pool of membrane-bound calcium (13). Although most of the studies have focused on calcium, it is clear that there are many second messengers in mast cells, and the other important ones are less known (14). Calcium ionophore A23187 complexes with calcium, can carry calcium in both directions across the cell membrane and induces histamine release from isolated human mast cells (15,16). In summary, Na^+-K^+ ATPase might play a role in the regulation of mast cell secretion mainly through the modulation of cytosolic and membrane-bound calcium stores and the accessibility of calcium to calmodulin-sensitive enzymes involved in secretion phenomena (8,13). Reactivating of Na^+-K^+ ATPase following histamine release has been suggested as a mechanism for cell recovery (16).

There are conflicting results concerning the effect of ouabain on the histamine release from rat mast cells and human basophils. Especially, the earlier reports suggested that ouabain did not modify histamine release from rat mast cells (17,18) or human basophils (19). Some other studies indicated that ouabain had an inhibitory effect on histamine release from guinea pig lung fragments (9) and human basophils (20). On the other hand, several recent reports showed that ouabain caused an increase in the release of histamine from rat mast cells in the absence of added calcium, but not in a medium containing normal (millimolar) calcium concentrations (8,10,13,21,22). Morever, some studies produced results that ouabain potentiated histamine release from rat mast cells in the presence of micromolar concentrations of calcium, and had no significant effect in the absence of added calcium and in the high calcium concentrations (from 10^{-3} M) (23). Recenly, our laboratory has documented that ouabain had an inhibitory effect on calcium ionophore-induced histamine release from rat basophilic leukemia cells (24) and had a stimulatory effect on immunologic histamine release from human basophils in allergic patients (25) in the presence of calcium. None of these earlier studies reported on the effect of ouabain in human mast cells (8,10,13,18,20-25). The purpose of this study was to investigate if ouabain, a potent and specific Na^+-K^+ ATPase inhibitor, modulates histamine release from human skin mast cells, in vitro. A unique model, human foreskin mast cells, was used.

MATERIALS AND METHODS

Cell isolation and purification. Mast cells were dispersed from human foreskin and purified by using techniques previously described (26-29).

Cell activation. 1000 mast cells (unpurified and purified) were used in each experimental tube. All experiments were performed in triplicate. Three kinds of experimental conditions were prepared:

Calcium (1 mM) added at the beginning of the ouabain incubation period. Samples of 80 µl containing 1000 mast cells were placed in 1.5 ml Eppendorf tubes and 10 µl of 10X $CaCl_2$ (1 mM final concentration) was added to each tube. Then 10 µl of diluent or 10 µl of appropriate ouabain concentration (10^{-4}, 10^{-6}, and 10^{-8} M final

concentrations) were added to the tubes. After incubation at 37°C for 1 hr, 10 µl of diluent (spontaneous histamine release) or 10 µl of calcium ionophore A23187 (1µM final concentration) (induced histamine release) were added. After second incubation for 30 min, reactions were terminated by the addition of 900 µl of ice-cold *histamine release buffer* containing PIPES 25 mM, NaCl 100 mM, KCl 5 mM, $MgCl_2$ 0.4 mM, glucose 5.5 mM, BSA 0.1%, and tubes were centrifuged at 2500 rpm for 2 min. Aliquots of supernatants were removed and stored at -70°C until histamine assay.

Calcium added after 1 hr ouabain incubation period. The same procedure was performed except that 10 µl of 10X $CaCl_2$ was added to the tubes only at the end of ouabain incubation period.

No added calcium. The same procedure as above, except that no calcium was added to the tubes at any time during the experiment.

Total histamine content was determined from samples (1000 mast cells in 1 ml of histamine release buffer) by boiling for 10 min to obtain all the histamine in the cells.

Histamine assay. Supernatants were assayed for histamine by using a commercial enzyme linked immunosorbent assay kit (Histamine ELISA kit, Immunotech, Westbrook, MA) (30,31). Data were processed and end results were calculated on computer by using Kineticalc and Lotus programs. The histamine in the supernatants was divided by the total histamine content of the mast cells to determine the percent released during experimental incubations. Spontaneous histamine release was subtracted from the induced histamine release to give the net histamine release. Results were expressed as the percentage of the total histamine content.

Cell viability. Cell viability was assessed by trypan blue exclusion in parallel with all experiments (32).

Statistical analysis. All results were expressed as mean±SEM of seven experiments. Differences between means were tested for significance by using two-tailed Student`s t-test for paired data. Differences were considered significant when the probability (p) was less then 0.05.

RESULTS

Average mast cell yield and purity. Digestion of neonatal foreskins with deoxyribonuclease, hyaluronidase, and collagenase yielded an average of 2.45±0.45 x 10^5 mast cells/g wet weight of tissue (n=20). Purity of mast cells was 4.2±0.7%, the major contaminating cells being fibroblasts and capillary endothelial cells. The average histamine content of unpurified mast cells was 6.5±2.3 pg/cell. Human foreskin mast cells were purified by cenrifugation through Percoll density gradients (n=7). The highest mast cell purity, 60.4±5.8%, was obtained at the bottom of the tubes. Approximately 20% of the total mast cells were recovered in the pellet, and they were contaminated with erythrocytes and some fibroblasts and endothelial cells. The average histamine content of purified mast cells was 6.1±1.5 pg/cell.

Mast cell viability assessed by trypan blue exclusion in both unpurified and purified mast cell preparations was higher than 95% in all experiments.

Ouabain effect on spontaneous and calcium ionophore-induced histamine releases from human skin mast cells.

In a media containing calcium (1 mM). The effect of various ouabain concentrations (10^{-4}, 10^{-6}, and 10^{-8} M) on spontaneous and stimulated histamine releases from unpurified and purified mast cells is outlined in Figure 1 a. Spontaneous histamine releases (% of total histamine±STD) from unpurified and purified mast cells were 33.94±4.01 and 10.39±2.84 whereas calcium ionophore-induced releases were 68.23±6.36 and 46.38±5.12, respectively. Spontaneous and stimulated histamine releases from unpurified mast cells were higher than releases from purified mast cells. Ouabain had no effect on spontaneous histamine release from both unpurified and purified mast cells, but had a slight but insignificant inhibitory effect on induced histamine release from unpurified mast cells and a slight but again insignificant stimulatory effect on induced histamine release from purified mast cells. As a result of these experiments, ouabain induced a slight but insignificant decrease in net histamine release from unpurified mast cells, and a slight but insignificant increase in net histamine release from purified mast cells, in a dose-response manner (Figure 1 b).

In a media that calcium (1 mM) was added at the end of the incubation period with ouabain. As shown in Figure 2 a**,** spontaneous histamine release values from unpurified and purified mast cells were 28.23±5.48 and 9.08±3.92 whereas stimulated releases were 51.19±7.42 and 42.59±6.88, respectively. Ouabain had no effect on spontaneous histamine release from both unpurified and purified mast cells under this circumstance too. But it had a slight but insignificant enhancing effect on ionophore-induced histamine release from both kinds of cells. Again, spontaneous and induced histamine releases from unpurified cells were higher than those of purified ones. Ouabain had a slight but insignificant potentiating effect on net histamine release from unpurified and purified human cutaneous mast cells, in a dose-response manner under this condition (Figure 2 b).

In a media containing no added calcium. Spontaneous histamine releases from unpurified and purified mast cells were 31.87±4.49 and 9.72±3.05, whereas induced histamine release values were 51.82±6.44 and 21.91±2.63, respectively. The results of these experiments showed that ouabain did not affect spontaneous and induced histamine releases from both unpurified and purified mast cells (Figure 3 a). Ouabain had no effect on net histamine release from unpurified and purified mast cells in the absence of calcium (Figure 3 b).

DISCUSSION

The effect of ouabain, a Na^+-K^+ ATPase inhibitor, was investigated on the spontaneous and the calcium ionophore A23187-induced histamine releases from unpurified and purified human cutaneous mast cells, under various experimental conditions. No significant effects were observed.

In some studies, it has been reported that ouabain had no effect on the antigen-induced secretion process from purified rat mast cells (17,18) and unpurified human basophils (19,25). On the other hand, some reports indicated that ouabain inhibited antigen-induced histamine release from unpurified rat lung mast cells (9), and unpurified human basophils (20), and ionophore-stimulated histamine release from rat basophilic leukemia cells (24). Conversely, most of some newer investigations suggested that ouabain had a stimulatory effect on immunologic (10) and non-immunologic histamine release from unpurified (8,13,23) and non-immunologic histamine release from purified (21) rat peritoneal mast cells.

Our results from unpurified and purified human skin mast cells in the presence of calcium are similar to several of these reports (17,18,19,25) and in the absence of calcium, they are parallel to those of some, but not the majority of others (23).

Some possible explanations for the differences in results of ours and previous studies can be considered: Most of the previous studies have been performed on animal models, especially rat mast cells and a few of them have been done by using human basophils. None of these earlier investigations has used human mast cells in their experiments. Mast cells from different species and even different tissues are highly heterogeneous. There are substantial differences between human and animal mast cells in morphology, mediator content, sensitivity to stimulatory and pharmacologic agents, and dependence on T cell-derived factors (1). The circulating basophil is distinctly different from the mast cell and may not be entirely representative of all the mast cells (29). Differences between species and tissues in Na^+-K^+ ATPase activity and sensitivity to modulating agents can also be considered (8). Although significant ATPase activity has been demonstrated in human mast cells and basophils (9), Na^+-K^+ ATPase represented only a small percentage of the total ATPase activity of mast cell membranes (8,18).

Additionally, there may be some methodologic and technical differences between present and previous studies. These include: differences in cell numbers, processing and assay procedures, concentrations of calcium, ionophore, and ouabain, period of incubation, secretagogue preference, contamination by cells other than mast cells, and histamine assay. In previous studies, mast cells were used in fairly high numbers as compare to ours. Our calculations and results might be affected by this factor. Other factors may be involved as well; eg, calcium and ouabain concentrations. Like some others, we observed no effect of calcium on histamine release in the presence of ouabain (8,10). The doses of ouabain that were used in our experiments were similar to those usually reported in secretion studies (10). However, this has not been a universal finding. One group showed that Na^+-K^+

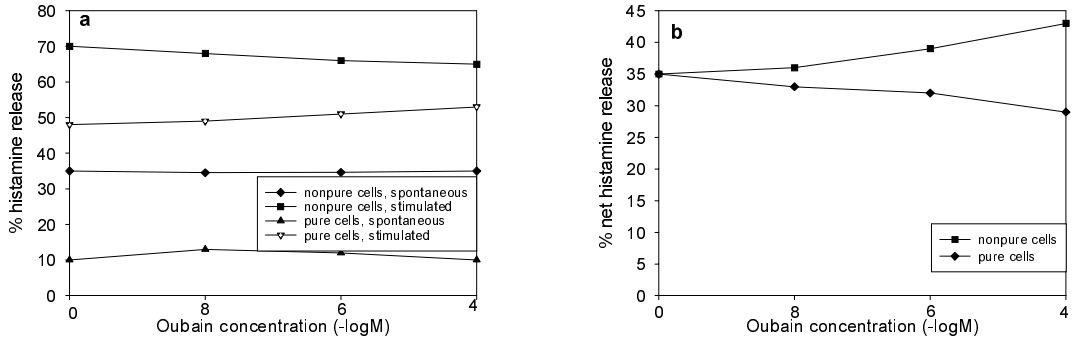

Figure 1. The effect of ouabain on (**a**) spontaneous and stimulated, and (**b**) net histamine releases from unpurified and purified human cutaneous mast cells in the presence of calcium (1 mM).

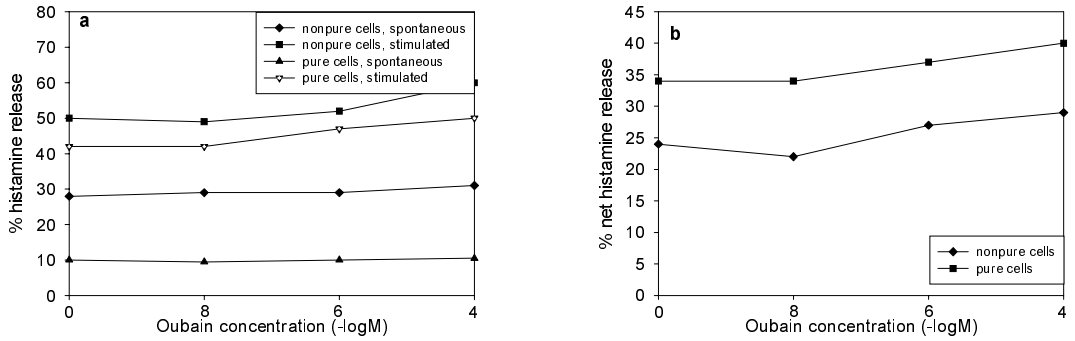

Figure 2. The effect of ouabain on (**a**) spontaneous and stimulated, and (**b**) net histamine releases from unpurified and purified human cutaneous mast cells in adding of calcium (1 mM) after the incubation period.

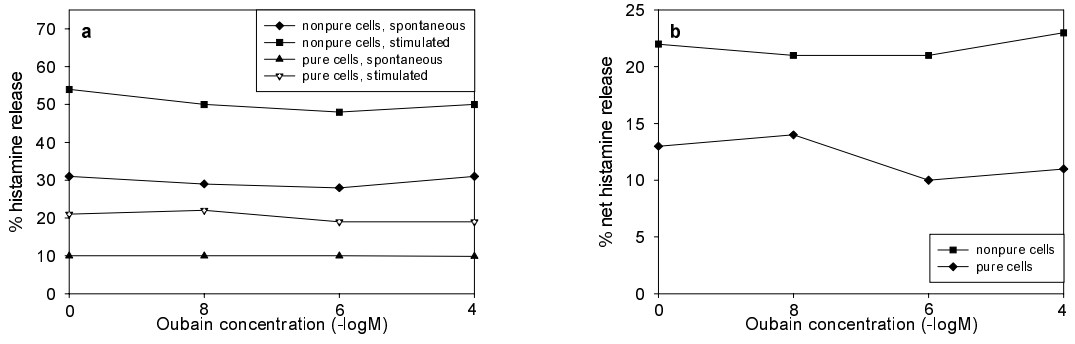

Figure 3. The effect of ouabain on (**a**) spontaneous and stimulated, and (**b**) net histamine releases from unpurified and purified human cutaneous mast cells in the absence of added calcium.

ATPase inhibition potentiated histamine secretion from rat mast cells when the extracellular calcium concentration was less than 0.5 mM (8). But ouabain, up to 10^{-3} M, failed to produce any

significant modification on histamine release in the presence of calcium (0.9 and 1 mM) (8,10). They concluded that the previous failures in observing an effect of ouabain were linked to the use of high calcium concentrations in the experiments. Prolonged exposure to calcium and increases in the concentration of extracellular calcium can have an inhibitory effect on histamine release (8,28,34,35).

In a calcium-free medium, it has been concluded that long term incubation of rat peritoneal mast cells increases the permeability of the plasma membrane to sodium. The consequent increase in the intracellular concentration of sodium causes an increase in the activity of the Na^+-K^+ pump (36). Magnesium may modify this process. For example, it has been demonstrated that preincubation of mast cells in a physiological concentration of magnesium caused a decrease in the secretory response and a less-marked inhibition of the pump was found (37,38). A mechanism for this effect has been postulated that magnesium might displace calcium from the plasma membrane (38). We used 0.4 mM magnesium in our experimental media.

The use of an unpurified or partly purified mast cell preparation raises the possibility that ouabain acted on other kind of cells such as fibroblasts or endothelial cells. Similarly, it is possible that ouabain bound to non-mast cells in the preparation, with a less-than-expected concentration of ouabain available to bind to mast cells. The net result would be an inability to directly test the primary hypothesis, i.e that ouabain modulates mast cell histamine release. It is unlikely that the cells in the mixture other than mast cells provided a significant contribution to the event because it is known that mast cell is the only histamine-containing cell in the human skin (1). In the other hand we observed no effect of ouabain in both unpurified and purified cell preparations.

The higher amounts of histamine releases from unpurified mast cells than those of purified ones can be explained by a direct interaction between mast cells and the other cells in the mixture. In a report, it has been indicated that cells other than mast cells in the preparation did not secrete effective humoral factors and molecules after challenge with antigen (4). However, the same study suggested that antigen-induced histamine release from rat peritoneal mast cells was potentiated through a direct physical contact with other cells via some cell surface molecules. In another study, it has been reported that mast cells possess the laminin receptor on their membrane and the signals mediated through VLA-4 potentiate the mediator release from the cells (33).

All of these facts and factors must be considered in the controversy of the results from different investigators. It is hoped that new studies on human skin mast cells by using various calcium and ouabain concentrations and various stimulatory agents including antigen will clarify the issue. As a result we concluded that Na^+-K^+ ATPase inhibition had no significant effect on non-immunologic histamine release from human skin mast cells in vitro.

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Correspondence: Mustafa ŞENOL, MD
İnönü Üniversitesi Tıp Fakültesi
Dermatoloji ABD
44300 MALATYA
Tel: 422-323 1753
Fax: 422-341 0036