

Ionic regulation of human basophil releasability.

II. Non-releasing basophils are converted into releasing basophils in a low- Na^+ medium

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Summary

The effects of different extracellular Na^+ and Ca^{2+} concentrations on histamine release from human basophils were investigated. Isosmotic replacement of extracellular Na^+ either with choline $^+$, a non-permeant Na^+ analogue, or glucose significantly increased spontaneous and anti-IgE-induced histamine release. Basophils from 12 of 49 normal subjects, which were found not to release histamine upon challenge with an optimal dose of anti-IgE in a 135 mM NaCl buffer, were converted into releasing basophils when stimulation with anti-IgE was performed in a low- Na^+ medium. The increase in Na^+ concentration in the extracellular medium was accompanied by a reduction in the magnitude of basophil response to anti-IgE, which was significantly more pronounced in non-releasers than in releasers (per cent inhibition by 70 mM NaCl 75.5 ± 3.2 vs 43.5 ± 9.0 , $P < 0.01$). At higher Na^+ concentrations a progressive and almost complete abrogation of histamine release was observed in non-releasers, but not in releasers (maximal per cent inhibition at 140 mM NaCl 97.3 ± 1.3 vs 50.4 ± 8.6). The Na^+/H^+ exchanger monensin had a dose-dependent inhibitory effect on anti-IgE-induced histamine release, and the concentration inhibiting 50% of histamine release was 1.5×10^{-7} M. When basophils were challenged in the presence of different Na^+ and Ca^{2+} concentrations, it was shown that the two cations have antagonistic effects, which is to say that they down-regulate and upregulate histamine release, respectively. Moreover, the requirement of extracellular Ca^{2+} was lowered in a low- Na^+ medium. These results suggest that Na^+ and Ca^{2+} ions contribute with opposite effects to the modulation of basophil response to anti-IgE and that non-releasing basophils are converted into releasing basophils in a low- Na^+ medium.

Clinical and Experimental Allergy, Vol. 24, pp. 66–72. Submitted 4 August 1992; revised 2 November 1992; accepted 10 September 1993.

Introduction

Basophils and mast cells can release preformed (as histamine) and newly generated (as peptide-leukotrienes) inflammatory mediators following interaction of membrane-bound IgE antibodies with specific antigen or anti-IgE [1]. IgE-mediated histamine release from human basophils is not strictly related to IgE density on cell

membrane [2], since several intracellular factors may influence the response to activating signals. One of the most intriguing observations is that 10% to 20% of donors have basophils which do not release histamine upon stimulation with anti-IgE in spite of the presence of cell-bound IgE [3]. It has also been reported that basophils from a few patients with a type I allergy do not respond following challenge with a specific antigen [4]. Recent work has elucidated some biochemical events involved in histamine release. Membrane phospholipid

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methylation, Ca²⁺ transmembrane flux, cyclic AMP generation, intracellular Ca²⁺ mobilization via inositol 1,4,5-triphosphate, and protein kinase C and calmodulin activation seem to be important steps involved in the histamine release process [5]. It has been recognized that Ca²⁺ ions play an important role in basophil activation, which is to say that they potentiate histamine release [6,7]. However, other ions, as, Sr²⁺, Na⁺ and Cs²⁺, can interfere with histamine release [8–13].

To investigate the role of Na⁺ and Ca²⁺ ions in the control of histamine release, we have evaluated the effects of different Na⁺ and Ca²⁺ concentrations on histamine release from human basophils showing a different response to anti-IgE. We conclude that Na⁺ and Ca²⁺ ions can regulate anti-IgE-induced histamine release from human basophils, and that non-releasing or low-releasing basophils respond to anti-IgE when stimulated in the presence of a low-Na⁺ medium.

Materials and methods

Blood sample collection and preparation of leucocyte suspensions

Peripheral venous blood (60–80 ml) was obtained from normal adult subjects belonging to the hospital staff (ages ranging from 18 to 40 years). All subjects were defined as normal and non-atopic on the basis of a detailed clinical history and physical examination. Informed consent was obtained from each subject prior to the blood collection.

Leucocyte suspensions were prepared by dextran sedimentation of peripheral venous blood, anticoagulated with 0.1 M Na₂EDTA and mixed with 6% dextran in saline solution (Solplex 70, Sifra, Verona, Italy) and 30 mM dextrose. The cells were allowed to sediment for 60–90 min at room temperature, according to Lichtenstein and Osler [14]. The leucocyte-rich plasma was centrifuged at 300 *g* for 15 min at 4°C, and the cell button was washed twice in Tyrode's buffer, pH 7.4, containing (mM): NaCl 135; dextrose 5.5; KCl 2.7; NaH₂PO₄ 0.36; NaHCO₃ 12. The cells were then suspended in Tyrode's buffer with 1.8 mM CaCl₂ and 0.5 mM MgCl₂. These suspensions in Tyrode's buffer were utilized to evaluate histamine release in control conditions. To investigate the effects of Na⁺ on histamine release, NaCl was replaced with the chloride salt of choline, a non-permeant Na⁺ analogue (Sigma Chemical, St. Louis, MO, USA), in a manner that when NaCl concentration was reduced, the concentration of choline chloride was increased correspondingly to maintain a constant 140 mM of the two cations together. Therefore, leucocytes were suspended in choline chloride solutions (140, 105, 70, 35 mM) with 1.8 mM CaCl₂, in the presence of different NaCl (0, 35, 70, 105 mM) concentrations. Leucocyte suspensions were

also prepared with isotonic glucose solutions (278 mM) (Monico, Venice, Italy) containing 1.8 mM CaCl₂. The osmolarity of the solutions employed was checked with an osmometer, and ranged between 270 and 300 mOsm/l.

Basophil concentration in leucocyte suspensions was evaluated by alcian blue staining [15] and subsequent counting in a Fuchs–Rosenthal chamber (Firma Walter Schrenck GmbH, Hoffheim am Taunus, Germany). Basophil concentration ranged between 0.5 and 2%; other contaminating cells were polymorphonuclear leucocytes, with eosinophils, lymphocytes, monocytes and platelets. The preparation obtained is referred to as 'basophils' in this paper. When cell viability was assessed by trypan blue exclusion, all leucocyte suspensions were greater than 95% viable.

Purification of human basophils

The basophils were purified from buffy coats of blood from healthy donors, according to the method described by De Boer and Roos [16], with minor modifications. Briefly, the buffy coat obtained from 500 ml of blood was divided in aliquots, layered over a Percoll gradient (Pharmacia, Uppsala, Sweden) obtained by two Percoll solutions with a specific gravity of 1078 and 1070 g/cm², and centrifuged (20 min, 2000 r.p.m., 20°C). The fraction at the interface between the two Percoll solutions was harvested, suspended in Tyrode's buffer without Ca²⁺ and Mg²⁺, and containing 5 g/l human serum albumin, and injected into an elutriator centrifugation system (Beckman J⁻²¹ centrifuge with a JE-6 elutriation rotor) (Beckman Instruments, Palo Alto, CA, USA). The elutriation medium was Tyrode's buffer without Na⁺ and Mg²⁺ and containing 13 mM trisodium citrate and 5 g/l human serum albumin. After cell injection, the centrifuge was started (400 r.p.m.). To separate the basophils the flow rate was maintained at 20 ml/min, while the rotor speed was reduced progressively. Fraction 1, with a volume of 120 ml, was collected between 4000 and 3700 r.p.m.; fraction 2, with a volume of 100 ml, was collected between 3700 and 2600 r.p.m. This fraction contained mainly lymphocytes and erythrocytes. Fraction 3 (100 ml, which was collected between 2600 and 1000 r.p.m.) contained the monocytes. The fourth fraction (100 ml, collected at 0 r.p.m.) contained mainly basophils, contaminated with monocytes and some lymphocytes. The last fraction was centrifuged at 1000 r.p.m. for 5 min at 20°C, and resuspended in Tyrode's buffer without Ca²⁺ with 13 mM trisodium citrate and 5 g/l human serum albumin. The cell suspension was layered on two Percoll solutions with a specific gravity of 1075 and 1068 g/cm², respectively, and centrifuged. The basophils contained in the lower interface were resuspended in Tyrode's buffer

and counted in a Fuchs-Rosenthal chamber after alcian blue staining. Two aliquots of each cell suspension were cytocentrifuged (Cytospin Shandon Ltd, Runcorn, UK, 10 min 500 r.p.m.), stained with May Grunwald Giemsa and examined under light microscope ($400\times$) for differential cell count. Average per cent purification of the basophils was $71\pm 6.2\%$ (mean $\pm$ SEM; $n=3$). Purified basophils were suspended in Tyrode's buffer or in choline chloride solutions and stimulated as described. Basophil stimulation was carried out no more than 2 h after elutriation.

Histamine release from leucocyte suspensions

Basophil stimulation was performed in 12 by 75 mm polyethylene tubes (LP Italiana, Milan, Italy). Briefly, $2-4\times 10^4$ basophils, suspended in 0.5 ml Tyrode's buffer, choline chloride or glucose solution, were added to prewarmed tubes (37°C) containing 0.5 ml of the same solution with anti-IgE (Sigma Chemical, final dilutions: 1/500 000, 1/50 000, 1/5000). After mixing and incubating for 30 min at 37°C , the reaction was stopped by centrifugation at $1000\times g$ for 20 min at 4°C . The supernatants were aspirated, mixed with an equal volume of 6% HClO_4 and centrifuged at $2000\times g$ for 15 min at 4°C . Histamine concentration in the supernatants was measured by an automated fluorometric method [17]. Spontaneous histamine release was evaluated by measuring histamine concentration in the supernatant of unstimulated cells; total histamine content was obtained adding 0.5 ml 6% HClO_4 to 0.5 ml cell suspension. Mean spontaneous histamine release was $2.1\pm 0.1\%$ in Tyrode's buffer and it never exceeded 5% of total histamine content.

Net histamine release was calculated as per cent of total histamine content after subtraction of spontaneous release. Every experiment was carried out in duplicate, and the average difference between replicate determinations was less than 5%. None of the reagents employed in this study, including monensin, interfered with the fluorometric determination of histamine concentration or modified cell viability, as assessed by trypan blue exclusion.

Effect of monensin on stimulated histamine release from human basophils

Basophils from 10 subjects, suspended in standard Tyrode's buffer with 1.8 mM CaCl_2 , were incubated for 30 min at 37°C with different concentrations of the Na^+/H^+ exchanger monensin (Sigma Chemical, 10^{-8} – 10^{-4} M) [18] and subsequently stimulated with an optimal dose of anti-IgE (dilution 1/5000). Histamine release in the supernatants was then measured as described.

Evaluation of Na^+ – Ca^{2+} interactions

To assess the effects of Na^+ and Ca^{2+} ions on histamine release, basophil stimulation with anti-IgE ($n=7$) was carried out in the presence of different NaCl and CaCl_2 concentrations in the extracellular medium (0, 70, 140 mM and 0, 0.45, 0.9, 1.8, 3.6 and 7.2 mM, respectively).

Statistical analysis

Results were expressed as mean $\pm$ SEM. Two-tailed Student's *t*-test for paired data and Mann-Whitney *U*-test for unpaired data were used for statistical analysis.

Results

Histamine release is enhanced in a low- Na^+ medium

Basophils of 12 of 49 normal adult donors showed no response (i.e. a histamine release $< 5\%$ of total histamine content) to anti-IgE. These cells failed to respond to an optimal dose of anti-IgE (1/5000) and even challenge with higher doses did not evoke any significant histamine release.

When leucocytes were suspended in a low- Na^+ medium (140 mM choline chloride solution), containing 1.8 mM CaCl_2 , a significant enhancement of anti-IgE-induced histamine release was observed in releasers (Fig. 1); moreover, non-releasing basophils showed a response to anti-IgE (Fig. 2). Histamine release induced by an optimal dose of anti-IgE (dilution 1/5000) in releasing

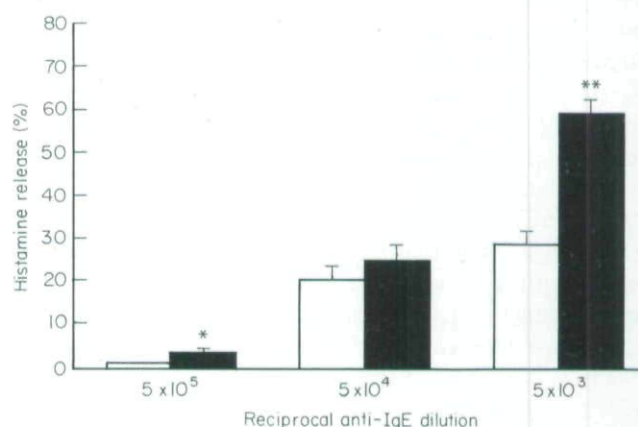


Fig. 1. Anti-IgE-induced histamine release from releasing basophils ($n=37$) in the presence of Na^+ -containing buffer (Tyrode's buffer with 135 mM NaCl) ($\square$) or in the presence of 140 mM choline chloride solution ($\blacksquare$). A significant enhancement of histamine release was found when challenge with anti-IgE was performed in a low- Na^+ medium. Results are expressed as the mean $\pm$ SEM. Statistical analysis was by Student's *t*-test for paired data. * $P < 0.05$, ** $P < 0.0001$.

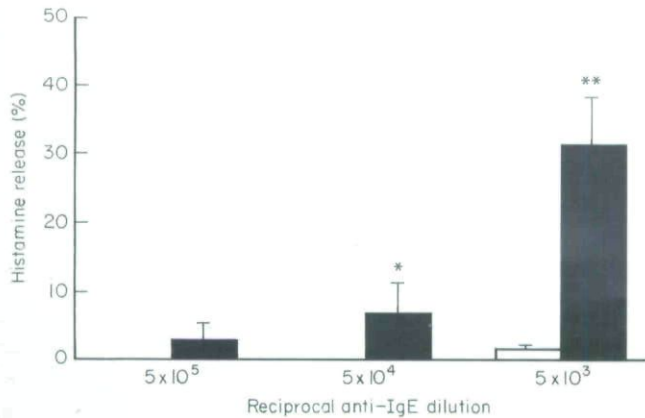


Fig. 2. Anti-IgE-induced histamine release from non-releasing basophils ($n=12$) in the presence of 135 mM NaCl buffer (□) or in the presence of 140 mM choline chloride solution (■). Non-releasing basophils were defined by the inability to release more than 5% of total histamine content after stimulation with an optimal dose of anti-IgE in a Na⁺-containing buffer. All the non-releasing basophils released >10% of total histamine content when challenge with an optimal dose of anti-IgE was carried out in a low-Na⁺ medium. Results are expressed as the mean $\pm$ SEM. Statistical analysis was by Student's *t*-test for paired data. * $P < 0.05$. ** $P < 0.0001$.

basophils was $29.1 \pm 2.4\%$ in a 135 mM NaCl buffer and $60.0 \pm 2.7\%$ in a 140 mM choline chloride solution ($n=37$, $P < 0.0001$). All the non-releasing basophils were converted into releasing basophils in the presence of a low-Na⁺ medium (mean release induced by anti-IgE diluted 1/5000 was $1.3 \pm 0.4\%$ in 135 mM NaCl buffer and $31.1 \pm 5.8\%$ in 140 mM choline chloride solution; $n=12$, $P < 0.00001$). Similar results were obtained with purified basophils (mean anti-IgE-induced histamine release was $26.3 \pm 6.3\%$ in Tyrode's buffer and $43.7 \pm 10.2\%$ in 140 mM choline chloride solution, $n=3$).

Spontaneous histamine release was enhanced when the cells were suspended in 140 mM choline chloride solution (mean $\pm$ SEM $3.8 \pm 0.3\%$ vs $2.1 \pm 0.1\%$; $n=49$, $P < 0.006$). A significant enhancement of spontaneous and stimulated histamine release was also observed when leucocytes were suspended in a 278 mM glucose solution (Table 1).

Increasing extracellular Na⁺ concentrations reduce anti-IgE-evoked histamine release

The increase in Na⁺ concentration in the extracellular medium was accompanied by a dose-dependent inhibition of anti-IgE-induced histamine release. As shown in Fig. 3, the inhibitory effect of Na⁺ was more pronounced in non-releasing ($n=6$) than in releasing basophils ($n=8$). At 70 mM NaCl, per cent inhibition of anti-IgE-induced

Table 1. Basophil releasability is enhanced when leucocytes are suspended in a glucose solution

Extracellular medium	Per cent histamine release	
	Spontaneous	Anti-IgE (1/5000)
NaCl buffer	2.0 ± 0.2	21.6 ± 3.0
Glucose solution	$5.2 \pm 0.8^*$	$39.2 \pm 4.0^{**}$

* $P < 0.01$.

** $P < 0.0001$.

Spontaneous and stimulated histamine release from human basophils suspended in 135 mM NaCl buffer and in an isotonic (278 mM) glucose solution containing 1.8 mM CaCl₂. A significant enhancement of spontaneous and stimulated histamine release was observed when the cells were suspended in a glucose solution. Results represent the mean $\pm$ SEM of 19 experiments.

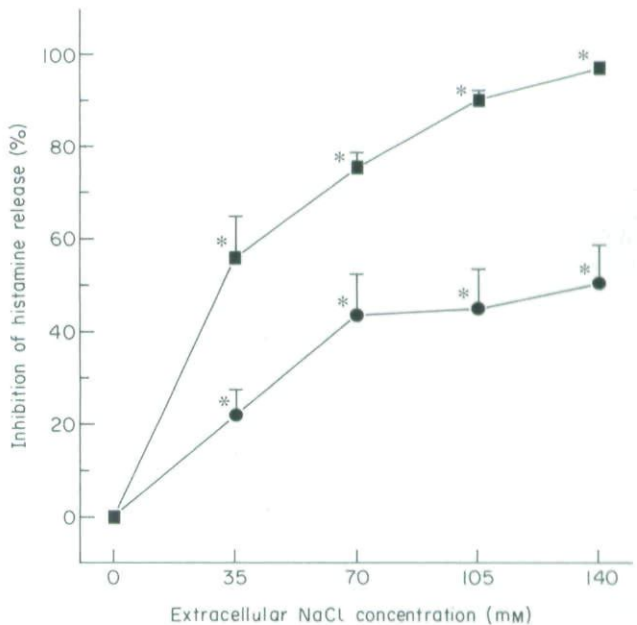


Fig. 3. Histamine release induced by an optimal dose of anti-IgE (1/5000) as a function of extracellular NaCl concentration. A significant dose-dependent inhibition of histamine release was found, both in releasers (●; $n=8$) and in non-releasers (■; $n=8$). The data obtained at 0 mM Na⁺ were considered as the control (histamine release was $55.3 \pm 6.6\%$ in releasers and $43.6 \pm 6.6\%$ in non-releasers). All other groups were compared statistically with the 0 mM Na⁺ group (Student's *t*-test for paired data). The inhibitory effect of Na⁺ was more pronounced in non-releasers (per cent inhibition at 70 mM NaCl 75.5 ± 3.2 and 43.5 ± 9.0 , respectively; $P < 0.01$ with Mann-Whitney *U*-test for unpaired data). * $P < 0.01$.

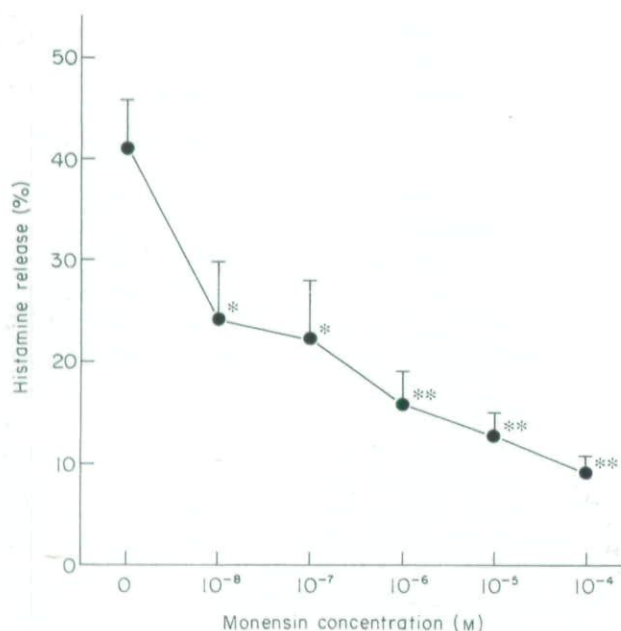


Fig. 4. Histamine release induced by an optimal dose of anti-IgE (1/5000) as a function of monensin concentration. This Na^+/H^+ exchanger exerted a dose-dependent inhibitory effect, with an IC_{50} of 1.5×10^{-7} M. Results represent the mean $\pm$ SEM of 10 different experiments. Statistical analysis was by Student's *t*-test for paired data. * $P < 0.001$. ** $P < 0.0001$.

histamine release was 43.5 ± 9.0 in releasers and 75.5 ± 3.2 in non-releasers ($P < 0.01$ between the two groups). At higher NaCl concentrations a progressive and almost complete abrogation of histamine release was observed in non-releasers, but not in releasers (maximal per cent inhibition at 140 mM NaCl 97.3 ± 1.3 vs 50.4 ± 8.6 , $P < 0.001$).

The Na^+/H^+ exchanger monensin inhibits anti-IgE-induced histamine release

The Na^+/H^+ exchanger monensin, in the concentration range of 10^{-8} – 10^{-4} M, displayed a significant inhibitory effect on anti-IgE-mediated histamine release from releasing basophils ($n=10$) (Fig. 4); the IC_{50} , estimated graphically, was 1.5×10^{-7} M. The inhibitory effect of monensin was observed also with purified human basophils (mean anti-IgE-induced histamine release was $32.6 \pm 15.3\%$ in control conditions and $17.1 \pm 8.7\%$ after incubation with 10^{-4} M monensin; $n=3$).

Evaluation of the effects of Na^+ and Ca^{2+}

Figure 5 shows anti-IgE-induced histamine release from human basophils as a function of extracellular Ca^{2+} concentration. IgE-mediated histamine release was potentiated by increasing extracellular Ca^{2+} concentra-

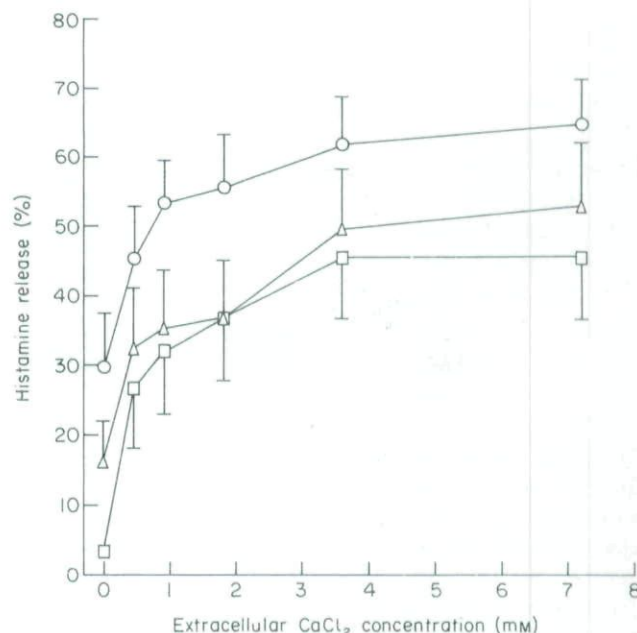


Fig. 5. The effect of different CaCl_2 concentrations on the inhibitory activity of Na^+ . Leucocytes were washed twice in ice-cold Tyrode's buffer and resuspended in Tyrode's buffer with different CaCl_2 (0, 0.45, 0.9, 1.8, 3.6, 7.2 mM) and NaCl (○—○, 0; △—△, 70; □—□, 140 mM) concentrations. In low- Na^+ solutions, NaCl was replaced by choline chloride. Anti-IgE was then added and the cells were incubated for 30 min. Histamine release was measured as described in materials and methods. Results are expressed as the mean $\pm$ SEM of seven different experiments.

tion; by contrast, Na^+ had a down-regulating activity, since the increase in extracellular Na^+ concentration produced a shift to the right of the dose-response curves. Removal of extracellular Na^+ lowered the requirement of extracellular Ca^{2+} for histamine release. For example, mean anti-IgE-induced histamine release in low Na^+ (140 mM choline chloride solution) and low Ca^{2+} (0.45 mM CaCl_2) overlapped with the value obtained in the presence of high Na^+ (140 mM) and high Ca^{2+} (7.2 mM) ($45.3 \pm 7.7\%$ and $46 \pm 9.0\%$, respectively).

Discussion

The results of this study indicate that Na^+ and Ca^{2+} ions have opposite effects on anti-IgE-induced basophil histamine release, that is to say they down-regulate and up-regulate histamine release, respectively. Non-releasing basophils are converted into releasing basophils in a low- Na^+ medium (choline chloride or glucose solution), and the increase in Na^+ concentration in the extracellular medium is accompanied by a decrease in the magnitude of basophil response to anti-IgE. Accordingly, the Na^+/H^+ exchanger monensin, which elevates intracellular Na^+

concentration, has an inhibitory activity on anti-IgE-induced histamine release.

In a previous paper, Hook and Siraganian [9] reported that replacement of extracellular Na⁺ with choline enhanced histamine release, but this effect was not further investigated. More recently, Beauvais *et al.* [10] have demonstrated that anti-IgE-induced histamine release from human basophils is inhibited by Na⁺ influx and potentiated by K⁺ efflux. A working hypothesis is that these effects are related to the modulation of membrane potential, which in turn could influence transmembrane Ca²⁺ flow. In human basophils membrane IgE cross linking by antigen or anti-IgE leads to an increase in cytoplasmic Ca²⁺ concentration [6], which is due to Ca²⁺ mobilization from intracellular stores and to Ca²⁺ entry from the extracellular to the intracellular medium. Previous results are not consistent with the presence of voltage-operated Ca²⁺ channels in human basophils, since the Ca²⁺ antagonists verapamil and nifedipine, at concentrations specific for voltage-operated Ca²⁺ channels, have no significant effects on histamine release [19–21]. Moreover, K⁺-rich solutions, alone or in combination with the K⁺ ionophore valinomycin, do not evoke histamine release from human basophils, indicating that membrane depolarization is not a sufficient signal for initiating histamine release [22]. Although human basophils seem not to bear true voltage-operated Ca²⁺ channels, it is conceivable that the intracellular concentrations of Na⁺ could influence Ca²⁺ entry via modulation of membrane potential. In keeping with this hypothesis it stands the observation that the requirement of extracellular Ca²⁺ was much lowered in a low-Na⁺ medium. According to this hypothesis, Na⁺ influx and membrane depolarization would hamper Ca²⁺ entry, whereas Na⁺ efflux and membrane hyperpolarization would facilitate it. Of course, this hypothesis needs further investigations and direct measurements of membrane potential.

An alternative mechanism of action of Na⁺ on histamine release could be the modulation of intracellular pH. The Na⁺/H⁺ exchanger monensin, which elevates intracellular Na⁺ concentration and causes cytoplasmic alkalization [18], inhibits anti-IgE-induced histamine release from human basophils. This suggests that the inhibitory effect of Na⁺ is related to its cytosolic level and, possibly, to cytoplasmic pH. Alterations in Na⁺/H⁺ exchange could in fact lead to changes in intracellular pH. Smith *et al.* [11] observed that amiloride derivatives that block Na⁺/H⁺ exchange and Na⁺/Ca²⁺ exchange can modulate histamine release, which is enhanced when intracellular pH is reduced or when intracellular Ca²⁺ concentration is increased.

One interesting feature which emerged from our study

is that non-responding basophils release histamine when challenge with anti-IgE is carried out in a low-Na⁺ medium. Up to now, it has not been elucidated why basophils of about 10–20% of normal donors fail to respond to anti-IgE [3]. Also, basophils from a few patients (6–7%) with a documented type I allergy do not release histamine upon *in vitro* stimulation with anti-IgE or antigen, in spite of the presence of circulating and membrane-bound IgE [4]. The failure to respond to IgE-mediated stimulation in these subjects was supposed to be related to an impaired protein kinase C (PKC) activation: non-releasing basophils were in fact demonstrated to release significant amounts of histamine when challenge with anti-IgE was preceded by incubation with the PKC activator 2-0-tetradecanoylphorbol-13-acetate (TPA) [4]. More recently, Nguyen *et al.* [23] suggested that non-releasing basophils have a defect in early signal transduction, possibly involving the influx of Ca²⁺. Warner and MacGlashan [24] found no elevation in cytosolic Ca²⁺ following challenge with anti-IgE in non-releasing basophils, whereas a small, but significant increase in PKC activity was detected. According to these authors, the main defect in non-releasing basophils would be the inability to elevate cytosolic Ca²⁺ concentration. Our experimental data show that non-releasing basophils release significant amounts of histamine when challenge with anti-IgE is carried out in a low Na⁺ medium. That is, in the absence of the inhibitory effect of Na⁺, anti-IgE can trigger the biochemical pathways leading to histamine release. The inhibitory effect of Na⁺ seems to be particularly efficient in non-releasing vs releasing basophils: at 70 mM NaCl per cent inhibition of anti-IgE-induced histamine release was significantly higher in non-releasing than in releasing basophils. Moreover, at higher NaCl concentrations a progressive and almost complete inhibition of histamine release was found in non-releasers, but not in releasers. Conversely, the inhibitory effect of Na⁺ on IgE-mediated histamine release has been shown to be impaired in allergic patients [25]: this impairment could account for the enhanced basophils releasability of these subjects. Our results confirm that Na⁺ has an inhibitory effect on IgE-mediated histamine release and demonstrate that non-releasing basophils respond to anti-IgE stimulation in a low-Na⁺ medium. Therefore, the inhibitory effect of Na⁺ seems to be an important factor influencing the ability of human basophils to respond to IgE-mediated stimulation.

Acknowledgement

We thank Mr M. Lorini for the technical assistance.

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