^{31}P and ^{23}Na nuclear magnetic resonance studies of resting and stimulated mast cells

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Exocytosis induced by crosslinking the type I receptor for Fc_ϵ domains present on rat mucosal mast cells (RBL-2H3-line) requires the influx of Ca^{2+} ions and is markedly influenced by the concentration of monovalent cations (K^+, Na^+ and protons) in their medium. We investigated the role of these ions in coupling the immunological stimulus to secretion using NMR spectroscopy to monitor simultanously intracellular pH, ATP and Na^+ concentrations and the secretory response of living adherent mast cells. Using this methodology we observed that: (i) ATP concentration and intracellular pH are highly regulated and no changes could be resolved in them upon stimulation and during exocytosis. (ii) In the absence of potassium ions in the cells' medium, a decrease is observed in the intracellular pH and ATP concentration and an increase in the Na^+ concentration. (iii) From the influx of extracellular Na^+ following inhibition of the Na^+, K^+-ATPase by ouabain, we estimated the inward Na^+ current of resting cells to 5×10^7 ions/(cell·s). This value does not vary by more than 10% during exocytosis.

RBL-2H3 cell; Nuclear magnetic resonance; Exocytosis; Cytosolic pH; Cytosolic [Na^+]; Cytosolic [ATP]

1. INTRODUCTION

Mast cells provide a useful and instructive system for investigating processes coupling the immunological stimulus, initiated by crosslinking their type I Fc_ϵ-receptors ($Fc_\epsilon RI$) via bound IgE by specific antigen, with the resultant mediator secretion [1,2]. Several biochemical coupling components such as Ca^{2+} ions have been identified so far [3-6]. Furthermore, intact energy metabolism was shown to be essential for secretion [7] and this process is in turn affected by parameters such as the extracellular concentrations of the monovalent ions Na^+ and K^+ [8-10] which are coupled tightly to cells' membrane potential [11-14]. NMR spectroscopy is a noninvasive tool for monitoring cellular metabolite concentrations and pH as well as intracellular concentrations of monovalent ions (for review see [15-17]). We adapted the recently developed methodology for studying living cultured cells by nuclear magnetic resonance (NMR) spectroscopy [18,19]. Here we present results of the use of ^{31}P and ^{23}Na NMR techniques to determine intracellular equilibrium concentration of phosphate metabolites and Na^+ ions in the rat mucosal mast cell line RBL-2H3. No detectable changes in intracellular ATP and Na^+ concentrations were observed upon immunological stimulation of the cells under regular conditions. However already in resting cells, the omission of potassium ions from the buffer under isoosmotic conditions - known to inhibit exocytosis - led to a marked decline in intracellular pH and ATP concentrations while the intracellular Na^+ concentrations ($[Na^+]_i$) increased to the external value. We have also determined the plasma membrane permeability for Na^+ ions by measuring the increase in $[Na^+]_i$ after treatment of resting cells with ouabain. From these experiments we derived an upper limit for conductivity changes following $Fc_\epsilon RI$ aggregation.

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Abbreviations: Ag, antigen; BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; BSA, bovine serum albumin; $Fc_\epsilon RI$, Fc_ϵ-receptor type I; IgE, immunoglobulin E; P_i, inorganic phosphate; PPP, tripolyphosphate; $[Na^+]_i$, intracellular sodium concentration; PME, phosphomonoesters

2. MATERIALS AND METHODS

2.1. *Cells*

Mucosal type mast cells of the rat basophilic leukemia secreting subline 2H3 [20] were maintained in monolayers in 80 cm^2 tissue culture flasks in Eagle's minimal essential medium (MEM, GIBCO, Grand Island, NY) supplemented with 10% heat inactivated fetal calf serum (BioLab, Jerusalem, Israel), 4 mM glutamine and antibiotics and were subcultured every 3 days. Cells for the NMR measurements were suspended from monolayers culture by treatment with 10 mM EDTA in MEM and 3×10^6 cells were seeded on 0.5 ml beads (Biosilon, NUNC, 160-300 μm diameter), placed in 9 cm diameter Petri dishes containing 12 ml of the FCS supplemented MEM. After 4 days of growth, the surface of the beads was covered with a monolayer of cells reaching a density of $3-4 \times 10^7$ cells per ml MEM

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with sedimented beads. The beads were harvested and 2 ml (approximately $6-8 \times 10^7$ cells) were transferred into a 10 mm diameter NMR-tube that was continuously perfused with growth medium equilibrated with 95% O_2, 5% CO_2 using the system described earlier in [19]. For different experiments, perfusion medium or Tyrode buffer were modified as follows:

(i) *Tyrode:* 137 mM NaCl, 2.7 mM KCl, 0.04 mM NaH_2PO_4, 10 mM Hepes, 1.4 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose and 0.1% bovine serum albumin (BSA).

(ii) *Dy(PPP)$_2$-Tyrode:* As the above except that $CaCl_2$ was omitted and supplemented with 12 mM Na_5 (PPP) and 6 mM $DyCl_3$. NaCl was added to yield a final Na^+-concentration of 137 mM and the osmolarity was adjusted by 36 mM glucose.

(iii) *Low sodium Dy(PPP)$_2$-Tyrode:* As (i) without NaCl and $CaCl_2$, yet supplemented with 6 mM Na_5(PPP), 3 mM $DyCl_3$ and 214 mM glucose. Final [Na^+] was 30 mM, the osmolarity was adjusted by the glucose.

In all buffers the pH was adjusted to 7.4 using a 2 M Tris solution.

2.2. *Exocytosis*

Monoclonal, DNP-specific, IgE class antibodies were added to the perfusion medium yielding a final concentration of 10 nM and allowed to bind to the cells during at least 1 h perfusion. The medium was then switched to the appropriate buffers required for the specific experiments. Exocytosis was triggered by adding antigen (Ag, 20 ng/ml final concentration) to the buffer reservoir outside the magnet. The secretion was monitored by measuring spectrophotometrically (using *para*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) as substrate) the activity of the granular enzyme β-hexosaminidase appearing in the perfusion buffer. This was done by taking samples of the efflux buffer and is expressed as percentage of the total cell content of β-hexoseaminidase determined by lysing the cells in the NMR tube at the end of the experiment (1% Triton). The values were corrected for the total buffer volume. For experiments in regular monolayer culture, cells were seeded in microwell plates (1×10^5 cels/well, 100 μl MEM, 0.01 nM IgE) and allowed to adhere by overnight inoculation. To simulate the conditions employed in the NMR tube, cells were treated with the same buffers as those employed in the respective NMR experiments. Whenever the buffer was changed, the cells were washed twice with the new buffer. Exocytosis was triggered by adding the antigen (20 ng/ml) yielding a total volume of 100-μl per well after adding all required reagents. The monoclonal dinitrophenyl-specific mouse IgE [21] was raised and purified in our lab. The Ag was a covalent conjugate of an average of ca. 11 DNP residues per molecule of bovine serum albumin.

2.3. *NMR experiments*

^{31}P and ^{23}Na NMR experiments were performed on a Bruker AM-500 spectrometer. The sample temperature was maintained at 36 ± 1°C. Composite pulse decoupling was employed in ^{31}P NMR experiments. The decoupler was not switched off when changing from ^{31}P to ^{23}Na NMR to avoid the requirement of a new temperature calibration which would have been otherwise necessary.

^{31}P NMR spectra were routinely recorded at 202.46 MHZ, using 2K data size, 15 000 Hz spectral width, 45° flip angle and 1 s repetition time. 600 scans were accumulated for each spectrum. Spectra recorded with 90° pulses and 10 s repetition time indicated partial saturation for P_i and PME under routine conditions. The data were digitally filtered by exponential multiplication with a line broadening of 25 Hz before Fourier transformation. The chemical shift scale was referenced to 85% phosphoric acid, using the α-signal of ATP as internal standard at -10.02 ppm.

^{23}Na NMR spectra were recorded at 132.26 MHZ using 1 K data size, 20 000 Hz spectral width, 90° flip angle and 0.062 s repetition time. 2000 scans were accumulated for each spectrum. Line broadening by exponential multiplication was 50 Hz. The chemical shift scale was referenced to the signal of intracellular Na^+. The areas of the intra and extracellular Na^+ signals were obtained by fitting the spectrum with the Glinfit program (Bruker, Abacus program library).

3. RESULTS AND DISCUSSION

RBL-2H3 cells were cultured on microcarriers (beads) to very high density ($3-4 \times 10^7$ cells/ml) suitable for NMR experiments. While in the NMR probe the continuously perfused cells were stimulated to secretion as determined by the activity of secreted β-hexosaminodase in the efflux buffer of cells (Fig. 1). At the indicated time point, specific Ag was added to the buffer reservoir and ca. 10 min afterwards a steep increase in the enzyme activity was observed (Fig. 1a). The final activity in the efflux buffer was calculated taking into account a correction due to the dilution in the total buffer volume. The apparent time-lag between the antigen addition and the observed activity increase was mainly due to the flow time to the efflux collecting point (10 min). Exocytosis was thus initiated when Ag reached the cells in the sample tube. The β-hexosaminidase activity reached a plateau at an activity which is typically about 40% to 50% of the activity of the total enzyme concentration (see Materials and Methods).

During the above experiments, ^{31}P NMR spectra were monitored consecutively every 10 min (Fig. 2). The main signals detected were phosphomonoesters (PME) at high field, cytoplasmic inorganic phosphate (P_i) and the three ATP signals. No significant changes in these signals were observed upon stimulation of the cells indicating that during exocytosis, the ATP concentration is practically constant ($\pm 10\%$). Also, this suggests that the intracellular pH (7.2) does not change within the experimental error of 0.1 units. In earlier experiments [22] carried out using the cytosolic fluorescent pH indicator BCECF in air saturated buffer (0.3% CO_2 concentration) smaller pH changes were observed (acidification of ca. 0.03 units) that are below the sensitivity detectable by the NMR experiments. The stability of the ATP-concentration is in agreement with the recent results of Lo et al. [23] who employed the luciferin-luciferase method. This suggests that under regular conditions, even during exocytosis, the intracellular ATP and the H^+ concentrations are highly regulated and no changes are resolved.

Exocytosis of RBL-2H3 cells depends on extracellular presence of Ca^{2+} and K^+ ions [3-6,8]. As shown in Fig. 1b this has been observed both for cells perfused in the NMR tube and for adherent cells in microwell plates using the same protocol. Exocytosis was induced only after the addition of $CaCl_2$ to the medium, yet none could be detected in K^+ free buffer. While no significant changes in ^{31}P NMR spectra were detected at different phases of the experiments in the K^+ containing buffer and all spectra were practically identical to that shown in Fig. 2, switching to K^+ free buffer, the ^{31}P NMR spectra of the cells exhibited a continuous decrease in pH and ATP concentration (not shown). After 2 h in the K^+ free buffer the cellular ATP concentration decreased to about 60% of its initial level

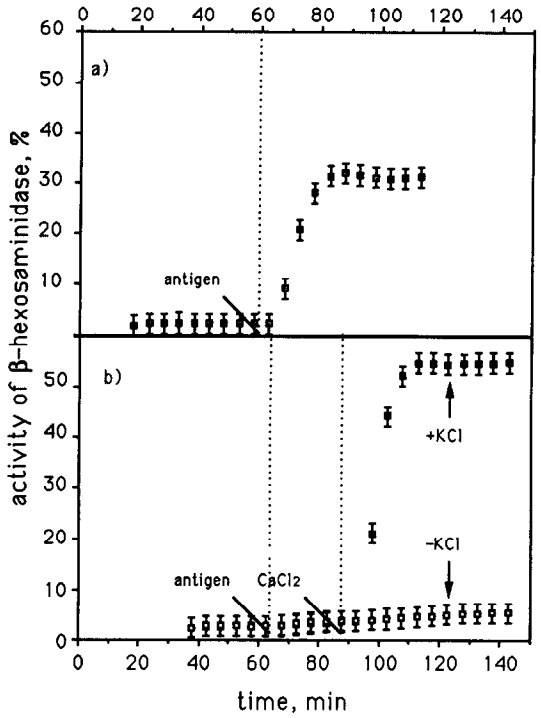

Fig. 1. Immunologically stimulated secretion of RBL-cells in the perfusion system. The plot shows the β-hexosaminidase activity measured in the efflux buffer and corrected for the total buffer volume. (a) Cells were perfused with Tyrode. (b) Cells were perfused with Tyrode lacking Ca^{2+} (■) and also K^+ ions (□). At the marked time points Ag (20 ng/ml) or $CaCl_2$ (1.4 mM) was added to the external reservoir. The β-hexosaminidase activity is presented as a fraction of the total contents of the cells.

and the signal of inorganic phosphate was shifted to high fields reflecting a decrease of at least 0.2 units in pH_i, indicating that K^+ is essential for the regulation of the latter parameters. Thus the inhibition of exocytosis by K^+ omission is most probably due to the marked decrease in either ATP or pH (or both) [7].

Extracellular Na^+ concentrations affect the immunologically induced secretion. Probably Na^+ fluxes modulate the secretion. This idea is also based on the observations that cell secretion induced by Ca^{2+} ionophore is enhanced by Na^+/H^+-exchange promotors such as phorbol myristate acetate (PMA) and inhibited by inhibitors of the Na^+ uptake via Na^+-channels like amiloride [24]. We measured $[Na^+]_i$ in both resting and $Fc_\epsilon RI$-stimulated cells by ^{23}Na NMR. In order to monitor $[Na^+]_i$, shift reagents had to be employed. $Dy(PPP)_2$ was chosen because of its ability to shift the signal more effectively than the other reagents. In order to use minimal concentrations of the shift reagent we also had to reduce the Na^+ concentration in the buffer to 30 mM (buffer 3, see Materials and Methods). In control experiments we established that the reduction of $[Na^+]$ to this value does not affect secretion significantly. The ^{23}Na NMR spectrum of an RBL cell sample perfused with buffer 3 without $CaCl_2$

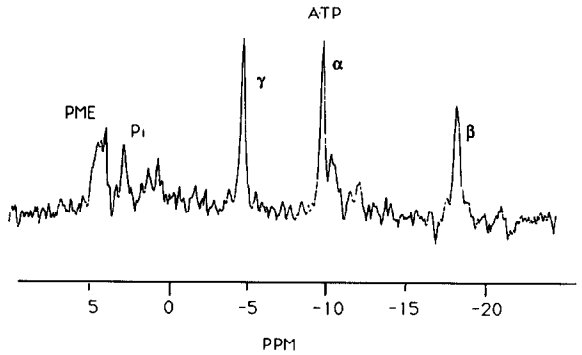

Fig. 2. ^{31}P-NMR-spectra of RBL-cells perfused as described in Materials and Methods. The spectrum was recorded during 10 min; signals can be assigned as follows: PME, phosphomonoesters; P_i, intracellular inorganic phosphate; α-, β-, γ-ATP: α-, β- and γ-phosphate of adenosine triphosphate.

is shown in Fig. 3a. The signal at 0 ppm is due to the intracellular Na^+ ions while that at -21 ppm reflects extracellular ones, shifted by $Dy(PPP)_2$. Both signals can be fitted to a lorentzian (Fig. 3a, dotted line). The area of the signal due to $[Na^+]_i$ amounts to about 2.5% of the extracellular Na^+ signal. Taking into account that 65% of the sample volume is taken up by the beads, 4% by the cells and 31% by the buffer and assuming that only 40% of the intracellular sodium is detected, a $[Na^+]_i$ = 15 mM can be calculated. Ca^{2+} competes with Dy^{3+} for the polyphosphate ions [26] leading to a drastic reduction in its effectivity as a shift reagent and therefore also to a smaller shift between the extra- and intracellular $^{23}Na^+$ signals. Hence we designed a com-

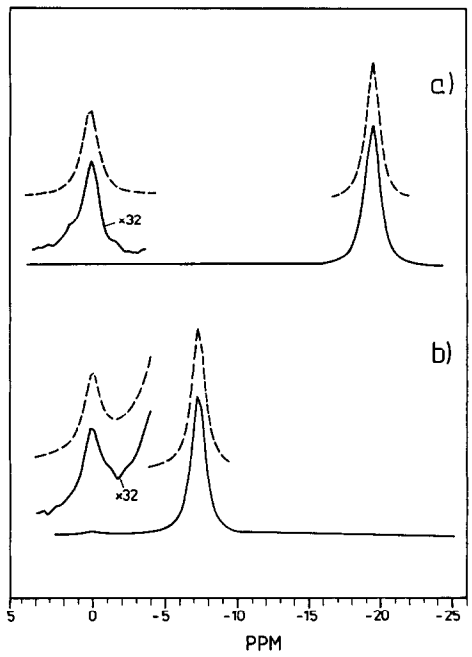

Fig. 3. ^{23}Na-NMR-spectrum of RBL-cells in $Dy(PPP)_2$-Tyrode without $CaCl_2$ (a) and with 2.25 mM $CaCl_2$ (b). The upper traces (dotted lines) show the fit to a lorentzian.

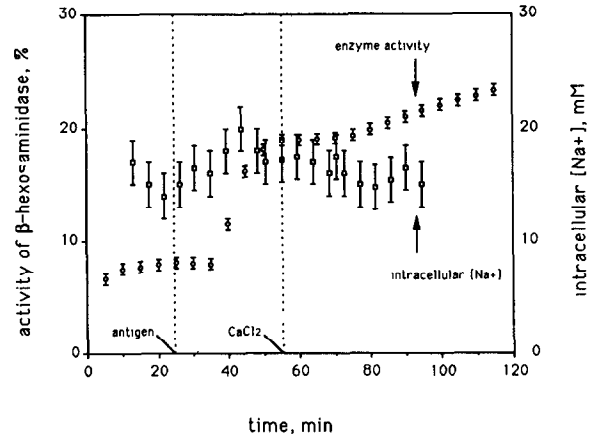

Fig. 4. Secretion of β-hexosaminidase (○) and intracellular Na^+-concentration (□) in RBL-cells perfused with low sodium $Dy(PPP)_2$-Tyrode ($[Na^+] = 30$ mM). At the marked time points, antigen or $CaCl_2$ (2.25 mM) were aded to the external reservoir. For determination of the β-hexosaminidase activity see Fig. 2.

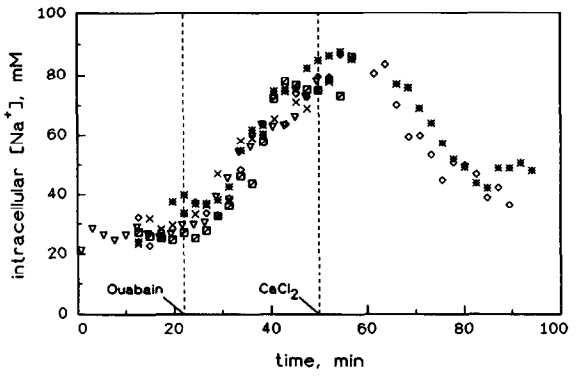

Fig. 5. Effects of ouabain and Ca^{2+} on the concentration of intracellular Na^+ in stimulated and resting RBL-cells in $Dy(PPP)_2$-Tyrode ($[Na^+] = 137$ mM). At the marked time points ouabain (0.1 mM) or $CaCl_2$ (4.5 mM) were added to the external reservoir. The symbols show several different, independent experiments. In experiments indicated with the symbols (□,◇,▽) Ag was added together with ouabain.

promise that enabled both, secretion and monitoring $[Na^+]_i$. Fig. 3b shows spectra of the same sample as in Fig. 3a but after addition of 2.25 mM $CaCl_2$. Using the PPP affinity constants for Dy^{3+} and Ca^{2+} [25], the concentration of free Ca^{2+} ions under these conditions can be calculated to be 100 μM, which already supports secretion. In spite of the decrease in the chemical shift distance between the Na^+ signals, they still were resolved and could be fitted to two lorentzians (Fig. 3b, dotted line) hence allowing $[Na]_i$ to be determined. Using these conditions, the protocol for addition of Ag and $CaCl_2$ was the same as in Fig. 1b and secretion as well as ^{23}Na NMR were monitored. Sequential addition of Ag and $CaCl_2$ (2.25 mM final concentration) did not induce resolvable changes in intracellular $[Na^+]_i$ (Fig. 4). While only a limited secretory response was observed following addition of Ag to the Ca^{2+} free buffer, the subsequent addition of $CaCl_2$ induced additional release. The maximal activity of released β-hexosaminidase reached however only about half that observed in experiments at higher free $[Ca^{2+}]$ (i.e. in regular tyrode). The same decrease in secretory response was observed in parallel experiments carried out on adherent cells in microwell plates. Hence the limited secretory response is most probably due to the lowered free Ca^{2+} concentration in the presence of the shift reagent. The finding that no change in the $[Na^+]_i$ is induced by the $Fc_\epsilon RI$-stimulus can be interpreted either by the lack of a change in Na^+ flux or by compensating changes in both influx and efflux. We tried to discriminate between these possibilities by experiments with cells where the Na^+,K^+-ATPase was inhibited by ouabain. In order to amplify the expected changes, maximal $[Na^+]$gradients across the plasma membrane were produced ($Dy(PPP)_2$-Tyrode, $[Na^+] = 137$ mM). The results for resting and stimulated cells are presented in Fig. 5. In three experiments both ouabain and Ag were added to the cells and in two experiments only ouabain was added. All results show $[Na^+]_i$ increase caused by ouabain, yet no significant difference in the extent or rate of that increase could be resolved between resting and Ag stimulated cells. These results provided an upper limit estimate for the Na^+ fluxes following stimulation. From the slope of the increase of the $[Na^+]_i$ (Fig. 5) we calculated a Na^+ flux of 8×10^{-5} mol/(l · s) indicating a current of 5×10^7 ions/(cell · s). This is in the order of the leakage conductance Fernandez and Lindau [26] observed in whole-cell clamp experiments. Since Ag did not modulate $[Na^+]_i$, the maximum Na^+ flux induced upon stimulation must be lower than the experimental error of 1×10^7 ions/(cell · s) (20%). This value fits data obtained for the Ca^{2+} influx [27].

An interesting finding is that Ca^{2+} addition 30 min after ouabain, leads to a decrease in the $[Na^+]_i$ almost to the initial value thus indicating activity of a Ca^{2+}/Na^+ antiport system. Omission of K^+ from buffer 2 was followed by an immediate increase in the $[Na^+]_i$ (data not shown). However in these experiments, subsequent addition of $CaCl_2$ did not reverse the Na^+ influx. Probably the Ca^{2+}/Na^+ transporter requires K^+ or an intact energy metabolism. These data are in agreement with Mohr and Fewtrell's results [8] where the Ag induced Ca^{2+} uptake by RBL-cells was inhibited in the absence of K^+ ions, in spite of maintaining the membrane potential. While normal Ag induced secretion was observed in ouabain treated cells upon $CaCl_2$ addition, it was suppressed in the absence of K^+. This again emphasizes the important role of Ca^{2+} influx for exocytosis of RBL-cells and, as mentioned above, that the inhibitory effect of the omission of K^+ is probably due to the essential role of this ion for the regulation of cellular parameters coupled to exocytosis.

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