Na^+-Dependent Ca^{2+} Transport Modulates the Secretory Response to the Fcε Receptor Stimulus of Mast Cells

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ABSTRACT Immunological stimulation of rat mucosal-type mast cells (RBL-2H3 line) by clustering of their Fcε receptors (FcεRI) causes a rapid and transient increase in free cytoplasmic Ca^{2+} ion concentration ($[Ca^{2+}]_i$) because of its release from intracellular stores. This is followed by a sustained elevated $[Ca^{2+}]_i$, which is attained by Ca^{2+} influx. Because an FcεRI-induced increase in the membrane permeability for Na^+ ions has also been observed, and secretion is at least partially inhibited by lowering of extracellular sodium ion concentrations ($[Na^+]_o$), the operation of a Na^+/Ca^{2+} exchanger has been considered. We found significant coupling between the Ca^{2+} and Na^+ ion gradients across plasma membranes of RBL-2H3 cells, which we investigated employing ^{23}Na-NMR, $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and the Ca^{2+}-sensitive fluorescent probe indo-1. The reduction in extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) provoked a $[Na^+]_i$ increase, and a decrease in $[Na^+]_o$ results in a Ca^{2+} influx as well as an increase in $[Ca^{2+}]_i$. Mediator secretion assays, monitoring the released β-hexosaminidase activity, showed in the presence of extracellular sodium a sigmoidal dependence on $[Ca^{2+}]_o$. However, the secretion was not affected by varying $[Ca^{2+}]_o$ as $[Na^+]_o$ was lowered to 0.4 mM, while it was almost completely inhibited at $[Na^+]_o = 136$ mM and $[Ca^{2+}]_o < 0.05$ mM. Increasing $[Na^+]_o$ caused the secretion to reach a minimum at $[Na^+]_o = 20$ mM, followed by a steady increase to its maximum value at 136 mM. A parallel $[Na^+]_o$ dependence of the Ca^{2+} fluxes was observed: Antigen stimulation at $[Na^+]_o = 136$ mM caused a pronounced Ca^{2+} influx. At $[Na^+]_o = 17$ mM only a slight Ca^{2+} efflux was detected, whereas at $[Na^+]_o = 0.4$ mM no Ca^{2+} transport across the cell membrane could be observed. Our results clearly indicate that the $[Na^+]_o$ dependence of the secretory response to FcεRI stimulation is due to its influence on the $[Ca^{2+}]_i$, which is mediated by a Na^+-dependent Ca^{2+} transport.

INTRODUCTION

Mast cells and basophils are immune effector cells that secrete mediators of inflammation in response to clustering of their surface type 1 Fcε receptors (FcεRI). Cellular events related to the coupling of this stimulus to the secretion have been studied in detail in RBL-2H3 cells, a line of rat mucosal-type mast cells. In these cells, FcεRI stimulation causes a biphasic change in intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$, a transient increase due to the release of Ca^{2+} from intracellular stores followed by a sustained elevation in $[Ca^{2+}]_i$. The sustained elevation, which is crucial for the secretory response, depends on an influx of extracellular Ca^{2+} ions, which is assumed to be initiated upon depletion of $InsP_3$-sensitive calcium stores (Putney, 1990). A corresponding Ca^{2+} current (designated I_{CRAC}) was observed using patch-clamp techniques (Hoth and Penner, 1992). Whereas in these experiments intracellular stores of rat peritoneal mast cells were depleted by their incubation in nominally Ca^{2+}-free buffers, a later study (Zhang and McCloskey, 1995) detected I_{CRAC} in immunologically stimulated RBL-2H3 cells. Polyvalent cations act on the level of the potential entry pathway and interfere with Ca^{2+} influx by blocking or competing with Ca^{2+} binding. The monovalent ions Na^+ and K^+ have also been reported to significantly influence the secretory response (Sussman et al., 1986; Putney, 1990; Gericke et al., 1995; Mohr and Fewtrell, 1987a; Pilatus and Pecht, 1993). One mechanism by which these ions affect the Ca^{2+} uptake may be related to their role in regulating the cells' membrane potential. Intact membrane potential has been reported to be essential for the FcεRI-stimulated Ca^{2+} uptake and secretion of RBL-2H3 cells (Mohr and Fewtrell, 1987c). Consequently, a high extracellular K^+ concentration, which depolarizes the membrane, was shown to inhibit the immunological response (Kanner and Metzger, 1984; Mohr and Fewtrell, 1987a,b). Stimulated RBL-2H3 cells show a transient depolarization (Sagi-Eisenberg and Pecht, 1983, 1984; Mohr and Fewtrell, 1987b) followed by a repolarization (Labrecque et al., 1989), indicating the operation of efficient mechanisms stabilizing the membrane potential during Ca^{2+} uptake. It has been speculated that these processes are coupled to K^+ and Na^+ transport (Labrecque et al., 1989, 1991), and therefore changes in extracellular concentration of these cations affects efficient Ca^{2+} uptake. While membrane potential-mediated effects suggest a rather indirect role of monovalent ions in immunologically stimulated secretion, Stump et al. (1987) postulated a mechanism that directly links Na^+ transport to the immunologically stimulated Ca^{2+}

Received for publication 1 March 2000 and in final form 8 September 2000.

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0006-3495/00/12/2975/12 $2.00

uptake via a Na^+/Ca^{2+} exchanger. Their hypothesis was based on findings that the cellular response is sensitive to the preferential inhibition of such a transporter and can be suppressed by the absence of extracellular Na^+ ions. Their results seem to contradict findings of Mohr and Fewtrell (1987a), who reported that substitution of extracellular Na^+ by glucose does not inhibit the secretory response. To address this problem we examined in detail the modulation of the cells' secretory response as a function of extracellular Na^+ ion concentrations (Rumpel et al., 1995). A minimal secretion was observed at low extracellular Na^+ concentration (10–20 mM). A further decrease partially restored the secretory response. Because this specific $[Na^+]_o$ dependence pattern of the secretory response to the FcεRI stimulus may reveal important insights into mechanisms involved in the stimulus-secretion coupling, we performed multiple experiments to characterize Ca^{2+} fluxes in relation to extra- and intracellular Na^+ and Ca^{2+} concentrations in resting and stimulated RBL 2H3 cells. The results are presented and discussed in this paper.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY). Fetal calf serum (FCS), glutamine, and the antibiotics were obtained from BioLab (Jerusalem, Israel). Bovine serum albumin (BSA), *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine, and Triton X-100 were from Sigma (St. Louis, MO). The monoclonal, -2,4-dinitrophenyl (DNP)-specific murine IgE class antibodies (A_2IgE) were raised and purified in our laboratory. BSA derivatized with an average of 11 DNP groups (DNP_{11}-BSA) per mole was prepared as described (Pilatus and Pecht, 1993; Gericke et al., 1995). $^{45}CaCl_2$ and $^{85}SrCl_2$ were purchased from DuPont (Boston, MA).

Cells

RBL-2H3 cells were grown in monolayers in DMEM, supplemented with 10% FCS, 2 mM glutamine, and antibiotics. For harvesting, the cells were exposed to 10 mM EDTA in DMEM for 15 min. The cells were suspended at 10^6/ml in DMEM containing DNP-specific A_2IgE (1 μl of ascites/10^6 cells) and seeded in multiwell plates (aliquots of 0.5 ml) or microtiter plates (aliquots of 0.1 ml) the day before the experiments. For experiments with perfused cell cultures, 3×10^6 cells were seeded on 0.5-ml spherical microcarriers (Biosilon beads, 160–300 μm diameter; Nunc, Copenhagen, Denmark) and placed in 9-cm-diameter Petri dishes containing 12 ml of the FCS-supplemented DMEM. The medium in the dishes was usually replaced every 2 days. After 4 days of growth the bead surface (150 cm^2/ml beads) was covered with a monolayer of cells as monitored by light microscopy, reaching a density of 2×10^7 to 4×10^7 cells/ml beads. In experiments where the calcium dependence of the cells' response was assayed, cells were maintained in modified Tyrode's solution: 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 5.6 mM glucose, 20 mM HEPES, 0.1% BSA, and $CaCl_2$ as indicated or 2 mM otherwise. In experiments examining the sodium concentration dependence, osmolarity was maintained by partly replacing NaCl with glucose. All of the buffers were adjusted to pH 7.3 with Tris or NaOH. Sodium ion concentrations were calculated according to the molecular weights of the ingredients.

Secretion assays

Cell monolayers grown in microtiter plates and saturated with IgE as indicated above, washed twice with buffered saline solution, and preincubated at 37°C for 30 min with the relevant modification of Tyrode's buffer. After preincubation, the cells were exposed to a final concentration of 25 or 50 ng/ml DNP_{11}-BSA at 37°C for 50 min. Equivalently treated cells (only omitting DNP_{11}-BSA) were lysed with Triton X-100 at a final concentration of 0.5%. Forty microliters of substrate solution (1.3 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine in 0.1 M sodium citrate, pH 4.5) were added to 15-μl aliquots of cell supernatant and incubated for 50 min at 37°C. The reaction was terminated by the addition of 150 μl of 0.2 M glycine (pH 10.7), and the light absorption of the solutions was measured at 405 nm in a microplate optical reader. The dose-response curve showed a maximum secretory activity when antigen was applied at concentrations between 20 and 200 ng/ml. Secretion assays were performed on nine replicates, each varying the extracellular calcium concentration at 0.4 mM Na^+ as well as at 136 mM Na^+ (six independent experiments) or varying the extracellular sodium concentration at 2 mM Ca^{2+} (17 independent experiments) or at 0.01 mM Ca^{2+} (four independent experiments). Calcium was replaced with strontium in experiments where either the strontium dependence of secretion was compared in 0.4 mM versus 136 mM Na^+ saline (four independent experiments, nine replicates each) or the sodium dependence of secretion was monitored in parallel in saline containing 8 mM or 0.04 mM Sr^{2+} (two independent experiments, nine replicates each).

Net $^{45}Ca^{2+}$ ion influx

Cell monolayers grown in and adherent to multiwell plates (24 wells/plate) were washed twice and loaded with ^{45}Ca by incubation (30 min) in Tyrode's supplemented with 10 μCi/ml ^{45}Ca. DNP_{11}-BSA (final concentration 50 ng/ml) was added to some of the wells, and the cells were incubated at 37°C for another 10 min. Supernatant aliquots were taken to determine the extent of mediator secretion, and the cells were then washed three times with ice-cold buffered saline solution. Special care was taken to expose the cells for identical periods of time to the washing buffer. Afterwards, the cells were lysed with Triton X-100 (final concentration 0.5%), and 0.4-ml lysate aliquots were taken and mixed with scintillation liquid to determine the β-activity of ^{45}Ca with a scintillation counter. The net calcium influx was monitored in three independent experiments (four replicates each). In another protocol the radioactive tracer was added together with the antigen to monitor the antigen-stimulated unidirectional ^{45}Ca influx.

$[Ca^{2+}]_i$ measurements with the fluorescent probe indo-1

IgE-saturated RBL-2H3 cells were washed and harvested with 10 mM EDTA. Excess EDTA solution was removed after centrifugation. Cell were resuspended with 5 μM indo-1 AM (10^7 cells/ml) and 250 μM sulfine pyrazone and incubated for 15 min at 37°C. After this period an identical volume of Tyrode's with sulfine pyrazone was added. After another 15 min of incubation, the cells were washed twice (centrifugation and resuspension in 10 ml Tyrode's buffer) and resuspended (10^6 cells/ml) in Tyrode's buffer (Na^+ concentration modified as indicated). In experiments where dye loading and measurement were performed in different saline solutions, an additional incubation period of 10 min was included in the period between the two washing cycles to allow equilibration with the test buffer. Ten minutes after final resuspension 2 ml of cells was transferred to a quartz cuvette. Fluorescence was excited at 355 nm and emission was monitored at 405 nm and 485 nm simultaneously with a home-built fluorescence spectrometer. In experiments where the secretory response was measured, cells were exposed to 200 ng/ml DNP_{11}-BSA as indicated. We found that antigen concentrations of 200 ng/ml were best suited for this

kind of experiment. Experiments were performed in triplicate; a total of four independent experiments were performed.

^{23}Na-NMR

^{23}Na-NMR experiments were performed using a Bruker AM-360 spectrometer. The spectra were recorded at 95.26 MHz, using 1 K of data, a 3817-Hz spectral width, a 90° flip angle, and a 0.240-s repetition time. A total of 2500 scans were accumulated for each spectrum. Biosilon beads covered with a monolayer of cells were harvested, and 2 ml of settled beads ($6-8 \times 10^7$ cells) were transferred into a 10-mm-diameter NMR tube. The temperature was maintained at 37°C. The cells were continuously perfused with a modified Tyrode's buffer, which was supplemented with 12 mM Na_5-tripolyphosphate (Na_5(PPP)), 6 mM $DyCl_3$ (final Na^+ concentration 60 mM), and $CaCl_2$ as indicated. The osmolarity was adjusted with 160 mM glucose. At the beginning and the end of each experiment (duration 5 h), the cell density was determined and the data were corrected for cell loss by linear interpolation. To suppress any further effect of cell loss, the order in which test solutions were applied was inverted every other experiment (four independent experiments). Intracellular sodium concentration was determined according to the expression $[Na^+]_i = A_i/A_o * V_m/V_c * [Na^+]_o$, where V_m represents the volume of the medium (31.5 ± 0.4%) and V_c represents the volume of the cells (3.5 ± 0.4%) (cf. Pilatus et al., 1990, for details). A_i and A_o denote the signal intensities of the peaks referring to intracellular and extracellular sodium, respectively. In calculating $[Na^+]_i$ we assumed a visibility of 100%, in accordance with the majority of literature on mammalian cells (Shinar and Navon, 1984; Gupta and Gupta, 1982; Ogino et al., 1985) (whereas a visibility of only 40% was observed for yeast cells (Ogino et al., 1983), algae (Bental et al., 1988), and bacteria (Nagata et al., 1995)). Our assumption is supported by the finding that increasing $[Na^+]_i$ by ouabain in endothelial cells correlates with an increase in the longitudinal relaxation time T_1 of intracellular sodium (Gruwel et al., 1995). These data show that elevated $[Na^+]_i$ is mainly represented by an increase in its nonbound state, and therefore 100% of intracellular sodium is visible. As a reduced extracellular calcium concentration (the typical condition of our experiments) also caused an elevated sodium concentration, a 100% visibility of intracellular sodium seems to be adequate.

$^{85}Sr^{2+}$ fluxes

$^{85}Sr^{2+}$ uptake was measured with a flow system developed previously for direct real-time measurement of $^{86}Rb^+$ fluxes in live adherent mast cells (Pilatus and Pecht, 1993). The bead-adherent cells (2×10^7 to 4×10^7/ml beads) were transferred to a specially designed glass sample holder that allows continuous perfusion of the cells under controlled temperature, and placing them in the detection chamber of a γ counter (GAMMAmatic I; Kontron Analytical) allows direct time-resolved monitoring of the γ-radiation associated with the sample. Taking into account an intracellular volume of 10^{-9} cm^3/cell, the total cellular volume in a sample containing 1 ml of settled beads is ~0.04 ml or 13% of the sample's buffer volume. (Only 31% of the total volume is water; the rest is taken up by the beads.) Because the Sr^{2+} ion content (free + bound) of the cells reaches the order of mM under optimal conditions, 10% of the total counts may originate from intracellular $^{85}Sr^{2+}$. Therefore a minimum count rate of at least 50,000 cpm is required to achieve a 10% accuracy in monitoring relative changes of the concentration of intracellular $^{85}Sr^{2+}$. For our experimental set-up, 1 μCi/ml ^{85}Sr in the perfusion buffer was found sufficient when we monitored the γ-photons at 513 keV (window: 500–600 keV). Special care was taken to avoid oscillations in the filling level of the sample tube caused by slight fluctuations in the efflux pumping rate. All experiments were performed at a sample temperature of 37°C and a perfusion rate of 1 ml/min. For the experiments, cell-covered beads were harvested and perfused for 30 min with the respectively modified Tyrode's buffer (Ca^{2+} replaced by 8 mM Sr^{2+}, Na^+ as indicated) without the radioactive isotope to allow equilibration of the cellular Sr^{2+} concentrations in the presence of Sr^{2+}-containing buffer. During this period the sample holder was mounted in the γ counter, and the background counts were registered every minute. Then the buffer was changed to that containing $^{85}Sr^{2+}$. The uptake by the resting cells was registered during the following 30 min before the cells were stimulated by changing to radioactively labeled buffer containing 50 ng/ml of the antigen. Aliquots of the efflux buffer were taken every 5 min and assayed for their β-hexosaminidase activity. Two independent experiments each were performed at three different Na^+ concentrations (0.4 mM, 17 mM, 136 mM).

RESULTS

Several distinct experimental protocols were employed to study the transport of calcium and sodium across the membrane of resting and stimulated RBL-2H3 cells and to investigate the role of these cations in coupling the immunological stimulus of RBL-2H3 cells to their secretory response. First, calcium- and sodium-dependent secretory response assays were carried out in parallel with $^{45}Ca^{2+}$ flux measurements. In a second approach, Ca^{2+} ions were replaced with Sr^{2+} in the secretion assays, and the γ-ray-emitting ^{85}Sr was employed in perfusion experiments to monitor its fluxes. Finally, to determine free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), separate sets of experiments were performed with the fluorescent probe indo-1.

At 140 mM $[Na^+]_o$ the secretory response shows a sigmoidal dependence on $[Ca^{2+}]_o$, whereas in the absence of $[N_a{}^+]_o$ the secretion is independent of $[Ca^{2+}]_o$.

The dependence of the FcεRI-induced secretory response on the extracellular calcium and sodium ion concentrations was investigated by β-hexosaminidase assays. Fig. 1 *A* shows the result of six independent experiments performed on cells that were incubated either in very low sodium (0.4 mM Na^+) Tyrode's buffer or in Tyrode's containing 136 mM NaCl. A control experiment with nonstimulated cells (control) did not resolve Ca^{2+}-dependent secretion (up to 5 mM). With a further increase in extracellular Ca^{2+} to 20 mM ($n = 2$) a slight secretion was monitored. No Na^+ dependence was observed under these conditions over the whole range from 0.4 to 136 mM ($n = 2$). In stimulated cells, the secretory response at very low sodium buffer did not depend on $[Ca^{2+}]_o$. In contrast, the presence of 136 mM NaCl induced a strong $[Ca^{2+}]_o$ dependence of the response. Although omitting $[Ca^{2+}]_o$ caused suppression of the cells' secretion, the absence of both Ca^{2+} and Na^+ ions in the external medium did yield a modest secretory response. The sigmoidal $[Ca^{2+}]_o$ dependence could also be monitored, when NaCl was replaced with sodium gluconate (two independent experiments, data not shown). Furthermore, the secretory response in nominally Na^+-free buffer did not depend on the choice of the substitute: the cell's secretory response expressed the same Ca^{2+} dependence in choline chloride or tetramethylammonium chloride as when glucose was used for NaCl substitution (three independent experiments, data not shown). The observation that the secretory response in nominally Na^+-free buffer did not show any $[Ca^{2+}]_o$ dependence indicates a Na^+-mediated Ca^{2+} transport.

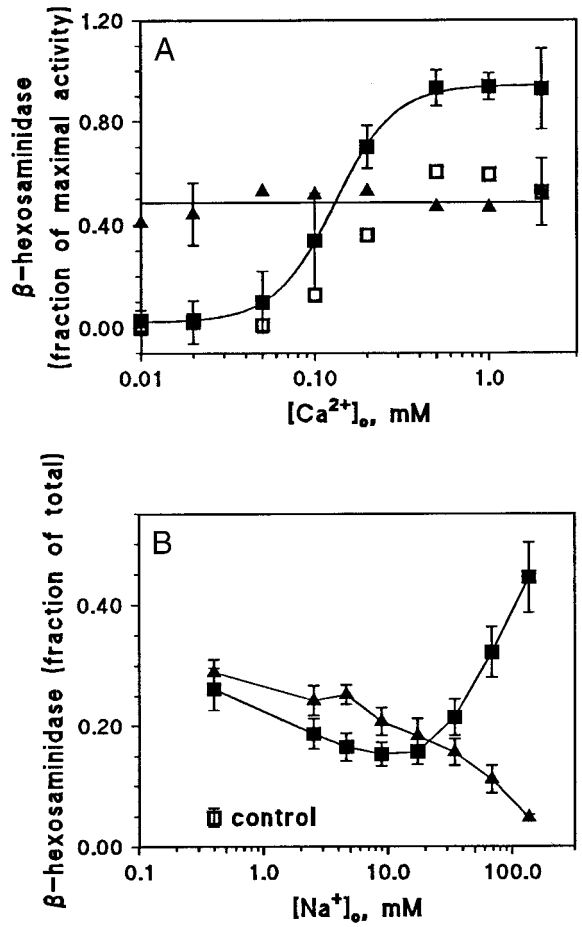

FIGURE 1 Antigen-stimulated secretion of β-hexosaminidase depends on extracellular concentrations of calcium and sodium ions. (*A*) $[Ca^{2+}]$ dependence of the secretory response of RBL-2H3 cells at $[Na^+]_o$ = 0.4 mM (▲) and 136 mM (□, ■). The results are the mean of six independent experiments (nine replicates each). The results of assays, where β-hexosaminidase activity in the cells' supernatants was measured, were normalized for each independent experiment by setting the maximum secretion to 1 (absolute values varied from 0.3 to 0.6). □, Secreted β-hexosaminidase activity in the presence of 2×10^{-5} M DCBA at $[Na^+]_o$ = 136 mM. (*B*) $[Na^+]_o$ dependence of the antigen-induced secretory response at physiological and at reduced $[Ca^{2+}]_o$. The figure presents data of 17 independent experiments carried out in the presence of $[Ca^{2+}]_o$ 2 mM (■) and of four independent experiments in the presence of $[Ca^{2+}]_o$ = 0.01 mM (▲). The error bars denote the standard deviation of nine replicates. Secretion is presented as a net percentage of the cells' total content of β-hexosaminidase activity.

The secretory response to Fc ϵ RI stimulus in the presence of $[Ca^{2+}]_o$ = 2 mM exhibits a significant minimum at $[Na^+]_o$ = 20 mM. In the nominal absence of $[Ca^{2+}]_o$ a decrease in $[Na^+]_o$ leads to an increased secretory response.

As shown in Fig. 1 *B*, the secretory response of RBL-2H3 cells to antigen exhibited a strong dependence on sodium concentration. When the extracellular sodium concentration was lowered in the presence of 2 mM $[Ca^{2+}]_o$ (Fig. 1 *B, squares*), the secretion decreased, went through a minimum at about $[Na^+]_o$ = 20 mM, and increased slightly to a modest secretory response, which was visible at the lowest $[Na^+]_o$ concentration of less than 0.4 mM (i.e., in HEPES/Tris-buffered Tyrode's solution without additional NaCl). The increase at very low sodium corresponds to the moderate non-Ca^{2+}-dependent secretion observed for the nominally Na^+-free buffer described above (Fig. 1 *A, triangles*). Consequently, a comparable secretion could still be observed at the lowest sodium concentration (0.4 mM), but at $[Ca^{2+}]_o$ = 0.01 mM (*triangles*). Under the conditions of low $[Ca^{2+}]_o$ the secretory response declined with increasing $[Na^+]_o$ and reached the level of basal secretion observed at physiological Ca^{2+} concentrations. The observed nonmonotonous $[Na^+]_o$ dependence of the secretory response suggests that at least two transport processes are involved in the stimulus-secretion coupling.

A significant ^{45}Ca uptake is observed in cells stimulated at $[Na^+]_o > 20$ mM.

To establish a protocol for measuring the ^{45}Ca uptake by $^{45}Ca^{2+}$-preloaded cells, 10^6 cells were incubated in 0.2 ml for 30 min with ^{45}Ca (10 μCi/ml) in Tyrode's buffer containing different sodium concentrations ($[Ca^{2+}]_o$ = 2 mM). Intracellular Ca^{2+} reached equilibrium after this period, inasmuch as further incubation with $^{45}Ca^{2+}$ in DMEM for up to 4.5 h did not significantly increase the intracellular $^{45}Ca^{2+}$ content (determined after the washing as described below; data not shown). Loss of intracellular Ca^{2+} during extensive washing with low-sodium Tyrode's ($[Na^+]$ = 0.4 mM) may cause a significant error in estimating intracellular ^{45}Ca, as shown in Fig. 2 *A*. ^{45}Ca-loaded cells (30 min) were exposed to the washing buffer (3 ml) either three times for 2 min or three times for 5 min. A decrease in cell-associated ^{45}Ca was observed upon increasing $[Na^+]_o$ and increasing the time interval between consecutive washing steps. To minimize the related error, a time interval of 30 s was chosen for further ^{45}Ca experiments.

An increased $^{45}Ca^{2+}$ influx was detected upon antigen stimulation at $[Na^+]_o > 20$ mM. Net ^{45}Ca uptake studies (Fig. 2 *B*) confirmed a marked increase in cell-associated Ca^{2+} after stimulation at $[Na^+]_o > 20$ mM. The data indicate a slight decrease in cell-associated Ca^{2+} upon antigen stimulation at $[Na^+]_o$ = 20 mM, whereas no change could be detected at $[Na^+]_o$ = 0.4 mM. The $[Na^+]_o$ dependence of the Ca^{2+} uptake paralleled the cells' secretory response pattern (Fig. 1 *B*), indicating that the $[Na^+]_o$-induced changes in $[Ca^{2+}]_i$ are related to the observed $[Na^+]_o$ dependence of the secretory response.

Decreasing $[Na^+]_o$ increases $[Ca^{2+}]_i$ in resting cells.

Results of the above tracer experiments (Fig. 2 *B*) show that resting RBL-2H3 cells accumulated an increasing amount of ^{45}Ca with decreased $[Na^+]_o$. Similar data were obtained

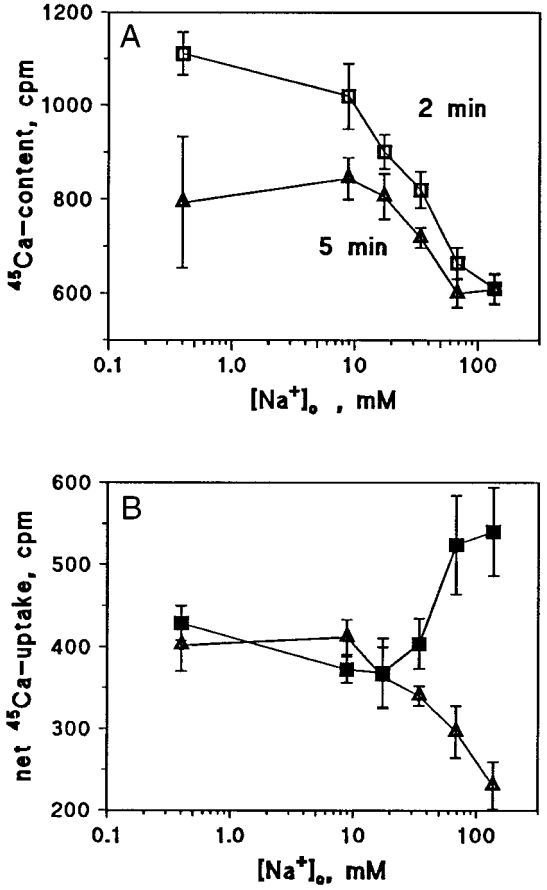

FIGURE 2 $^{45}Ca^{2+}$ uptake by RBL cells depends on $[Na^+]_o$. (*A*) Monitoring the influence of the washing procedure. Cells loaded with ^{45}Ca for 30 min at $[Na^+]_o$ as indicated in Materials and Methods were washed with Tyrode's (0.4 mM Na^+) either three times for a 2-min period (□) or three times for 5 min each (▲). (*B*) Antigen-stimulated net $^{45}Ca^{2+}$ uptake (in counts per minute (cpm) per 8×10^5 cells). Results of a representative experiment (from three independent experiments, four replicates each) are presented. After a 30-min preincubation with ^{45}Ca (10 μCi/ml) in Tyrode's buffer ($[Na^+]_o$ is indicated by the abscissa), antigen (DNP_{11}-BSA final concentration 50 ng/ml) was added to the wells, and the radioactivity associated with the cells was monitored (■). The basal uptake was determined by counting the radioactivity taken up by unstimulated cells (▲).

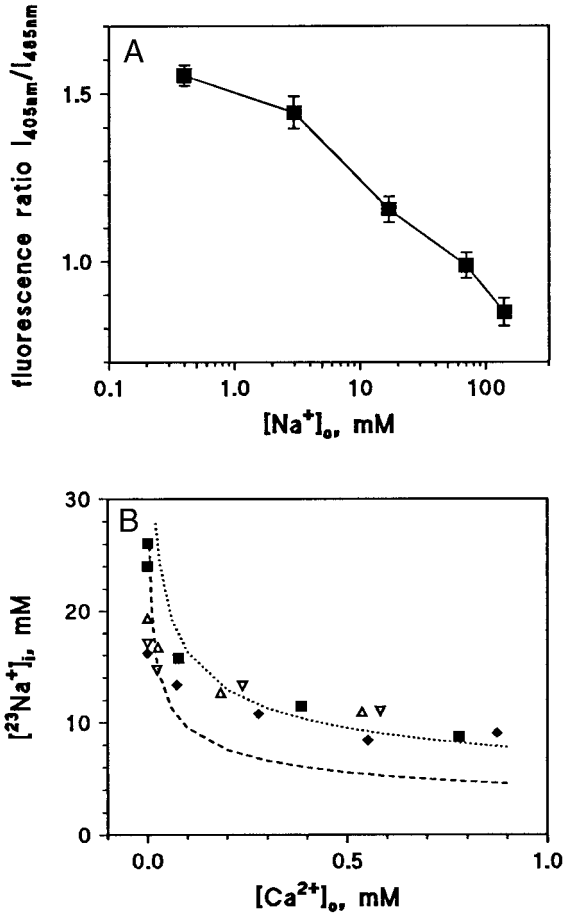

FIGURE 3 Coupling between sodium and calcium transport in resting cells. (*A*) $[Na^+]_o$ dependence of the free cytoplasmic calcium concentration in resting RBL-2H3 cells monitored with the fluorescent dye indo-1. Shown are mean values of triplicates representing one of four independent experiments. (*B*) $[Ca^{2+}]_o$ dependence of the cytoplasmic Na^+ concentration in resting RBL cells monitored by ^{23}Na-NMR. The symbols indicate the data of four independent experiments. Open symbols refer to experiments where the cells were subjected to a stepwise increasing, closed symbols to a stepwise decreasing concentration of extracellular calcium. $[Na^+]_i$ was calculated assuming the activity of a Na^+/Ca^{2+} exchanger for $[Ca^{2+}]_i$ = 100 nM (·····) and 20 nM (– – –), $[Na^+]_o$ = 60 mM.

in experiments where the calcium-sensitive dye indo-1 was employed as a fluorescent probe. Reducing $[Na^+]_o$ caused an increase in $[Ca^{2+}]_i$ of resting cells (Fig. 3 *A*). Changes in $[Ca^{2+}]_i$ after stimulation were monitored using RBL-2H3 cells loaded with indo-1 AM in Na^+-containing or Na^+-free Tyrode's in the presence of $[Ca^{2+}]_o$. For the experiments, cells were resuspended in the respective buffer, but in nominally Ca^{2+}-free conditions, and stimulated by the addition of 200 ng/ml antigen (Fig. 4 *A*). In the presence of extracellular Na^+ (*solid line*), these cells exhibited the short transient rise in $[Ca^{2+}]_i$ after FcεRI stimulation (Dar and Pecht, 1992). Subsequent addition of Ca^{2+} to the buffer caused a pronounced increase in $[Ca^{2+}]_i$ by Ca^{2+} uptake (three independent experiments). However, the short $[Ca^{2+}]_i$ transient was not observed, when the cells were preincubated in nominally Ca^{2+}-free buffer for ~30 min (i.e., while loading with the dye, two independent experiments; data not shown). As expected from data in Fig. 3 *A*, cells preincubated in Na^+-free Tyrode's showed increased $[Ca^{2+}]_i$ after resuspension in the respective Ca^{2+}-free buffers (*dotted line*). No transient increase of this already elevated $[Ca^{2+}]_i$ was observed upon stimulation in the absence of extracellular Ca^{2+}, and subsequent restoration of regular extracellular Ca^{2+} levels caused only a slight increase in the $[Ca^{2+}]_i$.

Decreasing the extracellular Ca^{2+} concentration increased $[Na^+]_i$ in resting cells.

The observed coupling between $[Ca^{2+}]_i$ and the extracellular Na^+ concentrations in resting cells may reflect the

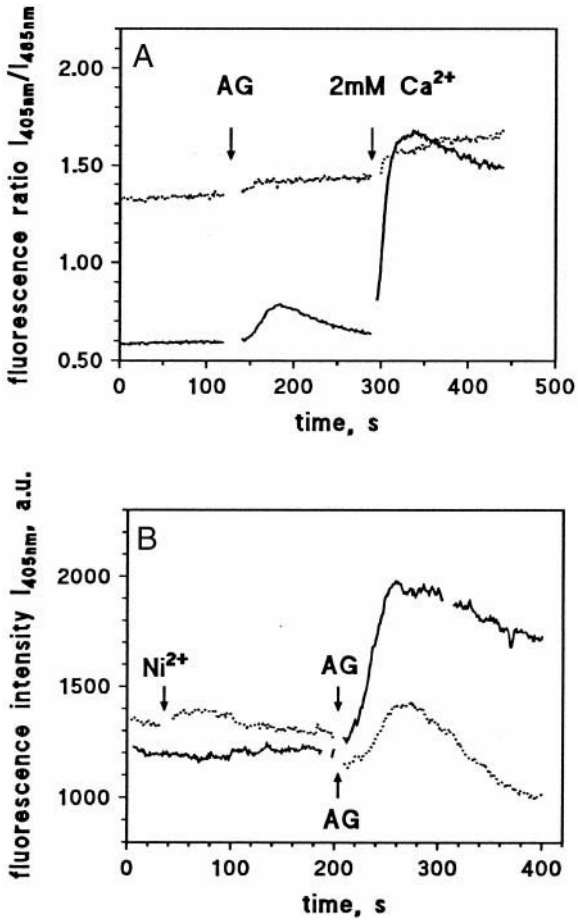

FIGURE 4 Changes induced in the free intracellular Ca^{2+} concentrations monitored by the fluorescent dye indo-1. (*A*) $[Ca^{2+}]_i$ response to antigen stimulation of RBL cells at $[Na^+]_o$ = 136 mM (——) and 0.4 mM (·····). Antigen (200 ng/ml) and Ca^{2+} (2 mM final concentration) were added at the indicated time points (results shown are for one of three independent experiments). Cells were loaded with the dye in Tyrode's buffer of the respective Na^+ concentration. Extracellular Ca^{2+} was removed by centrifugation and resuspension immediately before the experiment. (*B*) Calcium response to antigen stimulation of RBL cells in the absence and presence of 5 mM $NiCl_2$. $[Ca^{2+}]_o$ = 2 mM. The results shown represent one of two independent experiments.

activity of a Na^+/Ca^{2+} exchanger. To pursue further evidence for the role of such a transporter in regulating basal $[Na^+]_i$ and $[Ca^{2+}]_i$, we monitored the $[Ca^{2+}]_o$ dependence of the intracellular Na^+ concentration by ^{23}Na-NMR spectroscopy (Fig. 3 *B*). The formation of Ca^{2+} tripolyphosphate complexes ($CaPPP^{3-}$) causes the concentration of free calcium ions in the buffer to be much lower than its total Ca^{2+} concentration. Thus the $[Ca^{2+}]_o$ values depicted on the ordinate in Fig. 3 *B* are calculated numbers, determined by a procedure described in the Appendix. At $[Ca^{2+}]_o$ = 0.9 mM an intracellular sodium concentration of 10 mM was detected, and a further decrease in $[Ca^{2+}]_o$ caused an increase in $[Na^+]_i$. The broken lines in Fig. 3 *B* were calculated assuming that $[Na^+]_i$ and $[Ca^{2+}]_i$ are coupled by the activity of a Na^+/Ca^{2+} exchanger according to $[Na^+]_i = [Na^+]_o * ([Ca^{2+}]_i/[Ca^{2+}]_o * \exp(-VF/RT))^{1/3}$ (Eisner and Lederer, 1985), with $[Na^+]_o$ = 60 mM, T = 310 K, V = −80 mV (Sagi-Eisenberg and Pecht, 1983), and $[Ca^{2+}]_i$ = 100 nM or 20 nM as indicated. The comparison of these data indicates a) The $[Ca^{2+}]_o$ dependence of $[Na^+]_i$, as monitored by ^{23}Na-NMR, is described well by assuming a Na^+/Ca^{2+} exchange activity. b) At $[Na^+]_o$ = 60 mM and $[Ca^{2+}]_o$ = 0.9 mM the Na^+/Ca^{2+} exchanger appears to operate at equilibrium conditions. c) A constant basal level of $[Ca^{2+}]_i \approx$ 100 nM appears to be maintained for $[Ca^{2+}]_o > 0.1$ mM; only at very low extracellular calcium does a reduction of $[Ca^{2+}]_i$ have to be postulated to account for the $[Ca^{2+}]_o$ dependence of intracellular sodium.

Inhibitors of the Na^+/Ca^{2+} exchange reduced the secretory response of RBL-2H3 cells

Among several amiloride analogs known to act as Na^+-transport inhibitors, dichlorobenzamil (DCBA) was shown to exhibit the highest inhibition of the Na^+/Ca^{2+} exchanger (Cragoe et al., 1967). As previously reported, subjecting RBL cells to DCBA has reduced their secretory response (Fig. 1 *A*) (Stump et al., 1987). Because of its intense autofluorescence, DCBA could not be employed in experiments using the fluorescent probe indo-1. Ni^{2+} ions have also been used as inhibitors of the Na^+/Ca^{2+} exchange in a variety of cells (Ruknudin et al., 1996; He et al., 1996; Niggli and Lederer, 1991). Yet, Ni^{2+} ions do not block Ca^{2+}-activated nonspecific cation channels (Niggli, 1989). Stimulating RBL-2H3 cells by antigen in the presence of 5 mM Ni^{2+} abolished the sustained elevation in $[Ca^{2+}]_i$, whereas the fast transient was still maintained (Fig. 4 *B*).

RBL cells exhibit a similar secretory response pattern when Ca^{2+} ions are replaced with Sr^{2+}.

It has been shown that substituting Ca^{2+} with Sr^{2+} maintains the secretory response (Hide and Beaven, 1991; Foreman and Mongar, 1972, 1973; Pearce and Thompson, 1986). This provides a way to monitor in an independent manner the fluxes of this Ca^{2+} ion homolog and to examine whether Sr^{2+}-dependent secretion is coupled to extracellular Na^+ in a way similar to that described above for $[Ca^{2+}]_o$. First, secretion assays were performed while monitoring their dependence on extracellular Sr^{2+} concentrations $[Sr^{2+}]_o$ and $[Na^+]_o$ in the absence of $[Ca^{2+}]_o$. The results exhibit the same sigmoidal dependence on $[Sr^{2+}]_o$ at physiological $[Na^+]_o$, although a shift to higher $[Sr^{2+}]_o$ was observed: half-maximum was reached at $[Sr^{2+}]_o \approx$ 2 mM and maximum secretion at $[Sr^{2+}]_o >$ 10 mM (Fig. 5 *A*). In nominally Na^+-free buffer (0.4 mM), the $[Sr^{2+}]_o$ dependence of the secretory response was impaired. RBL-2H3 cells subjected to high (8, 10, or 20 mM) and low (0.04 mM) $[Sr^{2+}]_o$ exhibited a Na^+ dependence of antigen-stimulated secretion

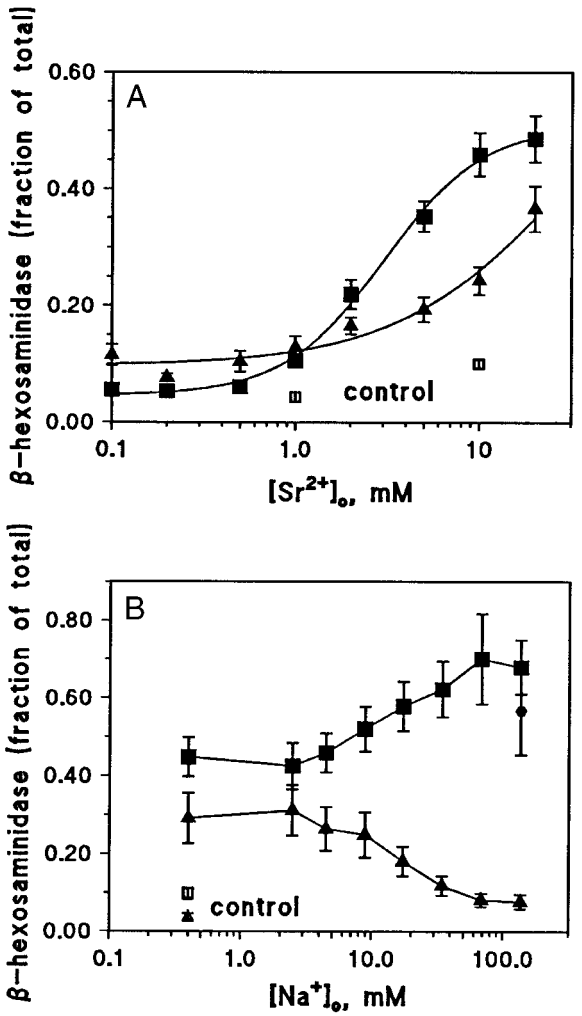

FIGURE 5 The secretory response is maintained when extracellular Ca^{2+} is replaced by Sr^{2+}. (*A*) Sr^{2+} concentration dependence of the antigen-stimulated response at $[Na^+]_o$ = 136 mM (■) and 0.4 mM (▲). Presented data are mean values of one of four independent experiments (nine replicates each). Open squares mark the basal secretion at indicated $[Sr^{2+}]_o$. (*B*) Na^+ dependence of the secretory response at 8 mM Sr^{2+} (■), 0.04 mM Sr^{2+} (▲), and 2 mM Ca^{2+} (●) (results represent one of two independent experiments, nine replicates each).

(Fig. 5 *B*) similar to that of cells exposed to high (2 mM) or low (0.01 mM) $[Ca^{2+}]_o$, respectively (Fig. 1 *B*). A $[Sr^{2+}]_o$ of 8 mM has been chosen for the experiments employing Sr^{2+} as a Ca^{2+} substitute, as an increase in spontaneous secretion could be observed at $[Sr^{2+}]_o > 10$ mM.

$^{85}Sr^{2+}$ perfusion experiments confirm the $[Na^+]_o$ dependence of antigen-stimulated Ca^{2+} fluxes.

Since the data (Fig. 2 *B*) did not enable us to establish the Ca^{2+} transport induced upon antigen stimulation at $[Na^+]_o < 30$ mM, the washing procedure (causing the main error) had to be avoided to gain a higher degree of accuracy. Thus, a different experimental protocol was adopted that employed the γ-ray-emitting tracer $^{85}Sr^{2+}$, which allowed on-line real-time monitoring of the changes in intracellular Sr^{2+} concentrations. Cells grown on microcarriers were perfused with ^{85}Sr-containing buffer, and radiation from the isotope taken up by the cell sample was registered on-line as described in Materials and Methods. When $^{85}Sr^{2+}$ uptake reached a steady-state level (Fig. 6 *A*) the cells were stimulated by antigen (25 ng/ml DNP_{11}-BSA final concentration). At $[Na^+]_o$ = 136 mM (Fig. 6 *B*) an increase in the cell-associated $^{85}Sr^{2+}$ ions was observed, while at $[Na^+]_o$ = 0.4 mM (Fig. 6 *D*) none could be detected. In contrast, at $[Na^+]_o$ = 17 mM a net efflux of $^{85}Sr^{2+}$ ions was monitored in response to FcεRI stimulation (Fig. 6 *C*).

DISCUSSION

Ca^{2+} influx is known to be an essential requirement for attaining an optimal secretory response of mast cells to an immunological stimulus. A sigmoidal dependence of the secretory response on $[Ca^{2+}]_o$ similar to that shown in Fig. 1 *A* had earlier been observed by monitoring the secretion of [^{3}H]histamine (Beaven et al., 1984a) or [^{3}H]serotonin (Mohr and Fewtrell, 1987a). The present observation that in nominally Na^+-free buffer, antigen-stimulated secretion of β-hexosaminidase does not show any $[Ca^{2+}]_o$ dependence suggests the involvement of a Na^+-mediated Ca^{2+} transport. Moreover, because subjecting the cells to DCBA, a blocker of Na^+ transport pathways, reduced the secretion of [^{3}H]serotonin (Stump et al., 1987) and of β-hexosaminidase (Fig. 1 *A*), the involvement of a Na^+ transport in the stimulus-secretion coupling of RBL cells has been considered (Stump et al., 1987). The similar Na^+ dependence of secretion (Fig. 1 *B*) and $[Ca^{2+}]_i$ (Fig. 2 *B*) indicates that it is the $[Na^+]_o$ dependence of $[Ca^{2+}]_i$ that is modulating the Na^+ dependence of the cells' secretory response. Therefore the interaction between intra- and extracellular Ca^{2+} and Na^+ concentrations in RBL cells and its impact on the secretory response deserve a more detailed consideration. We will first discuss the Na^+/Ca^{2+} exchange as a potential mechanism for the coupling of Na^+ and Ca^{2+} concentrations.

In excitable cells (e.g., neurons and different types of muscle cells) the Na^+/Ca^{2+} exchanger has been studied in great detail. In these cells the exchanger is used to export Ca^{2+} ions after a stimulus to restore the cell's basal $[Ca^{2+}]_i$. This exchange mechanism also appears to be operative in various nonexcitable as well as secretory cells, including platelets, pancreatic β-cells, lymphocytes, astrocytes, and neutrophils (Takuma et al., 1995; Simchowitz and Cragoe, 1988; Balasubramanyam et al., 1994; Herchuelz and Plasman, 1991; Kimura et al., 1994). Usually the transporter is characterized by a Na^+-dependent Ca^{2+} uptake, which is induced by the removal of extracellular sodium (Takuma et al., 1995; Balasubramanyam et al., 1994; Herchuelz and Plasman, 1991; Kimura et al., 1994) or an increase in $[Na^+]_i$ (Takuma et al., 1995; Balasubramanyam et al., 1994; Her-

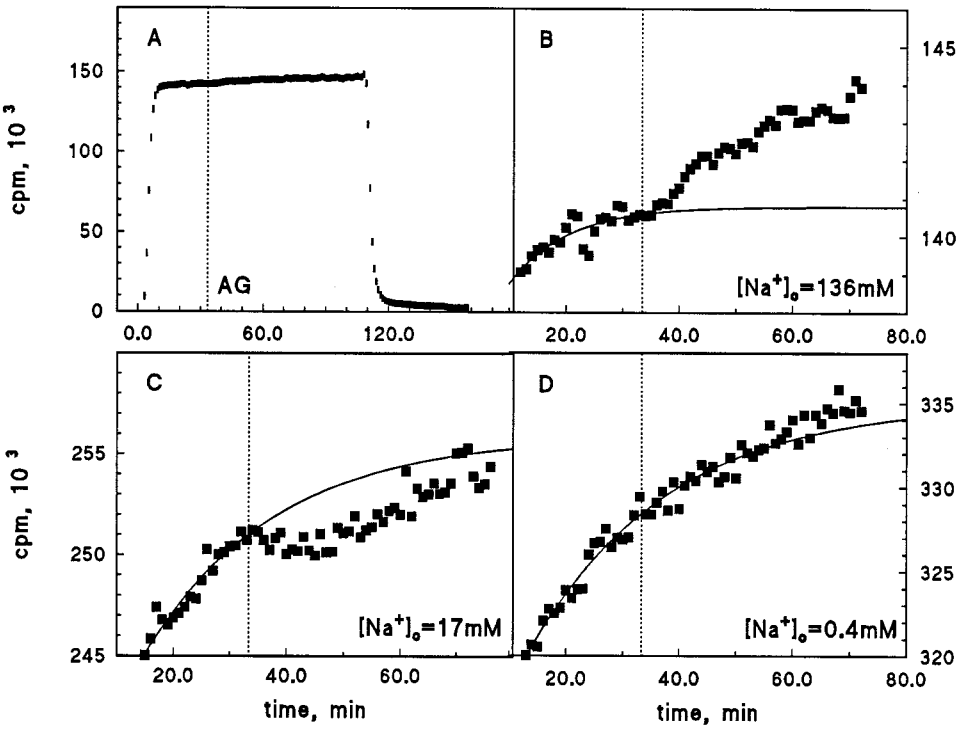

FIGURE 6 $[Na^+]_o$ dependence of the change in intracellular $^{85}Sr^{2+}$ content induced by FcεRI-IgE clustering (the *dotted line* indicates the time of antigen stimulation). (*A*) The time course of total counts from a representative experiment. (*B–D*) Counts near equilibrium at an expanded scale for data sets taken at 136 mM (*B*), 17 mM (*C*), or 0.4 mM (*D*) extracellular Na^+. The solid line extrapolates the $^{85}Sr^{2+}$ uptake of resting cells.

chuelz and Plasman, 1991). This dependence has also been observed for RBL-2H3 cells (Stump et al., 1987). Our measurements showed an increase in net ^{45}Ca uptake with decreasing $[Na^+]_o$ (Fig. 2 *B*), as well as an increase in $[Ca^{2+}]_i$ monitored by indo-1 (Fig. 3 *A*). Subjecting RBL-2H3 cells to the Na^+/H^+ ionophore monensin raised $[Na^+]_i$ (Kieselhorst and Pilatus, 1992) and subsequently also $[Ca^{2+}]_i$ (Stump et al., 1987), indicating that high intracellular sodium may stimulate the Na^+/Ca^{2+} antiporter to export sodium and therefore introduce calcium into the cell. Finally, cell treatment with ouabain in Ca^{2+}-free buffers resulted in an increase in $[Na^+]_i$, which could be reversed by raising $[Ca^{2+}]_o$ (Pilatus et al., 1990).

According to the equation describing the steady state of the Na^+/Ca^{2+} exchange process,

$$[Ca^{2+}]_i/[Ca^{2+}]_o = ([Na^+]_i/[Na^+]_o)^3 \cdot \exp(VF/RT)$$

a decrease in $[Ca^{2+}]_o$ should result in a decrease in $[Ca^{2+}]_i$ or an increase in $[Na^+]_i$. Although a positive correlation between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ was monitored in human neutrophils (Simchowitz and Cragoe, 1988), in resting RBL cells a 0.1–1 mM variation in $[Ca^{2+}]_o$ did not change $[Ca^{2+}]_i$ significantly. Only RBL cells that had been incubated at very low $[Ca^{2+}]_o$ (10 μM) for more than 1 h showed a reduced $[Ca^{2+}]_i$ of 57 nM (Beaven et al., 1984b). Consequently, if Na^+/Ca^{2+} exchange is operating in these cells and is coupling the Ca^{2+} gradients to Na^+ gradients, the intracellular Na^+ should be elevated, as is indeed shown in Fig. 3 *B*. These data also indicate that in resting RBL cells the steady-state concentration of $[Na^+]_i$ depends on $[Ca^{2+}]_o$. The lines were calculated for $[Ca^{2+}]_i$ = 100 nM (*upper*) and 20 nM (*lower*), assuming an exchange stoichiometry of 3 Na^+:1 Ca^{2+} (which has been established for several excitable cells (Eisner and Lederer, 1985; Reeves and Hale, 1984) and for neutrophils (Dale and Simchowitz, 1991; Simchowitz and Cragoe, 1988)). The good agreement between the calculated line for $[Ca^{2+}]_i$ = 100 nM and the experimental data obtained for $[Ca^{2+}]_o$ above 100 μM as well as for the $[Ca^{2+}]$ = 20 nM line for $[Ca^{2+}]_o$ below 100 μM supports the hypothesis that a Na^+/Ca^{2+} exchanger is responsible for coupling the intracellular calcium and sodium concentrations in RBL-2H3 cells.

The above-discussed relation between intra- and extracellular Na^+ and Ca^{2+} concentrations suggests the operation of an efficient coupling mechanism between the two ions in the plasma membrane of RBL-2H3 cells in a way that is specific to the action of a Na^+/Ca^{2+} exchanger. Because antigen-stimulated Ca^{2+} uptake is assumed to be mediated by capacitative Ca^{2+} entry (Fasolato et al., 1993b), the extracellular Na^+ concentration will influence this pathway via the related changes in the intracellular Ca^{2+} concentration. Reducing $[Na^+]_o$ below physiological levels will cause in this model a Ca^{2+} influx by the Na^+/Ca^{2+} exchanger. Increased $[Ca^{2+}]_i$ may prevent efficient depletion of intracellular Ca^{2+} stores, therefore attenuating store-operated Ca^{2+} entry. A decrease in the level of the sustained $[Ca^{2+}]_i$ elevation after immunological stimulation at low $[Na^+]_o$ was indeed observed (Fig. 2 *B*) for $[Na^+]_o >$ 10 mM. While $[Ca^{2+}]_i$ is elevated at low $[Na^+]_o$ above its basal levels under regular conditions (Fig. 4 *A*), it still may

be well below the $[Ca^{2+}]_i$ required for optimal secretory response. Consequently the secretor response will be reduced, as has indeed been observed (Fig. 1 *B*). At very low $[Na^+]_o$, however, the elevated $[Ca^{2+}]_i$ may be sufficient to support secretion, even without an additional Ca^{2+} influx. This may explain why a modest secretory response is observed at 0.4 mM $[Na^+]_o$ (Fig. 1 *B*).

Stump et al. (1987) have proposed that the Na^+/Ca^{2+} exchange may provide an alternative Ca^{2+} uptake pathway that is supporting the sustained elevated $[Ca^{2+}]_i$ after immunological stimulation. The antigen-activated Ca^{2+} entry pathway of RBL cells is known to show a Na^+ permeability in the absence of extracellular Ca^{2+} (Zhang and McCloskey, 1995; Kanner and Metzger, 1984; Mohr and Fewtrell, 1987b). According to more recent findings (Falcone and Fewtrell, 1995), subjecting RBL-2H3 cells to cyclopiazonic acid or thapsigargin caused a depolarization of the cell membrane, even in Ca^{2+}-free buffers. No depolarization, however, was observed in the absence of extracellular sodium, indicating a Na^+ permeability of the store-operated Ca^{2+} pathway. These results differ from those of studies in rat peritoneal mast cells, which expressed a high Ca^{2+} selectivity of a capacitative Ca^{2+} entry (Hoth and Penner, 1992). Therefore, a different or an additional transport pathway appears to be activated by store depletion in RBL-2H3 cells. The Na^+/Ca^{2+} exchanger offers such a possible mechanism, as has been shown by Chernaya et al. (1996). They studied Ca^{2+} and Na^+ fluxes across plasma membranes in transfected Chinese hamster ovary cells expressing the bovine cardiac Na^+/Ca^{2+} exchanger (thus containing both transport systems: store-operated Ca^{2+} channels and a Na^+/Ca^{2+} exchanger). By comparing the Na^+ dependence of store-operated calcium influx in these cells with the above vector-transfected cells, they found that Ca^{2+} release from intracellular stores induces a regulatory activation of Na^+/Ca^{2+} exchange activity. Catalytic activity of elevated $[Ca^{2+}]_i$ had been suggested before (Eisner and Lederer, 1985). A similar result was found in human lymphocytes, where Ca^{2+} entry, which is stimulated by elevated $[Na^+]_i$ and reduced $[Na^+]_o$, is enhanced by thapsigargin (Balasubramanyam et al., 1994). These findings indicate that the turnover rate of the Na^+/Ca^{2+} exchange is accelerated upon depletion of intracellular Ca^{2+} stores. The direction of the Na^+ or Ca^{2+} transport is determined by the ratio of the respective cation concentrations according to the following relations:

$$[Ca^{2+}]_i/[Ca^{2+}]_o > ([Na^+]_i/[Na^+]_o)^3 \cdot \exp(VF/RT)$$

Ca^{2+} efflux

$$[Ca^{2+}]_i/[Ca^{2+}]_o < ([Na^+]_i/[Na^+]_o)^3 \cdot \exp(VF/RT)$$

Ca^{2+} influx

According to these equations, the Na^+/Ca^{2+} exchanger should counteract the store-dependent Ca^{2+} influx, downregulating a temporarily elevated $[Ca^{2+}]_i$. Indeed, the above-mentioned transfected Chinese hamster ovary cells, which expressed the Na^+/Ca^{2+} exchanger, showed attenuation of the transient $[Ca^{2+}]_i$ rise after thapsigargin treatment in buffers containing physiological concentrations of Ca^{2+} and Na^+ (Chernaya et al., 1996). Extrapolating to Ca^{2+}-free buffer, one would expect a net efflux of Ca^{2+} mediated by the exchanger, because the influx through the store dependent pathway will decay. According to the stoichiometry of the Na^+/Ca^{2+} exchange, the resultant Na^+ influx will depolarize the membrane. In the presence of extracellular Ca^{2+} and the absence of extracellular Na^+ ions, Chernaya et al. noted that the thapsigargin-activated Ca^{2+} influx was significantly augmented by an additional Ca^{2+} entry into the cells via the Na^+/Ca^{2+} exchanger (operating in reverse mode) (Chernaya et al., 1996). Their findings clearly indicate that the Na^+/Ca^{2+} exchange can be stimulated by depleting intracellular Ca^{2+} stores. If, according to the conditions described above, the ratios of intra- to extracellular Na^+ and Ca^{2+} favor Ca^{2+} influx, the exchange-mediated Ca^{2+} influx may even exceed Ca^{2+} fluxes via the store-dependent pathway.

The antigen-stimulated Ca^{2+} influx pathway of RBL-2H3 cells was shown to be also permeable for other divalent cations like Mn^{2+} (Falcone and Fewtrell, 1995), Sr^{2+} (Zhang and McCloskey, 1995) (Fig. 6), or Ba^{2+} (Zhang and McCloskey, 1995). In the absence of calcium, Sr^{2+} ions can support antigen-stimulated secretion of 5-hydroxytryptamine (Hide and Beaven, 1991) and β-hexosaminidase (Fig. 5). However, I_{CRAC} is reported to be rather impermeable for these cations (Hoth and Penner, 1992). A second pathway for Ca^{2+} influx in mast cells (called the 50-pS channel; Fasolato et al., 1993a) is characterized as a receptor-operated nonspecific cation channel permeable for Mn^{2+}, but this mechanism cannot link the depletion of intracellular Ca^{2+} stores to the entry of Mn^{2+} cations, as has been observed in RBL cells treated with cyclopiazonic acid (Falcone and Fewtrell, 1995). In contrast, Sr^{2+}, Ba^{2+}, and Mn^{2+} appear to substitute for Ca^{2+} in several systems expressing the Na^+/Ca^{2+} exchanger and with very similar properties (Milanick and Frame, 1991; Trosper and Philipson, 1983). In summary, uptake of other divalent cations upon antigen stimulation or Ca^{2+} store depletion is likely to be mediated by Na^+/Ca^{2+} exchange, but, to test this hypothesis, further studies are required.

Na^+/Ca^{2+} exchange has been discussed as a potential pathway for Ca^{2+} influx being directly related to the response of RBL cells to FcεRI stimulus. Because this process links the uptake of one Ca^{2+} to the export of three Na^+ ions, the resulting net efflux of positive charge would hyperpolarize the membrane. Experimentally, membrane depolarization has been observed in response to the FcεRI stimulus (Sagi-Eisenberg and Pecht, 1983, 1984; Mohr and Fewtrell, 1987b; Labrecque et al., 1989), and membrane depolarization induced by elevated extracellular K^+ or administration of ionophores inhibits the secretory response (Kanner and

Metzger, 1984; Mohr and Fewtrell, 1987a). Thus, an activity of a Na^+/Ca^{2+} exchanger was considered unlikely (Zhang and McCloskey, 1995). However, if Na^+/Ca^{2+} exchange operates together with other transport mechanisms activated by antigen stimulation (like store-operated Ca^{2+} entry or Na^+/Ca^{2+} exchange and a Na^+ channel (as proposed by Stump et al., 1987), the resultant total charge transport across the membrane can cause depolarization, even with the exchanger operating in the Ca^{2+}-importing mode. Repolarization (and sometimes hyperpolarization) (Labrecque et al., 1989), as is observed within 5 min after stimulation, can then be ascribed to a sustained activity of Na^+/Ca^{2+} exchange while the activity of other transport systems is declining. The model proposed by Stump et al., which claims that FcεRI clustering induces a Na^+ uptake, which drives Ca^{2+} entry via Na^+/Ca^{2+} exchange, would especially be supported by our data for the $[Na^+]_o$ dependence of antigen-stimulated Ca^{2+} uptake. This is because Na^+ influx is required to maintain Ca^{2+} uptake, and a decrease in $[Na^+]_o$ will obviously inhibit antigen-stimulated Ca^{2+} uptake. The model is questioned by findings of Zhang and McCloskey, who reported that an increase in $[Na^+]_o$ from 0 to 88 mM did not affect the Ca^{2+} conductance in the presence of 10 mM $[Ca^{2+}]_o$ (Zhang and McCloskey, 1995). However, considering the impact of a nominal absence of extracellular Na^+ in the presence of an efficient Na^+/Ca^{2+} exchange on ion distribution and fluxes, the observed lack of Na^+-induced Ca^{2+} conductance does not necessarily exclude a Ca^{2+} entry via Na^+/Ca^{2+} exchange. As we have shown, resting RBL-2H3 cells exposed to nominally Na^+-free buffer accumulate Ca^{2+} ions (Figs. 2 *B* and 3 *A*). With a rising $[Na^+]_o$ concentration, the elevated $[Ca^{2+}]_i$ level will be reduced. If, at the same time, antigen-stimulated Ca^{2+} entry is also enhanced by the addition of extracellular sodium, the two fluxes may compensate for each other, and no net effect may be detectable.

CONCLUSIONS

The results of our experiments revealed a strong sensitivity of both the FcεRI-induced Ca^{2+} fluxes and secretion to variations in extra- and intracellular Na^+ and Ca^{2+} concentrations. These can be summarized as follows:

1. Decreasing $[Na^+]_o$ in media of resting cells increases $[Ca^{2+}]_i$, whereas decreasing the extracellular Ca^{2+} concentration increases $[Na^+]_i$.

2. A similar secretory response pattern is observed when Ca^{2+} ions are replaced with Sr^{2+}.

3. The secretory response to FcεRI in the presence of $[Ca^{2+}]_o = 2$ mM exhibits a significant minimum at $[Na^+]_o = 17$ mM. In the nominal absence of $[Ca^{2+}]_o$, a decrease in $[Na^+]_o$ causes an increased secretory response.

4. At $[Na^+]_o = 140$ mM the secretory response exhibits a sigmoidal dependence on $[Ca^{2+}]_o$, whereas in the absence of $[Na^+]_o$, secretion becomes independent of $[Ca^{2+}]_o$.

5. Inhibitors of the Na^+/Ca^{2+} exchange reduce the secretory response.

The results suggest the involvement of a Na^+/Ca^{2+} exchanger in supporting and modulating intracellular Ca^{2+} concentrations which couple the FcεRI stimulus to mediator secretion. The store-dependent Ca^{2+} influx pathways are probably affected by $[Ca^{2+}]_i$ via coupling of the Na^+ gradients to Ca^{2+} gradients. There is, in addition, evidence that the FcεRI-induced increase in $[Ca^{2+}]_i$ is enhanced by Ca^{2+} fluxes mediated by the Na^+/Ca^{2+} exchanger itself.

APPENDIX

The concentration of free calcium ions in buffer containing $DyCl_3$ and Na_5PPP was determined as follows. Regarding the equilibria

$$Dy(PPP)_2^{7-} \Leftrightarrow Dy(PPP)^{2-} + (PPP)^{5-}$$

$$(PPP)^{5-} + Ca^{2+} \Leftrightarrow Ca(PPP)^{3-}$$

the concentration of free calcium may be expressed according to the mass action equation,

$$[Ca^{2+}] = \frac{[Ca(PPP)^{3-}][Dy(PPP)^{2-}]}{[Dy(PPP)_2^{7-}]} \cdot K \qquad (1)$$

with

$$K = \frac{K_1}{K_2} \cdot \frac{f_{Ca(PPP)^{3-}} \cdot f_{Dy(PPP)^{2-}}}{f_{Dy(PPP)_2^{7-}}}$$

where K_1 and K_2 are the respective dissociation constants of the first and second reactions and f_i refers to the activity coefficients of the indicated reagents.
As

$$[Dy(PPP)^{2-}] + [Dy(PPP)_2^{7-}] = [Dy]_{tot}$$

and

$$\lfloor Ca^{2+} \rfloor + \lfloor Ca(PPP)^{3-} \rfloor = [Ca]_{tot}$$

Eq. 1 may be transformed into

$$[Ca^{2+}] = K \cdot \frac{([Ca]_{tot} - \lfloor Ca^{2+} \rfloor) \cdot ([Dy]_{tot} - \lfloor Dy(PPP)_2^{7-} \rfloor)}{[Dy(PPP)_2^{7-}]}$$

As the dissociation of $Dy(PPP)_2^{7-}$ is solely caused by the formation of $Ca(PPP)^{3-}$,

$$[Dy]_{tot} - [Dy(PPP)_2^{7-}] = [Ca]_{tot} - [Ca^{2+}]$$

Substituting

$$q = \frac{[Dy(PPP)_2^{7-}]}{[Dy]_{tot}}$$

$[Ca^{2+}]$ may be expressed by

$$[Ca^{2+}] = K \cdot [Dy]_{tot} \cdot \frac{(1-q)^2}{q} \qquad (2)$$

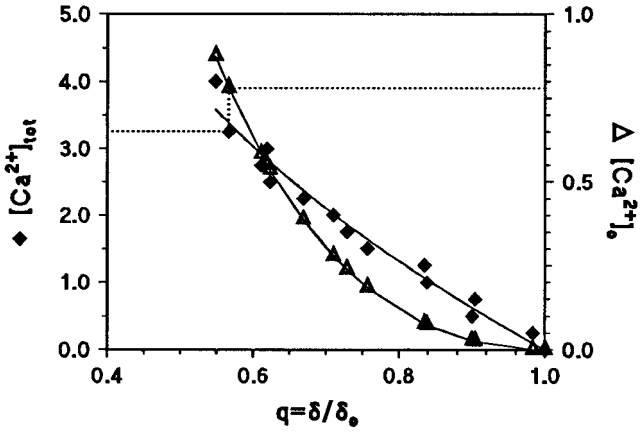

FIGURE 7 Measured resonance shifts at varying total calcium concentrations.

and the total concentration of calcium in the buffer as

$$[Ca^{2+}]_{tot} = [Dy]_{tot} \cdot (1 - q) \cdot \left(1 + K \cdot \frac{(1 - q)}{q}\right) \quad (3)$$

At constant sodium concentration the resonance shift is proportional to the concentration of the $Dy(PPP)_2^{7-}$ complex as long as [shift reagent]:$[Na^+] < 0.1$, and buffer containing x mM calcium is related to the dysprosium concentrations of Gupta and Gupta (1982) and Chu et al. (1984). Therefore the ratio of the resonance shifts in calcium-free buffer is

$$\frac{\delta(x \cdot \text{mM} \cdot Ca_{tot})}{\delta(Ca_{tot} = 0)} = \frac{[Dy(PPP)_2^{7-}]}{[Dy]_{tot}} = q$$

Fig. 7 depicts the measured resonance shifts at varying total calcium concentrations (*diamonds*). Fitting the data with Eq. 3, we obtained a value $K = 0.39 \pm 0.04$. Inserting K in Eq. 2, we calculated the concentration of free calcium in the buffer. The results are shown in Fig. 7 as well (*triangles*). The relative deviation of the calculated free calcium concentration is 13%.

Research reported in this paper has been supported by a Grant from The Israel National Science Foundation.

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