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A ROLE FOR Na/Ca EXCHANGE IN THE PANCREATIC B CELL

STUDIES WITH THAPSIGARGIN AND CAFFEINE

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Abstract—Sodium/calcium (Na/Ca) exchange is thought to play a role in Ca^{2+} extrusion from the pancreatic B cell. The aim of the present study was to provide direct evidence for such a role. The effect of extracellular Na^+ (Na_0^+) removal on cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in single pancreatic B cells was examined using fura 2 and dual wavelength microfluorimetry. Isosmotical replacement of Na_0^+ by sucrose increased $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} but failed to affect $[Ca^{2+}]_i$ in the absence of the divalent cation. Thapsigargin (1 μM), an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, induced a transient increase in $[Ca^{2+}]_i$ in the presence of Na_0^+. This increase was enhanced and more sustained in the absence of Na_0^+. In the absence of Na_0^+ and the presence of thapsigargin, reintroduction of Na_0^+ induced a rapid decrease in $[Ca^{2+}]_i$. A similar picture was observed when caffeine (10 mM) was used to release Ca^{2+} from the endoplasmic reticulum. The decrease in $[Ca^{2+}]_i$ induced by Na_0^+ reintroduction was accompanied by an important increase in ^{45}Ca outflow from perifused islets. In conclusion, this study provides direct evidence that Na/Ca exchange may regulate B cell $[Ca^{2+}]_i$ within physiological range.

In several types of cells, Na/Ca exchange is thought to represent an important mechanism for Ca^{2+} extrusion to the extracellular milieu [1–3]. Because in excitable cells the process is electrogenic and sensitive to membrane potential [4–5], it may reverse and also drive Ca^{2+} inflow during electrical activity [3]. In the pancreatic B cell, the existence of a Na/Ca exchange has been postulated for many years [6–8]. Moreover, based on indirect evidences, it has been proposed that the process of Na/Ca exchange could participate in Ca^{2+} extrusion from the B cell [6–9]. However, direct evidence for such a role is lacking.

The aim of the present study was to provide direct evidence for a modulatory role of Na/Ca exchange on $[Ca^{2+}]_i$ in the pancreatic B cell. For such a purpose, the effect of extracellular Na^+ removal on $[Ca^{2+}]_i$ in single rat pancreatic B cells was examined using fura 2 and dual wavelength microfluorimetry.

MATERIALS AND METHODS

Fluorescence measurement of single cells. The method used to isolate islet cells from rat pancreas has been described elsewhere [10]. The preparation has a cell viability of about 97% and responds satisfactorily to various insulin secretagogues [10, 11]. The cells were placed on glass coverslips and maintained in tissue culture for 48–72 hr before use, as described previously [12]. The cells were then incubated with Fura 2 AM (final concentration: 4 μM) for 1 hr and, after washing, the coverslips with the cells were mounted as the bottom of an open chamber placed on the stage of the microscope. Fura 2 fluorescence of single loaded cells was measured using dual excitation microfluorimetry with a Spex photometric system (Optilas, Alphen aan den Rijn, Holland). The system was coupled to an inverted fluorescence microscope (Diaphot TDM, Nikon, Tokyo, Japan) equipped with Fluor objectives (CF 40× and CF 100×, Nikon) for epi-fluorescence. The excitation wavelengths (340 and 380 nm) were alternated at a frequency of 1 Hz, the length of time for data collection at each wavelength being 0.05 sec. The emission wavelength was 510 nm. $[Ca^{2+}]_i$ was calculated from the ratios of the 340 and 380 nm signals after background subtraction using the equation:

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_f2}{S_b2}$$

where K_d is the dissociation constant for the Fura 2–Ca^{2+} complex (224 nM at 37°), R is the ratio of fluorescence at 340 over 380 nm, S_f2 is the fluorescence of free dye measured at 380 nm and S_b2 is the fluorescence of bound dye measured at 380 nm. R_{max}, R_{min} and S_f2/S_b2 were determined in separate experiments by recording Fura 2 fluorescence in the absence of extracellular Ca^{2+} or in the presence of a saturating Ca^{2+} concentration [12]. The open chamber (1 mL) was thermostated at 37° and perfused at a rate of 2 mL/min. In most cases, the stimuli were applied to the cells using the perfusing system. In the case of drugs available in very small amounts (thapsigargin), the compound was injected

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directly into the chamber as a small aliquot and the perfusion stopped. The drug was removed from the chamber by switching on the perfusion system. The period of equilibration before starting fluorescence measurements was 15 min.

^{45}Ca *outflow from perifused islets.* The method used to measure ^{45}Ca outflow from perifused islets has been described previously [7]. Briefly, groups of 100 islets were incubated for 60 min in the presence of 16.7 mM glucose and ^{45}Ca (0.02–0.04 mM, 100 μCi/mL). After incubation, the islets were washed three times and then placed in a perifusion chamber. The efflux of ^{45}Ca (cpm/min) was expressed as a fractional outflow rate (% lost per min).

The medium used to perfuse the cells and the islets was a Krebs–Ringer bicarbonate buffered solution having the following composition (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24. The solution was equilibrated against a mixture of O_2 (95%) and CO_2 (5%) and for the isolated cells also contained HEPES-NaOH (10 mM, pH 7.4). Some media contained no Ca^{2+} and were enriched with 0.5 mM EGTA. In some experiments, NaCl was isosmotically replaced by sucrose (200 mM) and $NaHCO_3$ by choline bicarbonate. The latter media also contained atropine 3.6 μM [7] to avoid cholinergic effects. In some other experiments, NaCl and $NaHCO_3$ were isosomotically replaced by sucrose (241 mM) or by choline chloride (139 mM) and HEPES-NaOH (10 mM) was replaced by HEPES-KOH (10 mM). The media also contained, when required, thapsigargin (Gibco BRL, Gent, Belgium) or caffeine (UCB, Leuven, Belgium). The media contained no glucose except for the experiments carried out in the presence of caffeine that contained 2.8 mM glucose.

The statistical significance of differences between mean data was assessed by using the Student's *t*-test. The area under the concentration–time curve (AUC) was measured using the trapezoïdal rule.

RESULTS AND DISCUSSION

As a first step, we have examined the effect of extracellular Na^+ (Na_0^+) removal on $[Ca^{2+}]_i$ of B cells perfused in the presence of extracellular Ca^{2+}. Isosmotical replacement of Na_0^+ by sucrose increased $[Ca^{2+}]_i$ (Fig. 1), the increase being variable from cell to cell. In a series of 45 cells, 24 (53%) displayed a biphasic increase in $[Ca^{2+}]_i$ (Fig. 1a), consisting of an initial phase followed by a plateau phase. The initial phase lasted 30–60 sec. Nine cells (20%) displayed a square wave increase in $[Ca^{2+}]_i$ (Fig. 1b), while 12 (27%) displayed a modest increase in $[Ca^{2+}]_i$ onto which distinct spikes were superimposed (Fig. 1c). In each case, the effect of Na_0^+ removal was rapidly reversible. Similar effects of Na_0^+ removal were observed whether in the presence or absence of bicarbonate, or whether Na^+ was replaced by sucrose or choline. When the same experiments were carried out in the absence of extracellular Ca^{2+}, Na_0^+ removal failed to affect $[Ca^{2+}]_i$ (Fig. 1d).

These data provide direct evidence that activation of reverse Na/Ca exchange by changing the Na^+ gradient may increase $[Ca^{2+}]_i$ in the B cell. Indeed, Na_0^+ removal has been shown to increase ^{45}Ca uptake in islet cells by reverse Na/Ca exchange [11]. The Ca^{2+} response was heterogeneous like that to many other secretagogues [12]. Incidentally, it cannot be excluded that the heterogeneity of the responses reflects in part the responses of different types of islet cells instead of B cells, although much care was taken to select only B cells according to their size [12]. The Ca^{2+} responses were also biphasic in the majority of cases. In Ca^{2+} uptake experiments, an initial fast component lasting 30–60 sec followed by a slower increase in Ca^{2+} inflow is also observed when Na_0^+ is removed [11]. That the increase in $[Ca^{2+}]_i$ resulted from a rise in Ca^{2+} inflow (by reverse Na/Ca exchange) was confirmed by the disappearance of the increase in the absence of extracellular Ca^{2+}. The latter observation also suggests that at a low $[Ca^{2+}]_i$, as induced by a prolonged exposure (±20 min) to a Ca^{2+}-depleted medium, Na/Ca exchange does not play a major role in Ca^{2+} extrusion from the B cell. Thus, if Na/Ca exchange played a significant role in the control of $[Ca^{2+}]_i$ below basal level (about 100 nM), the removal of Na_0^+ in the absence of $Ca^{2+}{}_0$ would be expected to increase $[Ca^{2+}]_i$ by inhibiting (forward) Na/Ca exchange. In the absence of $Ca^{2+}{}_0$, basal $[Ca^{2+}]_i$ averaged 65 ± 11 nM (N = 22) compared to 120 ± 10 nM (N = 58) in the presence of extracellular Ca^{2+}. This observation is in agreement with the low affinity characteristics of the Na/Ca exchanger for Ca^{2+} in many type of cells [1, 13].

In the next series of experiments, we have examined to what extent the presence of Na_0^+ could help to reduce $[Ca^{2+}]_i$. This was examined under raised $[Ca^{2+}]_i$. To raise $[Ca^{2+}]_i$, we used two drugs known to interfere with the Ca^{2+} uptake and release mechanisms in the endoplasmic reticulum: thapsigargin and caffeine. Thapsigargin in a specific blocker of the Ca^{2+}-ATPase of the endoplasmic reticulum [14]. Caffeine sensitizes the Ca^{2+}-induced Ca^{2+} release channels of the endoplasmic reticulum to resting Ca^{2+} levels [15]. In the presence of $Na^+{}_0$ (139 mM), thapsigargin (1 μM) induced a rapid but short-lived increase in $[Ca^{2+}]_i$, the increase being maximal during the first 3 min of exposure to thapsigargin (Fig. 2, upper panel). When the B cells were perfused in the absence of Na_0^+ from 20 min, basal $[Ca^{2+}]_i$ was not elevated compared to controls (Fig. 2, lower panel, $P > 0.05$). This was not unexpected since with increasing time of exposure to low Na^+ medium, a fall in the intracellular Na^+ concentration occurs that reduces the Na^+ gradient and hence Ca^{2+} entry into the cell by reverse Na/Ca exchange [11]. In the absence of Na_0^+, thapsigargin also increased $[Ca^{2+}]_i$ but the effect was more sustained (Fig. 2, lower panel) than in the presence of Na_0^+. Indeed, the increase in $[Ca^{2+}]_i$, as evaluated by measurement of the area under the curve, while not being affected during the first 3 min of exposure to thapsigargin ($P > 0.01$) was increased by 154 ± 46% (N = 7) and 127 ± 35% (N = 5) during the first 6 or 9 min, respectively ($P < 0.02$ and $P < 0.04$).

In further experiments, we examined the effect of a change in $[Na^+]_0$ on $[Ca^{2+}]_i$ of a cell exposed to thapsigargin. Figure 3 (lower panel) shows that normalizing the $[Na^+]_0$ induced a rapid drop of

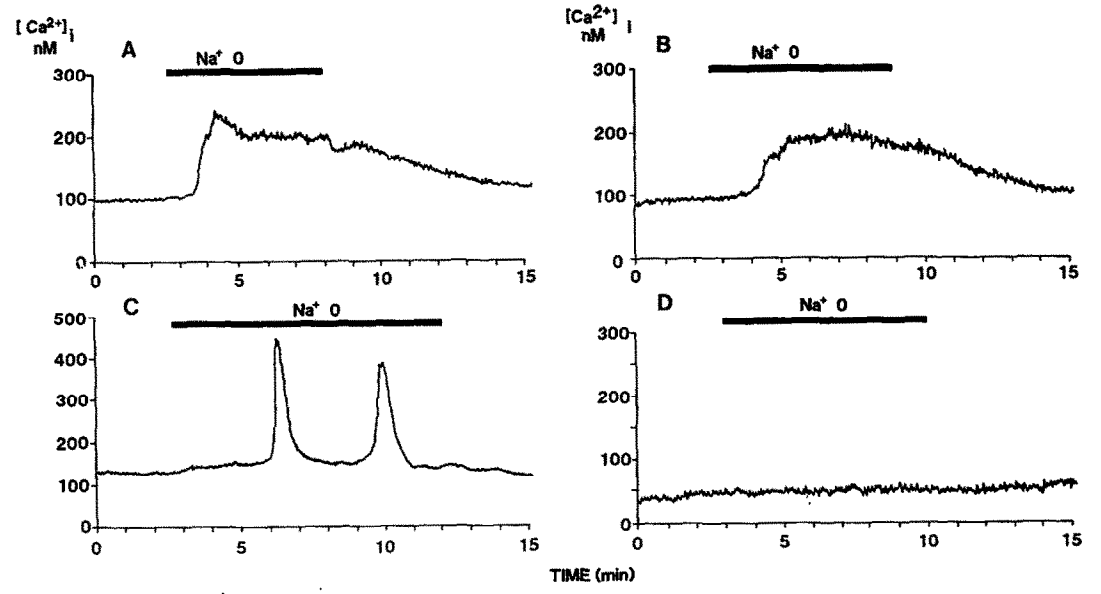

Fig. 1. Effect of Na_0^+ removal on $[Ca^{2+}]_i$ in single B cells. The cells were perfused in the presence (A, B, C) or the absence of extracellular Ca^{2+} (D). The panels A–C represent different typical patterns of $[Ca^{2+}]_i$ changes. The bar indicate the period of exposure to the absence of Na_0^+. The traces are representative of 24 (A), 9 (B), 12 (C) and 14 (D) cells.

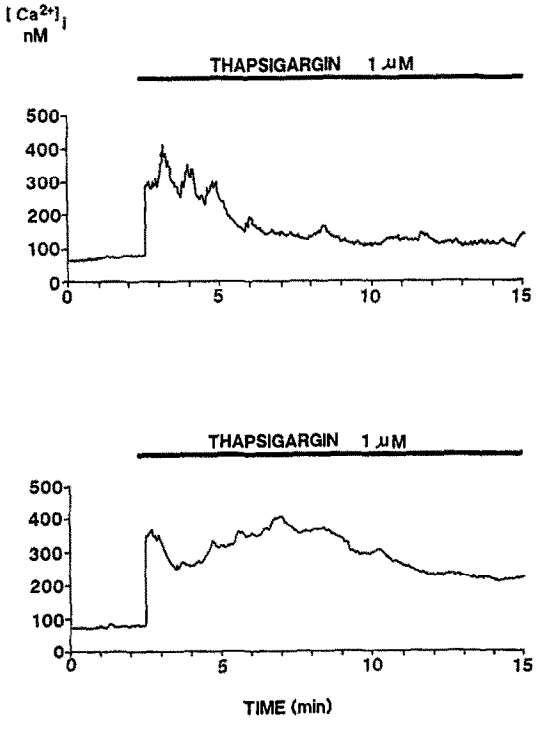

Fig. 2. Effect of thapsigargin (1 μM) on $[Ca^{2+}]_i$ in single B cells. The cells were perfused either in the presence (upper panel) or the absence (lower panel) of Na_0^+. The bar indicates the period of exposure to thapsigargin. The traces are representative of seven (upper panel) and five (lower panel) individual experiments.

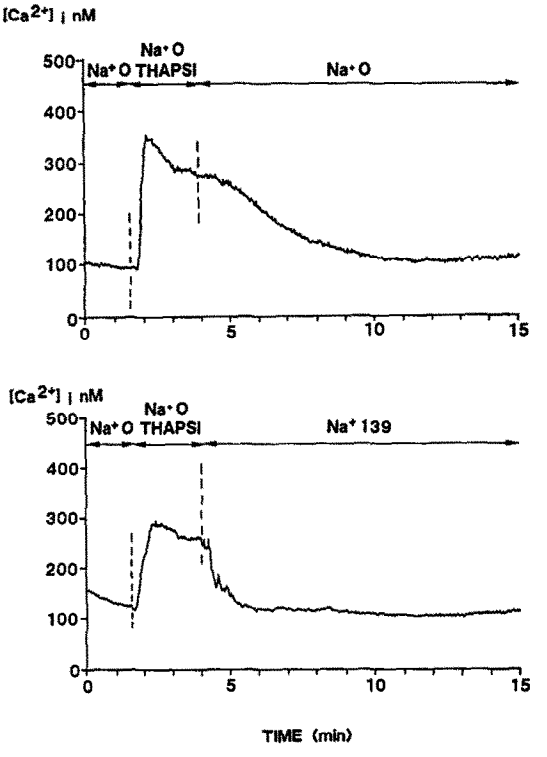

Fig. 3. Effect of thapsigargtin (thapsi) on $[Ca^{2+}]_i$ in single B cells perfused in the absence of Na_0^+. In the lower panel, Na^+ (139 mM) was reintroduced at the time indicated. The traces are representative of 16 (upper panel) and 21 (lower panel) individual experiments.

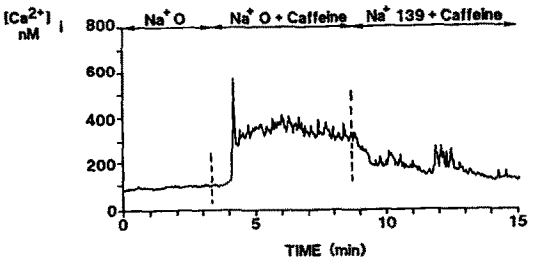

Fig. 4. Effect of caffeine (10 mM) on $[Ca^{2+}]_i$ in single B cells perfused in the absence of Na_0^+. Na^+ (139 mM) was reintroduced at the time indicated. Basal media contained 2.8 mM glucose. The trace is representative of 19 individual experiments.

very fast and short-lived spikes on an elevated Ca^{2+} level (data not shown). In the absence of Na_0^+, caffeine most frequently caused a biphasic increase in $[Ca^{2+}]_i$ with a short initial phase followed by a second plateau phase (Fig. 4). Reintroduction of Na_0^+ during this phase produced an immediate drop in $[Ca^{2+}]_i$. It is interesting to notice that after Na_0^+ reintroduction, caffeine still had a stimulatory effect on $[Ca^{2+}]_i$ in the form of sinusoidal oscillations on a slightly elevated $[Ca^{2+}]_i$ level (Fig. 4).

To ascertain that the changes in $[Ca^{2+}]_i$ seen on Na^+ reintroduction were indeed due to changes in Ca^{2+} outflow, we examined ^{45}Ca outflow from perifused islets exposed to caffeine. In the presence of both extracellular Ca^{2+} and Na^+, caffeine induced a rapid increase in ^{45}Ca outflow from perifused islets (Fig. 5, left panel). This increase was not suppressed in the absence of extracellular Ca^{2+} (Fig. 5, left panel), suggesting that it resulted from the release

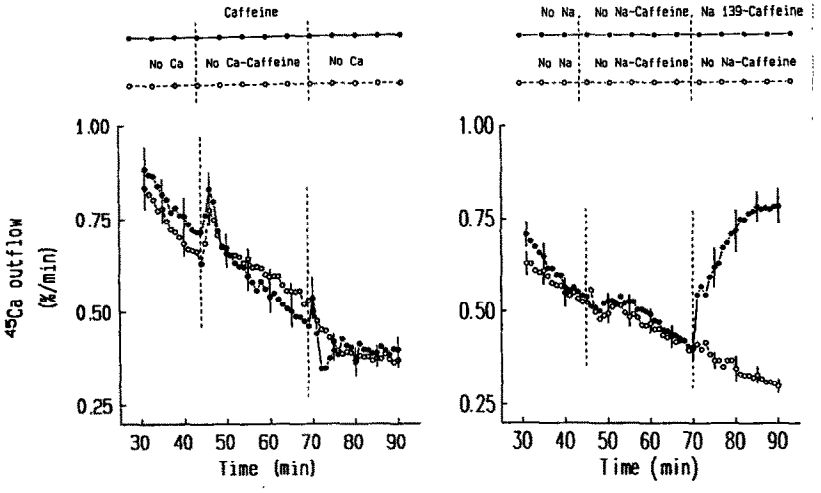

Fig. 5. Effect of caffeine on ^{45}Ca outflow from perifused islets. Left panel: The islets were perifused either in the presence (●) or the absence (○) of $Ca^{2+}{}_0$ (2.56 mM). Right panel: The islets were perifused in the presence of $Ca^{2+}{}_0$ but absence of Na_0^+. The closed circles and solid lines depict experiments in which NaCl was reintroduced in the extracellular milieu at the 70th min of perifusion. Basal media contained 2.8 mM glucose.

$[Ca^{2+}]_i$ in a cell perfused in the absence of Na_0^+ from 20 min and thapsigargin from about 3 min. In control experiments, where the absence of $[Na^+]_0$ was maintained throughout, such a rapid drop was not observed (Fig. 3, upper panel). The regression line characterizing the decrease in $[Ca^{2+}]_i$ before (1.5 min) and after (3 min) thapsigargin removal was examined in two series of cells displaying comparable basal $[Ca^{2+}]_i$ and peak increases in $[Ca^{2+}]_i$ in response to thapsigargin ($P > 0.1$ and 0.6, respectively). When Na^+ was reintroduced at the time of thapsigargin removal, a significant change in the slope was observed ($P < 0.001$). In contrast, no significant change in the slope was observed when the absence of extracellular Na^+ was maintained at the time of thapsigargin removal ($P > 0.4$).

As compared to that of thapsigargin, the effect of caffeine on $[Ca^{2+}]_i$ was more heterogeneous. It was also characterized by the frequent occurrence of of Ca^{2+} from intracellular stores [15]. By contrast, in the presence of Ca^{2+} and the absence of Na_0^+ (Fig. 5, right panel), the increase in ^{45}Ca outflow induced by caffeine was almost completely suppressed. The figure shows that reintroduction of Na^+ into the extracellular milieu induced a marked increase in ^{45}Ca outflow from islets exposed to caffeine as in the case of thapsigargin. These data indicate that in the presence of extracelluar Na^+, the Ca^{2+} released by caffeine from intracellular stores was extruded from the cell presumably by Na/Ca exchange. When such a process was blocked by the absence of extracellular Na^+, higher $[Ca^{2+}]_i$ levels were reached in response to caffeine as in the case of thapsigargin. When the process was reactivated by Na_0^+ readmission, ^{45}Ca outflow was stimulated (Fig. 5 right panel) and $[Ca^{2+}]_i$ dropped (Fig. 4).

The removal of extracellular Na^+ may not solely inhibit Na/Ca exchange but could also interfere with

other cellular processes, e.g. Na/H exchange, Na^+,K^+-ATPase, membrane potential. . . However, alterations in the latter processes do not appear to be responsible for the observed changes in $[Ca^{2+}]_i$ and ^{45}Ca outflow.

Indeed, at low glucose concentrations ($\leq$2.8 mM), extracellular Na^+ removal only barely affects membrane potential [16]. Na_0^+ removal could inhibit Na^+,K^+-ATPase, but this would not lead to Na^+_i accumulation and hence would not affect cellular Ca^{2+} movements [7, 17]. Na_0^+ removal could also inhibit Na/H exchange and result in a decrease in pH_i [18]. This acidification may reduce Ca^{2+} outflow but to a much lower extent than Na_0^+ removal [19]. In addition, the decrease in pH_i reduces ^{45}Ca outflow by inhibiting Na/Ca exchange [19].

Our data provide direct evidence that Na/Ca exchange, working in its Ca^{2+} efflux mode, may regulate the $[Ca^{2+}]_i$ of the pancreatic B cell. Thus, the presence of extracellular Na^+ favoured the decrease in $[Ca^{2+}]_i$ that had been raised by two different mechanisms. Our data strongly indicate that the presence of Na_0^+ probably acted by increasing Ca^{2+} outflow from the cell. This conclusion is strengthened by the observation that the absence of Na_0^+ affected the late instead of the immediate effects of thapsigargin on $[Ca^{2+}]_i$. In other words, the absence of Na_0^+ did not affect the action of the drug (blockade of Ca^{2+} uptake by the endoplasmic reticulum). Rather, it interfered with a cellular homoeostatic mechanism allowing the B cell to recover from a cytosolic Ca^{2+} load. Our data are in agreement with recent patchclamp experiments combined with microfluorimetric recordings in mouse pancreatic B cells showing that Na^+ removal produced both an elevation of resting $[Ca^{2+}]_i$ and an increased amplitude of depolarization-evoked $[Ca^{2+}]_i$ transients [20].

In conclusion, the B cell Na/Ca exchange appears as a Ca pumping system designed to extrude Ca^{2+} when its intracellular concentration is raised above basal levels, as in many other types of cell. Further work is required to determine the exact kinetic constants of the exchange and the possible participation of the process in Ca^{2+} uptake mechanisms.

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