Contribution of $\text{Na}^+/\text{Ca}^{2+}$ Exchanger in Maintaining $[\text{Ca}^{2+}]_c$ at a Stable State in Rat Pancreatic Islets

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Abstract: The effects of lowering extracellular Na^+ concentration, $[\text{Na}^+]_o$, on cytosolic Ca^{2+} concentration, $[\text{Ca}^{2+}]_c$, were examined by a microfluorimetric method using fura-2 in perfused preparations of isolated rat pancreatic islets. The total replacement of extracellular Na^+ (Na^+_o) by equimolar *N*-methyl-D(-)-glucamine caused a rapid rise in $[\text{Ca}^{2+}]_c$, and partial replacement of Na^+_o resulted in correlative rises in $[\text{Ca}^{2+}]_c$ in accordance with the magnitude of reduced $[\text{Na}^+]_o$. The rise in $[\text{Ca}^{2+}]_c$ induced by Na^+_o removal was strongly inhibited in the Ca^{2+}_o -deficient environment or by Ni^{2+} . The $[\text{Ca}^{2+}]_c$ rise, however, remained almost unchanged in the presence of

nifedipine or SK&F 96365, and was enhanced by the addition of ouabain. The electrochemical gradients for Ca^{2+} ($\Delta\mu\text{Ca}^{2+}$) and Na^+ ($\Delta\mu\text{Na}^+$) were calculated to be 39.08 and 12.8 kJ/mol, respectively, in this study, indicating a stoichiometry of $3\text{Na}^+ : 1\text{Ca}^{2+}$. These results indicate that, in rat pancreatic islets, the rise in $[\text{Ca}^{2+}]_c$ induced by lowering $[\text{Na}^+]_o$ is mainly due to Ca^{2+} entry mediated by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger operating with the stoichiometry of $3\text{Na}^+ : 1\text{Ca}^{2+}$, and that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger plays an important role in maintaining stable-state $[\text{Ca}^{2+}]_c$. [Japanese Journal of Physiology, 46, 473–480, 1996]

Key words: cytosolic Ca^{2+} concentration, islets of Langerhans, fura-2, microfluorimetry.

It has been well established that an increase in cytosolic Ca^{2+} concentration, $[\text{Ca}^{2+}]_c$, plays a signaling role in the stimulus-secretion coupling of pancreatic B-cells [1, 2]. The coupling consists of the six following steps. An increase in extracellular D-glucose results in: 1) an increase in the intracellular ATP level, 2) blockade of ATP-sensitive K^+ channels, 3) depolarization of the pancreatic B-cells, 4) activation of voltage-dependent Ca^{2+} channels located in the plasma membrane, 5) Ca^{2+} entry, and 6) Ca^{2+} -triggered insulin release.

The mechanism of $[\text{Ca}^{2+}]_c$ increase in a wide variety of secretory cells has been the focus of extensive investigations for many years, whereas the mechanism involved in the recovery process of increased $[\text{Ca}^{2+}]_c$ to the stable-state level after cessation of the stimuli has been studied to a lesser extent despite the fact that the recovery of $[\text{Ca}^{2+}]_c$ from an agonist-induced ele-

vation to a resting level is of vital importance in maintaining normal cell functions.

Like most other cells, the pancreatic B-cell is thought to be equipped with a dual system responsible for Ca^{2+} extrusion: Ca^{2+} -ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger [3]. It has been shown that the removal of extracellular sodium (Na^+_o) increases $^{45}\text{Ca}^{2+}$ uptake by pancreatic islets [4, 5], decreases $^{45}\text{Ca}^{2+}$ outflow from pancreatic islets [6, 7], and increases $[\text{Ca}^{2+}]_c$ measured using fura-2 and dual wavelength microfluorimetry [8, 9]. From these results, the authors proposed that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger plays an important role in the regulation of $[\text{Ca}^{2+}]_c$ in pancreatic islet cells. However, this view is based on qualitative data and, therefore, a quantitative analysis of the $\text{Na}^+/\text{Ca}^{2+}$ exchange activity in pancreatic islet cells at a stable-state remains to be made.

The aim of this study was to elucidate a possible

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contribution of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in maintaining the stable-state $[\text{Ca}^{2+}]_c$ level in rat pancreatic islets by recording changes in $[\text{Ca}^{2+}]_c$ through the replacement of extracellular Na^+ with equimolar *N*-methyl-D(-)-glucamine, a non-transportable substrate for the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, and to provide further insights into the properties of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger of pancreatic islet cells.

MATERIALS AND METHODS

Materials. Materials were purchased from sources as described in the following: adult male Sprague-Dawley rats (10–12 weeks old, 300–420 g in weight) from Nihon S.L.C. (Hamamatsu, Japan); RPMI 1640 and penicillin–streptomycin from GIBCO Laboratories (Grand Island, NY, USA); fetal bovine serum (FBS) from Irvine Scientific (Santa Ana, CA, USA); bovine serum albumin (BSA; Fraction V) from Sigma Chemical (St. Louis, MO, USA); collagenase Type IV from Worthington Biochemicals (Freehold, NJ, USA); ouabain, nifedipine and *N*-methyl-D(-)-glucamine (NMDG) from Wako Pure Chemical Industries (Osaka, Japan); and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), fura-2 acetomethoxymethyl ester (fura-2-AM), and *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA) from Dojindo Laboratories (Kumamoto, Japan).

Isolation and primary culturing of pancreatic islets. Rats were exsanguinated under ether anesthesia. The pancreas was excised, and the pancreatic islets were isolated by the collagenase digestion procedure detailed by Lacy and Kostianovsky [10] with a slight modification. HEPES-buffered Krebs–Henseleit's bicarbonate solution (KHB: HEPES, 10 mM; NaCl, 135 mM; KCl, 5 mM; CaCl_2 , 2.5 mM; MgCl_2 , 1 mM; NaHCO_3 , 5 mM; NaH_2PO_4 , 1 mM; glucose, 3 mM; BSA, 0.5%; pH 7.4) (15 ml) containing purified collagenase (160–300 U/ml) was injected into the common bile duct, and the pancreas was incubated at 37°C for 45 min with continuous shaking (120 cycles/min). After incubation, the pancreatic tissue was triturated several times with a polypropylene pipette (1-mm tip diameter). The mixture was diluted with 10 ml of enzyme-free KHB, centrifuged for 30 s at 500 rpm, and the supernatant discarded. The sediment containing islets was washed three times with KHB. The sediment was resuspended in 10 ml KHB, and 1–2 ml of the suspension was placed in a culture dish (100 mm in diameter). Islets were selected under a stereomicroscope to exclude any contaminating tissues and placed in another culture dish with a micropipette. Isolated islets were cultured 18–42 h under

an atmosphere of 95% air and 5% CO_2 at 37°C in RPMI 1640 tissue-culture medium containing 10% FBS, 1% penicillin, and 1% streptomycin.

Measurement of $[\text{Ca}^{2+}]_c$. Isolated islets were washed with KHB (0.1% BSA) and loaded by incubation for 60 min at 37°C with 3 μM fura-2-AM. The islets were then washed with KHB and transferred to a perfusion chamber, provided with a coverslip bottom, which was placed on the stage of an inverted microscope (TMD-2, Nikon, Tokyo, Japan). Perfusion was performed using a peristaltic pump, and the volume of the perfusion solution was held constant (about 100 μl) by adding and withdrawing the solution at a rate of 1 ml/min. The temperature of the perfusion solution was controlled precisely by circulating water around the perfusion chamber. Fura-2 fluorescence was measured using a P1 system (Nikon). In brief, light emitted from a 75-W xenon lamp was introduced into an inverted microscope after passing through excitation filters (380 and 340 nm, with a 10-nm bandwidth). The fluorescence emitted by the islets was passed through a 510-nm interference filter with a 10 nm bandwidth and detected by a photomultiplier. The fluorescence intensities at 340 nm (F_{340}) and 380 nm (F_{380}) were recorded and displayed on a monitor screen in the ratio F_{340} to F_{380} (R), and stored on a floppy disk. $[\text{Ca}^{2+}]_c$ was calculated from R using the following equation [11]:

$$[\text{Ca}^{2+}]_c = K_d \times (R - R_{\min}) / (R_{\max} - R) \times \beta,$$

where: K_d is the dissociation constant for the fura-2 binding to Ca^{2+} and assumed to be 224 nM [11]; $R_{\max}$ is the ratio when all the fura-2 is bound to Ca^{2+} ; $R_{\min}$ is the ratio when all the fura-2 is in the free-acid form; and β is the ratio of the fluorescence of free fura-2 divided by the fluorescence of Ca^{2+} -bound fura-2 with 380 nm excitation. In our system, $R_{\max}$, $R_{\min}$, and β were 2.537, 0.190, and 2.81, respectively.

Solutions. The final concentration of Na^+ in the standard solution (KHB) was 146 mM. The Na^+ -free solution (pH 7.4) had the following composition (in mM): 140 NMDG, 5 KHCO_3 , 1 MgCl_2 , 2.5 CaCl_2 , 10 HEPES, and 3 glucose. The Ca^{2+} -free solution (pH 7.4) contained (in mM): 0.1 EGTA, 135 NaCl, 5 KCl, 1 MgCl_2 , 5 NaHCO_3 , 1 NaH_2PO_4 , 10 HEPES, and 3 glucose. The Na^+ -, Ca^{2+} -free solution (pH 7.4) contained (in mM): 0.1 EGTA, 140 NMDG, 5 KHCO_3 , 1 MgCl_2 , 10 HEPES, and 3 glucose.

Statistics. Each calculated value was expressed as the mean $\pm$ SE, and the difference of the means was analyzed by Student's *t*-test.

Fig. 1. Effects of total or partial replacement of Na⁺ by equimolar NMDG on [Ca²⁺]_i in rat pancreatic islets. [Ca²⁺]_i dynamics were recorded continuously before, during, and after total or partial removal of Na⁺ from the perfusion solution. Periods of perfusion with solutions containing different [Na⁺]_o are indicated by the bars at the top. The traces are expressed as the mean (thick line) ± SE (thin line) of 20 (A), 3 (B), 5 (C), 3 (D), 4 (E), and 5 (F) experiments.

RESULTS

Changes in [Ca²⁺]_i induced by lowering [Na⁺]_o

The activity of Na⁺/Ca²⁺ exchanger in rat pancreatic islets was studied by recording the changes in [Ca²⁺]_i after replacement of Na⁺ by equimolar NMDG, a non-transportable substrate for the Na⁺/Ca²⁺ exchanger (Fig. 1). [Ca²⁺]_i rose rapidly from its resting level (130.7 ± 7.1 nM, $n=20$) to reach a peak level (277.2 ± 22.2 nM, $n=20$) within 60 s, followed by an initial sharp decline phase and a second plateau phase within 90 s after the replacement of Na⁺ with NMDG. It returned gradually to its resting level when NMDG was replaced totally with Na⁺ (Fig. 1A). Similar results were obtained when Na⁺ was replaced by equimolar Li⁺ or isotonic sucrose (data not shown).

The changes in [Ca²⁺]_i were quantitatively examined by replacing part of the Na⁺ with equimolar NMDG (Fig. 1B–F). When [Na⁺]_o was lowered to 10 mM, [Ca²⁺]_i rose rapidly to reach a peak level within 60 s, followed by a sharp decline to a plateau level within 90 s after lowering the Na⁺ concentration, and returned to the stable-state level. Similar results were obtained when [Na⁺]_o was lowered to 20 or 50 mM. However, the amplitude of the rise in [Ca²⁺]_i recorded in the environment containing 50 mM Na⁺ was 80% that recorded in a Na⁺-deficient environment. When [Na⁺]_o was lowered to 80 or 100 mM, [Ca²⁺]_i rose slightly to reach a plateau level in the absence of an initial peak phase and decreased toward

Fig. 2. Relation between [Na⁺]_o and the peak value of the rise in [Ca²⁺]_i induced by lowering [Na⁺]_o. Each value represents the mean ± SE of measurements from experiments 3–51 depicted in Fig. 1.

the stable-state level after lowering the Na⁺ concentration.

The peak values of the rise in [Ca²⁺]_i induced by lowering [Na⁺]_o were plotted against the corresponding [Na⁺]_o (Fig. 2). The relation thus obtained indicates that the peak values of [Ca²⁺]_i may directly correlate with [Na⁺]_o.

Effect of extracellular Ca²⁺ removal on the rise in [Ca²⁺]_i induced by Na⁺ removal

To determine the source of the rise in [Ca²⁺]_i, the change in [Ca²⁺]_i was recorded when Ca²⁺ was removed followed by Na⁺ removal from the perfusion

solution. $[Ca^{2+}]_c$ was decreased to 81.3 ± 4.1 nM ($n=4$) by the removal of Ca^{2+}_o . The rise in $[Ca^{2+}]_c$ induced by Na^+_o removal was completely inhibited when Ca^{2+}_o was removed from the perfusion solution and restored when Ca^{2+}_o was reintroduced to the Na^+_o -deficient environment (Fig. 3). This result indicates that the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal can be ascribed to Ca^{2+} entry through the plasma membrane.

Effect of Ni^{2+} on the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal

To further confirm the view that the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal can be ascribed to Ca^{2+} entry, the change in $[Ca^{2+}]_c$ was recorded when Na^+_o was removed from the perfusion solution in the presence of 2 mM Ni^{2+} , which has been reported to inhibit

the entry of Ca^{2+} as well as La^{3+} [5] in various types of cells [12–14]. When Na^+_o was removed in the presence of Ni^{2+} , the abrupt rise in $[Ca^{2+}]_c$ induced by Na^+_o removal was inhibited almost completely. $[Ca^{2+}]_c$ in the presence of Ni^{2+} and the absence of Na^+_o was 141.2 ± 15.0 nM ($n=4$). $[Ca^{2+}]_c$ increased again to 223.6 ± 29.2 nM ($n=4$) when Ni^{2+} was removed from the Na^+_o -deficient environment, but the amplitude of the rise in $[Ca^{2+}]_c$ was smaller than that obtained by Na^+_o removal before adding Ni^{2+} (Fig. 4). Similar results were obtained in three other experiments. The inhibitory effect of Ni^{2+} on the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal was further examined by varying Ni^{2+} concentrations in the range of 0.1–5 mM. The higher the Ni^{2+} concentration used, the smaller the magnitude of the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal obtained (Fig. 5).

Fig. 3. Effect of extracellular Ca^{2+} removal on the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal. Recordings of the $[Ca^{2+}]_c$ dynamics before, during, and after perfusion with Ca^{2+} -free solution, Ca^{2+} -free and Na^+ -free solution, and Na^+ -free solution are indicated by the bars. The traces are expressed as the mean (thick line) $\pm$ SE (thin line) of four individual experiments.

Fig. 4. Effect of Ni^{2+} on the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal. Examples of recordings of $[Ca^{2+}]_c$ dynamics before, during, and after perfusion with Na^+ -free solution (a control before), a solution containing 2 mM Ni^{2+} , Na^+ -free solution containing 2 mM Ni^{2+} , and Na^+ -free solution are indicated by the bars.

Effect of Ca^{2+} channel blockers on the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal

To exclude the possible involvement of Ca^{2+} channels in the pathway of Ca^{2+} entry induced by Na^+_o removal, the change in $[Ca^{2+}]_c$ was recorded when Na^+_o was removed from the perfusion solution in the absence or presence of nifedipine, which is known to inhibit voltage-dependent L-type Ca^{2+} channels [15, 16]; which in turn, inhibits insulin secretion induced by glucose [17], or SK&F 96365, which is known to inhibit receptor-mediated Ca^{2+} channels [18] (data not shown). $[Ca^{2+}]_c$ increased immediately after the removal of Na^+_o even in the presence of 10 μ M nifedipine (296.2 ± 24.5 nM, $n=3$) or 30 μ M SK&F 96365 (286.0 ± 11.7 nM, $n=5$). The amplitude of the rise in

Fig. 5. Relation between $[Ni^{2+}]_o$ and the rise in $[Ca^{2+}]_c$ induced by Na^+_o removal shown as a percent of controls before.

Fig. 6. Effect of ouabain on the rise in $[Ca^{2+}]_i$ induced by Na^+ removal. Recordings of $[Ca^{2+}]_i$ dynamics before, during, and after perfusion with Na^+ -free solution (a control before), a solution containing 3 mM ouabain, and Na^+ -free solution containing 3 mM ouabain are indicated by the bars. The traces are expressed as the mean (thick line) $\pm$ SE (thin line) of five individual experiments. * $p < 0.1$, the amplitude of the rise in $[Ca^{2+}]_i$ induced by Na^+ removal in the ouabain solution vs. the control solution; † $p < 0.05$, $[Ca^{2+}]_i$ in the ouabain solution vs. the control solution.

$[Ca^{2+}]_i$ was not significantly different from that of the controls obtained before the addition of the nifedipine or SK&F 96365.

Effect of ouabain on the rise in $[Ca^{2+}]_i$ induced by Na^+ removal

As the transmembrane electrochemical Na^+ gradient is maintained by Na^+-K^+ ATPase, the gradient would be inhibited by ouabain, which is known to inhibit Na^+-K^+ ATPase. $[Ca^{2+}]_i$ increased significantly (125.6 ± 5.7 nM, $n=5$) ($p < 0.05$) after 15 min after the addition of ouabain alone. $[Ca^{2+}]_i$ increased to 374.8 ± 54.1 nM ($n=5$) when Na^+ was removed in the presence of ouabain. The amplitude of the rise in $[Ca^{2+}]_i$ induced by Na^+ removal was significantly potentiated ($p < 0.1$) in the presence of 3 mM ouabain. When ouabain was removed and Na^+ was simultaneously reintroduced, $[Ca^{2+}]_i$ decreased toward the stable-state level (Fig. 6).

DISCUSSION

Na^+/Ca^{2+} exchanger participation in stable-state $[Ca^{2+}]_i$. The results obtained in this study showed that $[Ca^{2+}]_i$ increased rapidly after lowering $[Na^+]_o$, and that the amplitude of the rise in $[Ca^{2+}]_i$ depended on the degree of $[Na^+]_o$ reduction (Fig. 1). Plotting the peak level of $[Ca^{2+}]_i$ against $[Na^+]_o$ re-

vealed that there may be a reverse relation between these two values (Fig. 2). Increase in $[Ca^{2+}]_i$ in low Na^+ environments gradually diminished after prolonged exposure to low Na^+ solutions. This diminution may possibly be attributed to a Ca^{2+} -ATPase located in the plasma membrane. The Ca^{2+} -ATPase may be activated by the rise in $[Ca^{2+}]_i$ induced by Na^+ removal and, in turn, may decrease the level of $[Ca^{2+}]_i$ to an equilibration. The removal of Na^+ did not induce a rise in $[Ca^{2+}]_i$ in a Ca^{2+} -free environment or in the presence of Ni^{2+} . These results indicate that, in a low Na^+ environment, the Na^+/Ca^{2+} exchanger may operate in a reverse mode as a Ca^{2+} entry pathway. It was reported, in pancreatic B-cells [8], that $[Ca^{2+}]_i$ was increased by thapsigargin, which inhibits Ca^{2+} -ATPase located in Ca^{2+} deposits [19, 20] and depletes the Ca^{2+} deposits [21], in the absence of Na^+ . The rise in $[Ca^{2+}]_i$ induced by Na^+ removal, therefore, may not be due to Ca^{2+} release from intracellular Ca^{2+} deposits but to Ca^{2+} entry from extracellular milieu. The results reported here show that Ca^{2+} channel blockers, nifedipine, and SK&F 96365 did not inhibit the rise in $[Ca^{2+}]_i$ induced by the total replacement of Na^+ , suggesting that the Ca^{2+} entry induced by Na^+ removal was not mediated via Ca^{2+} channels. This view is compatible with a previous finding that $^{45}Ca^{2+}$ uptake induced by Na^+ removal was unaffected by nifedipine in pancreatic islet cells [5]. The observations that ouabain alone increased $[Ca^{2+}]_i$ slightly but significantly ($p < 0.05$) and that the amplitude of the rise in $[Ca^{2+}]_i$ induced by Na^+ removal was enhanced by the preceding application of ouabain agree well with a previous report that ouabain increased the uptake of $^{45}Ca^{2+}$ due to Na^+ removal in pancreatic islet cells [5]. Taken together, a transmembrane electrochemical Na^+ gradient may primarily be presented by the activity of the Na^+-K^+ ATPase. From these results, we conclude that the rise in $[Ca^{2+}]_i$ induced by Na^+ removal may mainly be due to Ca^{2+} influx mediated by the Na^+/Ca^{2+} exchanger, which is directly maintained by the Na^+ gradient.

The level of $[Ca^{2+}]_i$ rapidly increased upon Na^+ removal, and reached a peak level of 277.2 ± 22.2 nM as showed in Fig. 1A. The slope of the rise in $[Ca^{2+}]_i$ induced by Na^+ removal is almost equal to the respective slopes of rises in $[Ca^{2+}]_i$ induced by 15 mM glucose, 30 mM K^+ , or 100 μ M carbamylcholine shown in a previous study [22]. The peak level is similar to both peak levels of the rise in $[Ca^{2+}]_i$ induced by 100 μ M carbamylcholine (239 ± 28 nM, $n=4$) and 2 μ M glibenclamide (278 ± 24 nM, $n=4$) obtained with the same system [22]. These findings suggest that the Na^+/Ca^{2+} exchanger plays an important role in Ca^{2+}

extrusion from pancreatic islet cells, at least in the stable state.

Quantitative analysis of the role of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in maintaining $[\text{Ca}^{2+}]_c$.

To elucidate the quantitative relation between $[\text{Na}^+]_o$ and $[\text{Ca}^{2+}]_c$, we adopted the equation used to analyze the contribution of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in regulating $[\text{Ca}^{2+}]_c$ in kidney cells by Snowdowne and Borle [23], which is based on a theory by Blaustein and Hodgkin [24]. The direction of Ca^{2+} movement depends on the intracellular Na^+ concentration ($[\text{Na}^+]_i$) and $[\text{Ca}^{2+}]_c$ as well as on the cell membrane potential [25], which influences the electrochemical gradient for Ca^{2+} ($\Delta\mu\text{Ca}^{2+}$) and Na^+ ($\Delta\mu\text{Na}^+$) given by the following equations, respectively:

$$\Delta\mu\text{Ca}^{2+} = zF(E_{\text{Ca}^{2+}} - E_m),$$

$$\Delta\mu\text{Na}^+ = zF(E_{\text{Na}^+} - E_m),$$

where z , ionic valence; F , Faraday constant; and E_m , membrane potential. $E_{\text{Ca}^{2+}}$ and E_{Na^+} are the Nernst equilibrium potentials for Na^+ and Ca^{2+} , respectively, given by:

$$E_{\text{Ca}^{2+}} = (RT/zF) \ln(\text{Ca}_o/\text{Ca}_i),$$

$$E_{\text{Na}^+} = (RT/zF) \ln(\text{Na}_o/\text{Na}_i),$$

where R , gas constant; T , the absolute temperature. The $[\text{Na}^+]_i$ of the pancreatic B-cells, reported in a previous study, is 14 ± 1 mM [26]. The resting membrane potential ranges between -56 and -75 mV with a glucose concentration of 3 mM in mouse pancreatic B-cells [27] and averages -68 mV with 2.8 mM glucose in rat pancreatic B-cells [28]. Therefore, we assumed that $[\text{Na}^+]_i$ would be 14 mM and E_m would be -70 mV in this study. The mean value of a stable-state $[\text{Ca}^{2+}]_c$ in this study is 123.1 ± 4.7 nM, and $[\text{Na}^+]_o$ and $[\text{Ca}^{2+}]_o$ are 146 and 2.5 mM, respectively, as described in the MATERIALS AND METHODS section. In this study, $\Delta\mu\text{Ca}^{2+}$ and $\Delta\mu\text{Na}^+$ are thus calculated to be 39.08 and 12.80 kJ/mol, respectively. The driving force for Ca^{2+} influx ($\Delta\mu\text{Ca}^{2+}$), possibly reflects the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger while, on the other hand, the driving force for Na^+ influx ($\Delta\mu\text{Na}^+$) possibly reflects the forward mode of the exchanger. Since $[\text{Ca}^{2+}]_c$ remained in a stable state under normal physiological conditions, the net flux of Ca^{2+} may have been zero. If the transmembrane Ca^{2+} movement is operated only by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, the counter-movements of $\Delta\mu\text{Ca}^{2+}$ and $\Delta\mu\text{Na}^+$ may possibly balance each other: the ratio of the exchanger in reverse and forward modes should be in equilibrium. Thus, if the coupling ratio of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is $n\text{Na}^+ : m\text{Ca}^{2+}$, then

$$n\Delta\mu\text{Na}^+ = m\Delta\mu\text{Ca}^{2+},$$

substituting the values mentioned above into this equation:

$$12.80n = 39.08m,$$

$$n/m = 39.08/12.80 = 3.05.$$

The stoichiometry of $3\text{Na}^+ : 1\text{Ca}^{2+}$ thus obtained corresponded exactly to the ratio previously described in other cells [23, 29–31]. In glucose-responsive insulinoma cells, Hoenig *et al.* observed that Ca^{2+} efflux was dependent on $[\text{Na}^+]_o$, with a Hill coefficient of close to 3, and suggested the stoichiometry of $3\text{Na}^+ : 1\text{Ca}^{2+}$ [32]. In normal pancreatic islet cells, however, the stoichiometry of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger has not yet been elucidated. Therefore, the stoichiometry was calculated on the basis of thermodynamics in this study. The net driving force for Ca^{2+} influx on the $\text{Na}^+/\text{Ca}^{2+}$ exchanger can be expressed as $\Delta\mu\text{Ca}^{2+} - 3\Delta\mu\text{Na}^+$. The relation between $[\text{Na}^+]_o$ and the net driving force for Ca^{2+} influx ($\Delta\mu\text{Ca}^{2+} - 3\Delta\mu\text{Na}^+$) is shown in Fig. 7. If $\Delta\mu\text{Ca}^{2+}$ is less than $3\Delta\mu\text{Na}^+$, the ratio of the forward mode may increase, possibly causing Ca^{2+} to be driven out of the cell by the entry of Na^+ . If $\Delta\mu\text{Ca}^{2+}$ is larger than $3\Delta\mu\text{Na}^+$, the ratio of the reverse mode may increase, possibly causing Ca^{2+} to move into the cell in exchange for Na^+ . Thus, the peak value of $[\text{Ca}^{2+}]_c$ induced by Na^+ removal should be directly proportional to the Ca^{2+} entry through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and, therefore, directly proportional to its driving force. Figure 8 shows that there may be a linear relation between the peak value of $[\text{Ca}^{2+}]_c$ measured at various $[\text{Na}^+]_o$ in our experiments and the net driving force ($\Delta\mu\text{Ca}^{2+} - 3\Delta\mu\text{Na}^+$) calculated for the appropriate $[\text{Na}^+]_o$, indicating that the peak value increased proportionately

Fig. 7. Relation between $[\text{Na}^+]_o$ and the net driving force for Ca^{2+} influx on the $\text{Na}^+/\text{Ca}^{2+}$ exchanger ($\Delta\mu\text{Ca}^{2+} - 3\Delta\mu\text{Na}^+$).

Fig. 8. Relation between the net driving force for Ca²⁺ influx on the Na⁺/Ca²⁺ exchanger ($\Delta\mu\text{Ca}^{2+} - 3\Delta\mu\text{Na}^+$) and $[\text{Ca}^{2+}]_i$ obtained at different Na⁺_o concentrations depicted in Fig. 2. The relevant $\Delta\mu\text{Ca}^{2+} - 3\Delta\mu\text{Na}^+$ at a particular $[\text{Na}^+]_o$ was calculated as shown in Fig. 7.

with the elevation of the net driving force.

The magnitude of Ca²⁺ entry may depend on the relative electrochemical gradients for Na⁺ and Ca²⁺. If $\Delta\mu\text{Na}^+$ is decreased by lowering $[\text{Na}^+]_o$ or increasing $[\text{Na}^+]_i$, Ca²⁺ entry through the exchanger may be markedly activated. As a result, the activated Ca²⁺ entry may increase $[\text{Ca}^{2+}]_i$ to a level controlled by the driving force ($\Delta\mu\text{Ca}^{2+} - 3\Delta\mu\text{Na}^+$). Therefore, the rise in $[\text{Ca}^{2+}]_i$ induced by Na⁺_o removal may depend on the transmembrane Na⁺ gradient. This view is supported by the result that ouabain enhanced the rise probably due to the rise in $[\text{Na}^+]_i$ as shown in Fig. 6. When $\Delta\mu\text{Na}^+$ is decreased by increasing $[\text{Na}^+]_i$ or lowering $[\text{Na}^+]_o$, the reverse mode of the Na⁺/Ca²⁺ exchanger may dominate the forward mode and cause a rise in $[\text{Ca}^{2+}]_i$.

In conclusion, in rat pancreatic islets, the rise in $[\text{Ca}^{2+}]_i$ induced by lowering $[\text{Na}^+]_o$ is due to Ca²⁺ entry mediated by the Na⁺/Ca²⁺ exchanger, which operates with the stoichiometry of 3 Na⁺:1 Ca²⁺, and this exchanger plays an important role in maintaining the stable-state level of $[\text{Ca}^{2+}]_i$.

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REFERENCES

- Prentki M and Matschinsky FM: Ca²⁺, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiol Rev* 67: 1185-1248, 1987
- Wollheim CB and Sharp GWG: Regulation of insulin release by calcium. *Physiol Rev* 61: 914-973, 1981
- Herchuelz A and Plasman P-O: Sodium-calcium exchange in the pancreatic B cell. *Ann NY Acad Sci* 639: 642-656, 1991
- Donatsch P, Lowe DA, Richardson BP, and Taylor P: The functional significance of sodium channels in pancreatic beta-cell membranes. *J Physiol (Lond)* 267: 357-376, 1977
- Plasman P-O, Lebrun P, and Herchuelz A: Characterization of the process of sodium-calcium exchange in pancreatic islet cells. *Am J Physiol* 259: E844-E850, 1990
- Herchuelz A, Sener A, and Malaisse WJ: Regulation of calcium fluxes in rat pancreatic islets: Calcium extrusion by sodium/calcium countertransport. *J Membr Biol* 57: 1-12, 1980
- Hellman B, Andersson T, Berggren P-O, and Rorsman R: Calcium and pancreatic beta-cell function. XI. Modification of ⁴⁵Ca fluxes by Na⁺ removal. *Biochem Med* 24: 143-152, 1980
- Herchuelz A and Lebrun P: A role for Na/Ca exchange in the pancreatic B cell. Studies with thapsigargin and caffeine. *Biochem Pharmacol* 45: 7-11, 1993
- Hattori M, Kai R, and Kitasato H: Effect of lowering Na⁺ concentration on cytoplasmic pH and Ca²⁺ concentration in mouse pancreatic β-cells: Mechanism of periodicity of spike-bursts. *Jpn J Physiol* 44: 283-293, 1994
- Lacy PE and Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16: 35-39, 1967
- Gryniewicz G, Poenie M, and Tsien RY: A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440-3450, 1985
- Kass GEN, Llopis J, Chow SC, Duddy SK, and Orrenius S: Receptor-operated calcium influx in rat hepatocytes. Identification and characterization using manganese. *J Biol Chem* 265: 17486-17492, 1990
- Kwan C-Y and Putney JW Jr: Uptake and intracellular sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells. Dissociation by Sr²⁺ and Ba²⁺ of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. *J Biol Chem* 265: 678-684, 1990
- Silva AM, Rosário LM, and Santos RM: Background Ca²⁺ influx mediated by a dihydropyridine- and voltage-insensitive channel in pancreatic beta-cells. Modulation by Ni²⁺, diphenylamine-2-carboxylate, and glucose metabolism. *J Biol Chem* 269: 17095-17103, 1994
- Bean BP: Classes of calcium channels in vertebrate cells. *Annu Rev Physiol* 51: 367-384, 1989
- Nowycky MC, Fox A, and Tsien RW: Three types of neuronal calcium channels with different calcium antagonist sensitivity. *Nature* 316: 440-443, 1985
- Ohta M, Nelson J, Meglasson MD, and Erecińska M: Effect of Ca²⁺ channel blockers on energy level and stimulated insulin secretion in isolated rat islets of Langerhans. *J Pharmacol Exp Ther* 264: 35-40, 1993
- Merritt JE, Armstrong WP, Benham CD, Hallam TJ, Jacob R, Jaxa-Chamiec A, Leigh BK, McCarthy SA, Moores KE, and Rinks TJ: SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem J* 271: 515-522, 1990
- Lytton J, Westlin M, and Hanley MR: Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-

- ATPase family of calcium pumps. *J Biol Chem* 266: 17067-17071, 1991
20. Takemura H, Hughes AR, Thastrup O, and Putney JW Jr: Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J Biol Chem* 264: 12266-12271, 1989
21. Thastrup O: Role of Ca^{2+} -ATPase in regulation of cellular Ca^{2+} signaling, as studied with the selective microsomal Ca^{2+} -ATPase inhibitor, thapsigargin. *Agents Actions* 29: 8-15, 1990
22. Niwa K, Shibuya I, and Kanno T: Temperature dependent of processes proximal and distal to the glucose-induced $[\text{Ca}^{2+}]_i$ rise in stimulus-secretion coupling in rat pancreatic islets. *Biol Signals* 5: 30-43, 1996
23. Snowdowne KW and Borle AB: Effects of low extracellular sodium on cytosolic ionized calcium. Na^+ - Ca^{2+} exchange as a major calcium influx pathway in kidney cells. *J Biol Chem* 260: 14998-15007, 1985
24. Blaustein MP and Hodgkin AL: The effect of cyanide on the efflux of calcium from squid axons. *J Physiol (Lond)* 200: 497-527, 1969
25. Mullins LJ: The generation of electric currents in cardiac fibers by Na/Ca exchange. *Am J Physiol* 233: C103-C110, 1979
26. Saha S and Grapengiesser E: Glucose promotes turnover of Na^+ in pancreatic β -cells. *Biochim Biophys Acta* 1265: 209-212, 1995
27. Henquin JC and Meissner HP: The electrogenic sodium-potassium pump of mouse pancreatic B-cells. *J Physiol (Lond)* 332: 529-552, 1982
28. Akiyoshi M, Takei M, Nakazaki M, and Tanaka H: A new hypoglycemic agent, A-4166, inhibits ATP-sensitive potassium channels in rat pancreatic β -cells. *Am J Physiol* 268: E185-E193, 1995
29. Eisner DA and Lederer WJ: $\text{Na}-\text{Ca}$ exchange: Stoichiometry and electrogenicity. *Am J Physiol* 248: C189-C202, 1985
30. Rasgado-Flores H and Blaustein MP: Na/Ca exchange in barnacle muscle cells has a stoichiometry of $3 \text{ Na}^+ / 1 \text{ Ca}^{2+}$. *Am J Physiol* 252: C499-C504, 1987
31. Reeves JP and Hale CC: The stoichiometry of the cardiac sodium-calcium exchange system. *J Biol Chem* 259: 7733-7739, 1984
32. Hoenig M, Culbertson LH, Clement JM, and Ferguson DC: $\text{Na}^+/\text{Ca}^{2+}$ exchange in plasma membrane vesicles from a glucose-responsive insulinoma. *Cell Calcium* 13: 1-8, 1992