Effects of Lowering External Na^+ Concentration on Cytoplasmic pH and Ca^{2+} Concentration in Mouse Pancreatic β -Cells: Mechanism of Periodicity of Spike-Bursts

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Abstract The periodic spike-burst response of pancreatic β -cells varies in duration with an increase of external glucose within the range 5–20 mM. To elucidate the mechanism determining the length of spike-burst, we studied the low- Na^+ induced change in electrical response to glucose which is similar to the change induced by high glucose. Cytoplasmic pH (pH_i) and Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were measured by the micro-fluorometric method under normal and low- Na^+ conditions in mouse pancreatic islets. Lowering external Na^+ concentration from 135 to 25 mM by replacing Na^+ with Tris^+ induced progressive alkalinization in islet cells in the presence of 11.1 mM glucose. In contrast, reduction of external Na^+ by replacement with Li^+ caused intracellular acidification. Both manipulations described above caused a marked increase in $[\text{Ca}^{2+}]_i$, suggesting the presence of $\text{Na}^+/\text{Ca}^{2+}$ -antiport activity. Although the change in pH_i induced by decreasing external Na^+ varied in direction depending on the species of cations used for replacing Na^+ , the pattern of electrical activity consistently changed from the spike-burst type to the continuous spike-generation type without regard for the difference in species of cations replacing Na^+ . These findings lead to the following hypothesis: A decrease in Na^+ influx could cause a decrease in ATP-consumption by Na^+/K^+ -pumps that prevents the fall of intracellular ATP concentration. The resultant continuation of high concentrations of intracellular ATP may be responsible for the abolishment of the silent phase.

Key words: pancreatic β -cell, cytoplasmic pH, cytoplasmic Ca^{2+} .

Pancreatic β -cells respond with a depolarization to external glucose, and exhibit spike activity. As for the initial depolarization, it is widely accepted that the elevation of external glucose concentration causes an increase in ATP-production,

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leading to a rise in intracellular ATP levels which results in the closure of the ATP-sensitive K^+ channel [1-3]. The depolarization evokes Ca^{2+} spikes on it [4]. In the presence of around 10 mM of glucose, β -cells show periodic electrical activity consisting of a spike-burst phase (active phase) and a silent phase [5]. Elevation of the external glucose concentration prolongs the duration of the active phase, and shortens the silent phase. At glucose concentrations higher than 20 mM, a clear silent phase is no longer observed, but spikes are elicited continuously on a persistent depolarization [6]. Since the frequency of spike generation during the period of spike-bursts does not depend on the external glucose concentration, the number of spikes for a unit period of considerable length depends on the duration of spike-bursts [7]. Thus, the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) is related to the duration of the active phase. Since insulin release depends on $[Ca^{2+}]_i$ [8], insulin release is linked to the duration of the active phase. Although the mechanism of the depolarization induced by glucose has already been fully elucidated [1-3], the mechanism of repolarization that causes an interruption of the spike-burst phase still remains obscure.

A pattern of electrical activity similar to that observed at glucose concentrations higher than 20 mM can be induced by lowering external Na^+ concentration ($[Na^+]_o$) from 135 to 25 mM even at glucose concentrations lower than 12 mM [9, 10]. Amiloride, an inhibitor of the Na^+/H^+ antiport, has also been reported to induce a persistent depolarization with continuous spike activity [11, 12], whereas monensin which behaves like Na^+/H^+ -antiport in the plasma membrane [13] induces a repolarization [14]. Thus, we speculated that a fall of $[Na^+]_i$ causes a rise of intracellular ATP concentration ($[ATP]_i$) as a result of decreased ATP consumption by Na^+/K^+ -pumps, and that the resultant persistency of $[ATP]_i$ at high level prevents an increase in K^+ -conductance, leading to a continuous depolarization. To exclude the possibility that changes in pH_i and $[Ca^{2+}]_i$ may directly be involved in the change in electrical pattern observed on lowering $[Na^+]_o$, we microfluorometrically measured pH_i and $[Ca^{2+}]_i$ in isolated β -cells. Present experiments revealed that the change in pH_i induced by lowering $[Na^+]_o$ was not responsible for the shift of the pattern of electrical activity from the spike-burst type to the continuous spike-generation type. This finding implies that a decrease of Na^+ inflow plays an important role in the induction of the change in the pattern of electrical activity from periodic spike-burst type to continuous spike-generation type observed under low- Na^+ conditions.

MATERIALS AND METHODS

Isolation of pancreatic islets and preparation of β -cells. Male ICR mice aged 6 to 12 weeks were used throughout the experiments. Mice were fasted for 24-48 h with free access to water. The animals were killed by cervical dislocation. The pancreas was excised immediately after the death of the animal. Islets of Langerhans were isolated from the pancreas by collagenase digestion. They were then

cultured for 2–24 h at 36°C in RPMI 1640 medium containing 11.1 mM glucose supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The method used to isolate pancreatic islet cells has been described elsewhere [15]. In brief, after exposure to a Ca^{2+} - and Mg^{2+} -deprived medium, the islets were disrupted using dispase and were centrifuged through an albumin solution to remove debris and dead cells. Isolated single cells were seeded on glass coverslips. β -Cells were identified by confirming the typical response of $[\text{Ca}^{2+}]_i$ to 11.1 mM glucose or tolbutamide.

Recordings of β -cell membrane potential. A small piece of pancreatic tissue containing two or three islets was mounted on a small ball in a chamber. The islets were perfused with Krebs-Ringer-bicarbonate solution gassed with 95% O_2 + 5% CO_2 . The membrane potential of β -cell in the islet was recorded by using a glass microelectrode (250–400 M Ω). The electrode was connected to a high input-impedance bridge-amplifier (Nihon Kohden, MEZ-8301). The analog-output was stored in a videotape after it was digitized by a PCM processor (SONY, PCM-501 modified). The data were plotted on an XY-plotter (Roland, DXY-980A) after the experiment. The β -cell in the islet was identified by showing the typical electrical activity that they display in the presence of 11.1 mM glucose. The temperature of the solution was controlled at 36°C by a thermomodule. The flow rate was about 1 ml/min. The delay of solution-change due to the dead space was 100 s, and corrected in figures.

Measurement of pH_i . Islet cells cultured on coverslips were incubated with 1 μM 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (BCE-CF-AM) for 30–40 min. After loading, they were rinsed in standard solution and then transferred to a chamber for microfluorometry. The chamber was placed on the stage of an inverted microscope (Nikon, Diaphot-TMD) and the cells were perfused with a variety of test solutions at a flow rate of 2 ml/min. The dead space of the system corresponded to approximately 50 s. The delay due to the solution-change was corrected in the figures. Perfusion solutions were kept at 36°C in a water bath, and the temperature was always monitored by a thermistor. Measurements were performed using microspectrofluorometer (Nihon Bunko, CAM-220). The excitation and emission wavelengths were 490/440 and 530 nm, respectively. The ratio of the intensities of fluorescence excited at 490 nm to that at 440 nm (490/440 excitation ratio) was calibrated in terms of pH_i according to the nigericin/ K^+ method [16]. The concentration of Tris-HCl did not affect the 490/440 excitation ratio in buffered solution.

Measurement of $[\text{Ca}^{2+}]_i$. Isolated islets and dispersed islet cells were incubated with 2 μM Fura2 acetoxymethylester (Fura2-AM) for 30–40 min in an incubator containing 5% CO_2 at 36°C . After washing twice in Hanks' solution, the fluorescent intensity was measured using microspectrofluorometer (see above). The excitation and emission wavelengths were 340/380 and 510 nm, respectively. The ratio of the intensities of fluorescence excited at 340 nm to that at 380 nm (ratio F_{340}/F_{380}) was used as an indicator of $[\text{Ca}^{2+}]_i$ because the ratios obtained from the

experiments conducted on cells in islets were usually lower than those obtained from isolated cells.

Solutions and chemicals. The standard solution was composed of 110 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.1 mM MgCl_2 , 25 mM NaHCO_3 , 5 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), and 2.8 mM D-glucose, and gassed with 95% O_2 and 5% CO_2 . In low- Na^+ solution, tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) or LiCl was added instead of 110 mM NaCl. Nigericin was obtained from Calbiochem. Fura2-AM and BCECF-AM were purchased from Dojin (Kumamoto, Japan). Other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

RESULTS

Effect of glucose on membrane potential, $[\text{Ca}^{2+}]_i$, and pH_i

Figure 1A shows the typical example of electrical response of β -cell in an islet to external glucose. Elevating external glucose concentration from 2.8 to 11.1 mM induced a gradual depolarization followed by a spike-train and subsequent periodic spike-bursts, consistent with reports from many laboratories [9, 11, 17]. The response of $[\text{Ca}^{2+}]_i$ of cells in isolated islets are shown in Fig. 1B. $[\text{Ca}^{2+}]_i$ was stable at a glucose concentration of 2.8 mM. Upon raising glucose concentration to 11.1 mM, $[\text{Ca}^{2+}]_i$ changed in three phases: a small fall of $[\text{Ca}^{2+}]_i$ lasting for 30–90 s (arrow in Fig. 1B), a rapid and marked increase, and oscillations following the rapid increase. After the onset of the second phase, $[\text{Ca}^{2+}]_i$ reached a maximum within 30–90 s, and remained at that level for 1–3 min. Then, $[\text{Ca}^{2+}]_i$ declined slowly, associated with oscillation. The present result of the initial fall, subsequent increase and oscillation of $[\text{Ca}^{2+}]_i$ agrees with previous reports in mouse islets [18, 19] and single β -cell from the mouse [20] and rat [21].

Figure 1C shows the effect of glucose on pH_i . pH_i was about 7.0, and rose very slightly after the glucose concentration was increased from 2.8 to 11.1 mM or 22.2 mM. This result is consistent with previous reports [22, 23].

The isolated single islet cells differed from the cells embedded in an islet in response to glucose. The response of $[\text{Ca}^{2+}]_i$ of isolated single cells to external glucose is shown in Fig. 2. The initial fall of $[\text{Ca}^{2+}]_i$ lasted for 1–5 min (arrow in Fig. 2). The initial phase was followed by cyclic elevation of $[\text{Ca}^{2+}]_i$ with various durations from 1 to 20 min. Gap junctions between islet cells have been morphologically shown [24], and an electrical connection among cells in an islet has been demonstrated [25–27]. Taking these reports into account, the lack of an electrical connection between cells in an islet may be responsible for the difference in the time course of the response of isolated single cells from that of cells in an islet. The increase of the 340/380 nm excitation ratio in experiments on isolated single cell was much larger than that observed on cells in an isolated islet. This may come from the fact that isolated single β -cells are not covered by substances absorbing excitation and emitted lights.

Fig. 1. Typical effects of glucose on electrical activity in a single cell in an islet (A), $[\text{Ca}^{2+}]_i$ in the whole islet, and pH_i in whole islet (C). Glucose concentration was varied.

Fig. 2. The typical effect of 11.1 mM glucose on $[\text{Ca}^{2+}]_i$ in an isolated single islet cell.

Fig. 3. The effect of lowering $[Na^+]_o$ on the glucose-induced electrical activity in a β -cell in an islet. NaCl (110 mM) in perfusion solution was replaced with 110 mM Tris-HCl (A) or 110 mM LiCl (B). Traces A and B were recorded from different cells.

Relationship among changes in electrical activity, pH_i , and $[Ca^{2+}]_i$ induced by lowering $[Na^+]_o$

Figure 3 shows two examples of the change in electrical activity following reduction of $[Na^+]_o$ in the presence of 11.1 mM glucose. Replacement of external NaCl with Tris-HCl caused gradual depolarization and continuous spike activity (Fig. 3A). To confirm that the change in electrical activity observed in the replacement experiments with Tris-HCl is not specific to Tris-HCl but is due to low- Na^+ , monovalent cation species other than $Tris^+$ were used for replacement. A similar effect was observed on replacement of external NaCl with LiCl (Fig. 3B). In the next step, to examine whether the change in the pattern of electrical activity is mediated by a change in intracellular ionic conditions, we measured pH_i and $[Ca^{2+}]_i$ under the same conditions used to record electrical activity. Figure 4 shows a change in pH_i of an isolated islet upon lowering $[Na^+]_o$. Replacement of external NaCl with Tris-HCl in the presence of 11.1 mM glucose caused a progressive alkalinization without permissive delay: pH_i was raised by 0.27 ± 0.05 ($n=3$) units in 3 min after the decrease in $[Na^+]_o$ (Fig. 4A). The intracellular alkalinization induced by replacement with Tris-HCl may be due to the dissociation of penetrable form of tris(hydroxymethyl)aminomethane molecules as an alkali in the cytosol. In contrast, replacement of NaCl with LiCl induced a progressive acidification with little delay: pH_i lowered by 0.20 ± 0.06 ($n=3$) units in 4 min after the decrease in $[Na^+]_o$ (Fig. 4B). No obvious delay indicates that the pH_i change induced in replacing experiments is a direct result of the manipulations employed in these experiments.

Although $Tris^+$ and Li^+ had different effects on pH_i , the reduction of external Na^+ caused an identical change in the pattern of electrical activity independently of the species of cations used to replace Na^+ . This finding strongly supports the view that the change in the pattern of electrical activity caused by low- Na^+ manipulation

Fig. 4. The effect of lowering $[\text{Na}^+]_o$ on pH_i in islet cells. Glucose concentration was 11.1 mM. NaCl (110 mM) in perfusion solution was replaced with 110 mM Tris-HCl (A) or 110 mM LiCl (B).

Fig. 5. The effect of lowering $[\text{Na}^+]_o$ on the glucose-induced oscillation of $[\text{Ca}^{2+}]_i$ in the whole islet. NaCl (110 mM) in the medium was replaced with 110 mM Tris-HCl (A) or 110 mM LiCl (B).

is not due to the pH_i change but rather mediated by a decrease in the intracellular Na^+ concentration ($[\text{Na}^+]_i$).

It is expected that the lowered $[\text{Na}^+]_o$ suppresses the $\text{Na}^+/\text{Ca}^{2+}$ -counter-transport, leading to an elevation of $[\text{Ca}^{2+}]_i$. Figure 5 shows the time course of the change in $[\text{Ca}^{2+}]_i$ occurring upon a reduction of external Na^+ from 135 to 25 mM in the presence of 11.1 mM glucose. These traces were records from cells in an isolated islet. Replacement of external NaCl with Tris-HCl abolished the pulsation of $[\text{Ca}^{2+}]_i$, which had been observed in 135 mM Na^+ KRB solution, and brought about a clear increase in $[\text{Ca}^{2+}]_i$ to a level higher than that of initial increase in

$[\text{Ca}^{2+}]_i$ induced by the elevation of external glucose concentration (Fig. 5A). A similar effect was observed when replacing external NaCl with LiCl (Fig. 5B). Increasing $[\text{Na}^+]_o$ back to 135 mM caused $[\text{Ca}^{2+}]_i$ to return to its control level. This observation suggests that $\text{Na}^+/\text{Ca}^{2+}$ -countertransport is functioning, and that an elevation of $[\text{Ca}^{2+}]_i$ caused by an increased Ca^{2+} influx during spike-burst could result in a rise of $[\text{Na}^+]_i$.

DISCUSSION

Pancreatic β -cells exhibit periodic spike activity in the normal Krebs-Ringer-bicarbonate solution containing glucose at around 10 mM. The duration of glucose-induced spike-bursts becomes longer with the elevation of sugar concentration within the range 5–15 mM. The mechanism underlying the periodicity of spike-burst still remains to be clarified. Recently, some works on this mechanism have been reported [7, 28]. It has been once proposed that H^+ yielded in the oxidative breakdown of glucose is responsible for the depolarization induced by external glucose [29, 30]. There are plenty of works about the effect of glucose on pH_i in islet cells using various probes, reporting that no substantial change in pH_i is caused by elevating the external glucose concentration [22, 23]. In the present study, we demonstrated that both procedures of lowering $[\text{Na}^+]_o$ by replacing external Na^+ with Li^+ and with Tris^+ consistently caused a change in the electrical pattern from the spike-burst type to the continuous spike-generation type, whereas the change in pH_i induced by decreasing external Na^+ varied in direction depending on the species of replacing cations. This fact clearly indicates that the change in pattern of electrical activity induced by low- Na^+ is not due to the change in pH_i but rather due to the decrease of Na^+ influx.

The acidification brought about by the replacement of external Na^+ with Li^+ (Fig. 4B) is the first reported evidence directly indicating the presence of Na^+/H^+ -antiport in β -cell. This observation implies that Na^+/H^+ -antiport plays an important role in maintaining a constant pH_i . The replacement of external Na^+ with Tris^+ caused intracellular alkalinization (Fig. 4A). This may be accounted for by the permeation of electrically neutral tris(hydroxymethyl)aminomethane molecules across the plasma membrane. The electrically neutral molecules dissociate in the cytosol as alkali, and this alkalizing effect may overcome the acidifying effect of low $[\text{Na}^+]_o$. It has been reported that buffers causing intracellular alkalinization induces a hyperpolarization at normal $[\text{Na}^+]_o$ [11, 30]. It should be stressed that the replacement of external Na^+ with Tris^+ always induced a change in electrical activity similar to that induced by high glucose notwithstanding that pH_i moved toward high value. In other words, the effect on electrical activity of low $[\text{Na}^+]_o$ is greater than the hyperpolarizing effect of intracellular alkalinization resulting from the intracellular dissociation of neutral tris(hydroxymethyl)aminomethane molecules.

It is well known that the rate of ATP-consumption by Na^+/K^+ -pump or

transporting ATPase depend on $[\text{Na}^+]_i$ [31, 32]. On the other hand, during the period of spike-burst an increased Ca^{2+} influx causes an elevation of $[\text{Ca}^{2+}]_i$. The elevation of $[\text{Ca}^{2+}]_i$ accelerates $\text{Na}^+/\text{Ca}^{2+}$ -counter transport, resulting in a rise of $[\text{Na}^+]_i$. The rise of $[\text{Na}^+]_i$, which increases the rate of ATP breakdown at Na^+/K^+ -pump, promotes a fall in $[\text{ATP}]_i$. In contrast to the process occurring under normal conditions, lowering $[\text{Na}^+]_o$ causes a decrease of Na^+ -inflow, and may result in a fall of $[\text{Na}^+]_i$. Thus, the reduction of extracellular Na^+ is considered to decrease the rate of ATP consumption by Na^+/K^+ -pumps that prevents the fall in $[\text{ATP}]_i$ which could otherwise happen during spike-burst, and to cause high level of $[\text{ATP}]_i$ to be maintained provided that the production rate of ATP in mitochondria remains constant. Since pancreatic β -cell has specific ATP-sensitive K^+ channels, the maintenance of high levels of $[\text{ATP}]_i$ is expected to keep the ATP-sensitive K^+ channels closed state that causes a continuation of spike-generation. This may be a mechanism underlying the change in the pattern of electrical activity from the spike-burst type to the continuous spike-generation type. Recently, Gilon and Henquin [33] have measured $[\text{Na}^+]_i$ by using SBFI, an Na^+ -sensitive fluorescent probe, and reported that muscarinic stimulation induces a rise of intracellular Na^+ concentration. Since acetylcholine has a facilitating effect on spike-bursts [34], the observation reported by Gilon and Henquin [33] supports the view that $\text{Na}^+/\text{Ca}^{2+}$ -antiport is functioning during the period of electrically active phase.

In conclusion, the present study suggests that both the continuous electrical activity observed at high glucose concentrations and that induced by low $[\text{Na}^+]_o$ at lower glucose concentrations are due to the maintenance $[\text{ATP}]_i$ high enough to close ATP-sensitive K^+ channels. At glucose concentrations within the range 5–15 mM the ATP production rate is not so high as at concentrations above 20 mM. Under these conditions, a fall in $[\text{ATP}]_i$ can be induced by a rise in the ATP consumption rate due to acceleration of active Na^+/K^+ -transport caused by a progressive elevation of $[\text{Na}^+]_i$ through $\text{Na}^+/\text{Ca}^{2+}$ -countertransport during the period of spike-bursts. The fall in $[\text{ATP}]_i$ results in opening of the ATP-sensitive K^+ channels, leading to an initiation of the silent phase. During the silent phase, the level of $[\text{ATP}]_i$ gradually increases because no Ca^{2+} -spike is elicited. The gradual elevation of $[\text{ATP}]_i$ causes a depolarization. When the depolarization reaches a firing level, a spike-train is evoked again, then terminated by a hyperpolarization resulting from a fall in $[\text{ATP}]_i$. The cycle is repeated until the external glucose concentration drops below 4 mM. This may be the mechanism developing periodic spike-burst activity. To substantiate the hypothesis proposed above, further studies on $[\text{Na}^+]_i$ and $[\text{ATP}]_i$ are required.

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