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Abstract Pancreatic beta cells act as glucose sensors, in which intracellular ATP ($[ATP]_i$) are altered with glucose concentration change. The characterization of voltage-gated sodium channels under different $[ATP]_i$ remains unclear. Here, we demonstrated that increasing $[ATP]_i$ within a certain range of concentrations (2–8 mM) significantly enhanced the voltage-gated sodium channel currents, compared with 2 mM cytosolic ATP. This enhancement was attenuated by even high intracellular ATP (12 mM). Furthermore, elevated ATP modulated the sodium channel kinetics in a dose-dependent manner. Increased $[ATP]_i$ shifted both the current–voltage curve and the voltage-dependent inactivation curve of sodium channel to the right. Finally, the sodium channel recovery from inactivation was significantly faster when the intracellular ATP level was increased, especially in 8 mM $[ATP]_i$, which is an attainable concentration by the high glucose stimulation. In summary, our data suggested that elevated cytosolic ATP enhanced the activity of Na^+ channels, which may play essential roles in modulating β cell excitability and insulin release when blood glucose concentration increases.

Keywords ATP · Beta cell · Excitability · Kinetics · Na^+ channel · Pancreas

Na Zou and Xiao Wu contributed equally to this article, and both should be considered first author

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Introduction

The voltage-gated Na^+ channel is widely expressed in most excitable cells, including neurons, muscle cells and endocrine cells and plays important roles in action potential, cell excitability and signal transduction (Hille 1992). Several studies have reported Na^+ currents in pancreatic β cells (Plant 1988; Ashcroft and Rorsman 1989; Hiriart and Matteson 1988; Pressel and Misler 1990). It is well known that β cells express a series of ion channels and utilize electrical signals to couple various blood glucose concentrations to the insulin output (Henquin 1987; Bratanova-Tochkova et al. 2002; Rorsman and Renström 2003; Suckale and Solimena 2010). A considerable amount of evidence describes the characterizations and functions of Ca^{2+} and K^+ channels in β cells (Ashcroft et al. 1984; Rorsman and Trube 1986; Satin et al. 1994). However the

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property of Na^+ channels in β cells is poorly understood (Plant 1988; Pressel and Misler 1990; Lou et al. 2003).

Some work indicates that the contribution of Na^+ channels on the action potential of β cells are ignorable because Na^+ channels are completely inactivated in the resting potential (Ashcroft and Rorsman 1989; Hiriart and Matteson 1988; Pace 1979) and Na^+ channels has no effect on the insulin release in the regular glucose concentration (Hiriart and Matteson 1988). Recent experiments demonstrate that residual Na^+ channels are present in β cells and modulate Ca^{2+} influx-induced action potential (Vignali et al. 2006). Moreover, agonists of the Na^+ channel can regulate the electrical activity of β cells (Eberhardson and Grapengiesser 1999; Gonçalves et al. 2003). Interestingly, it is showed that the activation of Na^+ channels in β cell augments the insulin release in high glucose conditions (Gonçalves et al. 2003), suggesting that the function of sodium channels is related to the glucose concentrations (Hiriart and Matteson 1988; Pace 1979; Gonçalves et al. 2003). More importantly, a recent work reports that knockout of Scn1b causes the functional loss of NaV1.7 in β cells, resulting in defects of insulin secretion (Ernst et al. 2009). Therefore, Na^+ channels may participate in the modulation of the β cell activity and its function may be different under different glucose conditions.

Intracellular ATP plays critical roles in insulin secretion, which is under the control of the extracellular glucose concentrations (Hillaire-Buys et al. 1994; Sperling 2006). High glucose stimulates the elevation of $[ATP]_i$, which in turn inhibits ATP-sensitive K^+ (K_{ATP}) channels and triggers insulin secretion (Sperling 2006). Here, we investigated whether these increased ATP regulate Na^+ channels and the excitability of β cells. By using the whole-cell patch clamp in pancreatic tissue slice, we observed the actions of various concentrations of ATP in the pipette solution on Na^+ channel-mediated currents. We found that ATP, in a dose-dependent manner, modulates important properties of Na^+ channels, including the peak amplitude, current–voltage relationship, kinetics of inactivation and recovery time from inactivation. These data suggest that high glucose-induced ATP plays an important role to regulate Na^+ channel kinetics and thereby β cell excitability.

Materials and Methods

All animal experiments were specifically designed to minimize the number of animals used and approved by the Animal Experimentation Ethics Committee of Zhejiang University. Male mice at 8–10 weeks were used throughout the study. All drugs were from Sigma (St. Louis, MO), Tocris (Bristol, UK) or Ascent Scientific (Bristol) unless stated otherwise.

Pancreatic Slice Preparation

Pancreatic slices were prepared according to previous work (Speier and Rupnik 2003; Wu et al. 2012). In brief, the abdominal cavity of anesthetic mice was opened and warm (37 °C) low-gelling agarose (1.9 % wt/vol, Seaplaque GTG agarose, BMA Products) was injected into the distally clamped bile duct. The whole pancreas was immediately cooled with ice-cold extracellular solution (ECS, in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 Na-pyruvate, 0.5 ascorbic acid, 3 myo-inositol, 6 lactic acid, 1 $MgCl_2$, and 2 $CaCl_2$, adjusted to pH 7.3 and oxygenated with 95 % O_2/5 % CO_2. Four small cubes were cut from the agarose-embedded tissue and glued onto the sample plate of a vibrating tissue slicer (VT1000S; Leica, Wetzlar, Germany). Slices (140 μm) were kept in ice-cold ECS for at least 1 h before use.

Electrophysiology

Slices were placed in a submerged chamber and perfused with an ECS (30 °C) at 1.5 ml/min. Cells were visualized under an upright microscope (Zeiss Axioskop 2 FS, Carl Zeiss, Oberkochen, Germany) and a mounted CCD camera with 5 × digital amplification (Cohu, San Diego, CA). The β cells from the second or third layers in selected islets were used for whole-cell recording. Recording pipettes were pulled on an electrode puller (P-97, Sutter Instruments, Novato, CA) with a resistance of 2–4 MΩ in CsCl-based solution containing (in mM) 125 CsCl, 40 HEPES, 2 $MgCl_2$, 20 TEA-Cl, 2 $ATPNa_2$. Additional ATP magnesium salt was added to the internal saline for increasing the cytosolic concentration of ATP. The pH value was adjusted to 7.2 when the internal saline was filled with ATP. Electrical responses were filtered at 3 kHz and digitized at 10 kHz by an EPC10 amplifier (Heka, Lambrecht, Germany). There was no significant difference in cell series conductance when various concentrations of ATP were filled into cells (data not shown).

Data Analysis

Cells were excluded from the study if series resistance varied by >15 % over the course of the experiment. Offline analysis was done using Excel (Microsoft, Redmond, OR), SigmaPlot (Systat Software, San Jose, CA), and Igor Pro (WaveMetrics, Lake Oswego, OR). Statistical differences were determined using Student's *t*-test. A significant difference was accepted if P was <0.05 (*) or if P was <0.01 (**). Data in the text and figures are presented as mean ± SEM. Current–voltage (I–V) curves in Figs. 2 and 3 were fitted with the Boltzmann function:

$$I/I_{Max} = [1 - \exp(-V + V_{50})/k]^{-1} \quad (1)$$

where I is Na^+ current (I_{Na}), I_{max} is the maximum I_{Na}, V_{50} is the voltage producing half-maximal response, and k

is the slope factor (mV^{-1}). Recovery curves from inactivation were fitted with the single exponential function (Fig. 4):

$$I = I_{Max}[1 - \exp(-kV)] \quad (2)$$

where I is I_{Na}, I_{max} is the maximum I_{Na}, and k is the slope factor (mV^{-1}).

Results

Intracellular ATP Increases Depolarization-induced I_{Na}

We used pancreatic slices to study the kinetic of sodium current for physiological conditions. Supplemental Fig. S1 shows representative DIC images of pancreatic slices under different magnifications. The β cells are dominant in the center of islets (Speier and Rupnik 2003; Rupnik 2008). β cells were identified as described previously (Speier and Rupnik 2003; Rupnik 2008). Previous work indicates that I_{Na} is limitedly evoked at the resting membrane potential (Plant 1988). Hyperpolarization triggers a dramatic increase of I_{Na} (Plant 1988; Lou et al. 2003). Consistent with these findings, fast inward currents were obtained when we set −80 or −150 mV as the holding potential and 0 mV was set as the test potential (Fig. 1a). These inward currents were blocked by tetrodotoxin, indicating that they are mediated by voltage-gated Na^+ channels. We continued to record inward I_{Na} in elevated internal ATP (6, 8, and 12 mM) and found that all elevated ATP increased the I_{Na} amplitude (Fig. 1b). The optimum enhancement effect occurred at the 8 mM $[ATP]_i$. Too much internal ATP (12 mM) reduced the enhancement (Fig. 1b). Increased I_{Na} by ATP may significantly influence β cell excitation (Ashcroft and Rorsman 1989; Zhou and Misler 1995).

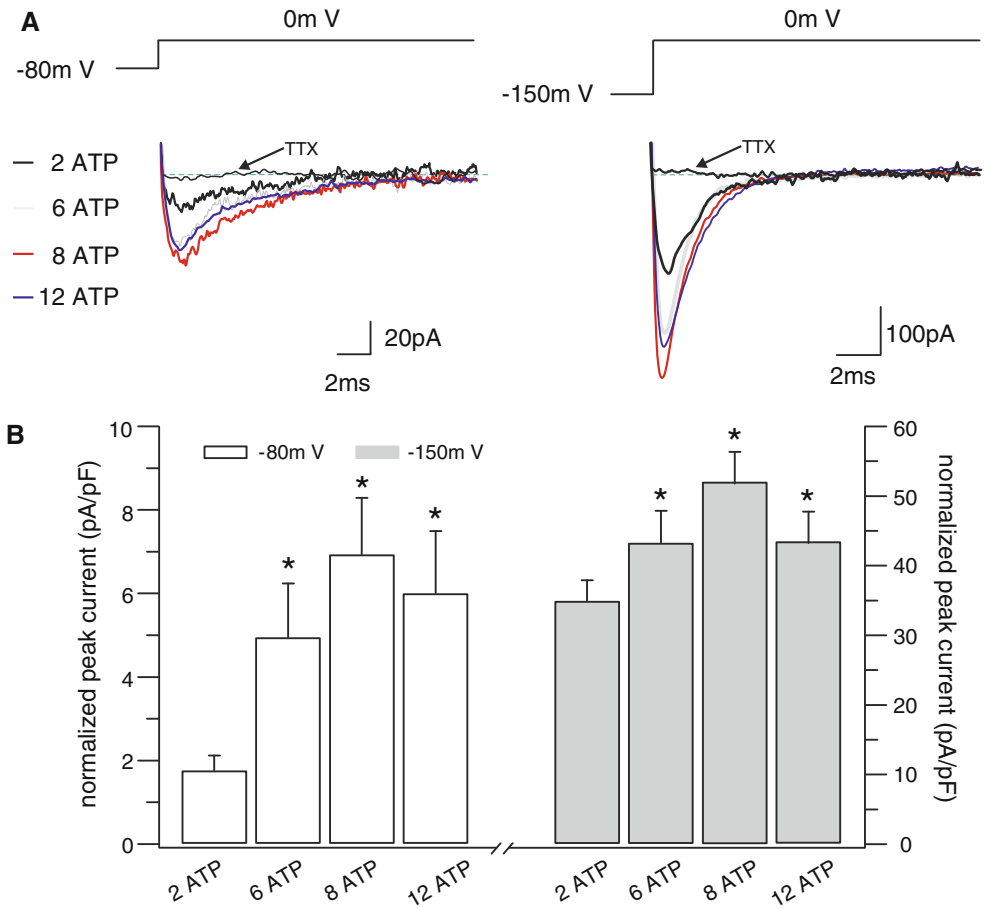

Fig. 1 ATP increased depolarization-induced I_{Na}. **a** Representative I_{Na} induced by voltage pulses from either −80 mV (*left*) or −150 mV (*right*) to 0 mV for 100 ms. Note that depolarization from a holding potential of −150 mV induced much larger I_{Na} than −80 mV. Control I_{Na} and I_{Na} in 6, 8 and 12 mM ATP are depicted in *black*, *gray*, *red*, and *blue*, respectively. **b** Statistics of ATP effects on either −80 mV-induced I_{Na} ($n = 10$) or −150 mV-induced I_{Na} ($n = 9$). The y-axis is the normalized value of peak currents divided by cell capacitance (pF). At −80 mV condition, normalized peak currents were 1.7 ± 0.4 pA/pF (2 ATP), 4.9 ± 1.3 pA/pF (6 ATP), 6.9 ± 1.3 pA/pF (8 ATP), and 5.9 ± 1.5 pA/pF (12 ATP). At −150 mV condition, normalized peak currents were 34.6 ± 2.9 pA/pF (2 ATP), 43.0 ± 4.5 pA/pF (6 ATP), 51.8 ± 4.6 pA/pF (8 ATP), and 43.1 ± 4.5 pA/pF (12 ATP). *$P < 0.05$ compared to 2 ATP

ATP Regulates I–V Curve of Na^+ Channels

We next tested whether the I–V relationship of I_{Na} is affected by ATP. A 100 ms hyperpolarizing prepulse (to −150 mV) was used to exclude the interference of Na^+ channel inactivation (Plant 1988). Representative traces recorded with 2 mM internal ATP was shown in Fig. 2a. I_{Na} was detectable at test pulse potentials with membrane potential more positive than −50 mV, consistent with previous work (Plant 1988). Normalized I–V curves derived with internal ATP levels of 2, 6, 8, and 12 mM are shown in Fig. 2b. I_{Na} increased to the maximal as the test pulse potential reached −20 mV and decreased when the potential was more positive than −20 mV (Plant 1988). Thus, we divided the I–V curve into two parts by the turning point in order to facilitate the fitting and data analysis, defined as $V_{hold}-1$ (−80 to −20 mV) and $V_{hold}-2$ (−20 to +50 mV), both of which were well fitted with Eq. (1) (Fig. 2b, c). These data demonstrated that 8 mM ATP significantly shifts the I–V curve of I_{Na} to the right. Slope factors (k) were also calculated, but no difference was found (Fig. 2d).

ATP Regulates Na^+ Channel Inactivation

We went on to compare the inactivation of Na^+ channels in different $[ATP]_i$. The inactivation of I_{Na} was measured using a test pulse to 0 mV, preceded by a 500 ms conditioning prepulses from −150 to −20 mV (Plant 1988). Transient inward currents were only observed with test pulses preceded by prepulses to potentials to −80 mV or below. When the prepulse was more negative, I_{Na} increased (Fig. 3a). For analysis, individual I_{Na} was normalized to the corresponding maximum current value. Mean values of

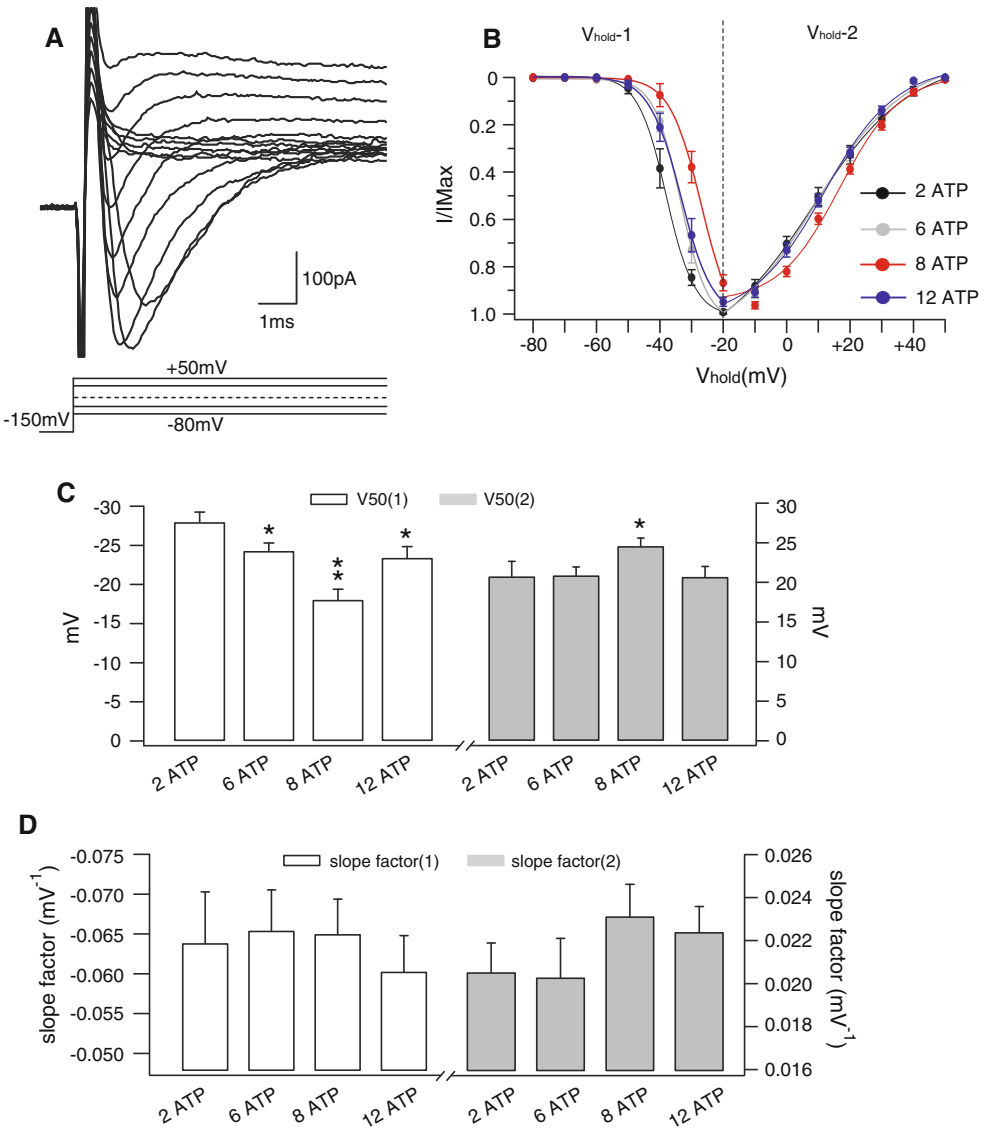

Fig. 2 ATP shifted the I–V curve of I_{Na}. **a** Representative I_{Na} recorded during 20 ms pulses to potentials from −80 to +50 mV following a 100 ms prepulse to −150 mV. **b** Normalized I–V curves of I_{Na} ($n = 10$). Individual I–V curves were normalized to the corresponding maximum current value. $^*P < 0.05$; $^{**}P < 0.01$ compared to 2 ATP. The two phases ($V_{hold}-1$ and $V_{hold}-2$) of the I–V curve are divided by a *dashed line*. **c** Statistics of V_{50}s in various ATP concentrations. V_{50}s in $V_{hold}-1$ were −27.9 ± 1.3 mV (2 mM), −24.3 ± 1.0 mV (6 mM), −18.0 ± 1.4 mV (8 mM), and −23.4 ± 1.5 mV (12 mM). V_{50}s in $V_{hold}-2$ were 20.7 ± 1.9 mV (2 mM), 20.9 ± 1.1 mV (6 mM), 24.6 ± 1.0 mV (8 mM), and 20.7 ± 1.3 mV (12 mM). **d** Statistics of slope factors (k) in various ATP concentrations. k values in $V_{hold}-1$ were −0.064 ± 0.006 mV^{-1} (2 mM), −0.065 ± 0.005 mV^{-1} (6 mM), −0.065 ± 0.004 mV^{-1} (8 mM), and −0.060 ± 0.005 mV^{-1} (12 mM). k values in $V_{hold}-2$ were 0.021 ± 0.001 mV^{-1} (2 mM), 0.020 ± 0.002 mV^{-1} (6 mM), 0.023 ± 0.001 mV^{-1} (8 mM), and 0.022 ± 0.001 mV^{-1} (12 mM)

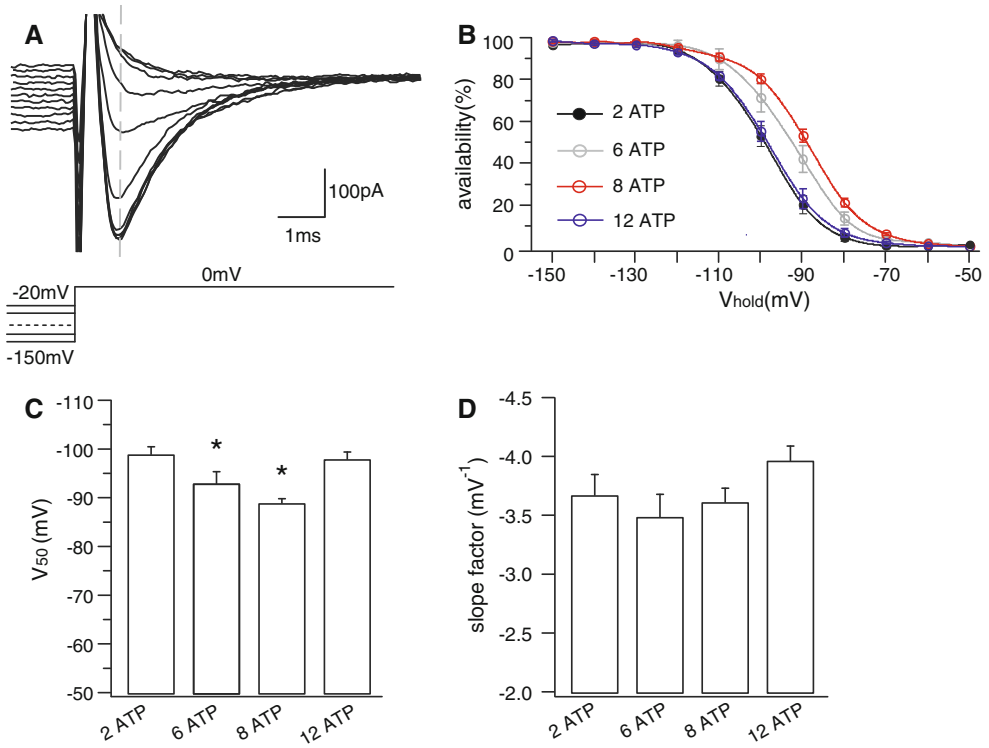

Fig. 3 ATP modulates I_{Na} inactivation in a dose-dependent manner. **a** I_{Na} derived from a single β cell that was given a test pulse to 0 mV preceded by prepulses (500 ms) to potentials between −20 and −150 mV. The internal ATP was 2 mM. **b** I_{Na} is fitted with Eq. (1). *Black*, *gray*, *red*, and *blue* traces represent I_{Na} in 2, 6, 8 and 12 mM ATP, respectively. **c** Statistics of V_{hold} for 50 % inactivation of I_{Na} (V_{50}). V_{50}s were −98.9 ± 1.5, −92.9 ± 2.4, −88.9 ± 0.9 and −97.9 ± 1.4 mV for 2, 6, 8 and 12 mM ATP, respectively. **d** Statistics of slope factors (*k*). *k* values were −3.7 ± 0.2, −3.5 ± 0.2, −3.6 ± 0.1, and −4.0 ± 0.1 mV^{-1} for 2, 6, 8 and 12 mM ATP. *$P < 0.05$ compared to 2 ATP

I_{Na} inactivation at different ATP levels were plotted against prepulse potentials (Fig. 3b). We fit all data with Eq. (1) and found that 6 and 8 mM ATP shifted the I_{Na} inactivation curve to the right, suggesting that ATP modulates the voltage-dependence of inactivation (Fig. 3b, c). Slope factors (*k*) were not affected by ATP (Fig. 3d).

ATP Accelerates Na^+ Channel Recovery From Inactivation

Na^+ channels recover from inactivation when the membrane potential is hyperpolarized (Hille 1992). We used a dual-pulse protocol to test the time required for the recovery (Lou et al. 2003). The dual pulse included two 10 ms pulses: the first prepulse was initiated from a holding potential of −120 to 0 mV and ended at −120 mV. The second (test) pulse was also from −120 to 0 mV and ended at −120 mV (Fig. 4a). Intervals between prepulses and test-pulses were increased during one recording. Cells were held at −120 mV for at least 5 s during the interval between two stimuli in order that the Na^+ channels reached steady-state before the next stimulus (Lou et al. 2003). Recovery was defined as the percentage of the peak amplitude of the second current versus that of the first current. I_{Na} induced by the first pulse was same for all dual-pulse stimuli, while currents induced by the second pulse increased with the increase of interpulse interval (Fig. 4a). We measured the recovery time courses at various internal ATP levels. The relationship between percentages of recovery versus interpulse intervals was depicted in Fig. 4b. All recovery curves from inactivation were well fitted by Eq. (2). The recovery time constants with 2, 6, 8 and 12 mM ATP were 23.5 ($n = 10$), 18.3 ($n = 13$), 15.5 ($n = 13$), and 17.0 ms ($n = 13$), indicating that the recovery of Na^+ channels became faster with high internal ATP and fastest recovery time at 8 mM $[ATP]_i$ (Fig. 4b).

Discussion

In the present work, we demonstrated that ATP increased the peak amplitude of I_{Na}, shifted both the I–V curve and inactivation of I_{Na} to the positive, and accelerated the recovery of I_{Na} in a dose-dependent manner. The resting concentration of $[ATP]_i$ is 3–5 mM in β cells (Detimary et al. 1998; Ainscow and Rutter 2002; Speier et al. 2005), which may increase by 20–40 % in response to the high-glucose stimulation (Ainscow and Rutter 2002; Speier et al. 2005). In the present work,

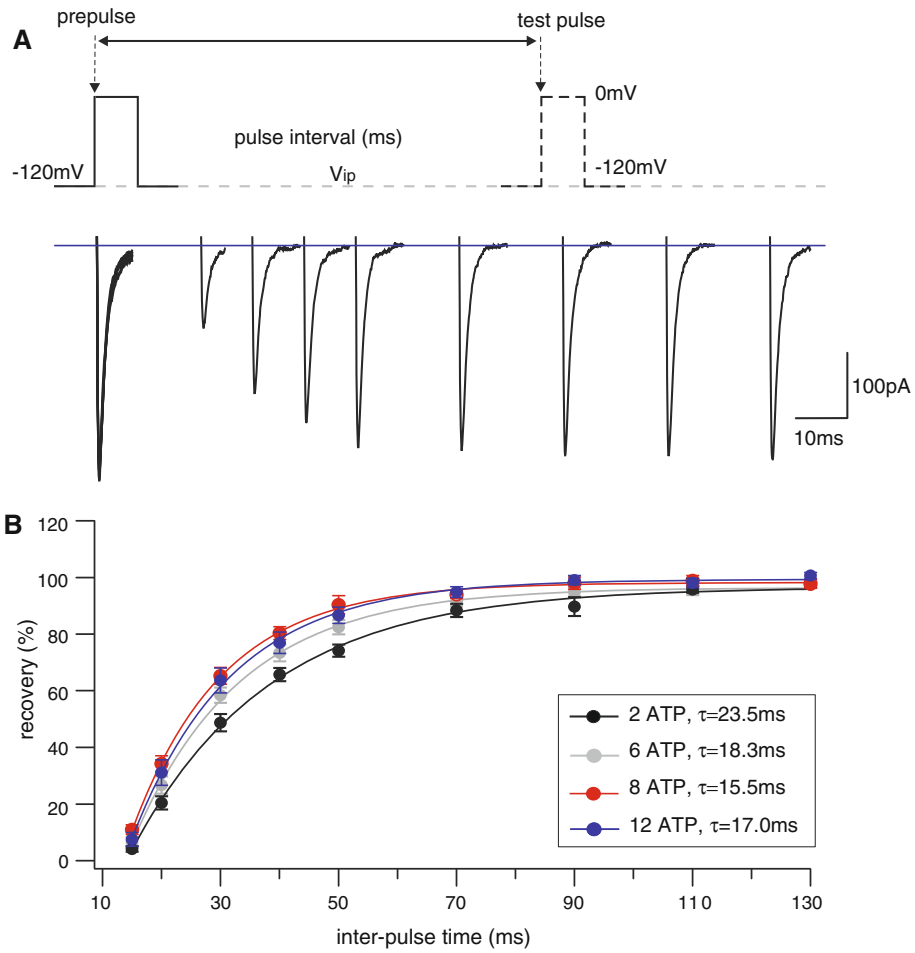

Fig. 4 ATP accelerates Na^+ channel recovery. **a** Time course of recovery of I_{Na} from inactivation. The dual-pulse protocol is shown in the top trace. By changing the interpulse interval, a series of dual pulse-induced currents were presented. Between two dual-pulse stimulations, the cell was held at the interpulse potential (V_{ip}) of −120 mV for at least 5 s. **b** ATP dependence of the recovery time course. *Black*, *gray*, *red*, *and blue traces* represent Na^+ responses in 2, 6, 8, and 12 mM ATP. Each trace was well fitted by Eq. (2) with a time constant (τ), as given in **b** [2 mM ATP ($n = 10$), 6 mM ATP ($n = 13$), 8 mM ATP ($n = 13$), and 12 mM ATP ($n = 13$)]. Note that ATP accelerated the recovery in a dose-dependent manner

we found that I_{Na} reached the maximum in 8 mM ATP, implying that high glucose-induced $[ATP]_i$ allows a large influx of extracellular Na^+ into β cells. Moreover, 8 mM ATP maximally shifted the inactivation of I_{Na} to the right, suggesting that Na^+ channels become more difficult to be inactivated and a stronger hyperpolarization is required for the closure of these channels. In accord with this speculation, we found that high intracellular ATP greatly facilitated the recovery of Na^+ channels from inactivation. On the basis of these data, we considered that β cells have an amplified and prolonged I_{Na} facing ATP elevation so that the membrane conductance and excitability of β cells are greatly enhanced. ATP-related Na^+ channel activation may thus regulate the electrical activity of β cells and participate in insulin secretion. Indeed, it is previously reported that inhibition of I_{Na} with tetrodotoxin reduces glucose-stimulated insulin secretion by 55–70 % (Braun et al. 2008). Our present work did not answer how ATP affects I_{Na}. Further experiments are needed to explore the mechanism of ATP modulation, for example, whether Na^+ channels contain ATP binding motif.

The most typical electrical activity of β cells in high-glucose stimulation is the voltage oscillation (Manning Fox et al. 2006). It is known that K_{ATP}, voltage-dependent Ca^{2+} channels (VGCC), voltage-dependent K^+ channels and Ca^{2+}-dependent K^+ channels participate in oscillation. High glucose-induced intracellular ATP elevation closes K_{ATP} and opens VGCC to produce voltage oscillations. Recent work indicates that closure of K_{ATP} alone is not sufficient to produce enough membrane depolarization and action potentials (Rorsman et al. 2011). Therefore, it is possible that, besides the K_{ATP}, I_{Na} may be involved in the voltage oscillation. High glucose induces oscillations of both intracellular ATP and Ca^{2+} in β cells (Ainscow and Rutter 2002), but whether and how these two oscillations are related remains unknown. Our present work suggested that Na^+ channels may offer to mediate the correlation between $[ATP]_i$ and Ca^{2+} oscillations, because elevated ATP increases Na^+ channel activity and affects the cell excitability.

Distinct from cultured cells (Plant 1988; Lou et al. 2003), pancreatic slice preparation keeps Na^+ channels in a more physiological condition (Speier and Rupnik 2003). Most of previous work was done in clonal cells or primary cultured β cells, in which enzymatic digestion, physical trituration, high-speed centrifugation and overnight culture are frequently used so that the structure and physiological environment of β cells are destroyed. The pancreatic slice

recording approach overcomes these shortcomings and is a useful technique to investigate the functions of endocrine and exocrine cells in the pancreas.

In summary, we demonstrated that intracellular ATP regulates the peak amplitude and kinetics of I_{Na} in a dose-dependent manner. ATP increased the amplitude of inward I_{Na} at a holding potential of either −80 or −150 mV (Fig. 1). ATP shifted the I–V curve of I_{Na} to the right when the holding potential was less than −20 mV (Fig. 2). ATP shifted the inactivation curve of I_{Na} to the right (Fig. 3). The recovery of Na^+ channels was facilitated by high concentrations of internal ATP. The maximal modulatory effects were obtained at 8 mM ATP (Fig. 4). Taken together, our study suggests that the modulation of Na^+ channels by ATP explains how Na^+ channels participate in glucose-stimulated electrical activity and insulin secretion.

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