Properties of voltage-gated Ca^{2+} currents measured from mouse pancreatic β-cells *in situ*

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ABSTRACT

We used the single-microelectrode voltage-clamp technique to record ionic currents from pancreatic β-cells within intact mouse islets of Langerhans at 37ºC, the typical preparation for studies of glucose-induced "bursting" electrical activity. Cells were impaled with intracellular microelectrodes, and voltage pulses were applied in the presence of tetraethylammonium. Under these conditions, a voltage-dependent Ca^{2+} current (I_{Cav}), containing L-type and non-L-type components, was observed. The current measured *in situ* was larger than that measured in single cells with whole-cell patch clamping, particularly at membrane potentials corresponding to the action potentials of β-cell electrical activity. The temperature dependence of I_{Cav} was not sufficient to account for the difference in size of the currents recorded with the two methods. During prolonged pulses, the voltage-dependent Ca^{2+} current measured *in situ* displayed both rapid and slow components of inactivation. The rapid component was Ca^{2+}-dependent and was inhibited by the membrane-permeable Ca^{2+} chelator, BAPTA-AM. The effect of BAPTA-AM on β-cell electrical activity then demonstrated that Ca^{2+}-dependent inactivation of I_{Cav} contributes to action potential repolarization and to control of burst frequency. Our results demonstrate the utility of voltage clamping β-cells *in situ* for determining the roles of ion channels in electrical activity and insulin secretion.

Key terms: islet of Langerhans, insulin, stimulus-secretion coupling, ion channel, inactivation kinetics, BAPTA.

PROLOGUE

I am honored to participate in this well-deserved homage to Eduardo "Guayo" Rojas, my mentor, colleague, role model and friend of the last twelve years. I honestly had no idea what a tremendous impact Guayo would have on my life when I first met him on a sweltering, late-spring day in Bethesda in 1993. I was a graduate student at Johns Hopkins University, and Guayo had generously offered to let me spend the summer in his lab learning electrophysiological techniques. Guayo immediately took me under his wing and taught me the essentials of ion channel biophysics and pancreatic β-cell physiology. I soon realized that Guayo was a 'hands-on' scientist who loved being in the lab to help solve problems or to test out new ideas. His knowledge, enthusiasm, and creativity were captivating, and my

'summer visit ' turned into seven years of graduate and postdoctoral training under Guayo's tutelage. Guayo was truly an outstanding mentor. He saw my potential and motivated me to challenge myself to the fullest. He nurtured my technical skills and taught me to think analytically. He shared his experience and vision, while always encouraging independent thought. But perhaps his greatest skill was inspiring confidence – I will always remember him as the first person to refer to me as an 'expert' in anything. Of course, as with everyone he knew, Guayo treated my wife and me like family, and some of my most cherished moments with him occurred outside the lab. In short, I will be forever indebted to Guayo for his commitment to my professional and personal development. I hope that by emulating his approach to research, mentoring, and to life itself, I can help to pass his legacy on to future

generations of scientists, so that they too can benefit from the masterpiece that was Eduardo Rojas's career.

INTRODUCTION

In the electrically excitable pancreatic β-cell, glucose metabolism is linked to insulin secretion by membrane depolarization and Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Mears, 2004; Rorsman, 1997). Deciphering the mechanisms that regulate β-cell membrane potential therefore is essential for understanding physiological control of insulin secretion. Beta cell electrical activity is typically studied using microdissected mouse islets of Langerhans, in which glucose induces a rhythmic electrical pattern known as 'bursting' (Dean and Matthews, 1968; Dean and Matthews, 1970; Henquin and Meissner, 1984). Bursting consists of membrane potential oscillations between 'active' phases, with Ca^{2+}-dependent action potentials arising from a depolarized plateau, and hyperpolarized 'silent' phases (see Figure 6). During the active phase of glucose-induced electrical activity, enhanced activity of voltage-dependent Ca^{2+} channels produces a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$), which serves as the principal trigger for insulin secretion by exocytosis (Gilon et al., 1993; Rosario et al., 1986; Santos et al., 1991)

The advent of the patch-clamp technique led to the identification and characterization of numerous ion channels in β-cell (reviewed in: Ashcroft and Rorsman, 1989; Mears and Atwater, 2000). The consensus model for glucose-induced electrical activity that has emerged from these efforts is as follows: glucose metabolism produces an increase in the intracellular ATP/ADP ratio, resulting in closure of ATP-sensitive K^+ (K_{ATP}) channels (Ashcroft et al., 1984; Cook and Hales, 1984). The membrane depolarization resulting from closure of K_{ATP} channels in turn activates voltage-dependent Ca^{2+} and K^+ channels, which are responsible for the upstroke and repolarization, respectively, of the action potentials of the active phase.

Conspicuously absent from this model is the mechanism that controls bursting and its exquisite glucose sensitivity, as well as explanations for the electrical effects of neurohormonal agents that affect insulin secretion.

The difficulty in fully elucidating the mechanisms of β-cell electrical activity may stem from differences in the conditions under which ion channel properties and β-cell membrane potential normally are studied. Unlike membrane potential recordings, most patch-clamp experiments have been performed on isolated, cultured β-cells or insulinoma cell lines, at temperatures below 37°. Islet-like bursting electrical activity and $[Ca^{2+}]_i$ oscillations (having a frequency of 1-6 min^{-1}) rarely are observed under these conditions (Bertram et al., 2000). The majority of single β-cells respond to glucose with very fast membrane potential oscillations or continuous spiking activity (Bertram et al., 2000; Falke et al., 1989; Kinard et al., 1999; Zhang et al., 2003), although very slow oscillations occasionally have been observed (Bertram et al., 2000; Larsson et al., 1996; Smith et al., 1990). Conversely, the $[Ca^{2+}]_i$ oscillations that result from glucose-induced electrical activity tend to be much slower in single β-cells than in islets (Gilon et al., 1994; Gylfe et al., 1998; Hellman et al., 1992; Jonkers et al., 1999; Ravier et al., 2005), but much faster patterns also have been reported (Zhang et al., 2003). The distinct behavior of isolated β-cells compared to intact islets motivates the development of methods to measure ionic currents from β-cells *in situ,* under conditions more closely resembling typical membrane potential recording experiments.

We previously demonstrated the feasibility of voltage clamping β-cells within intact mouse islets, using high-resistance intracellular electrodes attached to a conventional patch-clamp amplifier (Mears et al., 1995; Rojas et al., 1995). Voltage-gated K^+ currents measured with this technique were smaller than those observed in single, cultured β-cells with conventional whole-cell patch clamping and were modulated by glucose and HCO_3^- (Rojas et al., 1995). More recently, others

have applied the perforated patch-clamp technique to β-cells within enzymatically isolated islets in short-term culture (Goforth et al., 2002; Gopel et al., 1999a; 1999b; 2004). This approach offers the advantage of using low-resistance electrodes, but generally is limited to cells on the periphery of the islet, many of which are not β-cells. With this method, Gopel and co-workers (1999a; 1999b) found that the voltage-dependent Ca^{2+} current and a novel Ca^{2+}-activated K^+ current were larger in β-cells within intact islets than in single cells. Capacitance measurements of exocytotic granule fusion made with the same technique showed that the maximum rate of depolarization-evoked exocytosis was less than 5% of that observed in single β-cells (Gopel et al., 2004). Together, the available data support the premise that β-cells in their native environment are fundamentally different from isolated cells in culture, and as such, measurements of ionic currents from cells *in situ* could provide new insights into the mechanisms controlling β-cell electrical activity.

Owing to their well-appreciated role in stimulus-secretion coupling, voltage-gated Ca^{2+} channels in the β-cell have been studied extensively (Mears, 2004; Yang and Berggren, 2005). Whole-cell patch-clamp measurements from cultured mouse β-cells invariably reveal the presence of a high-voltage activated (HVA) Ca^{2+} current (I_{Cav}) with small amplitude (generally <15 pA/pF in 2.6 mM Ca^{2+}) (Barg et al., 2001; Hopkins et al., 1991; Jing et al., 2005; Kinard and Satin, 1996; Plant, 1988; Rorsman and Trube, 1986; Schulla et al., 2003). During sustained depolarizing voltage pulses, I_{Cav} displays rapid but incomplete Ca^{2+}-dependent inactivation (Gopel et al., 1999b; Plant, 1988; Satin and Cook, 1989), as well as a slower component of inactivation mediated by voltage (Hopkins et al., 1991; Satin and Cook, 1989). Cook and colleagues (1991) have suggested that slow inactivation and reactivation of I_{Cav} could mediate the phase transitions of bursting electrical activity.

Molecular and pharmacological studies reveal that mouse β-cells express several classes of HVA Ca^{2+} channels (Barg et al.,

2001; Gilon et al., 1997; Jing et al., 2005; Schulla et al., 2003; Seino et al., 1992; Yang et al., 1999). Of these, L-type, dihydropyridine-sensitive channels play the most direct role in regulation of insulin secretion. The α1 subunits of L-type channels physically associate with the cellular secretory apparatus, such that their activity leads to rapid exocytosis of a readily releasable pool of insulin granules (Barg et al., 2001; Schulla et al., 2003; Wiser et al., 1999). Non-L-type Ca^{2+} channels (R and P/Q) may carry up to 50% of I_{Cav} in β-cells, but their activity is not tightly coupled to granule exocytosis (Schulla et al., 2003). The non-L-type channels may be involved in controlling electrical activity, global $[Ca^{2+}]_i$ dynamics, or Ca^{2+}-dependent processes other than granule fusion, including replenishment of the readily releasable pool (Jing et al., 2005; Pereverzev et al., 2002; Schulla et al., 2003).

In this study, we used our previously described single-microelectrode voltage-clamp technique to measure I_{Cav} from β-cells in intact mouse islets of Langerhans. We report that, while the current is qualitatively similar to that observed in isolated cells with the patch-clamp technique, some quantitative differences are observed that may offer insights into the role of voltage-gated Ca channels in β-cell electrical activity. The *in situ* voltage-clamp technique is therefore a useful complement to traditional biophysical and molecular approaches in β-cell electrophysiology.

METHODS

Measurements of Ca^{2+} currents from β-cells in situ

The single-microelectrode voltage-clamp technique for intact islets is adapted from the classical method for recording β-cell membrane potential with intracellular microelectrodes and has been described in detail elsewhere (Mears et al., 1995; Rojas et al., 1995). Briefly, NIH Swiss mice (2-5 months old) were sacrificed in a CO_2

chamber and the pancreas removed. Intact islets of Langerhans were dissected from the pancreas and mounted in a sample chamber where they were perifused continuously with a modified Krebs solution (in mM: 120 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$; equilibrated with 95%: 5% O_2: CO_2 to obtain pH 7.4 at 37ºC). Cells within the islet were impaled with a glass microelectrode (~120-150MΩ when filled with a 1: 1 mixture of 1 M KCl: 1M K-citrate) using an amplifier equipped with a positive feedback "cell-puncture circuit." Beta-cells were identified based on bursting electrical activity in the presence of 11 mM glucose. Once a β-cell had been impaled, a switch was used to disconnect the preparation from the current-clamp amplifier and connect it to a patch-clamp amplifier that was then used for both voltage-clamp and current-clamp measurements.

Depolarizing voltage pulses were applied from a holding potential of -80 mV, and current signals were filtered at 2 kHz prior to being digitized and stored. Linear components of the current responses were removed from the records using a P, P/4 leak subtraction protocol. Sherman et al. (1995) have shown that this method effectively removes linear current components (including currents through K_{ATP} channels, gap junctions, and leaks), as long as the surrounding cells are not firing action potentials. Series resistance compensation was not used, but during off-line analysis the I-V curves were corrected for the voltage drop across the electrode tip, as described below.

Inward currents were recorded in isolation by replacing 20 mM NaCl with 20 mM tetraethylammonium (TEA) chloride in the perfusate to block voltage-dependent K^+ channels. The Ca^{2+} concentration of the Krebs solution was reduced to 1.3 mM in order to reduce the size of the Ca^{2+} current and improve the quality of the voltage clamp. Currents were recorded in the presence of 3 mM extracellular glucose so that surrounding cells would be electrically silent and the linear subtraction protocol would be effective. Nifedipine and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) were added to the perifusate from stock solutions in dimethylsulfoxide.

In the single-microelectrode voltage-clamp technique, current flowing across the series resistance of the electrode tip creates an error between the command voltage and the actual membrane potential achieved (Rojas et al., 1995). In order to estimate the actual membrane potential during each voltage pulse, a current record in response to the pulse was obtained without leak subtraction. The voltage drop across the electrode tip during the pulse was calculated by multiplying the magnitude of the current by the tip resistance, measured when the electrode was removed from the cell. The actual holding potential was then determined by subtracting the calculated tip voltage from the command potential.

The current records shown here are the average of at least 3 traces from the same cell. Because the voltage drop across the electrode tip differed in each experiment, the actual holding potentials during application of the pulse protocol also varied from experiment to experiment. Therefore, to construct the composite I-V curves shown in Figure 2, data points having similar membrane potential after correction for electrode resistance were binned and averaged. All other I-V curves represent data from individual cells but are representative of results from at least four experiments.

Whole-cell patch-clamp experiments

For comparison, inward currents were measured from cultured β-cells with the whole-cell configuration of the patch-clamp technique. Islets of Langerhans were isolated from the pancreas of NIH Swiss mice by collagenase digestion and dispersed into single cells in a Ca^{2+}- and Mg^{2+}-free medium supplemented with dispase (Mears and Zimliki, 2004). Cells were plated onto glass coverslips and cultured for 2-6 days at 37ºC in a 5% CO_2 atmosphere, using RPMI 1640 medium supplemented with 10% fetal bovine serum.

The coverslips formed the bottom of a sample chamber on the stage of an inverted microscope. Cells were bathed in an

extracellular solution containing (in mM): 140 TEA-Cl, 5 CsCl, 1.1 $MgCl_2$, 5 $CaCl_2$, 5 Na-Hepes, 11 glucose, pH 7.4. Pipettes were filled with (in mM) 100 Cs-aspartate, 20 CsCl, 1 $MgCl_2$, 3 Mg-ATP, 10 Cs-Hepes, 10 4-aminopyridine, 3 EGTA, pH 7.2, (resistance 2-4 MΩ). Seals were made on the membranes of individual cells and gentle suction was applied to obtain the whole-cell configuration. Voltage pulses were applied and current responses were recorded as described above for the experiments *in situ.* Series resistance was less than 10 MΩ and was not compensated.

Data analysis and statistics

Results are presented as mean ± SEM. Statistical significance was analyzed with paired and unpaired t-tests. In the I-V curves shown below, the points were connected using the 'spline' interpolation feature of the graphing software (Origin 6.1, OriginLab Corp., USA). The same software package also was used for curve fitting.

RESULTS

Voltage-dependent Ca^{2+} currents recorded from β-cells in situ

As we described previously (Rojas et al., 1995), in normal Krebs buffer with no TEA added, depolarizing voltage pulses from a holding potential of -80 mV yielded families of currents displaying initial small inward components that were rapidly overwhelmed by a delayed outward current (*data not shown*). When the islet was then perifused with Krebs solution containing 1.3 mM Ca^{2+} and 20 mM TEA^+, depolarizing voltage pulses elicited families of inward currents that rose to a peak and inactivated incompletely during a 100 msec pulse (Fig. 1A). In experiments lasting as long as 30 minutes, the magnitude of the inward current did not decrease ("run-down"), as is characteristic of experiments with the whole-cell configuration of the patch-clamp technique (Falke et al., 1989; Plant, 1988). Figure 1B shows the voltage dependence of both the peak current and the current remaining at the end of the 100 msec voltage pulses, from a representative *in situ* voltage-clamp experiment. The membrane potential values were corrected for the voltage drop across the tip of the electrode. Note that, while the pulse protocol was intended to reach a membrane potential as high as +60 mV, only about +20 mV actually was achieved, which was below the reversal potential of the current.

The majority of the inward current was carried by L-type Ca^{2+} channels, as demonstrated by the effect of nifedipine (Figure 1C). At a concentration of 10 μM, the dihydropyridine reversibly blocked 62 ± 10% (n=5) of the peak current elicited by 100 mV pulses. Higher concentrations of nifedipine did not induce further inhibition. The current also was insensitive to 1 μM tetrodotoxin, an antagonist of voltage-gated Na^+ channels (data not shown). From these experiments, we concluded that the inward current observed in the presence of 20 mM TEA is a voltage-dependent Ca^{2+} current (I_{Cav}), containing both L-type and non-L-type components.

Figure 2 compares the I-V properties of I_{Cav} measured from β-cells *in situ* (n=10) and from single β-cells with conventional whole-cell patch clamping (n=5). The most notable difference was the magnitude of the current, being larger when measured in intact islets. Thus, despite the extracellular Ca^{2+} concentration being four times lower for the *in situ* measurements, the peak current reached a maximum of 129 ± 6 pA, compared to only 44 ± 8 pA for the patch-clamp experiments (Fig. 2A). Because the relative inactivation occurring during the pulses was greater *in situ*, the sustained current did not differ as dramatically between the two techniques, with maximum values of 46 ± 5 pA *in situ* and 26 ± 9 with patch clamping (Fig. 2B). Nevertheless, considering the difference in extracellular Ca^{2+} concentration used in the two experimental procedures, the data indicate that the Ca^{2+} conductance was larger throughout the voltage pulse when the current was measured *in situ* (statistical analysis was not applied to the data in Fig. 2, since the membrane potential values were different for the two experimental methods).
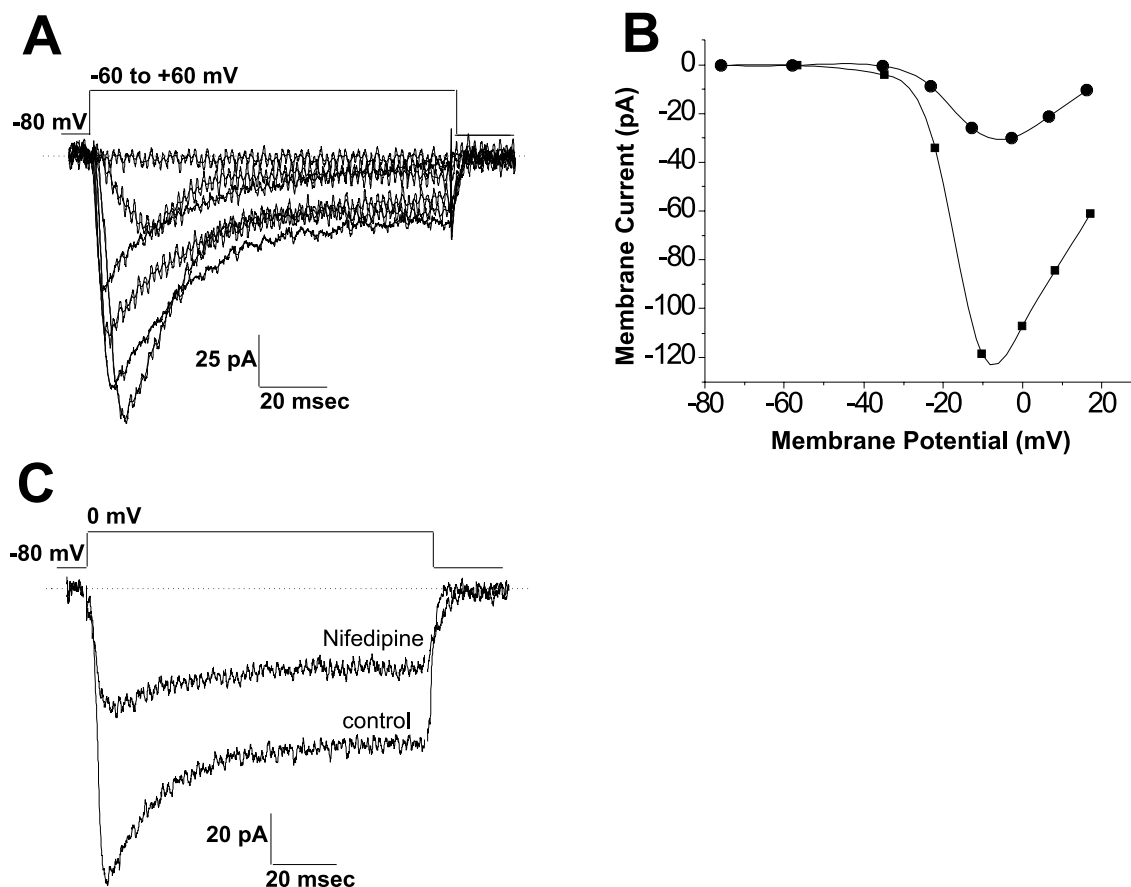
Figure 1. Voltage-dependent Ca^{2+} current recorded from β-cells in situ. A: Family of current traces recorded from a β-cell in situ, in response to the illustrated voltage pulse protocol. B: I-V curves of the peak current (■) and current remaining at the end of each 100 msec voltage pulse (●) for the experiment illustrated in part A. Membrane potential values were corrected for the voltage drop across the electrode tip. C: Inward currents recorded from a β-cell in situ in response to an 80 mV pulse, before and after addition of 10 μM nifedipine to the perifusate.

The I-V curves from the *in situ* experiments were also left-shifted compared to those from the patch-clamp experiments. Thus, the peak current reached its maximum at -8 ± 1 mV *in situ* and at +9 mV with patch clamping (Fig. 2A). Finally, although the I-V curves in Figure 2 do not indicate directly the reversal potential of I_{Cav}, extrapolation of the splines, connecting the data points in Figure 2A suggests a reversal potential of approximately +50 mV for both the patch-clamp and *in situ* measurements. Since the theoretical equilibrium potential for Ca^{2+} was much more positive in both experiments, the reversal of I_{Cav} at +50 mV

likely reflects permeation of ionic species other than Ca^{2+}.
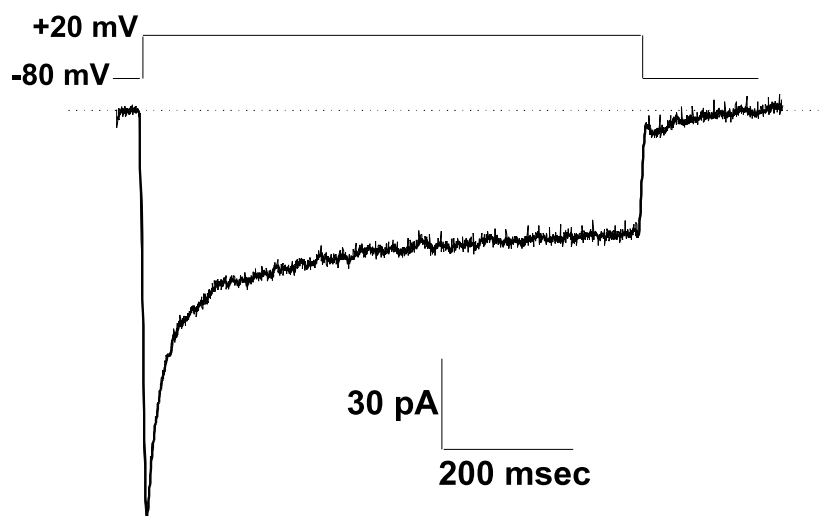
I_{Cav} displays fast and slow inactivation

Satin and Cook (1989) first showed that, in addition to the rapid inactivation occurring during a short voltage step, I_{Cav} in the β-cell continues to inactivate in a voltage-dependent manner throughout a prolonged pulse. Figure 3 shows a current trace recorded from a β-cell *in situ* in response to a 750 msec voltage step from -80 mV to +20 mV. As expected from the data of Figure 1, a portion of the current inactivated rapidly following the peak. The remaining current

then continued to inactivate at a slower rate throughout the pulse. For the trace shown in Figure 3, the time course of the current decay following the peak was best fit by a three-exponential model, with time constants of 29 msec, 115 msec, and 2700 msec. In each of six other experiments, the inactivation of I_{Cav} during a prolonged voltage pulse was described by a three-exponential model, with one time constant ranging from 15 to 30 msec, the second time constant between 100 and 400 msec, and the third time constant between 1000 and 3000 msec. The high variability in the time constants from experiment to experiment likely reflects differences in the voltage drop across the electrode tip, which caused the actual membrane potential during the pulse to differ in each recording. Nevertheless, the results demonstrate that multiple components of inactivation of I_{Cav}, which were first identified by patch clamping single β-cells in culture, are also observed with the *in situ* voltage-clamp technique.
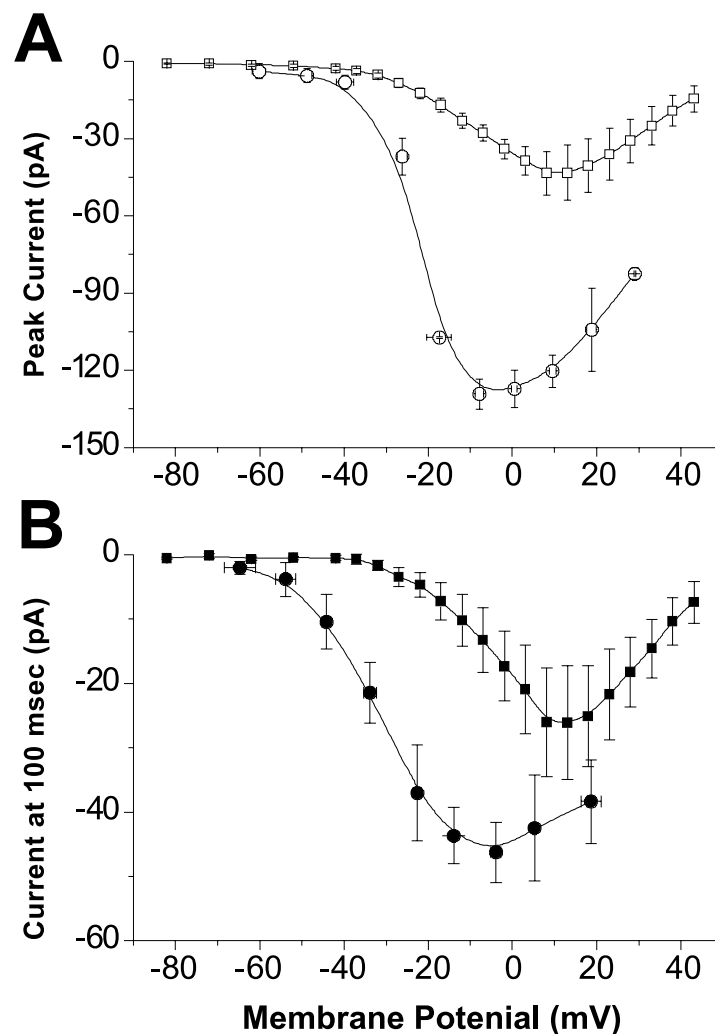
Figure 2. I-V curves of I_{Cav} measured with the *in situ* voltage-clamp method (O, n=10) and the whole-cell patch-clamp method (□, n=5). **A:** Peak current; **B:** Current remaining at the end of each 100 msec voltage pulse. Data from the *in situ* experiments were corrected for the voltage drop across the electrode tip, and points having similar membrane potential were binned to calculate individual points. Extracellular Ca^{2+} concentration was 1.3 mM for the *in situ* measurements and 5 mM for the patch-clamp measurements.

+20 mV
-80 mV

30 pA

200 msec

Figure 3. Fast and slow inactivation kinetics of I_{Cav}. Current trace elicited by a 100 mV voltage pulse of 750 msec duration. The initial rapid inactivation was followed by a slower component of inactivation that continued throughout the voltage pulse. The illustrated trace is representative of results from eight experiments.

The rapid inactivation is Ca^{2+} dependent

To study the properties of the rapid component of inactivation of I_{Cav}, we used a two-pulse protocol consisting of an initial voltage pulse of variable magnitude, followed by a brief return to the baseline holding potential, and finally, a test pulse to a constant potential (see inset in Fig. 4B). Figure 4A shows a trace from a representative experiment. In this case, both the initial and test pulses (not corrected for the electrode voltage drop) were to +40 mV. The magnitude of I_{Cav} was smaller during the test pulse, reflecting the inactivation that occurred during the initial pulse. In the inactivation curve of Figure 4B, the peak magnitude of I_{Cav} elicited by the test pulse (normalized to the peak observed in the absence of an initial pulse) is plotted versus the potential of the initial pulse. Due to the voltage drop across the electrode tip, few data points were obtained corresponding to very depolarized initial pulses. Nevertheless, a characteristic U-shape, reminiscent of the I-V curve of I_{Cav}, is apparent in Figure 4B, with maximum inactivation (62%) occurring for initial pulses between -10 mV and +10 mV. These data are indicative of Ca^{2+}-dependent inactivation in which the extent of inactivation is related to the magnitude of

the Ca^{2+} current (Gopel et al., 1999b; Plant, 1988; Satin and Cook, 1989). Similar results were observed in four other experiments.

We next sought to determine the effects of altering the Ca^{2+} buffering capacity of the β-cell on the properties of Ca^{2+}-dependent inactivation. To this end, I_{Cav} was recorded from β-cells *in situ*, before and after loading the cell with the membrane-permeable Ca^{2+} chelator, BAPTA-AM. As shown in Figure 5A, exposing the islet to 25 μM BAPTA-AM for 10 minutes did not affect the peak current elicited by a 100 mV depolarizing pulse. However, BAPTA did reduce the rate of inactivation, and as a result, the current measured at the end of the 100 msec pulse was larger after the cell was loaded with the Ca^{2+} chelator (77 pA control versus 115 pA after BAPTA exposure). In Figure 5B, a family of voltage pulses was applied, and the ratio of the current remaining at 100 msec to the peak current was calculated for each pulse. As with the double-pulse protocol, the inactivation curve was U-shaped, with maximum inactivation in this experiment occurring at approximately -10 mV. At all potentials, the ratio of sustained to peak current was greater after the cell was loaded with BAPTA-AM, indicating that less inactivation had occurred. Similar results were observed in four additional experiments.

The previous results suggest that membrane permeable Ca^{2+} chelators could be useful tools to study the functional role of Ca^{2+}-dependent inactivation of I_{Cav}. Therefore, we recorded glucose-induced electrical activity from β-cells in mouse islets before and after exposure to BAPTA-AM (25 μM for 10 minutes). In the experiment shown in Figure 6, the average magnitude of the action potentials of the active phase increased from 16 mV to 21 mV following exposure to BAPTA-AM. This change was accompanied by an increase in the frequency of the glucose-induced burst pattern, from 3 to 5 bursts per minute. In four experiments, exposure of mouse islets to BAPTA-AM increased the action potential height from 17 ± 1.1 mV to 23 ± 1.8 mV ($p<0.05$), and the burst frequency from 4.7 ± 0.6 min^{-1} to 6.9 ± 0.8 min^{-1} ($p<0.02$). These data indicate that rapid, Ca^{2+}-dependent inactivation of I_{Cav} contributes to the dynamic pattern of glucose-induced electrical activity in the β-cell.
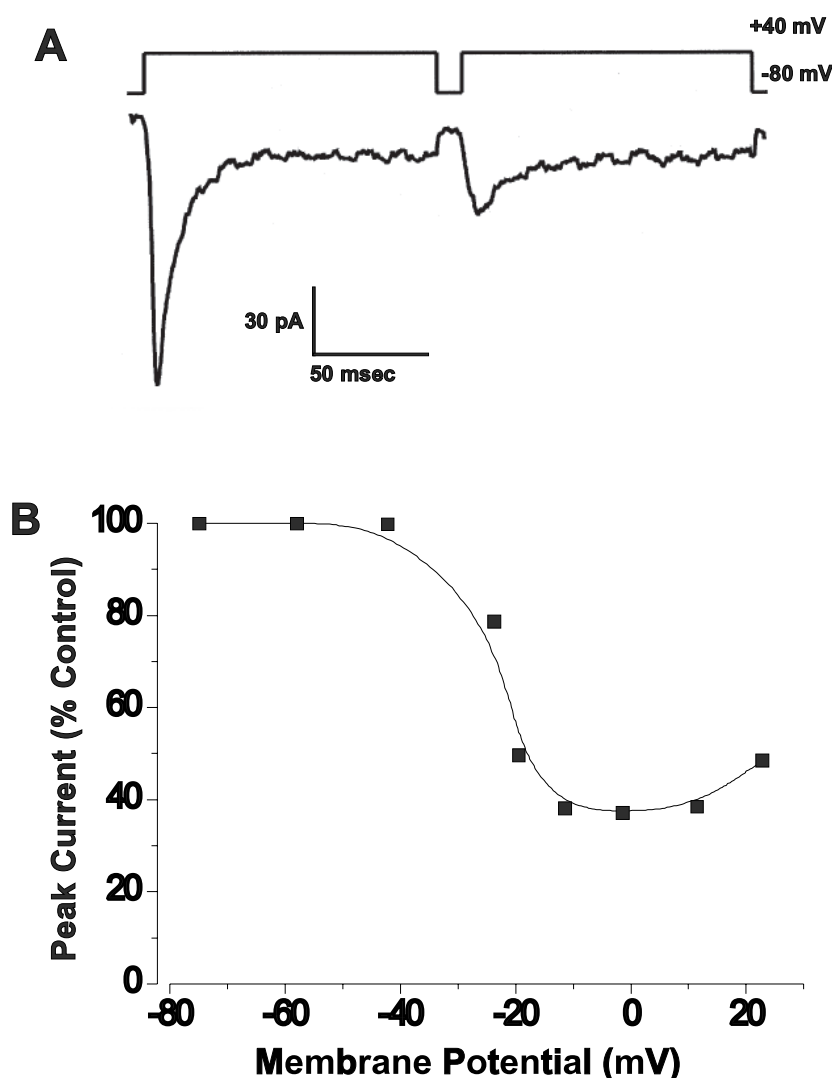
Figure 4. A: Current response elicited by the illustrated voltage pulse protocol, consisting of consecutive depolarizing pulses (interpulse interval = 10 msec). **B:** A two-pulse protocol (inset) was applied to a β-cell *in situ*. The peak current elicited by the second pulse is plotted against the membrane potential of the initial pulse (corrected for the voltage drop across the electrode tip). Data were normalized to the peak current elicited by the second pulse in the absence of an initial pulse.
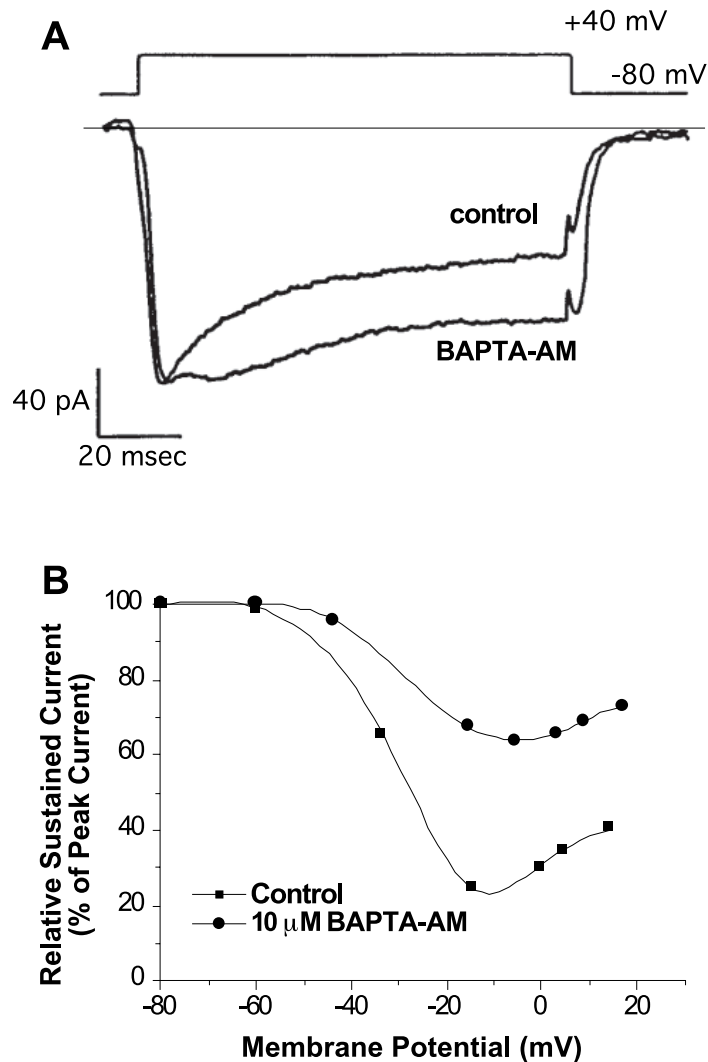
Figure 5. Rapid inactivation is attenuated by BAPTA. **A:** Superimposed current traces recorded from a β-cell *in situ* in response to a 100 msec voltage step to +40 mV, before (control) and after the islet was exposed to 25 μM BAPTA-AM for 10 minutes. **B:** A family of voltage pulses was applied to a cell before and after exposure to BAPTA-AM, and the ratio of sustained current (measured 100 msec into the voltage pulse) to peak current was plotted for each pulse. Following exposure to BAPTA-AM, the ratio was greater at all potentials (indicating less relative inactivation during the pulse).

Effect of temperature on voltage-dependent Ca^{2+} currents

Finally, we explored whether differences in temperature can account for the dramatically larger magnitude of I_{Cav} observed with the *in situ* voltage-clamp technique. Figure 7A shows current traces recorded from a β-cell in an intact islet at 37°C and 29°C. I_{Cav} activated more slowly at the lower temperature, peaking 8 msec into the pulse compared to 5 msec at 37°C. The peak current was also 36% smaller at 29°C. However, the rapid inactivation was less pronounced at 29°C, so the magnitude of the current at the end of the pulse was only 28% smaller than at physiological temperature. Figure 7B shows the voltage dependence of the peak currents observed when families of voltage pulses were

applied at the two temperatures. The peak current was up to 40% smaller at 29°C. However, I_{Cav} measured at 29°C with 1.3 mM extracellular Ca^{2+} was still larger than in patch-clamp experiments with 5 mM extracellular Ca^{2+} (compare Fig. 2A). Similar results were seen in three other experiments. These results indicate that the temperature dependence of I_{Cav} does not fully account for the current being larger when measured with the *in situ* technique.
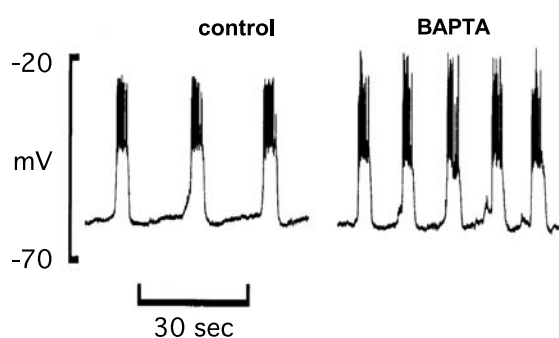
Figure 6. Effect of BAPTA on β-cell membrane potential. Glucose-induced electrical activity was recorded from a β-cell before and after the islet was exposed to 25 μM BAPTA-AM for 10 minutes. The glucose concentration was 11 mM throughout the experiment.

DISCUSSION

Single-microelectrode voltage-clamp method for β-cells in situ: Motivation, advantages and limitations

Electrical activity in the pancreatic β-cell has been the subject of considerable study for nearly four decades. During this time, it has become evident that the bursting electrical activity first described by Dean and Matthews (Dean and Matthews, 1968), with a characteristic frequency of 1-6 oscillations per minute, is a property of cells within intact islets of Langerhans (Bertram et al., 2000; Falke et al., 1989; Kinard et al., 1999; Zhang et al., 2003). In a comprehensive study of glucose-induced electrical activity in isolated mouse β-cells, Bertram and co-workers (2000) found that only 6% of the cells oscillated with 'islet-like' frequencies, whereas very fast bursting or non-oscillatory activity was observed in 92% of the cells. Therefore, efforts to elucidate the mechanisms governing 'islet-like' bursting will likely benefit from measurements of the biophysical properties of ion channels from β-cells within their native environment.

We previously demonstrated that, owing to their small size and relatively low resting membrane conductance, the membrane potential of pancreatic β-cells can be clamped with high-resistance intracellular microelectrodes (Rojas et al., 1995). This allows the measurement of ionic currents under the same conditions that are classically used to study β-cell electrical activity (i.e., cells within freshly microdissected islets, perfused with bicarbonate-buffered Krebs solution at 37°C). Furthermore, with this approach, β-cells can be identified based on their well-defined glucose-induced electrical response in intact islets, and currents can be measured from cells at various depths within the islet, without evidence of run-down during long experiments.

Despite the potential benefits of the method described here, voltage clamping through high-resistance electrodes sets limitations on the technique that must be considered. Most notably, current flowing across the electrode tip, which includes a substantial linear component through gap junctions and residual K_{ATP} conductance (Gopel et al., 1999b), produces a voltage drop that cannot be compensated easily with conventional patch-clamp amplifiers. We therefore devised a method to measure the electrode voltage drop and correct the I-V curves during off-line analysis. As observed in the I-V curves presented here, the electrode voltage drop makes it difficult to achieve very depolarized membrane potentials. However, we should point out that, while the behavior of the β-cell ionic currents at such high potentials may be of biophysical interest, physiologically the membrane potential rarely, if ever, exceeds 0 mV.
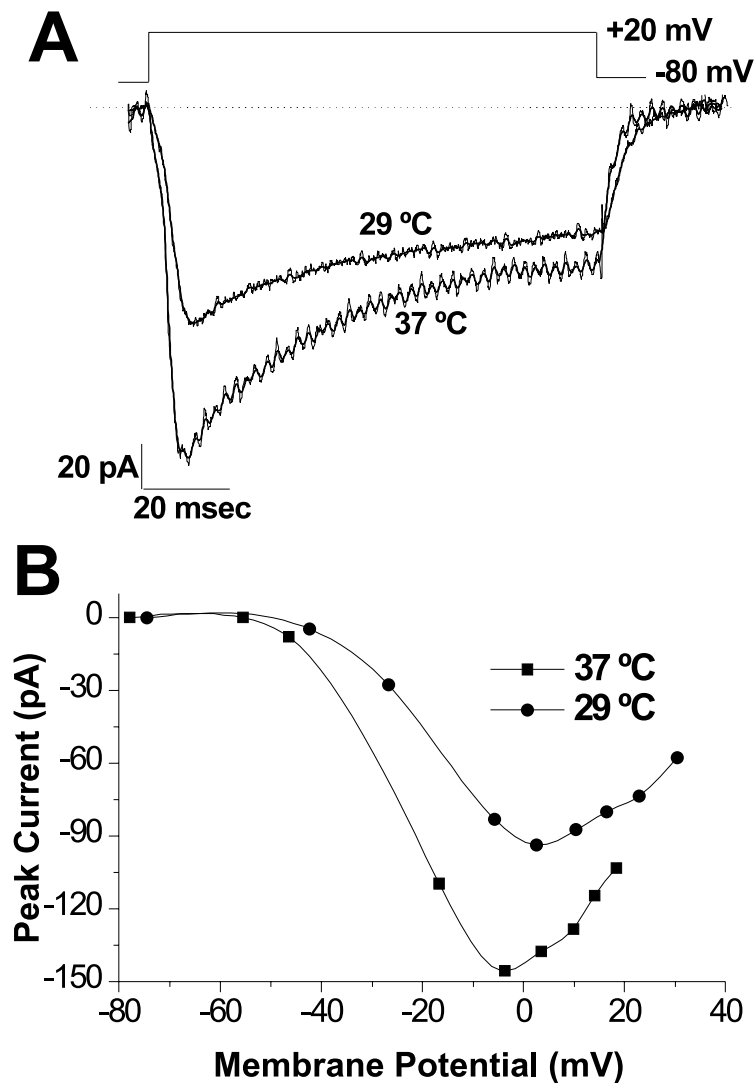
Figure 7. Effect of temperature on I_{Cav}. **A:** Current elicited by a 100 mV potential step, recorded at 37°C and 29°C from a β-cell *in situ*. **B:** I-V curves of I_{Cav} measured at 37°C and 29°C. Membrane potential values were corrected for the electrode voltage drop. Data for A and B are from the same experiment and are representative of results from 5 cells.

A second limitation relates to charging the β-cell membrane (capacitance ~ 10 pF) through intracellular microelectrodes, which is associated with a time constant of up to 1.5 msec. For this reason, we could not use the technique to study very fast processes associated with I_{Cav}, such as activation kinetics and tail current decay. However, processes with time constants greater than 10 msec, such as the inactivation kinetics studied here, can be resolved with this approach. In fact, in preliminary simulations with a mathematical model we found that time constants of >10 msec can be measured with less than 12% error when voltage clamping through resistances as high as 150 MΩ (not shown).

The magnitude of I_{Cav} is greater when measured in situ

As we showed previously (Rojas et al., 1995), depolarizing voltage pulses applied in the presence of the K+ channel antagonist TEA elicited a rapidly activating, voltage-dependent inward current with complex

inactivation kinetics. We previously identified this current as I_{Cav}, based on its dependence on Ca^{2+} in the extracellular medium. Here, we found that L-type (nifedipine-sensitive) Ca^{2+} channels carry about 60% of I_{Cav}. This result agrees with previous studies showing that L-type channels account for 50-90% of I_{Cav} in mouse β-cells (Gilon et al., 1997; Gopel et al., 1999b; Plant, 1988; Schulla et al., 2003). The nifedipine-resistant component of I_{Cav} in mouse β-cells has been attributed to R- and P/Q-type Ca^{2+} channels (Jing et al., 2005; Pereverzev et al., 2002; Takahashi et al., 2000). Influx of Ca^{2+} through these channels most likely drives the residual component of glucose-induced insulin secretion that is often observed in the presence of dihydropyridines. However, these channels are not sufficient to produce bursting electrical activity, which is completely suppressed by antagonists of L-type channels (Henquin and Meissner, 1984).

Gopel et al. (1999b) first reported that the voltage-gated Ca^{2+} current measured from β-cells *in situ* with the perforated patch technique was larger than that observed in single cells. We found a similar result when comparing our in situ measurements of I_{Cav} to measurements made with the whole-cell patch-clamp technique under 'typical' conditions (cultured β-cells, room temperature, Hepes buffer). We also found that the I-V curve of I_{Cav} measured *in situ* was slightly shifted to the left, probably because the lower extracellular Ca^{2+} levels used in the *in situ* experiments resulted in less surface charge screening. As a result of the left shift, the difference in the magnitude of I_{Cav} was most pronounced at potentials from -45 to -10 mV. Interestingly, this range covers the action potentials of glucose-induced β-cell electrical activity (see Fig. 6). This result suggests that Ca^{2+} influx during the spiking phase of bursting electrical activity is greater than would be predicted based on conventional patch-clamp experiments and provides a physiological basis for β-cells to achieve the $[Ca^{2+}]_i$ increases necessary to stimulate insulin secretion despite maintaining relatively hyperpolarized membrane potentials.

Since the magnitude of I_{Cav} in β-cells is temperature dependent (Kinard and Satin, 1996), we examined the possibility that temperature differences between our *in situ* experiments and whole-cell recordings could account for the difference in current magnitude observed with the two techniques. We found that the effect of temperature on I_{Cav} was too small to fully account for the data of Figure 2, showing that other mechanisms must be explored. For example, within the intact islet, β-cells are exposed to various intercellular and paracrine signals that could modulate Ca^{2+} current density. It also is possible that the process of dispersing islets into single cells or their maintenance in culture for patch-clamp experiments reduces the magnitude of I_{Cav} through an as yet unidentified mechanism.

I_{Cav} inactivates with fast and slow kinetics

The inactivation of I_{Cav} during prolonged voltage pulses followed a three-exponential model with fast, intermediate and slow components. The slow component of inactivation measured here is reminiscent of the slow, voltage-dependent inactivation of Ca^{2+} currents (time constant 2.75 sec) first observed in single β-cells (Hopkins et al., 1991; Satin and Cook, 1989). It was suggested that this component of inactivation could be the feedback mechanism that drives bursting electrical activity. However, mathematical models in which a single process drives bursting electrical activity suggest that burst frequencies of 1-6 min^{-1} require a process having a time constant of tens of seconds (Sherman, 1996). As such, the slow inactivation of β-cell Ca^{2+} currents observed both *in situ* and in single cells is probably too fast to be the sole mediator of islet-like bursts. However, since candidate processes with a time constant of tens of seconds are lacking, more recent models have explored the possibility that islet bursting could be driven by the interaction of fast (time constant 1-5 sec) and slow (time constant 1000-3000 msec) processes (Bertram et al., 2000; Bertram and Sherman, 2004). The 'slow' inactivation of I_{Cav} therefore could be considered a

candidate for one of the 'fast' processes involved in producing islet bursting.

In accord with several previous studies, we found that the fast component of inactivation of I_{Cav} was Ca^{2+} dependent (Gopel et al., 1999b; Plant, 1988; Satin and Cook, 1989). This process often has been studied by substituting extracellular Ba^{2+} for Ca^{2+}, but we found this approach difficult with intracellular electrodes, due to the development of a large leak current in the absence of Ca^{2+}. However, by using two-pulse protocols and studying the retarding effects of a membrane-permeable Ca^{2+} chelator, we were able to confirm that the rapid inactivation observed *in situ* is Ca^{2+}dependent. Since Ca^{2+}-dependent inactivation is mediated by Ca^{2+} in the microdomain near the mouth of the channel rather than bulk $[Ca^{2+}]_i$, the retarding effect of BAPTA-AM indicates that the chelator effectively buffers the Ca^{2+} level in the immediate vicinity of the channel.

Finally, we found that BAPTA-AM increased both the size of the action potentials of glucose-induced electrical activity and the frequency of the bursts. The former observation provides direct evidence that Ca^{2+}-dependent inactivation of I_{Cav}, which occurs on a time scale similar to a β-cell action potential, plays a role in action potential repolarization. However, since bursting occurs on a much slower time scale than Ca^{2+}-dependent inactivation of I_{Cav}, acceleration of the burst pattern by BAPTA is not intuitive. In fact, changes in $[Ca^{2+}]_i$ often have been proposed to control bursting electrical activity directly or indirectly (Atwater et al., 1979; Gopel et al., 1999a; Henquin, 1990; Rolland et al., 2002; Rosario et al., 1993). As such, slowing the rate of change of $[Ca^{2+}]_i$ by increasing the Ca^{2+} buffering capacity of the cell would be expected to reduce the frequency of the bursts. One possible explanation for the contrary result is that the BAPTA-mediated increase in burst frequency is a consequence of the enhancement of action potential amplitude. The enhancement has the effect of increasing the average membrane potential during the active phase, which in turn could activate an additional voltage-dependent process with fast kinetics. Indeed,

in a recent theoretical study, we showed that changes in action potential height were associated with dramatic changes in burst frequency (Zimliki et al., 2004). However, at this point we do not know the nature of the fast mechanism that could be activated by an increase in action potential magnitude. Another possibility is that the Ca^{2+} buffer causes a transient depletion of Ca^{2+} from the endoplasmic reticulum, which has been shown to increase burst frequency (Bertram et al., 1995; Worley et al., 1994).

In summary, we have shown that it is feasible to measure I_{Cav} from pancreatic β-cells *in situ*, under conditions that closely resemble those of typical membrane potential recording experiments. The properties of the currents measured with the in situ technique are qualitatively similar to those measured with conventional patch clamping, but quantitative differences in the size and voltage dependence are apparent that will likely provide new insights into roles of voltage-dependent Ca^{2+} channels in β-cell electrical signaling. Our results with Ca^{2+} chelators provide a specific example of how agents that affect the kinetic properties of ionic currents can be used for mechanistic studies of β-cell electrical activity and give unexpected evidence that burst frequency is partly controlled by fast, Ca^{2+}-dependent inactivation of I_{Cav}. We expect that this technique also will be useful for future studies of electrical signaling in human pancreatic β-cells and β-cells from animal models of obesity and diabetes.

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