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

DAVID MEARS and EDUARDO ROJAS

Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile

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

Correspondence: David Mears, Ph.D., Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Santiago, Chile, Tel.: (56-2) 978-6360, Fax: (56-2) 777-6916, E-mail: dmears@med.uchile.cl

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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 37º. 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).