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Single-Microelectrode Voltage Clamp Measurements of Pancreatic β-Cell Membrane Ionic Currents *In Situ*

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Abstract. A conventional patch clamp amplifier was used to test the feasibility of measuring whole-cell ionic currents under voltage clamp conditions from β-cells in intact mouse islets of Langerhans perifused with bicarbonate Krebs buffer at 37°C. Cells impaled with a high resistance microelectrode (*ca.* 0.150 GΩ) were identified as β-cells by the characteristic burst pattern of electrical activity induced by 11 mM glucose. Voltage-dependent outward K^+ currents were enhanced by glucose both in the presence and absence of physiological bicarbonate buffer and also by bicarbonate regardless of the presence or absence of glucose. For comparison with the usual patch clamp protocol, similar measurements were made from single rat β-cells at room temperature; glucose did not enhance the outward currents in these cells. Voltage-dependent inward currents were recorded in the presence of tetraethylammonium (TEA), an effective blocker of the K^+ channels known to be present in the β-cell membrane. Inward currents exhibited a fast component with activation-inactivation kinetics and a delayed component with a rather slow inactivation; inward currents were dependent on Ca^{2+} in the extracellular solution. These results suggest the presence of either two types of voltage-gated Ca^{2+} channels or a single type with fast and slow inactivation. We conclude that it is feasible to use a single intracellular microelectrode to measure voltage-gated membrane currents in the β-cell within the intact islet at 37°C, under conditions that support normal glucose-induced insulin secretion and that glucose enhances an as yet unidentified voltage-dependent outward K^+ current.

Key words: Islet of Langerhans — K^+ currents — Ca^{2+} currents — L-type Ca^{2+} channel — Glucose — Bicarbonate

Introduction

Glucose-induced pulsatile insulin secretion from a single perifused mouse islet of Langerhans in vitro is correlated with bursting oscillations of membrane potential in β-cells *in situ* within the islet of Langerhans (Atwater et al., 1980; Scott, Atwater & Rojas, 1981; Meda, Santos & Atwater, 1986; Rosario, Atwater & Scott, 1986). Our early attempts to resolve which ionic currents are involved in the burst pattern of electrical activity (Atwater, Ribalet & Rojas, 1978, 1979*b;* Atwater et al., 1981) was hampered by the inability to voltage-clamp a single β-cell in the islet. The present work explores the feasibility of a single-microelectrode voltage clamp system for β-cells *in situ* in the intact islet of Langerhans.

Using low resistance patch clamp microelectrodes, measurements of whole-cell membrane currents (Rorsman & Trube, 1986; Plant, 1988; Ashcroft, Rorsman & Trube, 1989; Hopkins, Satin & Cook, 1991) and single channel characteristics (Cook & Hales, 1984; Misler et al., 1986; Carroll et al., 1988; Bokvist, Rorsman & Smith, 1990*a,b;* Mancilla & Rojas, 1990; Rojas et al., 1990; Kelly, Sutton & Ashcroft, 1991) have allowed detection of specific membrane channels present in the β-cell (Atwater, Carroll & Li, 1989). Glucose-evoked electrical activity under current clamp conditions has also been measured from single cultured mouse islet β-cells either isolated or forming part of small clusters (Smith, Ashcroft & Rorsman, 1990*b*). However, despite these efforts, it has been impossible to explain the glu-

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cose-induced burst pattern of electrical activity which is only measured in β-cells in clusters or intact islets of Langerhans. It has been suggested that cell-to-cell coupling may be necessary for bursting (Sherman & Rinzel, 1991; Smolen, Rinzel & Sherman, 1993).

Owing to the difficult task of making the high resistance seal required to electrically fuse the pipette with the β-cell membrane at 37°C, the majority of experiments have been carried out at temperatures below 30°C and in the absence of bicarbonate buffer, experimental conditions that sustain glucose-induced electrical activity, with only subtle differences, but abolish glucose-stimulated insulin secretion (Henquin & Lambert, 1976; Atwater et al., 1984; Carroll et al., 1990). Thus, differences in experimental conditions and the as yet unknown effect that cell-to-cell coupling may have on β-cell ionic currents, suggest that there may be other currents involved in controlling the burst pattern. We therefore considered the possibility of using the single-microelectrode voltage clamp technique (Finkel & Redman, 1984) to measure the ionic currents from pancreatic β-cells in the intact islet at 37°C under perfusion conditions known to support glucose-induced insulin secretion.

We show here that it is possible to measure ionic currents from a β-cell *in situ* in a perifused islet of Langerhans using a single-microelectrode voltage clamp technique. We also show that it is possible to isolate the time-dependent ionic currents from the linear, time-invariant, current flowing between the impaled cell and electrically coupled cells using suitable subtraction protocols (Sherman, Xu & Stokes, 1995). The method has enabled us to measure specific ionic currents, and study for the first time the effects of glucose and $NaHCO_3$/$-HCO_3^-$ buffer on voltage-gated K^+ and Ca^{2+} currents at 37°C from β-cells *in situ* within a perifused islet of Langerhans.

Materials and Methods

A detailed description of the in vitro microelectrode recording technique for microdissected mouse pancreatic islets has been described previously (Atwater et al., 1978). Partly dissected mouse islets were continuously perifused with a modified Krebs solution (in mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 2.5 $CaCl_2$; 1.1 $MgCl_2$, equilibrated with 95% O_2/5% CO_2, pH 7.4 at 37°C. Tetraethylammonium (TEA) chloride was added to the Krebs solution when specified from a 1 M TEA-Cl stock solution. For the Ca^{2+}-deficient Krebs bicarbonate buffer, no $CaCl_2$ was added and $MgCl_2$ was augmented to 3.7 mM. For bicarbonate-deficient Krebs, 25 mM NaHEPES was used to adjust the pH and the solution was equilibrated with 100% O_2. Current measurements in 0-HCO_3 Krebs were made no sooner than 5 min after switching to Krebs-deficient solutions.

Membrane potentials were measured between two Ag-AgCl electrodes, one in the external solution and the other in the intracellular microelectrode. Microelectrodes were filled with a high K^+ solution (50% 1 M KCl, 50% 1 M K citrate) and had tip resistances of *ca.* 0.15 GΩ. The large potential difference between the two Ag-AgCl electrodes was compensated by electrically connecting the chamber Ag-AgCl electrode to a variable voltage source which had one terminal grounded (Fig. 1: *Ag-electrode offset adjust*). Prior to the penetration of the β-cell membrane, the output of the current clamp intracellular recording amplifier (indicated as *CC* in Fig. 1) was adjusted to read zero using the Ag-electrode offset voltage source (Fig. 1). The temperature of the perifusion solution was controlled at 37 ± 1°C and was continuously monitored by means of a miniature thermistor probe placed in the islet chamber.

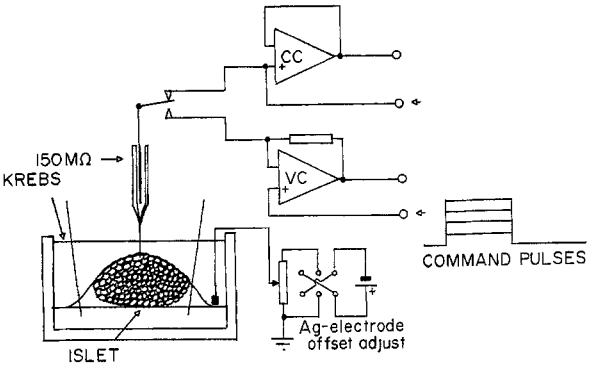

Fig. 1. Diagram of the amplifier arrangement used to measure membrane currents from an islet cell *in situ*. The solution (*KREBS*) in the perifusion chamber (volume = 40 μl) was exchanged at a rate of 50 μl/sec. Temperature was maintained at 37 ± 1°C using a peltier heating element placed in thermal contact with the solution and a thermistor for feedback to the controller (*not shown*). A microdissected mouse islet of Langerhans (*ISLET*) was fixed by entomological pins through adherent acinar tissue. *CC* represents the head stage of the amplifier used for current clamp measurements of V_m. *VC* represents the head stage of the patch clamp amplifier through which the *COMMAND PULSES* were delivered. The silver/silver chloride (*Ag*) *electrode* in the chamber was connected to the *offset adjust*.

Ionic currents under voltage clamp conditions were recorded using a List amplifier (EPC-7, List Electronics, Darmstadt-Eberstadt, Germany). The amplifier was used without series resistance compensation. A switch activated by a magnet enabled us to change from our standard current-clamp, membrane-potential-measuring amplifier, equipped with a "cell-puncture circuit" to impale the β-cells (*CC* in Fig. 1), to the patch clamp amplifier (*VC* in Fig. 1).

Voltage clamp protocols were under computer control. The control system (Lab Master DMA board and TL-1 125 kHz interface, Axon Instruments, Burlingame, CA) generated the voltage pulses (indicated as *command pulses* in Fig. 1) and digitized the voltage clamp currents. Current transients were fed through a low-pass filter set at 1–2 kHz (8-pole Bessel, Frequency Devices, model 902-LPF, Haverhill, MA) to the 12-bit analog-to-digital converter. Both on-line data acquisition and off-line analysis of the current records were carried out using a software package (pCLAMP, version 5.5.1, Axon Instruments) installed in the PC. Linear leak subtraction was accomplished by adding to each current record in response to a depolarizing pulse **P**, four current records in response to hyperpolarizing pulses of size **−P/4.** For comparison, this same pulse protocol was also applied to measure whole-cell currents using the nystatin-perforated membrane patch method in cultured rat pancreatic β-cells (Smith et al., 1990*b*).

Standard patch clamp experiments were performed on single cultured rat β-cells, comparing the activity of the glucose-sensitive K^+-channel (K_{ATP} channel) recorded with high potassium in the patch pipette (in mM: 135 KCl, 2.6 $CaCl_2$, 10 NaHEPES at pH 7.4) to the

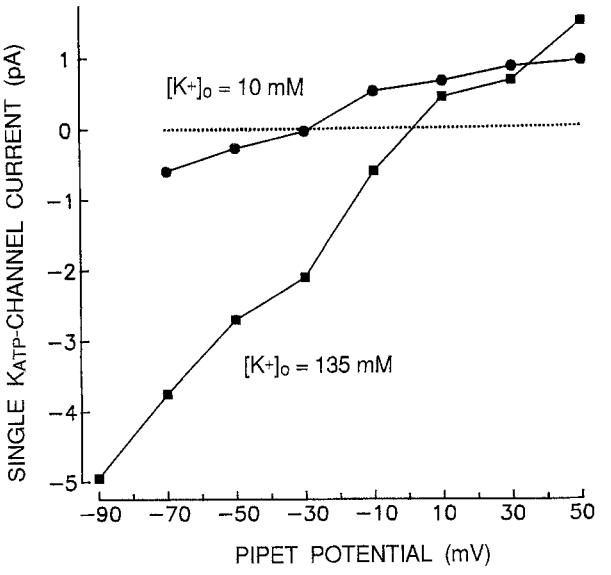

Fig. 2. Single K_{ATP} channel current-voltage relationships in low (10 mM) and high (135 mM) external potassium. K_{ATP} channel activity was recorded using the cell-attached configuration of the patch clamp technique. Symbol: (*) 10 mM K^+ and (■) 135 mM K^+ in the pipette (external aspect of the membrane). Dotted line indicates zero-current level.

activity recorded with a low potassium solution similar to the external medium (in mM: 10 KCl, 135 NaCl, 2.6 $CaCl_2$, 10 NaHEPES at pH 7.4). Under the latter conditions, the K_{ATP} channel conductance was greatly reduced, even for outward currents, and the rectification of the current disappeared, as illustrated in Fig. 2. Positive currents (graphed above the dotted line in Fig. 2) represent outward movement of K^+. Using high potassium in the pipette, conductances were *ca.* 55 pS for inward and *ca.* 26 pS for outward K^+ currents (■). These values are similar to those reported by others (Cook & Hales, 1984; Carroll et al., 1988; Bokvist et al., 1990*a,b;* Kukuljan, Li & Atwater, 1990; Fournier et al., 1992). However, using low external potassium, we report here that the current-voltage relationship was nearly linear (*) with a chord conductance of *ca.* 11 pS. Therefore, the pulse protocol used here effectively subtracts any contributions of currents flowing through K_{ATP} channels.

Classical feedback amplifiers for voltage clamping include a differential amplifier to measure the membrane potential (V_m) using two microelectrodes, one placed inside the cell and the other placed in the external medium. A third microelectrode, usually of low tip resistance (R_e), is used to inject the current (I_c) provided by the feedback amplifier to control V_m to the commanded potential (V_c). With the three-microelectrode voltage clamp system both V_m and I_c are measured independently.

The current injected by the voltage clamp amplifier (I_c) flows into the impaled cell through a microelectrode (with resistance, R_e) and out of the cell through a leakage pathway (R_L) and membrane-resident ion channels (R_{in}). Hence, the voltage drop across the resistance R_e of the microelectrode used to inject current plus the voltage drop across the membrane V_m will be equal to the command V_c,

$$V_c = V_m + V_e \tag{1}$$

where

$$V_e = R_e \cdot I_c. \tag{2}$$

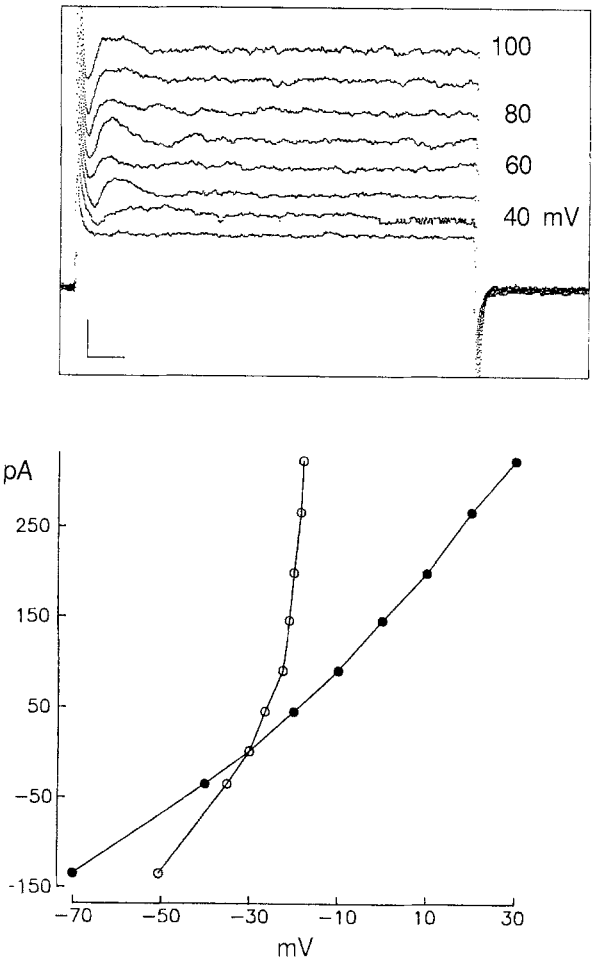

Fig. 3. Correction for the effects of R_e on calculated *I-V* curves. Records made in the absence of glucose at a command holding potential, $V_{c,h}$, of −70 mV requiring a current I_h of −130 pA. Electrode resistance, R_e, was 0.15 GΩ. (Upper panel) Superimposed current records in response to depolarizing pulses, $V_{c,P}$, of increasing amplitude in 10 mV steps. Vertical calibration, 70 pA; horizontal calibration, 10 msec. Values of the commanded potentials are given in mV next to the corresponding record. (Lower panel) Uncorrected (*) and corrected (○) *I-V* curves.

From Eqs. (1) and (2) we get

$$V_m = V_c - R_e \cdot I_c. \tag{3}$$

Using the three-microelectrode voltage clamp configuration, if R_e is known one can correct the command voltage V_c on-line (Eq. 3). However, the single-microelectrode system does not allow simultaneous and independent measurements of V_m and I_c. Therefore, on-line correction based on Eq. (3) is not available in the voltage clamp system used here.

To correct the error due to V_e, at least in part, we acquired a family of current records in response to depolarizing pulses of increasing size (10 mV increments, no subtraction). The current records ($I_{c,P}$ records) depicted in Fig. 3 (upper panel) acquired using this pulse protocol exhibit, in addition to the linear components of the current, nonlinear components. Linear components of $I_{c,P}$ include the current flowing into the electrically coupled cells, leak currents and, in the absence of glucose, the current flowing through open K_{ATP} channels.

At each commanded $V_{c,P}$ the change in current $I_{c,P}$ was measured towards the end of the pulses. The equivalent resistance R_c (= $R_{in} + R_e$) was calculated as the ratio

$$R_c = V_{c,P}/I_{c,P}. \quad (4)$$

The whole-cell equivalent resistance R_{in} (= $R_c - R_e$) was estimated as

$$R_{in} = V_{c,P}/I_{c,P} - R_e. \quad (5)$$

Membrane potential values between pulses $V_{m,h}$ and during depolarizing pulses $V_{m,P}$ were calculated as follows,

$$V_{m,h} = V_{c,h} - I_h \cdot R_e, \quad (6)$$

$$V_{m,P} = V_{m,h} + [V_{c,P}/R_c) \cdot R_e] \quad (7)$$

where I_h represents the current required to hold the potential at a commanded value $V_{c,h}$.

To illustrate how we used Eq. (7) to correct $V_{c,P}$ to take into account the effects of R_e, we deliberately selected the worst possible situation: an islet in the absence of glucose, with a β-cell exhibiting a low resting potential of *ca.* −34 mV (instead of *ca.* −65 mV). Plots of I_c as a function of uncorrected $V_{c,P}$ (*) and corrected $V_{m,P}$ (○) as defined in Eq. (7) are shown in Fig. 3. Adjusting the command of $V_{c,h}$ to −70 mV required a holding current I_h of −130 pA. Since the electrode resistance R_e was *ca.* 0.15 GΩ, we estimate that the β-cell membrane was held at −50.5 mV ($V_{c,h} - V_e = -70 + 19.5$). Thus, the example illustrates that, in the worst possible situation, voltage corrections amounted to a 20 mV shift for the holding potential. The corrections used are given in the figure legends for each experiment illustrated. the *I-V* curve (*) corresponding to the steady-state uncorrected membrane current ($I_c = I_h + I_{c,P}$; Fig. 3, lower panel) was used to estimate the input resistance R_{in} as 0.12 GΩ ($R_c - R_{elec}$), found here to be within the range of values previously reported (Atwater et al., 1978).

Finally, in data reported beyond Fig. 3, the nonlinear voltage-dependent components of the total ionic currents (Fig. 3) were isolated on-line using the standard **P-P/4** protocol to remove linear currents. It should be noted that the linear components of the current, which account for a substantial proportion of the total current, include the current flow owing to cell-to-cell coupling (Sherman et al., 1995) and the current flowing through open K_{ATP} channels and other nonvoltage gated channels. From the measurements of coupling currents (Eddlestone et al., 1984; Perez-Armendariz et al., 1991), the current flowing into electrically coupled cells may account for about half of the subtracted linear portion.

Each type of experiment presented here was repeated at least three times on different islets. Superimposed current records shown in the remaining figures represent the average of at least three families from the same cell and were digitally filtered (low-pass set at 2 kHz).

Results

Glucose-induced Periodic Bursting Recorded under Current Clamp and Voltage Clamp Conditions

The majority of the islet cells successfully impaled responded to glucose (11 mM) as illustrated in Fig. 4. A segment of a continuous recording of the membrane potential V_m (upper trace) together with the corresponding segment of the current injected by the feedback amplifier I_c are depicted (lower trace). In the current clamp mode (indicated as *CC*), with the current I_c held at zero, V_m during the silent phases was *ca.* −54 mV. Switching the circuit to the voltage clamp mode (indicated as *VC*) and adjusting the command $V_{c,h}$ to −80 mV required the injection of a negative holding current I_h equal to −25 pA in the silent phase. For this experiment R_e was *ca.* 0.18 GΩ and, therefore $V_{m,h}$ was only −75.5 mV (this represents a shift of 4.5 mV, Eq. 6). As defined in Eq. (4), R_c can be calculated as the difference between $V_{c,h}$ (−80 mV) and V_m (−54 mV), i.e., −26 mV, divided by the size of the injected current I_h (−25 pA), i.e., 1.1 GΩ. In the presence of glucose (11 mM), and including only those experiments in which R_e before and after the impalement changed by less than 26%, we noted that R_{in} varied from cell to cell over a wide range of values (0.1 to 1.5 GΩ).

To remove the linear components from the records depicted in Fig. 3 (upper panel), we applied the **P-P/4** pulse sequence as shown in Fig. 4 (upper trace). Our protocol consisted of eight depolarizing pulses **P** of increasing size (in 10 mV steps), each pulse preceded by four hyperpolarizing prepulses of size **−P/4.** A chart record of I_c in response to these pulses is also depicted in Fig. 4 (lower trace).

In the presence of stimulatory concentrations of glucose (≥6 mM), under voltage clamp conditions we always observed that I_c exhibited periodic bursting (Fig. 4, lower trace). These bursts are presumably due to current flow from neighboring coupled cells that continue to burst (Sherman et al., 1995). To avoid contamination of the records of current transients in response to rectangular depolarizing pulses, we selected those records acquired between the bursts. Sherman et al. (1995) present a model analysis of these coupling currents that supports this selection.

Effects of Glucose on Outward Ionic Currents

In the absence of glucose, application of depolarizing pulses from a commanded holding potential $V_{c,h}$ of −70 mV ($V_{m,h}$ of −67 mV) generated records which exhibited both inward and outward currents (Fig. 5*A*). The outward current reaches a peak value and then declines towards a steady-state level. The corresponding *I-V* curve measured towards the end of the pulses is depicted in Fig. 5*C* (*). The horizontal axis represents the potential during the pulses corrected for the voltage across R_e (V_e). In the presence of glucose (Fig. 5*B*) setting $V_{c,h}$ at −70 mV ($V_{m,h}$ of −63 mV) the net outward currents are larger than in its absence and the transient component of the outward currents is virtually absent from the records. The corresponding *I-V* curve corrected for the V_e error is also shown in Fig. 5*C* (○).

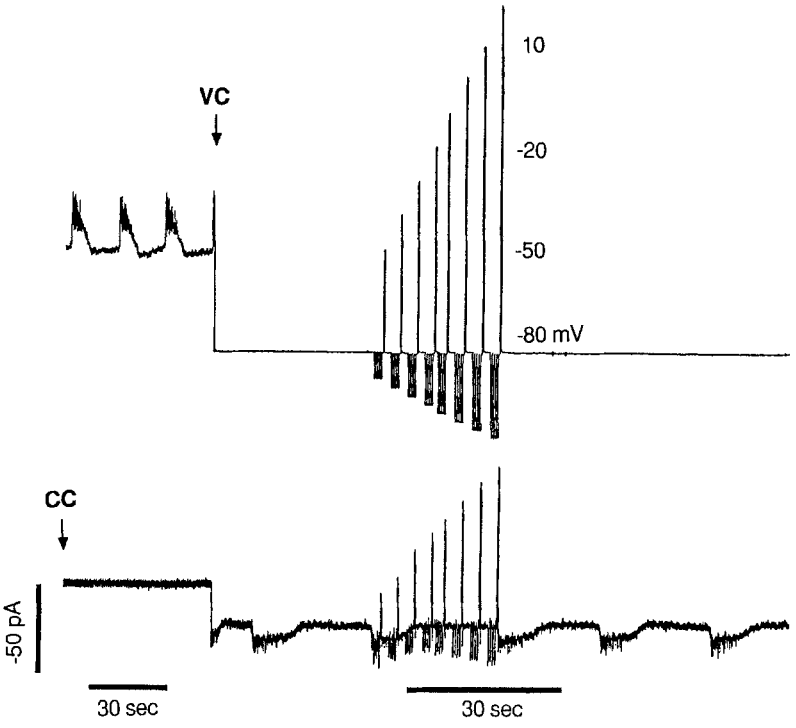

Fig. 4. Standard protocol to measure V_m under current clamp and I_c under voltage clamp conditions. (Upper trace) Left side of V_m record was obtained under current clamp (*CC*) conditions (holding current was set to 0) and then under voltage clamp (*VC*) conditions, as indicated by the arrow. (Lower trace) I_c record was obtained under CC and VC conditions. Under VC conditions the command holding potential $V_{c,h}$ was set to −80 mV and this required a holding current, I_h, of −25 pA during the silent, inter-burst phases. I_c records in response to the application of **−P/4** prepulses and **P** pulses are shown in the middle. Some V_c values during the **−P/4** prepulses and the **P** pulses are indicated in mV on the upper trace for orientation. The duration of both hyperpolarizing prepulses and depolarizing pulses was 108 msec. R_e = 0.18 GΩ.

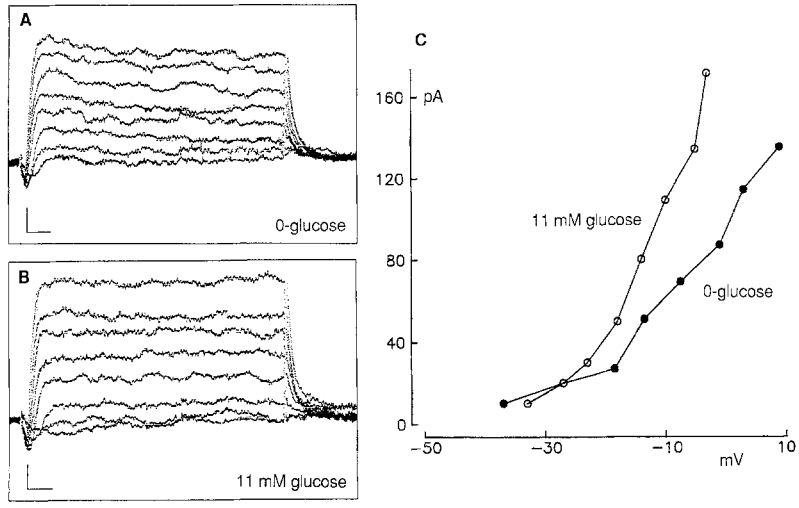

Fig. 5. Inward and outward currents in the presence or absence of glucose (11 mM). Each family of superimposed β-cell membrane current records in *A* and *B* represents the average of 3–4 series of records. Calibrations (lower left side corner in *A* and *B*): Vertical, 30 pA and horizontal, 10 msec. R_e = 0.21 GΩ. V_m in the absence of glucose was −61 mV and, in the presence of glucose (silent phase) was −46 mV, $V_{c,h}$ was set at −70 mV throughout; the corresponding I_h values were −14 and −33 pA, and the calculated $V_{m,h}$ values (Eq. 6) were −67 and −63 mV. In the absence and presence of glucose the calculated R_c values (Eq. 4) were 0.27 and 1.7 GΩ, respectively. *C* shows the *I-V* curves after correction for V_e: symbols, (*) 0-glucose, (○) 11 mM glucose.

Figure 6 depicts three records of whole-cell currents from Fig. 5 at $V_{c,P}$ of 10, 20 and 30 mV in the absence (*A*) and presence (*B*) of glucose (11 mM). After correction for V_e, the true membrane potentials during the pulses were −1, 3 and 9 mV in the absence (*A*) or −10, −5 and −3 mV in the presence (*B*) of glucose. Only the end of the current record during and tail currents after the pulse are shown on an expanded time base. After the corrections in $V_{c,h}$ and $V_{c,P}$, for the voltage drop across the microelectrode (V_e), the comparison that best reflects the true cell behavior is between the lowest value in Fig. 6*A*, 47 pA ($V_{m,P}$ = −1 mV, $V_{m,h}$ = −67 mV), and the highest value in Fig. 6*B*, 145 pA ($V_{m,P}$ = −3 mV, $V_{m,h}$ = −63 mV). Initial values for tail currents were significantly larger in the presence of glucose.

Effect of Bicarbonate on Net Currents in the Presence or Absence of Glucose

Because most experiments on islet cell cultures are done using pH buffers other than bicarbonate, we repeated the protocols described in the previous section in the presence and absence of bicarbonate. The effects of glucose on net currents in bicarbonate-free medium (20 mM NaHEPES equilibrated with 100% O_2, pH 7.4 at 37°C)

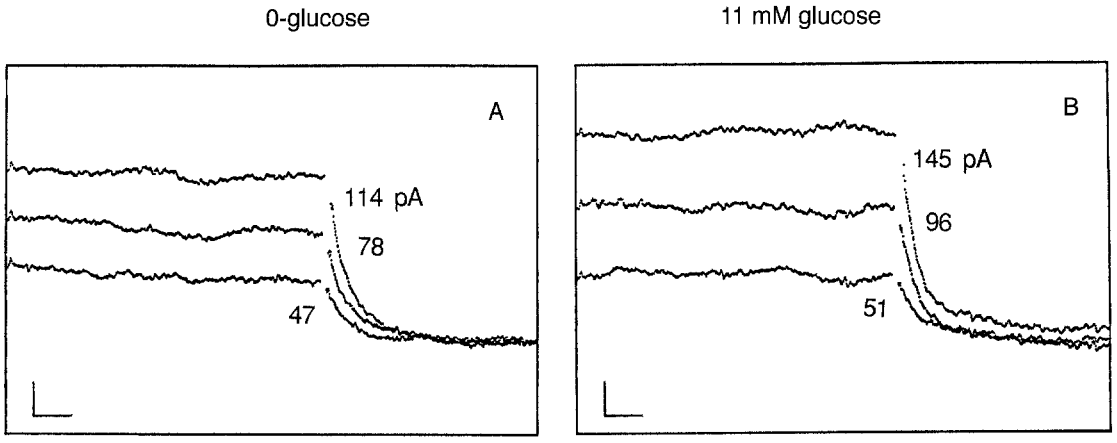

Fig. 6. Tail currents in the absence (*A*) and presence (*B*) of glucose (11 mM). *A* and *B* depict the last part of three records (corresponding $V_{c,h} - V_{c,P}$ values of 10, 20 and 30 mV) from Fig. 5 on an expanded time base to highlight the time course and the direction of the tail currents. Calibrations: vertical, 30 pA; horizontal, 5 msec. Initial value of the tail current is given in pA next to the corresponding record.

are shown in Fig. 7 (left side). Addition of bicarbonate at constant external pH, regardless of the presence of glucose, increases the net outward current (Fig. 7, compare left and right-side panels). The tail currents are also larger when bicarbonate is used as buffer (Fig. 7).

The outward currents were inhibited to the same extent after glucose removal in both the absence (Fig. 7, left-side panels) and presence (Fig. 7, right-side panels) of bicarbonate medium. Tail currents were affected by glucose (11 mM) in much the same way as described for the experiment illustrated in Figs. 5 and 6 both in the presence and absence of bicarbonate. From these data we conclude that net outward currents are reduced in the absence of bicarbonate buffer. Furthermore, outward currents are increased in β-cells *in situ* after addition of glucose (11 mM) regardless of the presence or absence of physiological bicarbonate buffer.

Effects of Glucose on Membrane Currents from Single Isolated Cultured Rat β-Cells

To compare the effects of glucose on a mouse β-cell *in situ*, in ideal physiological conditions (i.e., 37°C perfused with a bicarbonate-buffered Krebs' solution), with those on a single, isolated cultured rat β-cell in ideal patch clamp conditions (i.e., 20°C bathed in HEPES-buffered Kreb's saline), we repeated the experiment illustrated on the left-side panels of Fig. 7 in cultured rat pancreatic β-cells. Using the nystatin-perforated patch to achieve the whole-cell configuration, we acquired families of current records in response to **P-P/4** pulses increasing **P** in 15 mV steps from a holding potential of −80 mV (Fig. 8). In the single whole-cell voltage clamp configuration, the holding potential can be accurately set at the potassium equilibrium potential because the microelectrode resistance is negligible. Thus, the tail currents are almost absent from the records suggesting that the $V_{m,h}$ was set precisely at the reversal potential, V_{rev}, for the tail currents. In isolated cultured rat β-cells glucose had only marginal effects on both the shape and the amplitude of the currents as recorded with the **P-P/4** protocol. These results differ from our *in situ* data presented here, where glucose (11 mM) was found to increase the net outward currents.

Inward Currents Measured from β-Cells *In Situ*

Exposure of the islet cells to Krebs solution containing TEA (20 mM) in the absence of glucose effectively blocked outward currents. As shown on the upper panel of Fig. 9, only inward currents were observed in the presence of TEA.

Each inward current record exhibits an early transitory component with fast activation-inactivation kinetics and a delayed sustained component with slow inactivation (Fig. 9, upper panel). The *I-V* relationships obtained from measurements of maximum inward current (○) and inward current values at 100 msec (*) are shown in Fig. 9 (lower panel). Filled squares (■) were obtained by multiplying the 100 msec current values (*) by 3. These values approximate the measured peak values reasonably well, providing evidence that the inactivation occurring over the first 100 msec of the pulse is largely voltage independent. Correction of the V_c values to account for the effects of the microelectrode resistance (R_e = 0.2 GΩ) would shift the entire *I-V* curve towards more negative values (by about 20 mV for small current values).

Replacement of the extracellular Ca^{2+} by Mg^{2+} completely abolished the inward component of the currents at all potentials examined from *ca.* −50 to 30 mV (*data not shown*). Furthermore, subtraction of current records ob-

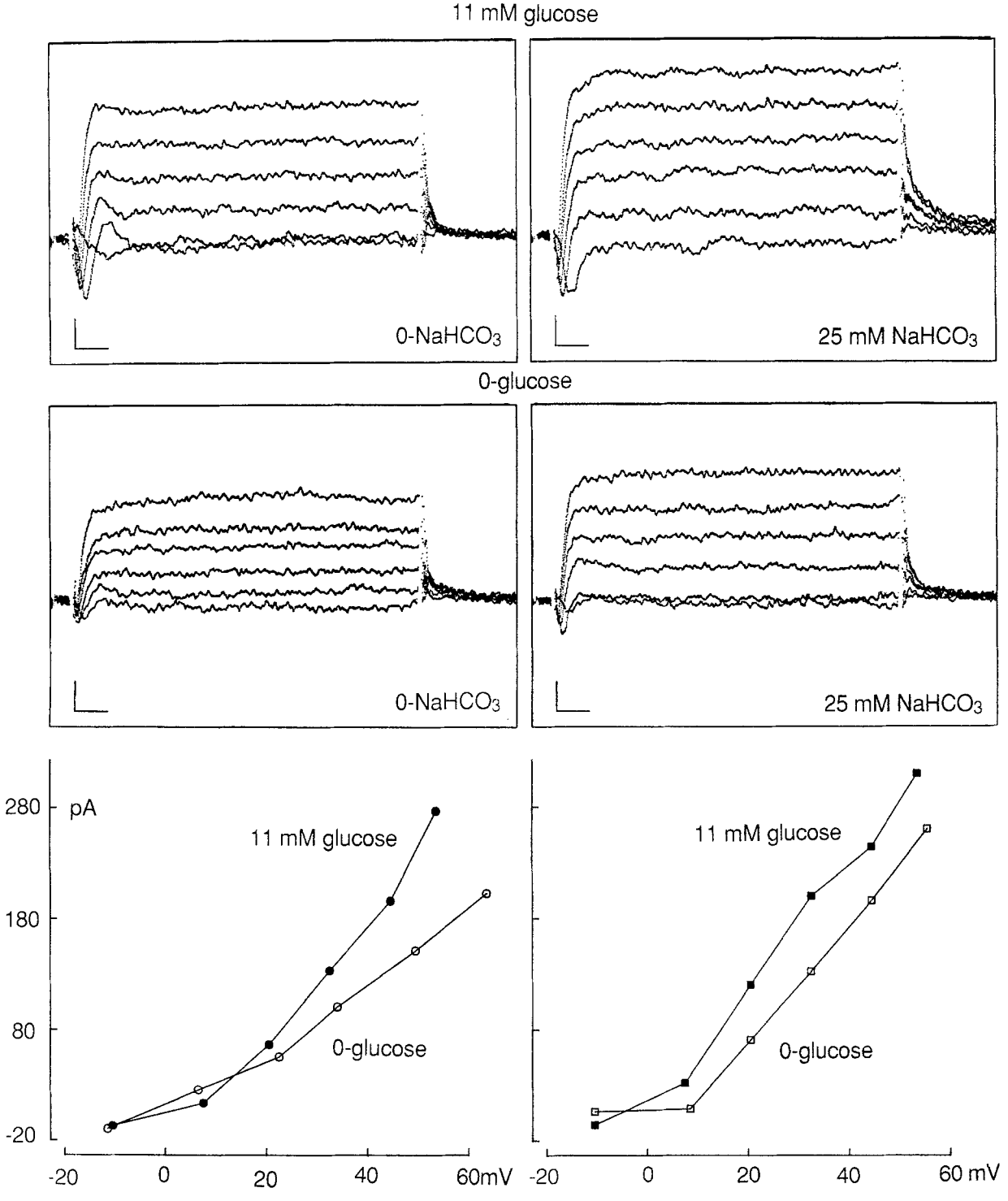

Fig. 7. Effects of bicarbonate on inward and outward currents in the presence or absence of glucose. Left-side panels (0-$NaHCO_3$; solutions saturated with 100% O_2): records were made in the presence of 11 mM glucose (*A*), and in the absence of glucose (*C*). Right-side panels (25 mM $NaHCO_3$; solutions saturated with 95% O_2/5% CO_2 mixture): Records were made in the presence of 11 mM glucose (*B*) and in the absence of glucose (*D*). All records from the same cell. Calibrations: vertical, 60 pA; horizontal, 10 msec. R_e was 0.13 GΩ. In the presence of glucose, V_m (during the silent phase) was −63 and −65 mV in the absence (*A*) or presence (*B*) of bicarbonate, respectively; in the absence of glucose V_m was −68 mV, and was unaffected by bicarbonate. $V_{c,h}$ was set at −70 mV throughout. The holding current was between −105 and −150 pA. Lower panels give *I-V* curves after corrections for R_e in the absence of bicarbonate (*E*) and in the presence of bicarbonate (*F*); filled symbols in the presence of 11 mM glucose, open symbols in the absence of glucose.

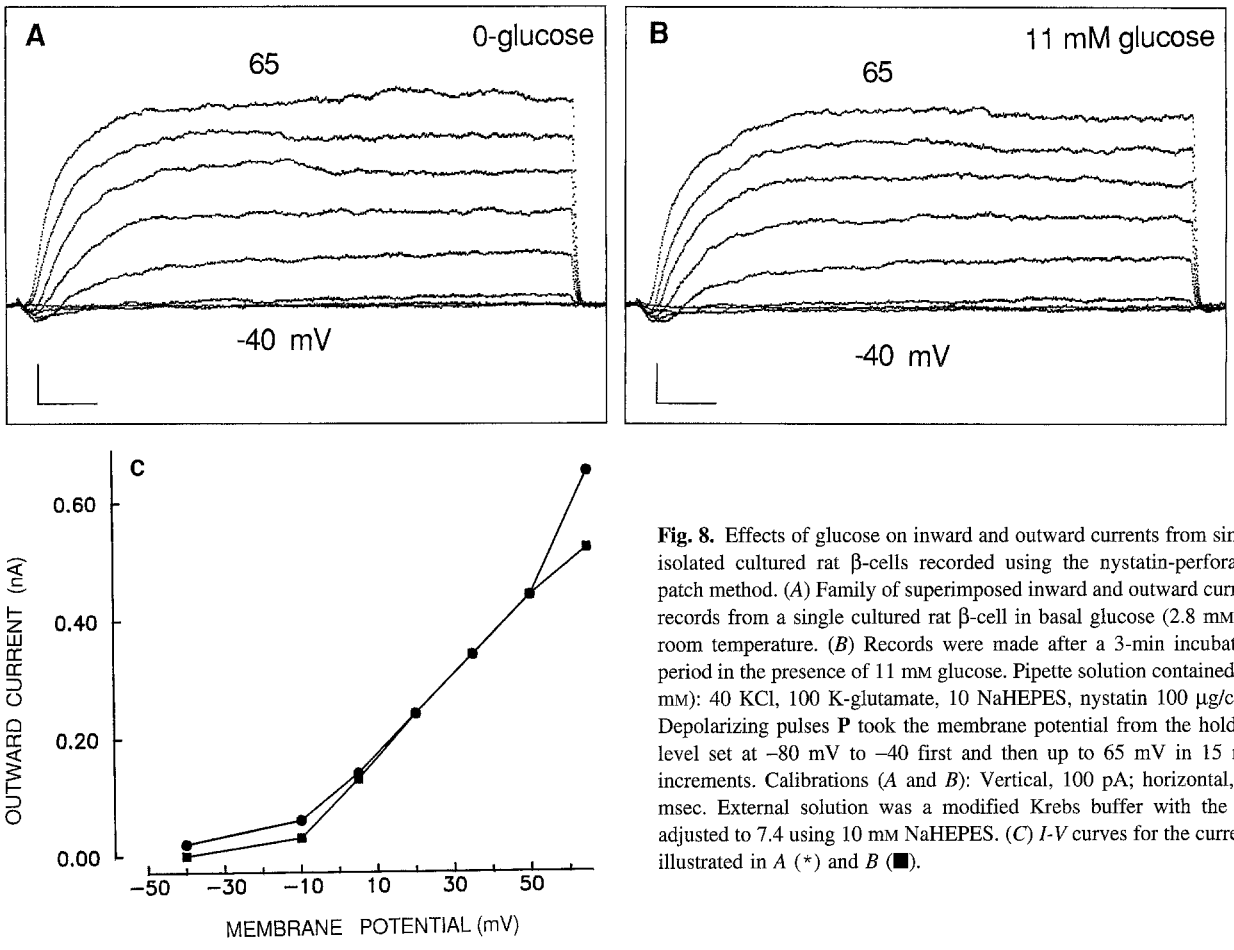

Fig. 8. Effects of glucose on inward and outward currents from single isolated cultured rat β-cells recorded using the nystatin-perforated patch method. (*A*) Family of superimposed inward and outward current records from a single cultured rat β-cell in basal glucose (2.8 mM) at room temperature. (*B*) Records were made after a 3-min incubation period in the presence of 11 mM glucose. Pipette solution contained (in mM): 40 KCl, 100 K-glutamate, 10 NaHEPES, nystatin 100 μg/cm^3. Depolarizing pulses **P** took the membrane potential from the holding level set at −80 mV to −40 first and then up to 65 mV in 15 mV increments. Calibrations (*A* and *B*): Vertical, 100 pA; horizontal, 10 msec. External solution was a modified Krebs buffer with the pH adjusted to 7.4 using 10 mM NaHEPES. (*C*) *I-V* curves for the currents illustrated in *A* (*) and *B* (■).

tained in the absence of extracellular Ca^{2+} from corresponding records made in the presence of physiological $[Ca^{2+}]_o$ generated a family of currents (*data not shown*) remarkably similar to those recorded in the presence of TEA (Fig. 9).

Discussion

We have shown here for the first time that it is possible to measure membrane ionic currents from mouse pancreatic β-cells *in situ* in intact perifused islets of Langerhans under physiological conditions that support insulin secretion stimulated by glucose. Using this novel combination of traditional methods, we have examined the effects of glucose on net currents and concluded that outward K^+ currents are greater in the presence than in the absence of glucose. We also gathered evidence in support of the idea that in mouse pancreatic β-cells, Ca^{2+} is the main cation carrying inward currents through L-type voltage-gated Ca^{2+}-channels.

Rationale for the Use of a Single-Microelectrode Voltage Clamp

The microelectrodes traditionally used to measure membrane potential from mouse pancreatic β-cells (Atwater et al., 1978; Atwater et al., 1979*a,b;* Atwater et al., 1980), which are relatively small (5–6 μm; Dean, 1973), have a diameter of *ca.* 0.1 μm and a large tip resistance, R_e, of *ca.* 0.15 GΩ. To achieve adequate control of the membrane potential V_m, however, using a single microelectrode both to measure the potential and to inject current, the microelectrode should have the smallest possible tip resistance regardless of the voltage clamp system used. The reason is that, while the potential to be controlled is that of the cytosol, the feedback amplifier is in

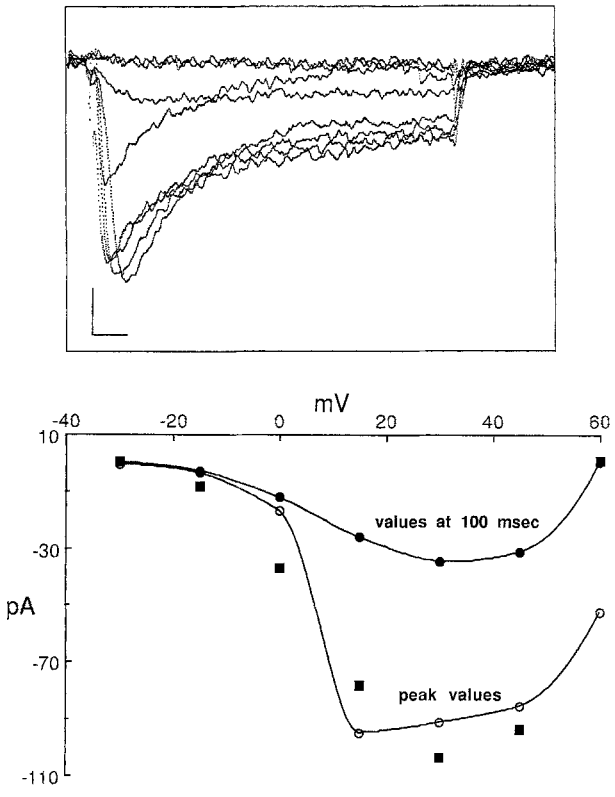

Fig. 9. Inward currents recorded in the presence of TEA (20 mM). (Upper panel) Eight superimposed inward current records made in the absence of glucose and in the presence of TEA (20 mM). R_e was 0.2 GΩ. Calibrations: Vertical, 20 pA; horizontal, 10 msec. (Lower panel) (*) Current measurements made towards the end of the pulses (100 msec); (○) maximum inward current values; Filled squares (■) were obtained by multiplying the 100 msec current values (*) by 3. Horizontal axis, $V_{c,h} + V_{c,P}$ values (uncorrected for R_e). $V_{c,h}$ set to −70 mV. Splines were drawn through the symbols representing either maximum inward current values (○) or inward current at 100 msec (*).

fact controlling the potential of the solution filling the microelectrode. Since both the cell interior and the solution in the pipette are electrically connected through the microelectrode tip resistance (R_e), the current injected by the feedback amplifier (I_c) would produce a voltage drop across R_e. Thus, there is a difference between V_c and the actual cytosolic potential (or V_m), which is calculated with Eq. (2). Since the maximum errors are *ca.* 20 mV for holding potential and up to 40 mV during the depolarizing pulses, it should be possible to control the membrane potential of a β-cell within the intact islet with a standard patch clamp amplifier and intracellular microelectrodes used for membrane potential measurements. We have presented a method to correct for these errors. Furthermore, it should be possible to isolate the time-dependent ionic currents from the linear, time-invariant current flowing into the coupled cells from the impaled cell using suitable subtraction protocols (Sherman et al., 1995). In the present work, we tested this concept and determined that it is possible to voltage-clamp a single β-cell in the intact islet at 37°C, and that it is possible to measure β-cell membrane currents in isolation using our technique.

With the method reported here, we expect to narrow the gap between the conditions required for the measurement of specific currents under voltage clamp and the physiological conditions for insulin secretion. The intact microdissected islet of Langerhans from mouse is an ideal system in which to study stimulus-secretion coupling under physiological conditions. This preparation has enabled us and others to carry out simultaneous recordings of intracellular membrane potential and intracellular pH (Rosario & Rojas, 1986) as well as Ca^{2+} signals (Valdeomillos et al., 1990) and insulin secretion responses to secretagogues (Scott et al., 1981; Dawson, Atwater & Rojas, 1982). We conclude here that the perifused intact islet of Langerhans can also be used to study the modulation of ionic currents by secretagogues.

Effects of Cell-to-Cell Coupling

To hold the membrane of the impaled β-cell at a constant potential (*ca.* −60 mV) in the presence of glucose (11 mM), requires the injection of negative (or inwardly directed) current that exhibits bursts strikingly similar to the burst pattern of electrical activity (*see* lower record in Fig. 4). The simplest explanation for this result is that, while the single-electrode voltage clamp system used here is adequate to control the potential of the impaled cell at a constant level, neighboring electrically coupled cells continue to burst. Hence, a significant fraction of the current flows via linear gap junction channels between the electrically coupled cells (Eddlestone et al., 1984; Sherman et al., 1995).

In an intact islet, β-cells are known to be electrically coupled among one another (Eddlestone et al., 1984). Thus, we cannot exclude the possibility that the current flowing between coupled cells might affect the measurement of the ionic currents measured here. The paper by Sherman et al. (1995) considers a mathematical model that provides the basis to understand, at least in principle, how the method used here works. This model describes an islet with electrically coupled cells and examines the effects of cell-to-cell coupling on I_c. Sherman et al. (1995) state that "the leak-subtraction method will work reasonably well if one uses only data taken when the unclamped [coupled] cells are in the silent phase" i.e., between bursts of glucose-induced spike activity or at rest in the absence of glucose. Further analysis of this point is beyond the scope of this paper, except to reiterate that the coupling of β-cells together in the intact islet,

while a complication for voltage clamp analysis, seems to be a physiological requirement for normal glucose-induced electrical bursting and insulin secretion.

In Mouse Pancreatic β-Cells *In Situ* Net Outward Currents Are Carried by K^+

TEA is a well-documented K^+-channel blocker (Rudy, 1988) in pancreatic β-cells (Atwater et al., 1979*b*; Findlay, Dunne & Petersen, 1985; Rosario & Rojas, 1986; Atwater et al., 1989; Bokvist et al., 1990*a,b;* Kukuljan et al., 1990), but is inactive on Ca^{2+} channels (Lebrun & Atwater, 1985). We have shown here that TEA (20 mM) also effectively blocked outward currents recorded from β-cells *in situ.* It is thus safe to conclude that the outward currents observed in our records from β-cells *in situ* flow through membrane K^+ channels. Likely candidates for the currents recorded here include the delayed rectifier K^+ channel (K-dr) (Rorsman & Trube, 1986) and the family of Ca^{2+}-activated K^+ channels (K-Ca) previously found in pancreatic β-cells (Atwater et al., 1979*a;* Atwater et al., 1980; Atwater, Rosario & Rojas, 1983; Findlay et al., 1985; Ribalet, Eddlestone & Ciani, 1988; Atwater et al., 1989; Kukuljan, Goncalves & Atwater, 1991).

Voltage-gated Outward K^+ Currents from Mouse β-Cells *In Situ* and the Single Rat β-Cell Are Different

Historically, studies of β-cell membrane potential response to different secretagogues have been carried out almost exclusively on mouse β-cells *in situ,* while the bulk of information on β-cell ionic currents comes from studies on isolated rat β-cells in culture. In attempts to reconcile burst activity with known ionic currents, it has often been assumed that data from the single rat β-cell apply to mouse β-cells *in situ.* However, our results show that, at least for outward K^+ current, the two types of β-cells behave quite differently. While in single isolated rat β-cells the outward K^+ currents are unaffected by 11 mM glucose (Fig. 8), in the mouse β-cell *in situ* the outward K^+ currents are significantly increased by the hexose (Fig. 7). The dissimilar behavior of membrane currents in the two experiments may be caused by species variation or differences in experimental conditions such as temperature or pH. Contamination of the records with coupling current cannot explain the observations since current from a neighboring coupled cell, when depolarized by glucose, would tend to reduce the apparent outward currents, the opposite effect from that seen here. The results illustrate the importance of using mouse β-cells and physiological conditions when attempting to elucidate the ionic currents involved in glucose-induced bursting.

Effect of Glucose and Bicarbonate on Outward K^+ Current

As mentioned above, the outward K^+ currents in the mouse β-cell *in situ* are significantly increased by 11 mM glucose, suggesting that at least one of the above-mentioned channels (K-dr or K-Ca) is stimulated by glucose. Indeed, glucose is known to increase substantially intracellular $[Ca^{2+}]$ in intact mouse islets (Santos et al., 1991; Santos et al., 1992; Rosario et al., 1993) and to a lesser extent in isolated rat β-cells (Rojas et al., 1994), thus lending support to the possibility that activation of a K_{Ca} channel by glucose may explain the increase in outward currents observed here in intact islets at 37°C. Since both in the presence and in the absence of glucose the tails of current after the pulses are in the outward direction, the reversal potential V_{rev} for the ion carrying the current must be more negative than the true potential at which the β-cell membrane potential was held, i.e., $V_{rev} < V_{m,h}$. We observed that the initial value of the tail current increased in the presence of glucose. This observation can be partially explained by the difference between the true holding potentials ($V_{m,h} = -67$ *vs.* -63 mV, *see* Figs. 5 and 6), but can also be interpreted to indicate that the reversal potential for K^+ is increased (becomes more negative) in the presence of glucose. Indeed, it has been observed in rat islets that glucose increased intracellular $[K^+]$ (Boschero et al., 1977).

Interestingly, the amplitude of the outward currents measured during the pulse as well as after (tail currents) were increased during perfusion of the islet with bicarbonate (HCO_3^-/CO_2) buffered solutions, similarly as with glucose. In cultured rat pancreatic β-cells bicarbonate buffer inhibits the activity of the K_{ATP} channel (Carroll et al., 1988) proposed to control the β-cell membrane potential response to glucose (Cook & Hales, 1984). Inadequacy of the pulse subtraction protocol to eliminate the current owed to K_{ATP} channels would have increased the outward currents measured in the absence of bicarbonate, the opposite effect from that seen here. Removal of bicarbonate has been reported to inhibit insulin secretion from islets (Henquin & Lambert, 1976). Bicarbonate is probably involved in the regulation of intracellular pH in β-cells (Carroll et al., 1988; Carroll et al., 1990) and therefore its removal could indirectly affect any one of the membrane K^+ channels involved in stimulus-secretion coupling.

With the data at hand we cannot yet identify the mechanism by which bicarbonate and glucose enhance the outward currents; however, the observation is interesting since the enhancement is only observed when recording from β-cells *in situ.*

Ca^{2+} is the Main Cation Carrying Inward Currents in Mouse β-Cells *In Situ*

Since TEA blocks the outward K^+ currents in the pancreatic β-cells, the macroscopic currents recorded using the **P-P/4** protocol in the presence of TEA (Fig. 9) must represent inward current, probably carried by Ca^{2+}. This conclusion is supported by the observation that replacement of the extracellular Ca^{2+} by Mg^{2+} abolished the inward component from the current records. These results provide evidence that both the transitory and the delayed components of the inward currents are carried by Ca^{2+}.

Records of inward Ca^{2+} current exhibit two components with difference kinetic characteristics. This suggests either the presence of more than one type of voltage-gated Ca^{2+} channel, or a single type of channel with both fast and slow inactivation. At the single channel level only one type of Ca^{2+} channel has been detected in cultured mouse pancreatic β-cells (Smith, Ashcroft & Fewtrell, 1993). These authors also characterized the gating properties of the channel together with the sensitivity to dihydropyridines. Previous studies of the effects of dihydropyridines on V_m in pancreatic β-cells *in situ* (Lebrun & Atwater, 1985) and I_{Ca} in cultured β-cells (Rorsman & Trube, 1986; Plant, 1988; Smith et al., 1990*a;* Smith et al., 1993) have already shown that the dihydropyridine-sensitive Ca^{2+}-channel is present in mouse β-cells. Recent reports in cultured islet β-cells from mouse (Ashcroft, Kelly & Smith, 1990; Hopkins et al., 1991) and from rat (Hiriart & Matteson, 1988; Sala & Matteson, 1990), however, have shown the presence of two types of voltage-gated Ca^{2+} channels. The kinetics of the inward current recorded here are in good agreement with Smith et al. (1993), suggesting that both components could be carried through a single, voltage-gated channel, probably the L-type Ca^{2+} channel. Plant (1988) and Smith et al. (1993) also showed that the delayed component of I_{Ca} depends on the size of the Ca^{2+} influx during the depolarizing conditioning prepulse in primary cultures of mouse pancreatic β-cells. Hence, the apparent slow inactivation observed in our I_{Ca} records may also be caused by a depolarization-induced rise in intracellular $[Ca^{2+}]_i$. The observed increase in inward currents during exposure to glucose (as shown in Fig. 7) was not a consistent observation. Modulation of inward currents by glucose and other secretagogues, and further characterization of the Ca^{2+} currents in isolation are the subjects of on-going studies using the *in situ* single microelectrode technique.

In conclusion, we have successfully developed a technique for applying whole-cell voltage clamp analysis to single β-cells within an intact islet of Langerhans under physiological conditions. We have shown that, after subtraction of the linear, nonvoltage dependent currents, glucose, as well as bicarbonate-containing physiological buffers, increase the outward K^+ currents. We suggest that these currents may reflect increased activity of a voltage- and Ca^{2+}-activated K^+ channel. Finally, we show that the nonlinear inward currents are carried mainly by Ca^{2+} in mouse islets probably through L-type Ca^{2+} channels showing fast activation-inactivation kinetics and slow inactivation kinetics.

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