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THE RELATIONSHIP BETWEEN GLUCOSE-INDUCED K^+_{ATP} CHANNEL CLOSURE AND THE RISE IN $[Ca^{2+}]_i$ IN SINGLE MOUSE PANCREATIC β-CELLS

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SUMMARY

1. Intracellular calcium $[Ca^{2+}]_i$ and channel activity were simultaneously recorded in single, dissociated mouse β-cells kept in culture for 1–3 days. $[Ca^{2+}]_i$ was estimated from microfluorometric ratio methods using Indo-1. Channel activity was measured using the cell-attached configuration of the patch-clamp technique.

2. At low glucose concentrations (0·3 mM), resting K^+_{ATP} channel activity was prevalent. Increasing glucose up to 16 mM, produced a gradual decrease in K^+_{ATP} channel activity over a time course of 90–120 s (temperature = 23 °C) and an increase in $[Ca^{2+}]_i$.

3. In the majority of experiments, glucose elicited biphasic action currents (action potentials) which preceded the rise in $[Ca^{2+}]_i$. There was a close correlation between spike frequency and the levels of $[Ca^{2+}]_i$.

4. The sulphonylurea tolbutamide (1 mM) blocked K^+_{ATP} channels in 10–20 s. K^+_{ATP} channel blockade was associated with a quick rise in $[Ca^{2+}]_i$.

5. When K^+_{ATP} channel activity was stimulated in the presence of diazoxide (100 μM), increasing the glucose concentration from 3 to 16 mM produced a *decrease* in $[Ca^{2+}]_i$. Only when diazoxide was removed did glucose produce an increase in $[Ca^{2+}]_i$.

6. In a small population of cells, glucose (16 mM) produced a small decrease in K^+_{ATP} channel activity but not an increase in $[Ca^{2+}]_i$. In such cells, tolbutamide blocked K^+_{ATP} channels and produced an increase in $[Ca^{2+}]_i$.

7. These results demonstrate a close correlation between K^+_{ATP} channel activity and $[Ca^{2+}]_i$ in β-cells. The findings are consistent with the model in which glucose metabolism produces a rise in $[Ca^{2+}]_i$ through the blockade of K^+_{ATP} channels, membrane depolarization and calcium current activation.

INTRODUCTION

It is well known that glucose-induced insulin release from pancreatic β-cells is mediated by an increase in intracellular calcium ($[Ca^{2+}]_i$) (see Prentki & Matschinsky,

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1987, for a review). According to the current model for stimulus–secretion coupling, under resting conditions the β-cell membrane potential is kept close to the K^+ equilibrium potential due to the activity of ATP-dependent K^+ channels (K^+_{ATP}; Ashcroft, Harrison & Ashcroft, 1984; Cook & Hales, 1984; Ashcroft, Ashcroft & Harrison, 1988). When glucose increases, its metabolism produces a rise in the ATP level (or an increase in the ATP/ADP ratio) leading to the closure of K^+_{ATP} channels and membrane depolarization. Voltage-dependent calcium channels would then activate, leading to an increase in $[Ca^{2+}]_i$ levels and insulin-containing granule exocytosis (Ashcroft & Rorsman, 1991). From this scheme, the effects of glucose on $[Ca^{2+}]_i$ would be mediated only by changes in membrane potential. Indeed, a close correlation between bursting electrical activity and $[Ca^{2+}]_i$ oscillations has been found in whole islets of Langerhans (Santos, Rosario, Nadal, Garcia-Sancho, Soria & Valdeolmillos, 1991), but those experiments did not completely exclude other effects of glucose on $[Ca^{2+}]_i$.

An important problem when assessing the contribution of ionic channels in stimulus–secretion coupling, is related to the integrity of cell metabolism. This is particularly important in the case of the β-cell, as the activity of the K^+_{ATP} channel fades when recorded in any 'open' patch-clamp configuration. This is due to the wash-out of intracellular modulators due to cell disruption or dialysis. For these reasons we choose the cell-attached configuration of the patch-clamp technique, whilst simultaneously measuring $[Ca^{2+}]_i$ from the patched cell.

Several lines of evidence suggest that β-cells are a heterogeneous population when analysed at the single-cell level, in terms of both the $[Ca^{2+}]_i$ response to secretagogues and insulin release (Salomon & Meda, 1986). Such heterogeneity may arise at different levels, from different capacities in glucose metabolism and ATP synthesis, to differences in K^+_{ATP} channel modulation.

The experiments presented in this paper were designed to correlate K^+_{ATP} channel activity and $[Ca^{2+}]_i$ in single cells, and their changes in response to glucose and tolbutamide. Our results are consistent with a hypothesis in which glucose-induced changes in $[Ca^{2+}]_i$ are mediated by the closure of K^+_{ATP} channels and subsequent activation of voltage-dependent calcium channels. Our results also provide new insights into the β-cell population heterogeneity.

A preliminary account of some of the present results has appeared (Nadal, Contreras, Soria & Valdeolmillos 1992).

METHODS

Cell isolation and cell culture

Cells were dispersed from mouse islets of Langerhans isolated by collagenase digestion (3·5 mg/ml for 7 min) as previously reported (Lernmark, 1974). Once isolated, the islets (typically forty to sixty per pancreas) were incubated for 1 h at 37 °C in modified Krebs medium (solution A) supplemented with 11 mM-glucose and 3% bovine serum albumin before starting the dissociation procedure.

Isolated islets were dispersed into single cells by enzymic digestion in the presence of 0·05% trypsin plus 0·02% EDTA (Flow Laboratories) for 3 min. Before the trypsin step, the islets were washed three times in Krebs–Ringer (solution B) in the absence of either added calcium or EGTA. After the trypsin treatment the cells were mechanically dispersed by gentle passage through a narrow Pasteur pipette. The cells were then centrifuged, re-suspended in culture medium (RPMI 1640) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (0·1 mg/ml) and 11 mM-glucose and plated on glass coverslips.

Only 100 μl of the cell suspension was plated on each coverslip in a central drop limited by a rim made of sterilized vacuum grease. The cells were kept at 37 °C in a humidified atmosphere of 95% air, 5% CO_2 and used within 1–3 days of plating. The culture medium was renewed every day.

Fluorescence and electrophysiological recording

Intracellular calcium was monitored by measuring the fluorescence emitted by the fluorophore Indo-1 (Grynkiewicz, Poenie & Tsien, 1985) as previously described (Valdeolmillos, Santos, Contreras, Soria & Rosario, 1989; Santos *et al.* 1991). Cells were loaded with Indo-1 by incubation for 1 h at room temperature with 3 μM of the acetoxymethyl derivative, Indo-1 AM (Molecular Probes, OR, USA). This was added as a concentrated stock solution in dimethyl sulphoxide (DMSO; final DMSO concentration 0·3% v/v). In some experiments, the mixture also contained 10% (w/w) pluronic F127 (Molecular Probes, OR, USA). We could not detect any clear loading improvement in the presence of the dispersing agent. Loading was done either in medium buffered by HEPES or bicarbonate (both supplemented with 3% albumin and 11 mM glucose) with similar results

Once loaded, the coverslip was transferred to the experimental bath (vol = 300 μl) mounted on the stage of a Nikon epifluorescence inverted microscope and perfused at a range of 1–2 ml/min.

Indo-1 was excited at 350±5 nm by means of a 100 W mercury lamp and a narrow bandpass filter. The level of ultraviolet (UV) excitation light compatible with a good signal-to-noise ratio was adjusted by placing neutral density filters in the light pathway. This was done to reduce photobleaching and photodamage of the preparation. UV light was focused using a 40 × Neofluar (Nikon) oil immersion objective (numerical aperture = 1·3).

The emitted fluorescence was split into two beams by means of a dichroic mirror (450 nm), filtered respectively at 410±5 and 480±5 nm and detected by two photomultipliers (Thorn EMI 9924B). An increase in $[Ca^{2+}]_i$ produces a rise in Indo-1 fluorescence at 410 nm and a decrease in the fluorescence measured at 480 nm (Grynkiewicz *et al.* 1985). The ratio of the fluorescence ($F_{410/480}$ at both wavelengths was determined on-line, filtered at 10 kHz and stored on digital tape (Biologic DTR-1800) for further analysis. The $[Ca^{2+}]_i$ given in Fig. 1 at rest and during glucose stimulation has to be taken as an approximation. This is due to the fact that the fluorescence signals were calibrated using an '*in vitro*' calibration curve. A further problem is possible asymmetry in $[Ca^{2+}]_i$ due to non-homogeneous distribution, especially when the cells are stimulated (O'Sullivan, Cheek, Moreton, Berridge & Burgoyne, 1989). In this respect, it should be stressed that our fluorescence measurements represent the mean value from the whole cell, therefore underestimating the signal coming from any restricted space.

The final steps of the patch-pipette approach to the cell and seal formation were monitored on a TV screen from a camera connected to the lateral port of the microscope. For this purpose the cell was transilluminated with red light (700 nm), thereby avoiding cross-talk between fluorescence signals and transillumination. A detailed description of the optical set-up can be found elsewhere (Valdeolmillos & Eisner, 1991).

The methods for preparing patch pipettes and recording single-channel currents were similar to those already described (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes were pulled from haematocrit glass capillaries using a two-stage puller (Mecanex BB-CH). They had resistances in the range of 2–10 MΩ when filled with standard intracellular solution (solution C).

In these experiments we used the cell-attached configuration of the patch-clamp technique. Unitary currents were measured using a Biologic RK 300 patch-clamp amplifier. The experiments shown in the figures were filtered at 1–5 kHz. Given the high potassium concentration of the pipette-filling solution and the resting membrane potential of the cells (around −70 mV), K^+ channel openings are seen as inward currents and conventionally displayed as downward deflections in the records. In the majority of experiments, the pipette potential was held at 0 mV, therefore the potential across the patch membrane was due only to the cell membrane potential. In some experiments a positive potential was applied to the inside of the pipette producing a membrane hyperpolarization. Changes in the patch potential (V_p) are indicated in the figure legends. Channel activity was digitized in samples of 10–20 s duration and open probability (P_o) estimated as P_o = total open time/total time.

Solutions. Solutions A and B were indiscriminately used for cell perfusion. The main difference was their pH buffer. Solution A had the following composition (mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 1·1 $MgCl_2$ and 2·5 $CaCl_2$. This solution was gassed with a mixture of 95% O_2 and 5% CO_2 for a final pH of 7·35. The composition of solution B was (mM): 130 NaCl, 4·7 KCl, 1·1 $MgCl_2$, 2·5 $CaCl_2$ and 10 HEPES–NaOH, with a final pH of 7·3.

Solution C was used for patch-pipette filling and had the following composition (mM): 140 KCl, 10 NaCl, 1·1 $MgCl_2$, 2·5 $CaCl_2$ and 10 HEPES–NaOH, with a final pH of 7·3. In some experiments calcium was omitted from the solution without appreciable change in the behaviour of the K^+_{ATP} channel. All the solutions were filtered before the beginning of the experiments.

The majority of the experiments were done in single isolated cells. Some were carried out in small clusters of three to five cells with essentially the same results. The experiments were carried out at room temperature (22–23 °C).

RESULTS

The effects of glucose on K^+_{ATP} channel activity and $[Ca^{2+}]_i$

Figure 1 shows the effects of changing extracellular glucose concentration from 3 to 16 mM on $[Ca^{2+}]_i$ and channel activity using the cell-attached configuration of the patch-clamp technique in a single β-cell. As previously reported by other authors, under resting conditions (0–3 mM-glucose) a high activity of K^+_{ATP} channels was observed in all the studied patches. Channel openings are represented by downward deflections, reflecting inward currents due to the high potassium content of the pipette. In this experiment, the apparent number of active channels is five, but a large proportion of K^+_{ATP} channels are already blocked at this glucose concentration (Ashcroft *et al.* 1988). The number of active channels under the pipette was very variable in different cells, ranging from one up to seven. The criteria used for identification of K^+_{ATP} channels was their unitary conductance (52 pS ($n = 3$) in the linear range of voltages, from −40 to +60 mV (not shown)), inward rectification and glucose and tolbutamide sensitivity. The resting $[Ca^{2+}]_i$ concentration in this cell was around 200 nM, but this figure has to be taken only as an approximation due to the

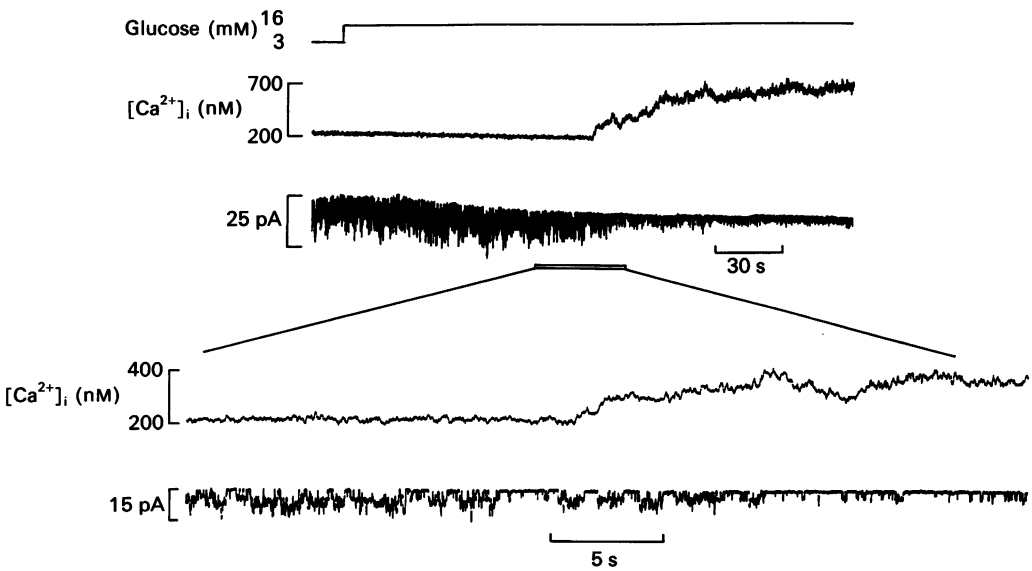

Fig. 1. Effects of changing glucose concentration from 3 to 16 mM on $[Ca^{2+}]_i$ and K^+_{ATP} channel activity using the cell-attached configuration in a single isolated β-cell. Pipette potential (V_p) was held at 0 mV throughout the experiment. Inward potassium currents are represented as downward deflections. The lower part of the figure represents, at a faster time scale, the part of the upper record corresponding to the rise in $[Ca^{2+}]_i$.

uncertainties derived from the calibration procedure (see Methods). For this reason in the next figures only the ratio of fluorescence ($F_{410/480}$ nm) will be given. When extracellular glucose concentration is increased from 3 to 16 mM, after a lag time of approximately 1·5–2 min, K^+_{ATP} channel activity starts to decrease and eventually

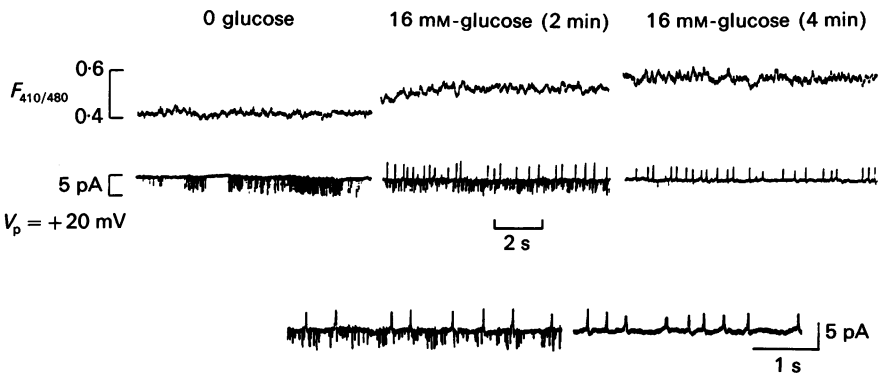

Fig. 2. Time course of the effects of glucose on $[Ca^{2+}]_i$ (upper record) and membrane current (middle record). The record on the left was taken just before increasing the glucose concentration. The middle record was taken 2 min after changing glucose to 16 mM. The current trace shows action currents (upward deflections) and K^+_{ATP} channel activity (downward deflections). After 4 min in 16 mM-glucose, only action currents are seen. The pipette potential was held at +20 mV throughout the experiment. The lower record shows, at a faster time scale, the current traces after 2 and 4 min in 16 mM-glucose.

$[Ca^{2+}]_i$ starts to rise, reaching a concentration of approximately 700 nM. The part of the record corresponding to the time when $[Ca^{2+}]_i$ starts to rise is displayed at a faster time scale in the lower part of the figure. Although there is a clear continuous decrease in K^+_{ATP} channel activity in the presence of glucose, it is also clear that there is some remaining activity even when $[Ca^{2+}]_i$ has increased. Associated with the change in glucose, there is a current shift in the inward direction. This current shift was seen in some experiments, and could be due to the membrane depolarization induced by glucose (Atwater, Ribalet & Rojas, 1978).

Given the bursting activity of K^+_{ATP} channels and the transient nature of the experiments, a precise correlation between the levels of channel activity and $[Ca^{2+}]_i$ can only be tentative. However, this experiment seems to indicate that there is a parallelism during the time when $[Ca^{2+}]_i$ starts to rise and the gradual closure of K^+_{ATP} channels. In other words, during the time when K^+_{ATP} channel activity is changing, the levels of $[Ca^{2+}]_i$ are also changing and a stable $[Ca^{2+}]_i$ level is only reached when a stable level of K^+_{ATP} closure is attained.

In approximately two-thirds of the experiments, biphasic spikes (action currents) were seen in the presence of stimulatory glucose concentrations. Biphasic currents are action potentials seen via the capacitance and resistance of the patch (Fenwick, Marty & Neher, 1982); their recording, therefore, is critically dependent on the resistance of the patch. We could consistently record biphasic spikes in high glucose concentration using pipettes of 2–5 MΩ. An example is shown in Fig. 2. In the absence of extracellular glucose, the burst activity of the K^+_{ATP} channel is clearly

seen. Two minutes after changing glucose to 16 mM, there is a mixed pattern of channel activity (downward deflections) and simultaneous spike activity (upward deflections). There is, at this time, a concomitant increase in $[Ca^{2+}]_i$ (upper panel). After 4 min in high glucose concentration, the electrical pattern consists almost

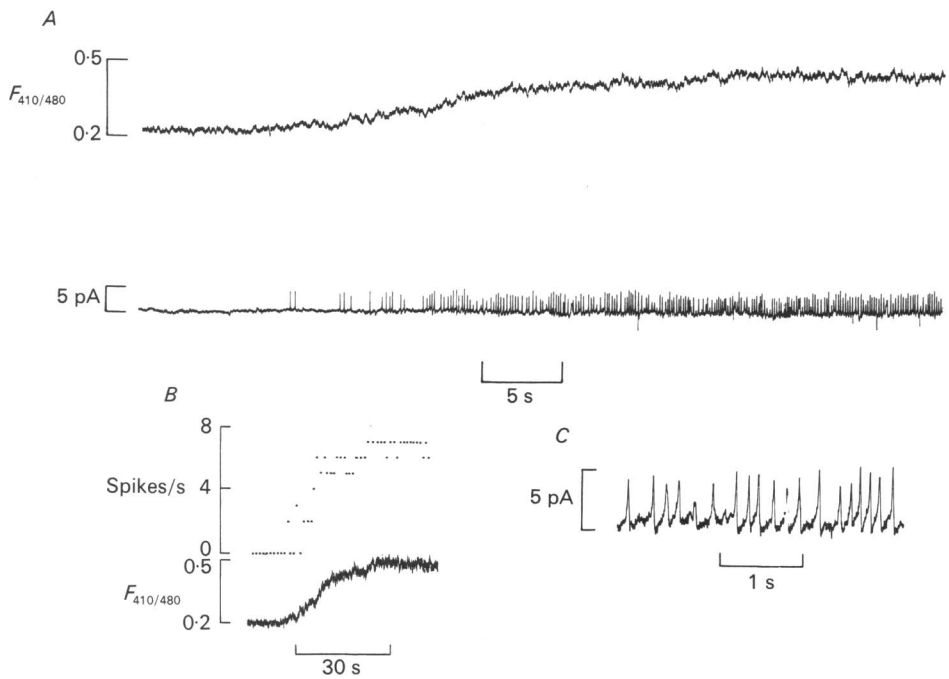

Fig. 3. *A*, effects of glucose (16 mM) on $[Ca^{2+}]_i$ and membrane current. Glucose was increased from 0 mM 100 s before the beginning of the record. Only action currents are seen in the presence of glucose. The lower part of the figure (*B*) shows a plot of spikes/s and fluorescence on a slower time scale. *C* shows a specimen record of the current trace once the spike frequency had reached a steady value.

entirely of action currents with rare K^+_{ATP} channel openings. During this period, $[Ca^{2+}]_i$ rose even higher than before. The co-existence during a certain period (2 min in the presence of glucose) of K^+_{ATP} channel activity and action potentials clearly indicates that the blockade of a small percentage of K^+_{ATP} channels by glucose is enough for membrane depolarization and calcium channel activation. This mechanism may provide a fine control by glucose upon membrane potential and therefore upon $[Ca^{2+}]_i$. This result helps to explain results like the one shown in Fig. 1, in which $[Ca^{2+}]_i$ starts to rise even when an appreciable activity of K^+_{ATP} channels is still present.

A more precise temporal correlation between the appearance of biphasic currents and the rise in $[Ca^{2+}]_i$ can be demonstrated in experiments like the one depicted in Fig. 3. In this experiment the K^+_{ATP} channel activity was very low under resting conditions (not shown). After 90 s in 16 mM-glucose, there is an abrupt start to biphasic spikes, reaching a maximum frequency of seven spikes/s. $[Ca^{2+}]_i$ only starts

to rise after the beginning of the electrical activity. The lower part of the figure (*B*) shows a plot of number of spikes/s and the fluorescence ratio at a much slower time scale, where the close correlation between both parameters can be clearly appreciated. Part *C* of the figure shows a specimen record of action potentials on a

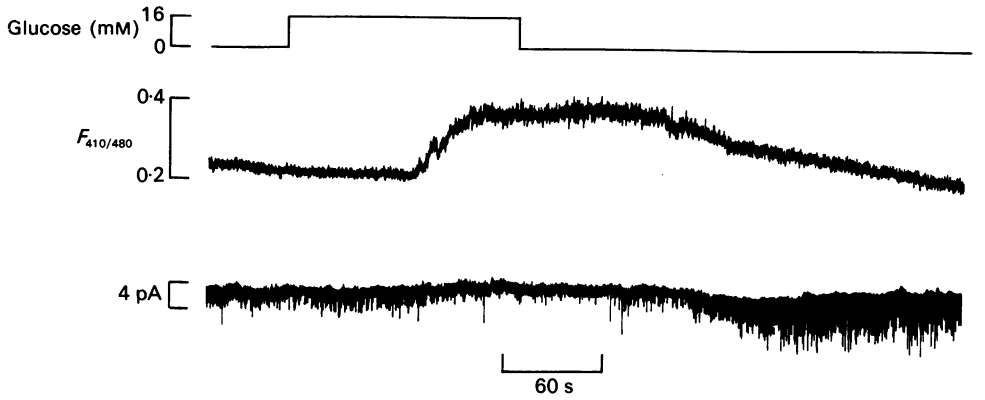

Fig. 4. Reversibility of the effects of glucose on $[Ca^{2+}]_i$ and channel activity.

faster time scale when their frequency is constant. No clear $[Ca^{2+}]_i$ transients were associated with each spike, which probably reflects the low amplitude of the calcium currents elicited under normal conditions during the electrical activity in the β-cells (Rorsman & Trube, 1986; Plant, 1988). This may also explain the slow rise in $[Ca^{2+}]_i$ that reaches a steady value only 20 s after the beginning of the electrical activity, once the frequency of spikes has also reached a steady value.

The effects of glucose on $[Ca^{2+}]_i$ and channel activity were reversible, as shown in Fig. 4. The experiment started in 0 mM-glucose, and an increase in glucose to 16 mM produced a blockade of K^+_{ATP} channels and a rise in $[Ca^{2+}]_i$ that slowly recovered towards control values upon glucose withdrawal. The decrease in $[Ca^{2+}]_i$ is associated with the recovery of K^+_{ATP} channel activity. We generally found that the recovery of the channel activity after longer times in high glucose concentrations took up to 15 min. In general the activity after the recovery was higher than during the control period. This may reflect the refreshment of the K^+_{ATP} channel after a period in high glucose concentration.

The effect of glucose on $[Ca^{2+}]_i$ and K^+_{ATP} channel activity was checked in thirty-two cells, obtaining a clear correlation in twenty-five experiments. In the seven remaining experiments, some degree of K^+_{ATP} channel closure was achieved in the presence of glucose, but not associated with a change in $[Ca^{2+}]_i$. The significance of these experiments will be addressed in the last part of this section.

The effects of tolbutamide on K^+_{ATP} *channel activity and* $[Ca^{2+}]_i$

In the next series of experiments, we checked the ability of the sulphonylurea tolbutamide to increase $[Ca^{2+}]_i$. It is known that this drug blocks the K^+_{ATP} channel by a mechanism not dependent on glucose metabolism (Trube, Rorsman & Ohno-

Shosaku, 1986). Figure 5 shows the effect of tolbutamide on $[Ca^{2+}]_i$ and K^+_{ATP} channel activity. Glucose was absent from the extracellular medium throughout the experiment. Before tolbutamide, two channel levels are evident. After a short delay, tolbutamide was able to completely block the channel activity leading to an increase

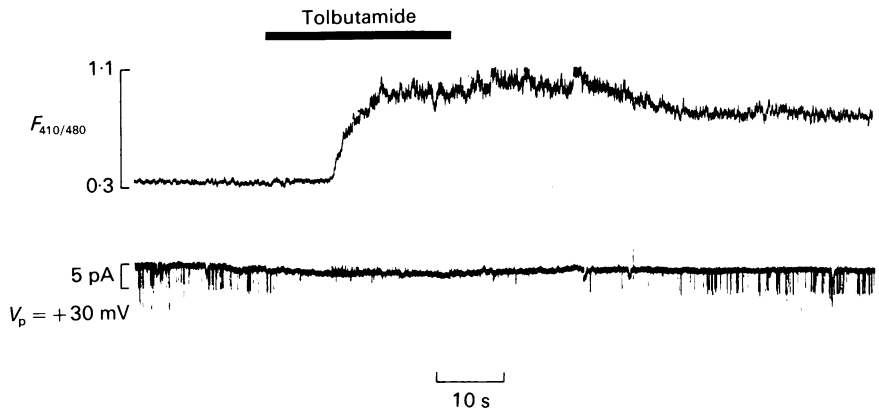

Fig. 5. Effects of tolbutamide (1 mM) in the absence of glucose on $[Ca^{2+}]_i$ and channel activity. Tolbutamide was added during the time indicated by the bar. The rise in $[Ca^{2+}]_i$ coincides with small action currents shown as upward deflections in the current trace. $V_p = +30$ mV.

in $[Ca^{2+}]_i$. After removal of tolbutamide, channel activity reappears and $[Ca^{2+}]_i$ tended to decrease. The poor reversibility of the effect on $[Ca^{2+}]_i$ may have been due to the lack of glucose, leaving the cell in an impaired state as far as the $[Ca^{2+}]_i$ sequestering/removal mechanism(s) is concerned. The effect of tolbutamide was very consistent, being positive in twelve out of thirteen tested cells.

The effects of glucose on $[Ca^{2+}]_i$ when depolarization is prevented

The experiments presented so far are consistent with the idea that the glucose-induced rise in $[Ca^{2+}]_i$ is mediated by K^+_{ATP} channel closure membrane depolarization, and activation of calcium currents. However, there are reports in the literature showing effects of glucose metabolism on phospholipid metabolism (Turk, Wolf & McDaniel, 1986), giving rise to the possibility that effects of glucose on $[Ca^{2+}]_i$ are different from those produced by calcium entering due to cell depolarization (Biden, Prentki, Irvine, Berridge & Wollheim, 1984). In order to study this problem further we looked at the effects of glucose under conditions when depolarization was not possible. An example of such an experiment is shown in Fig. 6. The membrane potential was 'clamped' by means of the drug diazoxide. It is known that diazoxide directly activates K^+_{ATP} channels (Trube *et al.* 1986; Dune, Illot & Petersen, 1987; Sturgess, Kozlowski, Carrington, Hales & Ashford, 1988) therefore hyperpolarizing the cells. Studies in excised patches have shown that the activation of the channel can only be reversed by very high ATP levels. In this experiment, addition of 100 μM-diazoxide to a cell in the presence of 3 mM-glucose produced a significant increase in K^+_{ATP} channel activity with no apparent change in $[Ca^{2+}]_i$ levels. When glucose was

increased to 16 mM in the presence of diazoxide, even after 4 min K^+_{ATP} channel activity, as expected, did not decrease whereas there was a small but clear *decrease* in $[Ca^{2+}]_i$. Only when diazoxide was removed did K^+_{ATP} channel activity and amplitude decrease and $[Ca^{2+}]_i$ rise. This point is more clearly appreciated in the

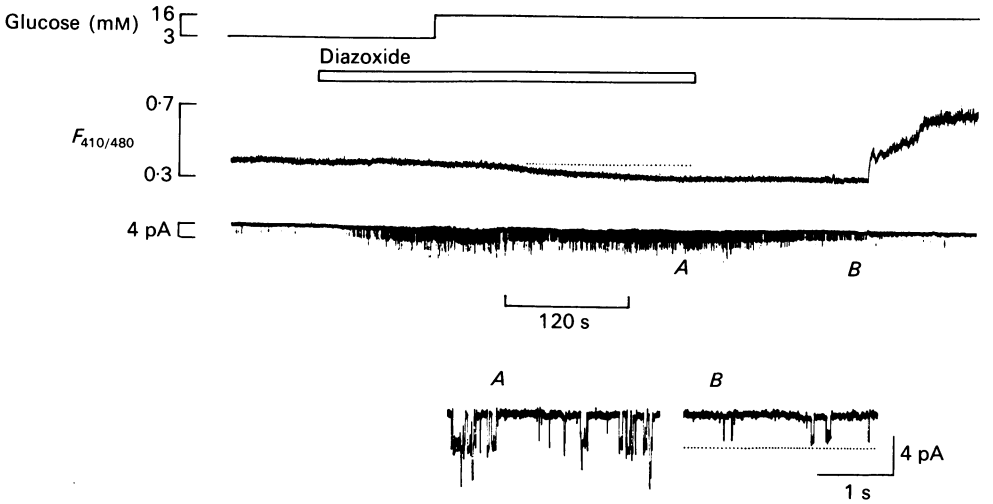

Fig. 6. Effects of glucose on $[Ca^{2+}]_i$ and channel activity in the presence and absence of diazoxide (100 μM). At the beginning of the experiment the glucose concentration was 3 mM. In the presence of diazoxide, the increase in glucose produced a decrease in $[Ca^{2+}]_i$ (dotted line). The lower part of the figure shows specimen records of channel activity before the withdrawal of diazoxide (*A*) and just before the rise in $[Ca^{2+}]_i$ (*B*). The dotted line in *B*, corresponds to the open channel level in *A*.

lower panel of the figure, where the channel amplitude before the diazoxide withdrawal (*A*) is compared to the amplitude just before the rise in $[Ca^{2+}]_i$ (*B*). We interpret the decrease in channel amplitude in *B* as a consequence of membrane depolarization due to diazoxide removal in the presence of high glucose.

The effects of diazoxide were checked in three cells, the same results being obtained both in case of the decrease of $[Ca^{2+}]_i$, when glucose was increased in the presence of diazoxide, and in the correlation between K^+_{ATP} channel closure and the rise in $[Ca^{2+}]_i$ in the absence of the drug.

Heterogeneous response of single β-cells to glucose

As pointed out before, a small number of cells did not respond to glucose with changes in $[Ca^{2+}]_i$. An example of this kind of result is shown in Fig. 7. In this experiment, three channel levels are seen in the absence of glucose. After 4 min in the presence of 16 mM-glucose, channel activity is barely affected and there is no change in $[Ca^{2+}]_i$. The experiment provides some evidence, however, of the effect of glucose on membrane potential, manifested by a decrease in the channel amplitude, as expected by a decrease in the driving force for potassium entry into the cell due to membrane depolarization. This point is more clearly appreciated in the lower part of

the figure, where the amplitude of the channel a few seconds before increasing glucose concentration (*A*) is compared with the amplitude of the channel 3 min after changing glucose to 16 mM (*B*) and at the end of the period with glucose (*C*). Channel amplitude approximately halved (compare *A* and *C*) corresponding to a membrane

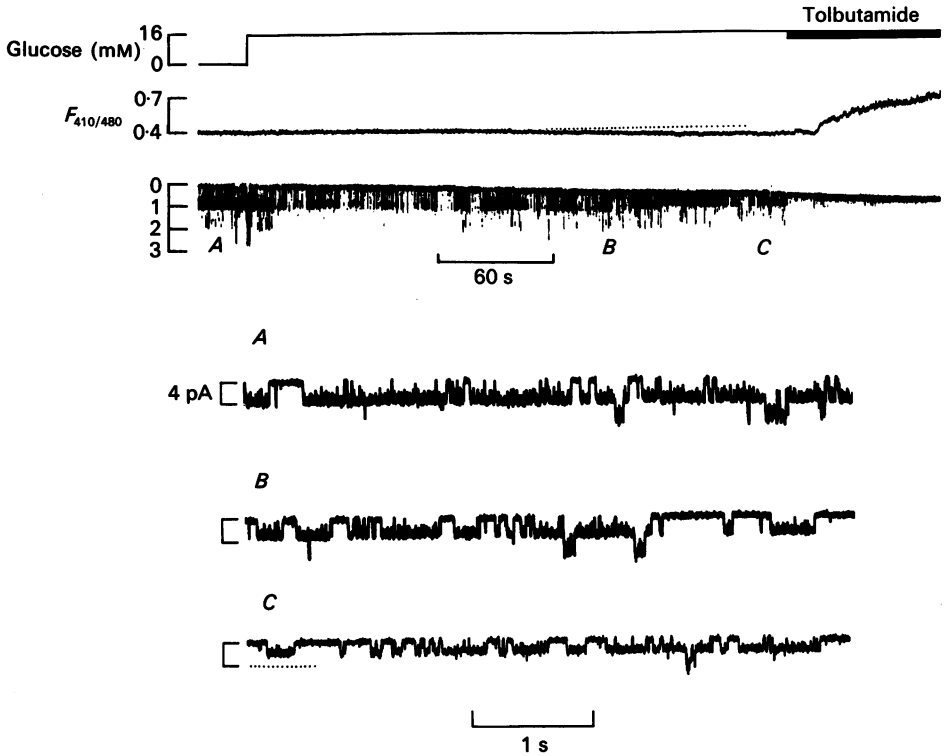

Fig. 7. Effects of increasing glucose on $[Ca^{2+}]_i$ and channel activity in a 'non-responsive' cell. Glucose produced a decrease in $[Ca^{2+}]_i$ (dotted line in the fluorescence record). After 5 min in 16 mM-glucose, tolbutamide (1 mM) was added producing the closure of K^+_{ATP} channels and a rise in $[Ca^{2+}]_i$. The lower part of the figure shows channel activity at the periods indicated. The dotted line in *C* corresponds to the open channel level in *A*.

depolarization of about 30 mV. However, channel activity remained almost constant. The P_o measured in 10 s samples were 0·32 (*A*), 0·18 (*B*) and 0·20 (*C*). A second piece of evidence for the effect of glucose is provided by the fact that there is a gradual decrease in $[Ca^{2+}]_i$ in the presence of 16 mM-glucose (dotted line; compare with Fig. 6). When tolbutamide was added to the medium, K^+_{ATP} channel activity was rapidly blocked with a concomitant increase in $[Ca^{2+}]_i$, suggesting that this cell would be able to respond to glucose in terms of $[Ca^{2+}]_i$ if enough K^+_{ATP} channels are blocked.

In the seven experiments where glucose failed to increase $[Ca^{2+}]_i$, it was possible to see either a decrease in channel amplitude – as shown in Fig. 6 – or a discrete decrease in K^+_{ATP} channel activity. Tolbutamide was added in four of such experiments, obtaining in all cases the complete closure of the channel and a rise in $[Ca^{2+}]_i$.

DISCUSSION

The experiments presented in this paper directly demonstrate the correlation between glucose-induced K^+_{ATP} closure and the rise in $[Ca^{2+}]_i$ in pancreatic β-cells. The results are consistent with the proposed role of glucose in membrane depolarization and the increase in $[Ca^{2+}]_i$. No evidence for any glucose-induced increase in $[Ca^{2+}]_i$ different from that mediated by the generation of calcium action potentials has been found. A close correlation between electrical activity and $[Ca^{2+}]_i$ was also found in studies in intact islets of Langerhans (Santos *et al.* 1991).

As reported by other authors, at low glucose concentrations (0–3 mM), the prevailing channel activity found in β-cells is due to the K^+_{ATP} channel (Trube *et al.* 1986; Ashcroft *et al.* 1988). Although the increase of glucose concentration from 0 to 3 mM may appreciably decrease K^+_{ATP} channel activity, it does not have any effect in terms of $[Ca^{2+}]_i$ (not shown). This is consistent with previously published data (Ashcroft *et al.* 1988) showing a marked effect of glucose concentrations in the range of 0–5 mM both in terms of decreased channel activity and glucose oxidation, but not in terms of insulin release. This would suggest that at glucose concentrations below 5 mM, the hyperpolarizing effect of the remaining K^+_{ATP} channels is enough to counteract any depolarizing influence due, for instance, to a Na^+ leak permeability or another resting inward current (Atwater *et al.* 1978).

Glucose-induced oscillations in electrical activity (Dean & Matthews, 1968) and $[Ca^{2+}]_i$ (Valdeolmillos *et al.* 1989; Santos *et al.* 1991) are critically dependent on the recording temperature. For instance, using the nystatin technique, it has been shown that isolated β-cells undergo the characteristic burst electrical pattern when the recording is made at 30 °C (Smith, Ashcroft & Rorsman, 1990). $[Ca^{2+}]_i$ oscillations in isolated β-cells are also dependent on the recording temperature (Hellman, Gylfe, Grapengiesser, Panten, Schwanstecher & Heipel, 1990), being inhibited below 30 °C. The fact that our experiments were done at 23 °C would explain why we did not see $[Ca^{2+}]_i$ oscillations. Another reason may be related to the high glucose concentration used, enough for continuous electrical activity, therefore producing a maintained rise in $[Ca^{2+}]_i$.

The closest correlation between $[Ca^{2+}]_i$ and channel activity was observed when the cells exhibited biphasic currents in the presence of stimulatory glucose concentrations (see Figs 2 and 3). It was also very clear that $[Ca^{2+}]_i$ never starts to rise before the first spike appears. It was impossible, however, to correlate discrete calcium transients with any given spike.

In some experiments it was possible to observe a rise in $[Ca^{2+}]_i$ even when not all the K^+_{ATP} channels were blocked (Fig. 1) or to record simultaneously action currents and K^+_{ATP} channel activity (Fig. 2). This finding may indicate that blocking a certain percentage of K^+_{ATP} channels is sufficient for membrane depolarization and calcium current activation. The counter balance between K^+_{ATP} channel activity and membrane depolarization – due to calcium and other depolarizing currents – may provide the basis to explain the different burst duration at different glucose concentrations seen in whole islets.

The time taken for glucose to increase $[Ca^{2+}]_i$ ranged from 90 to 120 s, reflecting the time required for glucose transport, metabolism and blockade of K^+_{ATP} channels.

Due to the different mode of action, tolbutamide very quickly blocked the channels and induced a faster increase in $[Ca^{2+}]_i$. The same temporal differences have been previously reported (Ashcroft *et al.* 1984; Trube *et al.* 1986).

In the experiment (Fig. 6) where glucose was increased in the presence of diazoxide, $[Ca^{2+}]_i$ *decreased*. This may be due to the dual role of glucose in $[Ca^{2+}]_i$ handling by β-cells. On one hand, glucose-induced blockade of K^+_{ATP} channels provides the basis for calcium increase. On the other hand, β-cells have active calcium sequestering/removing mechanisms that, as in any other cells, are fuelled by metabolism. The same effect has been put forward by other authors (Grapengiesser, Gylfe & Hellman, 1988; Gylfe, 1988). This experiment provides strong evidence in favour of the role of glucose in cell depolarization and calcium current activation as the mechanism for the $[Ca^{2+}]_i$ increase. On the time scale of our experiments, other effects of glucose, like calcium release from intracellular stores, do not seem to play an important role. It is also interesting to note that diazoxide itself did not have any effect on $[Ca^{2+}]_i$, despite the fact of producing a membrane hyperpolarization.

In those cells where the closure of K^+_{ATP} channels was not sufficient to depolarize the cells up to the threshold for calcium channel activation (Fig. 7), glucose did not increase, but rather decreased $[Ca^{2+}]_i$. The fact that in these cells tolbutamide elicited an increase in $[Ca^{2+}]_i$ demonstrates that they have all the ionic machinery necessary for glucose-induced depolarization.

The finding that some cells were not fully responsive to glucose – in terms of increased $[Ca^{2+}]_i$ – is consistent with previous reports in isolated cells indicating a heterogeneous cell population (Salomon & Meda, 1986). Also consistent with our results is the fact that both responsive and non-responsive cells had the full set of ionic channels (Soria, Chanson, Giordano, Bosco & Meda, 1991). It is suggested that in the intact islet of Langerhans such heterogeneity is masked by intercellular coupling mechanisms (i.e. gap junctions; Meda, Kohen, Kohen, Rabinovitch & Orci, 1982; Perez-Armendariz, Roy, Spray & Bennett, 1991) leading to a homogeneous and synchronized response in terms of electrical activity (Meissner, 1974) and $[Ca^{2+}]_i$ (Santos *et al.* 1991).

In conclusion, our results demonstrate the link between glucose metabolism, K^+_{ATP} channel closure and the ensuing rise in $[Ca^{2+}]_i$ due to calcium channel activation. The combined use of microfluorometric measurements and channel activity in the cell-attached configuration can be used to study other glucose-induced metabolic changes, for instance monitoring cell autofluorescence (Pralong, Bartley & Wollheim, 1990). The same technique can be also applied to other cells in order to study the functional significance of specific binding sites for sulphonylureas in different areas of the CNS (Mourre, Ben Ari, Bernardi, Fosset & Lazdunski, 1989) or the role of K^+_{ATP} channel activation during cardiac hypoxia (Lederer, Nichols & Smith, 1989; Gasser & Vaughan-Jones, 1990).

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