Substrate-dependent changes in mitochondrial function, intracellular free calcium concentration and membrane channels in pancreatic β -cells

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Microfluorimetric and patch-clamp techniques have been combined to determine the relationship between changes in mitochondrial metabolism, the activity of K_{ATP} channels and changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) in isolated pancreatic β -cells in response to glucose, ketoisocaproic acid (KIC) and the electron donor couple tetramethyl *p*-phenylenediamine (TMPD) and ascorbate. Exposure of cells to 20 mM glucose raised NAD(P)H autofluorescence after a delay of 28 ± 1 s (mean $\pm$ S.E.M., $n = 30$). The mitochondrial inner membrane potential, $\Delta\psi_m$ (monitored using rhodamine 123 fluorescence), hyperpolarized with a latency of 49 ± 6 s ($n = 17$), and the $[Ca^{2+}]_i$ rose after 129 ± 13 s ($n = 5$). The amplitudes of the metabolic changes were graded appropriately with glucose

concentration over the range 2.5–20 mM. All variables responded to KIC with shorter latencies: NAD(P)H autofluorescence rose after a delay of 20 ± 3 s ($n = 5$) and rhodamine 123 changed after 21 ± 3 s ($n = 6$). The electron donor couple, TMPD with ascorbate, rapidly hyperpolarized $\Delta\psi_m$ and raised $[Ca^{2+}]_i$. When $[Ca^{2+}]_i$ was raised by sustained exposure to 20 mM glucose, TMPD had no further effect. TMPD also decreased whole-cell K_{ATP} currents and depolarized the cell membrane, measured with the perforated patch configuration. These data are consistent with a central role for mitochondrial oxidative phosphorylation in coupling changes in glucose concentration with the secretion of insulin.

INTRODUCTION

The secretion of insulin by pancreatic β -cells is stimulated by glucose and by a variety of other substrates for intermediary metabolism. The steps that underlie transduction, from a rise in substrate supply (most notably glucose) to the secretion of insulin, involve processes that result in the closure of a class of potassium channels known as K_{ATP} channels. These channels are closed by an increase in the concentration of ATP, probably as ATP^{4-} , at the inner face of the plasma membrane (for a review, see Ashcroft and Rorsman, 1990). As the K_{ATP} channels are the major determinant of the resting membrane potential in the β -cell, their closure results in depolarization of the cell membrane, the opening of voltage-activated calcium channels and Ca^{2+} influx, which promotes the secretion of insulin. There remains some debate about the precise coupling between substrate metabolism and K_{ATP} channel closure, although it is currently thought that this is predominantly mediated by an increase in intracellular [ATP] and a concomitant reduction in [ADP]. This hypothesis requires that changes in [ATP] and [ADP] are closely entrained to glucose oxidation. The rate of oxygen consumption by β -cells rises in response to an increase in glucose concentration (Hellerstrom, 1967; Hutton and Malaisse, 1980), and this response can be prevented by inhibitors of mitochondrial electron transport (Hutton and Malaisse, 1980). Several observations suggest that the ATP that regulates the K_{ATP} channels is derived, at least in part, from mitochondrial metabolism. First, inhibitors of mitochondrial metabolism induce marked activation of the K_{ATP} channel (Ashcroft et al., 1985). Secondly, the deamination product of leucine, 2-ketoisocaproic acid (KIC), which is metabolized directly via the tricarboxylic acid cycle within the mitochondria, is a potent insulin secretagogue, increases oxygen

consumption (Hutton and Malaisse, 1980) and inhibits K_{ATP} channel activity (Ashcroft et al., 1987). Finally, bongkreic acid, an inhibitor of the mitochondrial ATP/ADP translocase, is a potent inhibitor of K_{ATP} channel activity (Kiranadi et al., 1991).

Mitochondrial ATP production is driven by the proton-motive force which is generated by the electron transport chain. The proton-motive force consists largely of a membrane potential ($\Delta\psi_m$), estimated in the mitochondria of most cells to lie between -150 and -200 mV, and a pH gradient. The rate of ATP synthesis is steeply dependent on $\Delta\psi_m$ (see, for example, Zoratti et al., 1982). Thus the increased supply of reducing equivalents from substrate oxidation might reasonably be expected to increase the rate of electron transport, and to increase $\Delta\psi_m$. The increased proton-motive force will in turn drive an increase in the rate of ADP phosphorylation. In the experiments described here, microfluorimetric techniques were used to monitor changes in mitochondrial function in response to changes in the concentration of either glucose or KIC to enable examination of the temporal correlations between changes in metabolism, in electrical activity and in the intracellular free calcium concentration ($[Ca^{2+}]_i$).

Some of this work has been presented to the Physiological Society (Duchen et al., 1993).

MATERIALS AND METHODS

Preparation of cells

Briefly, the pancreas was removed from adult male Balb/c mice and isolated islets were prepared by collagenase digestion as previously described (Lernmark, 1974). The islets were dissociated into isolated cells and small clusters of cells by further incubation in a Ca^{2+} -free trypsin/EDTA solution. Cells were

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; $[Ca^{2+}]_m$, intramitochondrial calcium concentration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Rh 123, rhodamine 123; $\Delta\psi_m$, mitochondrial membrane potential; KIC, ketoisocaproic acid. TMPD/asc, tetramethyl *p*-phenylenediamine plus ascorbic acid; DMSO, dimethyl sulphoxide; TPP^+ , tetraphenylphosphonium ion.

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then plated on to glass cover-slips and maintained in RPMI 1640 medium supplemented with 11 mM glucose, 10% (v/v) foetal calf serum (Gibco) and 50 µg/ml gentamycin (Gibco), and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were maintained in culture for up to 1 week, but most experimental data were obtained within 4 days in culture.

Fluorescence measurements

Microfluorimetric experiments were carried out using standard fluorescence microscopy as previously described (e.g. Duchen, 1992a,b; Duchen and Biscoe, 1992a,b). Autofluorescence derived from NAD(P)H was excited by light at 350 nm and measured with a bandpass filter combination between 400 and 500 nm (Chance, 1976). Autofluorescence from FAD²⁺ was excited using a 10 nm bandwidth interference filter at 450 nm and measured between 510 and 590 nm (see Chance et al., 1979). For measurements of [Ca²⁺]_i, Fluo-3 was used in preference to Indo-1, to avoid interference from the changes in autofluorescence which are a significant problem with Indo-1 (see, for example, Biscoe and Duchen, 1990). Cells were loaded with the acetoxymethyl (AM) ester of Fluo-3 for 30 min in the presence of 5 mM glucose and then incubated for another 30 min before recordings were made, to allow completion of ester hydrolysis. Fluo-3 fluorescence was excited at 490 nm and measured at 530 nm. We have not attempted to calibrate these signals, and data are presented only as relative changes in signal. Rhodamine 123 (Rh 123) was used as an indicator of mitochondrial potential (Emaus et al., 1986; Duchen and Biscoe, 1992b). Cells were loaded by incubation with 10 µg/ml Rh 123 for 10 min (in 5 mM glucose) and were then washed. The localization of fluorescence to mitochondria is obvious under standard microscopic conditions (Johnson et al., 1980). Rh 123 fluorescence was excited at 490 nm and measured at 530 nm as previously described (Duchen and Biscoe, 1992b; Duchen, 1992b).

The use of Rh 123 to follow changes in $\Delta\psi_m$ with time has not been widely documented. Experiments using Rh 123 are the equivalent of more familiar experiments performed on isolated suspensions of mitochondria, in which the partitioning of a lipophilic cation such as tetraphenylphosphonium (TPP⁺) is used to monitor changes in $\Delta\psi_m$. A TPP⁺-sensitive electrode is used to follow the distribution of the ion. Addition of substrate, e.g. succinate, to the mitochondria leads to the generation of a potential, and thus to the uptake of TPP⁺. In the current study, the distribution and subsequent quenching of the fluorescent lipophilic cation Rh 123 was used to monitor changes in $\Delta\psi_m$. An increase in Rh 123 fluorescence indicates depolarization of $\Delta\psi_m$, as shown by the observation that mitochondrial uncouplers increase the Rh 123 fluorescence signal, as do agents that block respiration (Duchen and Biscoe, 1992b; Duchen, 1992b). This is interpreted in terms of the well-established biophysics of a variety of lipophilic cations in response to partitioning into negatively charged organelles. The concentration of the dye by mitochondria leads to the forced aggregation of dye molecules and to an associated quenching of the fluorescence signal (see Emaus et al., 1986; Bunting, 1992). Some dye must remain in the cytosol, prevented from leaving the cell by the plasma membrane potential. Thus hyperpolarization of $\Delta\psi_m$ will increase the partitioning of dye into the mitochondria, and increase the concentration-dependent quenching. Depolarization of $\Delta\psi_m$ allows dye to redistribute from the mitochondria into the cytosol, increasing the signal. Voltage clamp experiments (Duchen, 1992b) have shown that changes in Rh 123 fluorescence following depolarization of the plasma membrane in neurons are due solely to changes in $\Delta\psi_m$, secondary to changes in [Ca²⁺]_i, and that the

dye does not signal changes in plasma membrane potential *per se*.

Data were digitized and stored for off-line analysis. Estimates of the latencies of responses were obtained by fitting a linear regression line to the baseline, extrapolating that line throughout the response and then identifying the time at which the divergence of the data from the line was significant. All values are given as means $\pm$ S.E.M.

Drugs and solutions

During experiments, cells were continuously superfused with a solution that contained (mM): 132 NaCl, 5.6 KCl, 2.6 CaCl₂, 1.1 MgCl₂, 1.2 NaH₂PO₄, 4.2 NaHCO₃ and 10 Hepes (pH 7.4). Concentrations of glucose or KIC were varied as indicated in the text. For perforated patch-clamp recordings, the pipette solution contained (mM): 70 K₂SO₄, 10 KCl, 10 NaCl, 1 MgCl₂, 40 sucrose and 10 Hepes, plus 0.1 mg/ml amphotericin B and 0.1% dimethyl sulphoxide (DMSO), pH 7.4 (Smith et al., 1990). Drugs used included tolbutamide (100 µM), rotenone (1 µM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 1 µM) and tetramethyl *p*-phenylenediamine (TMPD; 20 µg/ml), with 2 mM sodium ascorbate (all from Sigma). All experiments were performed at 30–32 °C. The perfusion systems used had dead-times of 5–10 s for full equilibration of solution changes.

Patch-clamp recordings

Patch-clamp recordings were made using conventional techniques and a List EPC-7 amplifier. Data were stored on video tape (PCM 8; Medical System Corp.) for off-line analysis using pClamp software (Axon instruments).

Whole-cell permeabilized patch-clamp techniques were employed to record whole-cell K_{ATP} currents, using amphotericin B to permeabilize the patch membrane. This approach minimizes interference with cellular metabolism (see Smith et al., 1990).

RESULTS

Change in [Ca²⁺]_i in response to glucose

The viability and identity of the cells was initially tested by examination of the change in [Ca²⁺]_i in response to glucose. After incubation in glucose-free saline for about 20 min, exposure of cells to 20 mM glucose raised [Ca²⁺]_i with a mean delay of 128 $\pm$ 13 s (n = 15) (Figures 1a and 1b). Complex fluctuations of [Ca²⁺]_i were seen in some, but not all, cells (Figure 1). Depolarization of the cells by blockade of K_{ATP} channels with 100 µM tolbutamide (Figure 1b) or by the brief application of 50 mM K⁺ (isotonic replacement of Na⁺) (Figure 1c) also raised [Ca²⁺]_i. Responses to depolarization recovered completely when the stimulus was removed (Figures 1b and 1c). In contrast, [Ca²⁺]_i responses to glucose usually failed to recover to baseline levels on removal of glucose over a period of 10 min, although levels fell from the peak obtained during glucose application (Figures 1a and 1b). This is clearly not simply a failure of [Ca²⁺]_i homeostasis in the absence of glucose, as the responses to high potassium and to tolbutamide both recovered to the original resting levels. These measurements serve (i) to demonstrate that the cells show the responses expected of β -cells, and (ii) to establish the average latency between exposure of the cells to glucose and the rise in [Ca²⁺]_i.

Changes in NADH autofluorescence in response to substrate

The basic premise of these experiments is that the supply of substrate to the β -cell ultimately effects a rise in [ATP], and that

Figure 1 Changes in $[Ca^{2+}]_i$ on addition of glucose and on membrane depolarization

Fluo-3 fluorescence was measured in small clusters of cells as an indicator of $[Ca^{2+}]_i$. In (a), the cells were initially bathed in glucose-free saline, which was switched as indicated to saline containing 20 mM glucose. After a delay of about 150 s, the $[Ca^{2+}]_i$ rose and then showed substantial fluctuations. In (b), 100 μ M tolbutamide was applied in glucose-free saline from a micro-pipette placed close to the cells. $[Ca^{2+}]_i$ rose rapidly, and recovered after a short delay when the drug was removed. The response is compared with the response to 20 mM glucose. In (c) the response to a 1 s application of 50 mM K^+ from a puffer pipette is shown. The rapid increase and biphasic recovery were characteristic of these cells.

this is the consequence of an increased supply of reducing equivalents for mitochondrial respiration. We therefore measured autofluorescence under conditions that evoke fluorescence of NAD(P)H (see the Materials and methods section), as this is derived predominantly from mitochondrial NADH (for review, see Balaban and Mandel, 1990). Under these conditions, an increase in fluorescence signals an increase in the reduced state of the pyridine nucleotide [i.e. NAD(P)H], and a decrease indicates increased oxidation to NAD(P) $^+$. Increased activity of the tricarboxylic acid cycle in response to increased substrate supply should lead to an increase in the NAD(P)H/NAD(P) $^+$ ratio. Exposure of β -cells to glucose was invariably followed by a reversible increase in autofluorescence (Figure 2a), and the steady state reached was graded with glucose concentration (Figure 2b). Changes in fluorescence were detectable on switching from glucose-free saline to glucose concentrations as low as 2.5 mM. The mean latency of the response to 20 mM glucose was 28 ± 1 s (mean $\pm$ S.E.M., $n = 30$). An increase in autofluorescence was

Figure 2 Changes in NAD(P)H autofluorescence with substrate supply

These records show the fluorescence recorded from unloaded cells in response to illumination with light at 350 nm, measured between 400 and 500 nm. In (a), the full reversible increase in fluorescence seen in response to 20 mM glucose is shown. In (b), the onset of responses to superfusion of another group of cells with glucose at a range of concentrations (as indicated adjacent to each trace) have been superimposed, showing the graded response to graded glucose concentrations. The trace in (c) shows the response of the same cell group to 20 mM KIC. Note the shorter latency to the onset of this response compared with the records shown in (b). The dotted line indicates the baseline.

also seen in response to 20 mM KIC (Figure 2c), a well-established stimulant of insulin secretion, which is metabolized entirely within the mitochondria. At a concentration of 20 mM, KIC evoked a response of similar amplitude to that produced by 20 mM glucose, but the latency was shorter (20 ± 3 s; $n = 7$), consistent with the shorter metabolic pathway. These changes in autofluorescence in response to glucose were unaffected by removal of extracellular Ca^{2+} ($n = 5$), suggesting that they cannot in any way reflect a response secondary to Ca^{2+} influx.

The fluorescence signal measured under these conditions is derived from both mitochondrial and cytosolic NADH and NADPH. As the spectra of NADH and NADPH overlap, it is not possible to differentiate between the signals originating from each source; for this reason we refer to NAD(P)H, indicating that the signals are derived from either NADH or NADPH, or from both. A further measure of mitochondrial respiration can be obtained from flavoprotein fluorescence, as oxidized flavoproteins are fluorescent (see Panten and Ishida, 1975; Chance et al., 1979). Under conditions appropriate to elicit flavin fluorescence (see Materials and methods section), a signal was measurable which decreased reversibly on switching to KIC ($n = 5$; Figure 3) or to a higher glucose concentration. This is

Figure 3 Changes in flavoprotein fluorescence in response to KIC

Fluorescence was excited by illumination with light at 450 nm and measured between 510 and 590 nm from cells that had not been loaded with dye. A decrease in fluorescence signals an increase in the reduced state of flavins in the cells. Here, 20 mM KIC was applied for the period shown, causing a slow and reversible fall in fluorescence.

consistent with the increased provision of reduced flavoproteins by the tricarboxylic acid cycle. As the signals were relatively small, and the signal-to-noise ratio was low, we did not attempt any further analysis of these signals, but they do support our interpretation that the autofluorescence signals described above originate from mitochondrial respiration.

Changes in $\Delta\psi_m$ in response to substrate

The increased supply of reducing equivalents is expected to increase the rate of electron transport, which should increase proton efflux and hyperpolarize $\Delta\psi_m$. This would in turn serve to increase the rate of oxidative phosphorylation, raising the rate of ATP synthesis. Figure 4 shows measurements of Rh 123 fluorescence from a small cluster of β -cells. In response to an increased glucose concentration, the signal decreased after a mean delay of 49 ± 6 s (mean $\pm$ S.E.M., $n = 17$). The decrease in signal is consistent with an increase in $\Delta\psi_m$ (e.g. see Duchen and Biscoe, 1992b). As in the case of the NADH signal, the amplitude of the response varied with the glucose concentration (Figure 4b). The responses to 20 mM KIC had a shorter latency (21 ± 3 s; mean $\pm$ S.E.M., $n = 6$) (Figure 4c), but a similar amplitude, to the peak responses to 20 mM glucose.

That Rh 123 fluorescence recorded in β -cells behaves similarly to that in other cells was established by examining the fluorescence response to inhibition of electron transport with CN^- (results not shown) and to the uncoupler FCCP (Figure 5). Both CN^- and FCCP increased Rh 123 fluorescence, indicating depolarization of $\Delta\psi_m$. While the Rh 123 signals are not readily calibrated in terms of $\Delta\psi_m$, in most cell types, in the presence of physiological glucose concentrations, FCCP (a proton ionophore which should dissipate $\Delta\psi_m$ completely) increases Rh 123 fluorescence by about 80% (Duchen and Biscoe, 1992b). Interestingly, in β -cells FCCP increased fluorescence by about 35–45% in the absence of glucose ($n = 5$; Figure 5). This suggests that in β -cells the mitochondrial potential is significantly depolarized in the absence of glucose and is restored in a graded manner as the substrate concentration is increased. If it is assumed that the fully polarized potential is about -180 mV in the presence of 20 mM glucose, and that FCCP depolarizes $\Delta\psi_m$ to zero, then the resting level in the absence of glucose is about 60% of the fully polarized state, i.e. about -100 mV. This of course assumes a linear calibration of Rh 123 fluorescence with potential, a condition that is fulfilled in isolated mitochondria (Emaus et al., 1986).

Figure 4 Changes in Rh 123 fluorescence with substrate supply

These records are equivalent to those shown in Figure 2, but here Rh 123 fluorescence was measured. Application of 20 mM glucose led, after a delay, to a decrease in the signal (a). On removal of the glucose the signal continued to decrease for about 20 s, but then increased slowly towards the original baseline. In (b), the onset of responses of another cell cluster to three different glucose concentrations have been superimposed, while in (c) the response to KIC of the same cell cluster as in (b) is shown. The time course of recovery was typically very slow. Note also that Rh 123 fluorescence frequently shows significant bleaching, as indicated by the dotted lines especially in (a).

Figure 5 Relative changes in Rh 123 fluorescence with FCCP and glucose

Rh 123 fluorescence was recorded from a group of β -cells. FCCP ($1 \mu M$) was applied to the glucose-free superfusate close to the cells for about 10 s from a puffer pipette, and the signal increased accordingly, recovering as the FCCP was washed away. The superfusate was then switched to saline containing 20 mM glucose for the time indicated by the bar, causing a reversible decrease in signal. The signal was slowly bleaching, hence the slow decline in basal fluorescence (dotted line).

Figure 6 Effects of the TMPD/Asc couple

(a) In zero glucose, application of TMPD/Asc produced a reversible decrease in Rh 123 fluorescence, indicating a hyperpolarization of $\Delta\psi_m$. The action of TMPD/Asc was further tested as shown in (b) and (c). Rotenone (1 μ M) was used to block electron transfer at complex I and caused a slow and slowly reversible depolarization of $\Delta\psi_m$ (b). (c) TMPD/Asc reversed the depolarization when applied at its height. Residual action of rotenone was apparent on removal of the TMPD/Asc couple.

An elevation in $[Ca^{2+}]_i$ through membrane depolarization by 100 μ M tolbutamide or 50 mM K^+ had no effect on either the NAD(P)H autofluorescence or the Rh 123 signals in the absence of glucose ($n > 5$ for each manipulation), consistent with a depolarized $\Delta\psi_m$ and impaired mitochondrial Ca^{2+} uptake (see Duchen, 1992b).

β -cell responses to the electron donor couple, tetramethyl *p*-phenylenediamine and ascorbate (TMPD/Asc)

These experiments suggest that the supply of reducing equivalents to the mitochondria of β -cells should by itself lead to an increase in electrical activity, and thence to insulin secretion. In order to examine this idea further, we used TMPD/Asc as an electron donor. TMPD bypasses all but the final step of the electron transport chain and acts by directly reducing cytochrome *c* oxidase (Dutton et al., 1970; Duchen and Biscoe, 1992b). This increases the availability of the reduced form for oxidation by oxygen, increasing the respiratory rate and hyperpolarizing $\Delta\psi_m$. Ascorbate acts as an electron donor (with a higher redox potential) and so regenerates the reduced form of the TMPD and serves to prevent oxidation of the TMPD by atmospheric O_2 . Figure 6(a) shows that TMPD/Asc hyperpolarized $\Delta\psi_m$ in β -cells in the absence of glucose. TMPD/Asc restored $\Delta\psi_m$ after blockade of the electron transport chain at complex I by rotenone,

Figure 7 Fluo-3 signals recorded from a small cluster of β -cells

(a) Application of TMPD/Asc from a puffer pipette provoked an almost immediate rise in $[Ca^{2+}]_i$, which recovered only slowly back toward basal levels. In (b), another small cluster of cells was exposed continuously to 20 mM glucose. The $[Ca^{2+}]_i$ rose and remained high throughout this time, showing some fluctuations. Application of TMPD/Asc failed to raise $[Ca^{2+}]_i$ further.

consistent with the above scheme. Rotenone alone caused a slow depolarization of $\Delta\psi_m$ that usually lasted for several minutes after removal of the drug before being reversed (Figure 6b). Application of TMPD/Asc rapidly reversed the effect of rotenone, decreasing the Rh 123 signal below its initial baseline, and on withdrawal of TMPD/Asc the residual effect of rotenone was still apparent (Figure 6c).

In the absence of glucose, TMPD/Asc alone caused a decrease in resting Rh 123 fluorescence (a hyperpolarization of $\Delta\psi_m$) and raised $[Ca^{2+}]_i$ after a delay of less than 5 s (Figure 7a; $n = 8$). Since TMPD/Asc was applied locally from a pipette, the latencies are not strictly comparable with those found with superfused substrates. That these changes in Fluo-3 responses represent changes in $[Ca^{2+}]_i$ and not some fluorescence artefact is supported by the observation that application of TMPD/Asc to cells already stimulated with 20 mM glucose had no additional effect on the signal (Figure 7b; $n = 5$). The changes in $[Ca^{2+}]_i$ in response to TMPD/Asc resembled those in response to glucose or KIC in that recovery was only partial, and the Fluo-3 signal invariably remained raised for prolonged periods after the stimulus was washed away.

The electrophysiological correlate of these experiments is shown in Figure 8. K_{ATP} currents were measured using the perforated-patch whole-cell configuration according to the procedure of Trube et al. (1986). The cell membrane potential was clamped at -70 mV and alternate hyperpolarizing and depolarizing pulses of 10 mV amplitude and 200 ms duration were applied at a frequency of 0.5 Hz. Most of the resulting current during these pulses flows through ATP-sensitive K^+ channels, since voltage-dependent currents are not activated by these pulse

Figure 8 Whole-cell K_{ATP} currents measured using the amphotericin permeabilized patch technique

The figure shows traces obtained from each of three single β -cells. In each case, the holding potential was -70 mV, and pulses of ± 10 mV were applied alternately at 0.5 Hz to test the resting conductance, predominantly a K_{ATP} . In (a), $100 \mu\text{M}$ tolbutamide decreased the holding current and suppressed the resting conductance. In (b), application of 20 mM glucose led to the reversible suppression of the resting conductance. Finally, TMPD/Asc caused a very rapid and reversible suppression. The asterisks indicate the times at which the amplifier was switched to current clamp to measure membrane potential. The cells were significantly depolarized in each case. Values for the resting membrane potential were -64 mV in the absence of substrate. During application of tolbutamide the potential was continuously spiking from -44 mV to $+2$ mV; during application of glucose, from -47 mV to $+2$ mV, and during application of TMPD/Asc, from -54 mV to -3 mV.

amplitudes. Tolbutamide ($100 \mu\text{M}$), a selective blocker of K_{ATP} currents, rapidly and reversibly inhibited the currents resulting from the pulses, and reversibly decreased the holding current, confirming that they result from current flow through ATP-sensitive K^+ channels (Figure 8a). The addition of 20 mM glucose reversibly blocked the K_{ATP} current after a mean latency of 42 ± 3 s (Figure 8b; $n = 9$). Cells responded similarly but more rapidly to 20 mM KIC (results not shown; mean latency of 16 ± 5 s, $n = 3$). The TMPD/Asc couple blocked the K_{ATP} currents almost immediately (Figure 8b, latency 10 ± 2 s, $n = 6$). In this case, the TMPD was bath-applied, and so the latencies are directly comparable with those for glucose, KIC and tolbutamide. This metabolic response is comparable in speed to the pharmacological block by tolbutamide (latency 6 ± 1 s, $n = 6$). TMPD/Asc had no effect on single channel currents in isolated inside-out membrane patches ($n = 3$; results not shown). This is consistent with the proposed metabolic action of the TMPD/Asc couple, and indicates that it is not acting to block the K_{ATP} channel directly.

DISCUSSION

We have shown that application of glucose to isolated β -cells initiates a sequence of events summarized in Table 1, which starts with an increased reduction of the mitochondrial pyridine nucleotide and flavin pool. This is followed by stimulation of the electron transport chain and hyperpolarization of the mitochondrial membrane potential, as indicated by the decrease in Rh 123 fluorescence. Inhibition of whole-cell K_{ATP} currents occurs after or around the same time as mitochondrial hyperpolarization and is then followed by an increase in the intracellular Ca^{2+} concentration. Our data are therefore consistent

with a central role for mitochondrial oxidative phosphorylation in the transduction process underlying glucose-stimulated insulin secretion in pancreatic β -cells.

The same sequence of events is initiated by KIC, although the latency of each response is shorter than that measured for glucose (Table 1). It seems likely that at least part of the explanation for this finding is that KIC is metabolized directly by the mitochondria. Clearly, we must also take into account the rates of transport for these substrates in accounting for the delays on the transduction pathway. The transport of glucose across the β -cell membrane is extremely rapid, and equilibration is complete within a few seconds (Hellman et al., 1971). Thus the rate of transport of glucose is not generally believed to be rate-limiting. However, KIC transport is much slower, and full equilibration may take up to 10 min (Best et al., 1992). Thus KIC transport appears to be rate-limiting, and the rate of metabolism may in this case reflect the rate of uptake. However, the speed of the responses to KIC that we have seen here suggests that the subsequent steps must be rapid, and perhaps that the concentrations we have used are supramaximal, so that the responses are generated more quickly than those to glucose. TMPD crosses cell membranes freely.

We have generally implied throughout this paper that the autofluorescence signal we measure reflects changes in mitochondrial NAD(P)H. The arguments about the origins of such signals have been extensively rehearsed in the literature (see Chance et al., 1962; Jöbsis and Duffield, 1967; Chapman, 1972; Chance, 1976; Balaban and Mandel, 1990; Duchen and Biscoe, 1992a; Balaban, 1992). While cytosolic and mitochondrial NADH and NADPH have similar spectral properties and both must make some contribution to the signal, several factors suggest that the bulk of labile signal originates from the mitochondrial pool of NADH. The cytosolic enzyme which most strongly binds NADH, glyceraldehyde phosphate dehydrogenase, is one of the few enzymes that actually quenches NADH fluorescence (Velick, 1961). In contrast, the binding of mitochondrial NADH to the inner membrane enhances the fluorescence signal some 6–8-fold (Avi-Dor et al., 1962). In most tissues, calculations of mitochondrial and cytosolic NADH concentrations also suggest that the concentration present in the cytosol is insufficient to contribute significantly to the fluorescence signal (Williamson, 1965; Chapman, 1972). In any cell type in which mitochondria represent a substantial proportion of the total cell volume, the bulk of autofluorescence elicited under the conditions we have used is therefore likely to be mitochondrial.

Table 1 Relative time courses of the variables measured

The mean latencies (means $\pm$ SEM) for each of the main groups of measurements reported here are shown to indicate the relative time courses of the events. Recordings of the whole-cell K^+ currents are referred to as WC-Ik, and autofluorescence at 450 nm [NAD(P)H] as AF. Responses to KIC and to glucose refer to responses to each at 20 mM.

Measured variable (agent)	Latency (s)
WC-Ik (tolbutamide)	6 ± 1 ($n = 6$)
WC-Ik (TMPD/Asc)	10 ± 2 ($n = 6$)
AF (KIC)	20 ± 3 ($n = 7$)
Rh 123 (KIC)	21 ± 3 ($n = 6$)
WC-Ik (KIC)	16 ± 5 ($n = 3$)
AF (glucose)	28 ± 1 ($n = 30$)
Rh 123 (glucose)	49 ± 6 ($n = 17$)
WC-Ik (glucose)	42 ± 3 ($n = 9$)
$[\text{Ca}^{2+}]_i$ (glucose)	129 ± 13 ($n = 5$)

Our data on NADH autofluorescence resemble those obtained from whole islets (Panten et al., 1973) and from single rat β -cells (Pralong et al., 1990), with two exceptions. First, we did not see fluctuations in NADH autofluorescence in response to 20 mM glucose as reported by Pralong et al. (1990) and, secondly, we only occasionally observed an initial fall in autofluorescence. In general the exposure to glucose in the experiments reported here was much briefer than in the experiments described by Pralong et al. (1990), and it is conceivable that fluctuations may have been seen over a slower time base.

In the present experiments, exposure of β -cells to glucose was generally kept relatively short (2–3 min). This was done to limit the resulting rise in $[Ca^{2+}]_i$, which typically had a 2–3 min latency. It has been suggested that the onset of various metabolic and functional responses may be influenced by the conditions and duration of the period preceding the stimulation (Lenzen, 1978). We have attempted to examine this in some detail throughout these experiments by varying the sequence of substrate applications and their timing, and have found only slight variability in the response times of any given cell in relation to its history; indeed, the consistency of the responses for any given cell have been rather striking, despite multiple applications of substrate for variable periods. Nevertheless, the interactions between mitochondrial responses to substrate and the changes in $[Ca^{2+}]_i$ could provide a potential mechanism to generate a metabolic 'memory' of previous substrate exposures. It is well established that a rise in the intramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) stimulates matrix dehydrogenases, further increasing the supply of NADH to the electron transport chain (for reviews, see Denton and McCormack, 1990; Duchen, 1992b). A rise in $[Ca^{2+}]_i$ might therefore, by raising the $[Ca^{2+}]_m$, increase the NADH/NAD⁺ ratio through stimulation of the dehydrogenases. A rise in $[Ca^{2+}]_m$ has also been shown to promote the dissociation of an ATPase inhibitor protein (Das and Harris, 1990). This could also serve as a positive feedback step, promoting further ATP synthesis. Such effects might be expected to complicate attempts to examine repeated glucose exposures in single cells, and could warrant further, more detailed study. However, in our experiments, we found no effect of $[Ca^{2+}]_i$ elevation, produced by membrane depolarization by either tolbutamide or potassium, or NAD(P)H autofluorescence or Rh 123 fluorescence. This is in contrast with sensory neurons or chromaffin cells, in which an elevation in $[Ca^{2+}]_i$ leads to striking changes in both of these variables (Duchen, 1992b). The major difference is that the present experiments were performed in glucose-free salines in which the resting mitochondrial membrane potential is relatively depolarized, probably to levels that limit mitochondrial Ca^{2+} uptake. If this is the case, glucose-stimulated hyperpolarization of $\Delta\psi_m$, coupled with activation of $[Ca^{2+}]_i$ influx by the closure of K_{ATP} channels, may together act to increase $[Ca^{2+}]_m$ and thereby modulate further ATP synthesis.

One observation for which we cannot yet account is the consistent failure of $[Ca^{2+}]_i$ to fall back to resting levels following metabolic stimulation, even over periods during which the metabolic and electrophysiological variables have fully recovered. This cannot represent a failure of $[Ca^{2+}]_i$ buffering due to low resting ATP/ADP_i ratios resulting from the absence of metabolic substrate, as these cells were fully able to remove $[Ca^{2+}]_i$ loads imposed by membrane depolarization with either K^+ or tolbutamide. This suggests that metabolic stimulation, by glucose, KIC or TMPD, plays some other role in $[Ca^{2+}]_i$ homeostasis. This phenomenon clearly warrants further investigation.

The glucose-dependence of both NADH autofluorescence and Rh 123 fluorescence resemble that for glucose oxidation (Ashcroft

et al., 1988), in that sugar concentrations as low as 2.5 mM elicited a significant response. A similar dose-response curve is observed for K_{ATP} channel activity (Ashcroft et al., 1988), consistent with the idea that mitochondrial metabolism regulates K_{ATP} channel activity. That insulin secretion is not initiated below about 5 mM glucose (Henquin, 1978) is explained by the finding that suppression of K_{ATP} channel activity is insufficient to elicit electrical activity and Ca^{2+} influx below these glucose concentrations.

There remains some debate in the literature regarding the identity of the second messenger which couples glucose metabolism to K_{ATP} channel closure. Our data provide further support for the idea that the rate of ATP turnover serves this role. The rate of mitochondrial ATP synthesis is steeply dependent on $\Delta\psi_m$ over a relatively narrow range (see Zoratti et al., 1982), so that relatively small changes in $\Delta\psi_m$ will have marked effects on the rate of ATP synthesis. The close correlation between the time course of the hyperpolarization and that of inhibition of whole-cell K_{ATP} currents is also consistent with a coupling role for ATP. Finally, the experiments with TMPD and ascorbate demonstrate that only the final step of mitochondrial oxidative phosphorylation is required to hyperpolarize $\Delta\psi_m$ and close K_{ATP} channels. Since the TMPD/Asc couple bypasses all intermediary metabolic steps, and will directly stimulate ATP synthesis, ATP must provide the primary link between β -cell metabolism and K_{ATP} channel inhibition.

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