Two sites of glucose control of insulin release with distinct dependence on the energy state in pancreatic B-cells

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The energy state of pancreatic B-cells may influence insulin release at several steps of stimulus–secretion coupling. By closing ATP-sensitive K^+ channels (K^+ -ATP channels), a rise in the ATP/ADP ratio may regulate the membrane potential, and hence Ca^{2+} influx. It may also modulate the effectiveness of Ca^{2+} on its intracellular targets. To assess the existence of these two roles and determine their relative importance for insulin release, we tested the effects of azide, a mitochondrial poison, on mouse B-cell function under various conditions. During stimulation by glucose alone, when K^+ -ATP channels are controlled by cellular metabolism, azide caused parallel, concentration-dependent (0.5–5 mM), membrane repolarization, decrease in cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ and inhibition of insulin release. When K^+ -ATP channels were closed pharmacologically (by tolbutamide in high glucose), azide did not repolarize the membrane or decrease

$[Ca^{2+}]_i$, and was much less effective in inhibiting insulin release. A similar resistance to azide was observed when K^+ -ATP channels were opened by diazoxide, and high K^+ was used to depolarize the membrane and raise $[Ca^{2+}]_i$. In contrast, azide similarly decreased ATP levels and increased ADP levels, thereby lowering the ATP/ADP ratio under all conditions. In conclusion, lowering the ATP/ADP ratio in B-cells can inhibit insulin release even when $[Ca^{2+}]_i$ remains high. However, this distal step is much more resistant to a decrease in the energy state of B-cells than is the control of membrane potential by K^+ -ATP channels. Generation of the signal triggering insulin release, high $[Ca^{2+}]_i$, through metabolic control of membrane potential requires a higher global ATP/ADP ratio than does activation of the secretory process itself.

INTRODUCTION

Glucose is by far the most important physiological regulator of pancreatic B-cell function. The mechanisms by which it stimulates insulin release are not completely understood, but there is a general consensus that the regulation of certain ionic events by changes in B-cell metabolism is essential for stimulus–secretion coupling [1–7].

It is now widely accepted that glucose entry in B-cells is followed by a stimulation of glycolysis and glucose oxidation. This acceleration of metabolism generates one or several signals, which close ATP-sensitive K^+ channels (K^+ -ATP channels) in the plasma membrane. This closure causes a decrease in K^+ conductance that leads to membrane depolarization with subsequent opening of voltage-dependent Ca^{2+} channels. Ca^{2+} influx through these channels then increases, leading to a rise in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), which eventually triggers exocytosis of insulin granules. K^+ -ATP channels are thus a key target on which changes in B-cell metabolism act to control insulin release. Although the definitive proof is still lacking, a number of arguments support the view that variations of the ATP/ADP ratio serve as a coupling factor at this level [5–9].

Recently, however, we presented evidence that glucose can also influence insulin release independently from its action on K^+ -ATP channels and membrane potential [10]. This effect of glucose does not involve changes in B-cell $[Ca^{2+}]_i$, but rather an amplification of the effectiveness of Ca^{2+} on its intracellular targets [11]. Like the control of membrane potential, this novel effect of glucose is tightly linked to changes in B-cell metabolism. Its biochemical nature has yet to be established, but several lines of evidence suggest that changes in the ATP/ADP ratio are also involved [11].

The aim of the present study was to investigate which of the

two mechanisms of regulation of insulin release by glucose is most dependent on the energy state of B-cells: that requiring control of the membrane potential (through K^+ -ATP channels), or the second, more distal, site of action. To this end, mouse islets were poisoned by azide, an inhibitor of oxidative phosphorylation at the level of cytochrome oxidase, during stimulation by various agents: first, during stimulation by 25 mM glucose alone, i.e. when K^+ -ATP channels, the membrane potential and Ca^{2+} influx are entirely controlled by glucose metabolism; second, in the presence of 15 mM glucose and of a high concentration (200 μ M) of tolbutamide, which closes K^+ -ATP channels directly [12,13]; third, in the presence of 10 mM glucose, of 100 μ M diazoxide which opens K^+ -ATP channels [12], and of high extracellular K^+ , which restores depolarization [10]. Different concentrations of glucose were used because we wished to produce a similar stimulation of insulin release under these three conditions. The pharmacological closure and opening of K^+ -ATP channels by tolbutamide and diazoxide, respectively, should make the membrane potential, the influx of Ca^{2+} , and hence $[Ca^{2+}]_i$ in B-cells, much less dependent on the energy state than in the presence of glucose alone. It thus becomes possible to compare the energy requirement of the two mechanisms of control of insulin release by glucose, that depending on the regulation of membrane potential and that which is independent of changes in membrane potential.

MATERIALS AND METHODS

Solutions

The medium used was a bicarbonate-buffered solution which contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 24 mM $NaHCO_3$. It was gassed with O_2/CO_2 (47:3) to maintain pH 7.4 and was supplemented with BSA (1 mg/ml).

When the concentration of KCl was increased to 30 mM, that of NaCl was decreased accordingly to maintain iso-osmolarity.

Measurements of insulin release

All experiments were performed with islets isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g), killed by decapitation.

In one type of experiment, the islets were first preincubated for 60 min in a control medium containing 15 mM glucose. The islets were then placed in batches of 25 in parallel perfusion chambers and perfused at a flow rate of 1.25 ml/min [14]. Effluent fractions were collected at 1 min intervals, and insulin was measured by a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark).

In another type of experiment, after the initial preincubation of 60 min the islets were distributed in batches of five. Each batch of islets was then incubated for 60 min in 1 ml of medium containing appropriate concentrations of glucose and test substances. A portion of the medium was withdrawn at the end of the incubation and appropriately diluted for insulin assay.

Measurements of ATP and ADP

After the initial preincubation for 60 min in a control medium, batches of five islets were incubated for 60 min in 0.4 ml of medium containing the appropriate concentrations of glucose and test substances. The incubations were stopped by addition of 0.6 ml of ice-cold trichloroacetic acid to a final concentration of 5%. The tubes were then vortex-mixed, left at room temperature for 15 min, and centrifuged for 5 min in a Microfuge (Beckman). A fraction (400 μ l) of the supernatant was then thoroughly mixed with 1.5 ml of diethyl ether, and the ether phase containing trichloroacetic acid was discarded. This step was repeated three times to ensure complete elimination of trichloroacetic acid. The extracts were then diluted with 400 μ l of a buffer containing 40 mM Hepes, 3 mM $MgCl_2$ and KOH to adjust pH to 7.75. They were then frozen at $-20^\circ C$ until the day of the assay. ATP was assayed by a luminometric method [15]. A portion (50 μ l) of each sample was mixed with 190 μ l of the above buffer and 60 μ l of a commercially available ATP monitoring reagent containing firefly luciferase and luciferin (LKB-Wallac, Turku, Finland). The emitted light was measured in a luminometer (Biocounter M 2000; Lumac, Landgraaf, The Netherlands). Another 50 μ l portion of each sample was first incubated for 30 min with 190 μ l of buffer containing 1.5 mM phosphoenolpyruvate and 2.3 units/ml pyruvate kinase to convert ADP into ATP. ATP was then assayed as described above and ADP content was calculated by difference. Appropriate blanks, ATP, and ADP standards were run through the entire procedure, including the extraction step.

Recordings of B-cell membrane potential

A piece of mouse pancreas was fixed in a perfusion chamber, and a few islets were partially microdissected by hand. The membrane potential of a single cell within the islet was continuously measured with a high-resistance electrode [16]. B-cells were identified by the typical electrical activity that they display in the presence of 10–15 mM glucose. The perfusion medium was similar to that described above except for the absence of BSA.

Measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by microspectrofluorimetry in whole islets cultured for 1–2 days in RPMI 1640 medium containing 10 mM

glucose and then loaded for 40 min with fura-2 (Molecular Probes, Eugene, OR, U.S.A.). The tissue was excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured by a CCD camera (Photonic Science, Tunbridge Wells, U.K.). The images were analysed with the system MagiCal (Applied Imaging, Sunderland, U.K.). The technique has been described in detail previously [17].

Materials

Diazoxide was obtained from Schering-Plough Avondale (Rathdrum, Ireland); tolbutamide was from Hoechst A.G. (Frankfurt, Germany); dinitrophenol and oligomycin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ATP, ADP, phosphoenolpyruvate and pyruvate kinase were from Boehringer (Mannheim, Germany); azide and all other reagents were from Merck A.G. (Darmstadt, Germany).

Presentation of results

Measurements of B-cell membrane potential and certain measurements of $[Ca^{2+}]_i$ are illustrated by recordings which are representative of results obtained with the indicated number of islets. Otherwise, results are presented as means $\pm$ S.E.M. for the indicated number of experiments (different animals or islet preparations) or batches of islets. The statistical significance of differences between means was assessed by analysis of variance, followed by Dunnett's test for comparison of test samples with controls, or by Newman-Keuls test for multiple comparisons [18].

RESULTS

Effects of azide on the membrane potential of B-cells

The first step of the study was to verify the prediction that azide produces distinct effects on the membrane potential of B-cells under the different experimental conditions.

Figure 1 Effects of azide on the membrane potential of a mouse B-cell perfused with a medium containing 25 mM glucose

At the beginning of the experiment, the concentration of glucose (G) was raised from 15 to 25 mM. Azide was then added at the indicated concentrations and was withdrawn as shown by the arrows. The three records show a single experiment without interruption, except for a 6 min break in record (a). These records are representative of results obtained in 7 similar experiments with different mice.

Figure 2 Effects of azide on the membrane potential of mouse B-cells

In (a) and (b), the medium contained 15 mM glucose (G15) and 200 μ M tolbutamide (Tolb). Record (a) starts 8 min after addition of tolbutamide to the medium. Azide was then added at the indicated concentrations, and withdrawn as shown by the arrows. Record (b) is the direct continuation of (a), in which the break corresponds to 6 min. In (c), the medium containing 15 mM glucose and 4.8 mM K^+ was first replaced by a medium containing 10 mM glucose (G10), 30 mM K^+ (K30) and 100 μ M diazoxide (Dz). Azide was then added at the indicated concentrations and was withdrawn as shown by the arrows. The three breaks correspond to periods of 7.5 min. These records are representative of results obtained in 6 (a, b) and 4 (c) similar experiments with different mice.

In the presence of 15 mM glucose and 4.8 mM K^+ , B-cells exhibited a rhythmic electrical activity consisting of slow waves of the membrane potential with Ca^{2+} spikes superimposed on the plateau (Figure 1a). Raising the concentration of glucose to 25 mM was followed by a depolarization to the plateau potential. This depolarization was sustained and the spike activity was continuous in 2/7 cells, whereas brief repolarizations occurred in the other 5 cells (Figure 1a). Addition of 0.5 mM azide caused a marked but transient repolarization of the membrane. The electrical activity that subsequently re-appeared was intermittent, with an intensity intermediate between those in 15 and 25 mM glucose. Raising the concentration of azide to 2 mM abolished all electrical activity and repolarized the membrane to a potential similar to the resting potential. These effects of azide were completely reversible on removal of the drug (Figure 1).

When the medium contained 15 mM glucose and 200 μ M tolbutamide, the B-cell membrane was persistently depolarized at the plateau potential and the spike activity was continuous (Figure 2a). Azide did not affect the membrane potential at a concentration of 0.5 mM, but caused a slight repolarization of the plateau with a decrease in spike amplitude at a concentration of 2 mM. There also occurred a fall in spike frequency, by 32% and 47% in the presence of 0.5 and 2 mM azide respectively. When azide was withdrawn from the medium, a paradoxical transient repolarization occurred, with a decrease in spike activity in certain cells (Figure 2b) or even a suppression in others (results not shown). Thereafter, the amplitude of the spikes again became similar to that recorded before application of azide, but the frequency remained about 40% lower. It should be noted that the fall in spike frequency that occurred during treatment with azide cannot be entirely ascribed to the poison, because it also occurs spontaneously with time in B-cells stimulated by tolbutamide alone [19].

When the control medium containing 15 mM glucose and 4.8 mM K^+ was replaced by a medium containing 10 mM glucose,

Figure 3 Effects of azide on $[Ca^{2+}]_i$ in islet cells stimulated by 25 mM glucose (a and b), by 10 mM glucose, 30 mM K^+ and 100 μ M diazoxide (c), and by 15 mM glucose and 200 μ M tolbutamide (d)

Azide was added at concentrations of 0.5 and 2 mM as indicated by the hatched bars. In the presence of 25 mM glucose (G25), the experiments could not be averaged because of the oscillatory nature of the response. (a) and (b) are thus representative of similar responses observed in 15 islets. In (c) and (d), the traces correspond to the mean responses ($\pm$ S.E.M.) in 9 islets. Other abbreviations are as in Figure 2.

30 mM K^+ and 100 μ M diazoxide, the rhythmic electrical activity of B-cells disappeared and was replaced by a steady depolarization of the membrane, without spikes (Figure 2c). Azide did not affect the membrane potential under these conditions.

These results thus show that poisoning B-cells with azide progressively repolarizes the membrane in the presence of 25 mM glucose alone, but has little or no effect in the presence of tolbutamide, or of high K^+ and diazoxide.

Effects of azide on $[Ca^{2+}]_i$ in B-cells

In the presence of 25 mM glucose alone, B-cell $[Ca^{2+}]_i$ was steadily elevated, as shown in Figure 3(b) (5/15 islets), or this plateau was intermittently interrupted by brief decreases in $[Ca^{2+}]_i$, as shown in Figure 3(a) (10/15 islets). On addition of 0.5 mM azide to the medium, $[Ca^{2+}]_i$ fell and started to oscillate in all islets. At a concentration of 2 mM, azide caused a more pronounced and sustained decrease in $[Ca^{2+}]_i$, to an average level (105 ± 5 nM) which remained slightly higher than the basal level

Figure 4 Effects of azide on insulin release from perfused mouse islets stimulated by 25 mM glucose (a), by 10 mM glucose, 30 mM K^+ and 100 μM diazoxide (b), and by 15 mM glucose and 200 μM tolbutamide (c) from 30 min

The experiments were always performed in parallel chambers, and azide (0.5 and 2 mM) was added to one of the chambers (●) between 40 and 60 min. Values are means ($\pm$ S.E.M.) for 5–6 experiments. For abbreviations see Figure 2 legend.

of $[Ca^{2+}]_i$ (75 ± 3 nM) measured in the presence of 3 mM glucose [17]. This difference might be due to a slight mobilization of intracellular Ca^{2+} , because azide was found to increase $[Ca^{2+}]_i$ by about 20 nM in islets perfused with a medium containing 3 mM glucose, or 15 mM glucose and no Ca^{2+} (results not shown). The effect of azide on $[Ca^{2+}]_i$ was completely reversible upon removal of the poison (Figures 3a and 3b).

When the islets were perfused with a medium containing either 10 mM glucose, 30 mM K^+ and 100 μM diazoxide (Figure 3c) or 15 mM glucose and 200 μM tolbutamide (Figure 3d), $[Ca^{2+}]_i$ was high and stable in B-cells. It was unaffected by 0.5 mM azide and transiently increased by 2 mM azide, probably because of the mobilization of cellular Ca^{2+} . On removal of azide, a marked but transient decrease in $[Ca^{2+}]_i$ occurred when islets were perfused with glucose and tolbutamide (Figure 3d), whereas only a small and inconsistent fall was observed in the presence of high K^+ and diazoxide (Figure 3c).

Effects of azide on insulin release from perfused islets

Figure 4 shows the increase in insulin release that occurred when islets were stimulated by 25 mM glucose, by 10 mM glucose, 30 mM K^+ and 100 μM diazoxide, or by 15 mM glucose and 200 μM tolbutamide. It also shows that azide did not inhibit release to the same extent under these different conditions. The effect of 25 mM glucose was partially inhibited by 0.5 mM azide and almost abolished by 2 mM azide (Figure 4a). This contrasts with the lack of effect of 0.5 mM azide, and the very partial (30%) inhibitory effect of 2 mM azide under the other experimental conditions (Figures 4b and 4c). The inhibition by azide was consistently reversible upon washing out the poison. However, the reversal was preceded by a transient further inhibition in the presence of tolbutamide.

Effects of azide on insulin release from incubated islets

The inhibitory effect of various concentrations of azide was tested under four different conditions. The first three were the same as above, and stimulated insulin release to a similar extent in the absence of poison: 12.9 ± 0.7 ng/h per islet in 25 mM glucose; 15.3 ± 0.7 ng/h per islet in 10 mM glucose, 30 mM K^+ and 100 μM diazoxide; 13.5 ± 0.8 ng/h per islet in 15 mM glucose and 200 μM tolbutamide. These values are not significantly different. As shown in Figures 5(a) and 5(b) the dose-response curve illustrating the inhibitory effect of azide was clearly shifted to the right under the last two conditions (IC_{50} above 2 mM) as compared with 25 mM glucose alone (IC_{50} below 1 mM). The fourth condition, a combination of 25 mM glucose and 30 mM K^+ in the presence of diazoxide, stimulated control insulin release (24 ± 1.6 ng/h per islet; $P < 0.01$) more than did 25 mM glucose alone. Although this makes the relative inhibitory effects of azide less readily comparable, the dose-response curve was also shifted to the right in the presence of high K^+ and diazoxide (Figure 5c). When a high concentration of azide (5 mM) was used, the degree of inhibition of insulin release was much less dependent on the nature of the stimulus.

Effects of azide on adenine nucleotide levels in islet cells

Adenine nucleotide levels were measured in islets incubated under conditions similar to those used for measuring insulin release (Figure 6).

In the presence of 25 mM glucose alone, azide produced a concentration-dependent decrease in the ATP/ADP ratio. Between 0.5 and 2 mM azide, this decrease was due to the fall in ATP and the rise in ADP without a change in the sum ATP+ADP. At 5 mM azide, however, ATP levels and the sum ATP+ADP fell markedly (Figure 6).

Azide also produced a concentration-dependent decrease in the ATP/ADP ratio in islets incubated under the other three conditions. This was also the result of opposite changes in ATP and ADP levels, but the fall in the sum ATP+ADP started at lower azide concentration (1–1.5 mM) than in the presence of 25 mM glucose alone. At all concentrations of azide between 0.5 and 2 mM, the ATP/ADP ratio was higher in the presence of 25 mM glucose than under the other conditions (Figure 6). The reversibility of the effects of azide on adenine nucleotide levels was not investigated.

Effects of dinitrophenol and oligomycin

To exclude the possibility that the above results could be due to peculiar properties of azide, we also compared its effects with those of dinitrophenol and oligomycin. Both poisons similarly

Figure 5 Effects of various concentrations of azide on insulin release from mouse islets incubated under various conditions

The results are expressed as a percentage of control insulin release in the absence of azide. The islets were stimulated by 25 mM glucose (□) or by 10 mM glucose, 30 mM K^+ and 100 μ M diazoxide (▲) (a), by 15 mM glucose and 200 μ M tolbutamide (◆) (b), or by 25 mM glucose, 30 mM K^+ and 100 μ M diazoxide (●) (c). Values are means ($\pm$ S.E.M.) for 18–24 batches of islets from 4–6 experiments. For abbreviations see Figure 2 legend.

Figure 6 Effects of various concentrations of azide on ATP and ADP levels (lower panels) and on the ATP/ADP ratio (upper panels) in mouse islets stimulated by various agents

The stimulatory conditions were similar to those described in Figure 5. The broken lines in the upper panels show the changes in the ATP/ADP ratio in islets incubated in the presence of 25 mM glucose alone. Black symbols denote a statistically significant difference from controls in the absence of azide. Values are means ($\pm$ S.E.M.) for 16–22 batches of islets from 4–6 experiments. For abbreviations see Figure 2 legend.

lowered the ATP/ADP ratio in islets incubated in 25 mM glucose alone or in 10 mM glucose, 30 mM K^+ and diazoxide (Figure 7, upper panels), but they inhibited insulin release less readily in the latter than in the former condition (Figure 7, lower panels). These results therefore show that the data obtained with azide are not specific to this poison.

DISCUSSION

The mitochondrial poison azide was used as a tool to decrease ATP production in mouse B-cells, with the aim of comparing the energy-dependence of two identified steps of regulation of insulin release: the control of the B-cell membrane potential through K^+ -ATP channels, and the modulation of Ca^{2+}_i action on the releasing process itself. Two series of experimental conditions were selected. In the presence of glucose alone, the stimulation of insulin release is tightly dependent on the metabolic control of

membrane depolarization, Ca^{2+} influx and $[Ca^{2+}]_i$ rise. In the second series, tolbutamide or high K^+ with diazoxide were used to make the membrane potential and $[Ca^{2+}]_i$ independent of metabolic control, and to restrict the influence of the metabolic state of B-cells to the action of Ca^{2+} on the releasing process itself.

The first, essential, step of the study was to ascertain that poisoning B-cells with azide had distinct effects on the membrane potential under the selected experimental conditions. In the presence of glucose alone, azide repolarized the B-cell membrane and inhibited the electrical activity, as expected from previous studies also with azide [20,21] or with other mitochondrial poisons [20,22]. That this repolarization is due to the opening of K^+ channels is indicated by the acceleration of K^+ efflux from islet cells [23] and the increase in electrical conductance of the B-cell membrane [20] during poisoning. Experiments using the cell-attached configuration of the patch-clamp technique sub-

Figure 7 Effects of dinitrophenol and oligomycin on the ATP/ADP ratio in mouse islets and on insulin release from mouse islets stimulated by 25 mM glucose (□) or by 10 mM glucose, 30 mM K⁺ and 100 μM diazoxide (▲)

The results of insulin release are expressed as a percentage of control insulin release in the absence of poison. Values are means ($\pm$ S.E.M.) for 16–20 batches of islets (insulin release) or 10–16 batches (ATP/ADP). For abbreviations see Figure 2 legend.

sequently showed that the K⁺ channels that open during poisoning are K⁺-ATP channels [5,13]. Sulphonylureas such as tolbutamide or glibenclamide prevent or reverse the opening of K⁺-ATP channels and the attendant increase in K⁺ efflux that are produced in B-cells by mitochondrial poisons [24–26]. This explains why azide did not repolarize the membrane when glucose and tolbutamide were combined. Diazoxide, on the other hand, opens K⁺-ATP channels [12] and repolarizes the B-cell membrane in the presence of a physiological concentration of extracellular K⁺ [27]. When the concentration of K⁺ is increased, the membrane, whose K⁺ conductance is high, depolarizes to follow the change of E_K . This depolarization is independent of the energy state of the cell. Azide was indeed without effect on the B-cell membrane potential in the presence of diazoxide and high K⁺.

The $[Ca^{2+}]_i$ in B-cells is essentially controlled by changes in Ca²⁺ influx through voltage-dependent Ca²⁺ channels [17]. One could thus expect that Ca²⁺ would change or not, according to the effect of azide on the membrane potential. This, however, required direct verification, because cell poisoning might paradoxically increase $[Ca^{2+}]_i$ by releasing Ca²⁺ sequestered in intracellular organelles [2,3,23,26]. A small mobilization of cellular Ca²⁺ was produced by 2 mM azide, but it was much smaller than that caused by 3 mM azide in clumps of human B-cells incubated in 3 mM glucose [28]. The difference is probably due to a larger fall in ATP levels in the latter experiments, which were performed at 30 °C only, a condition which itself already inhibits B-cell metabolism [29]. In our experiments performed in the presence of

Figure 8 Relationship between the ATP/ADP ratio in mouse islets and insulin release from mouse islets stimulated by 25 mM glucose (□), by 10 mM glucose, 30 mM K⁺ and 100 μM diazoxide (▲), by 15 mM glucose and 200 μM tolbutamide (◆), or by 25 mM glucose, 30 mM K⁺ and 100 μM diazoxide (●)

From right to left along each curve, the different points correspond to results obtained in the absence of azide and in the presence of 0.5, 1, 1.5, 2 and 5 mM azide respectively. These data are taken from Figures 5 and 6.

glucose alone, $[Ca^{2+}]_i$ decreased in parallel with the progressive repolarization of the B-cell membrane. In contrast, $[Ca^{2+}]_i$ remained elevated and stable in B-cells stimulated by tolbutamide or by high K⁺ in the presence of diazoxide, except for a transient rise produced by 2 mM azide.

The control experiments thus show that mitochondrial poisoning decreases the triggering signal of secretion ($[Ca^{2+}]_i$) in the presence of 25 mM glucose, but does not decrease it in the presence of tolbutamide or high K⁺ plus diazoxide. The selected experimental conditions are thus suitable to compare the energy-dependence of the two sites of glucose control of insulin release.

It has long been known that mitochondrial poisons inhibit insulin release induced by glucose or other agents [30–34], but it had not been reported that the inhibitory potency of a given poison depended on the nature of the stimulus. The present study clearly shows that azide, dinitrophenol and oligomycin are less potent inhibitors of insulin release when glucose is combined with tolbutamide or with high K⁺ and diazoxide, than when it is used alone. The results not only show that there is a shift in the dose-response curves, but they also establish that the relationship between insulin release and the ATP/ADP ratio in islet cells is different, under the different conditions.

Figure 8 shows that insulin release was much more strongly and rapidly inhibited by small decreases in the ATP/ADP ratio in the presence of glucose alone than in the presence of tolbutamide or high K⁺ and diazoxide. When the ratio fell to about 2.2, insulin release was abolished in the first situation, whereas it was barely affected in the others. Thus, when the membrane potential of B-cells was clamped pharmacologically, a linear relationship was found between insulin release and the ATP/ADP ratio, as long as this ratio did not fall below 1.3–1.4. The key difference between the two sets of situations is that the inhibition of insulin release parallels the changes in membrane potential and $[Ca^{2+}]_i$ in the presence of glucose alone, whereas it occurs in spite of the clamping of membrane potential and $[Ca^{2+}]_i$ in the presence of tolbutamide and high K⁺. The present demonstration, that a decrease in the energy state of intact B-cells can inhibit insulin release even when $[Ca^{2+}]_i$ is high, is in keeping with several reports showing that ATP provision is

necessary for a rise in $[Ca^{2+}]_i$ to sustain insulin release by permeabilized islets [35,36] or RINm5F cells [37]. Generation of the triggering signal, high $[Ca^{2+}]_i$, through a metabolic control of membrane potential is, however, more energy-dependent than activation of the distal steps of the secretory process by this triggering signal.

It has been reported that lowering the O_2 partial pressure of the medium similarly inhibited insulin release induced by 16 mM glucose or by 40 mM K^+ and 5 mM glucose (without diazoxide) [38]. This observation is difficult to compare with ours, because the decrease in O_2 partial pressure from 21 to 1 % was surprisingly without effect on the ATP/ADP ratio in the presence of high K^+ , whereas it produced the expected decrease in the presence of high glucose. In addition, a much older study has reported that pieces of rabbit pancreas had to be incubated under anoxic conditions for a longer period of time to inhibit KCl - than glucose-induced insulin release [32].

One last observation, which may seem surprising at first sight, deserves a brief comment. Removal of azide from the perfusion medium was followed by a transient repolarization of the B-cell membrane and a decrease in electrical activity, which may explain the transient fall in $[Ca^{2+}]_i$ and further inhibition of insulin release. This phenomenon could result from a transient re-activation of the Na^+ pump, because it was prevented by ouabain (J.-C. Henquin and M. Henquin, unpublished work). The Na^+ pump is electrogenic [39], but the current that it produces repolarizes the membrane only if the electrical resistance is high enough. This is the case in the presence of tolbutamide, which closes K^+ -ATP channels, but not in the presence of high K^+ and diazoxide, because K^+ -ATP channels are open. Re-activation of the pump might also be stronger in the presence of tolbutamide than of high K^+ , because intracellular Na^+ is slightly increased by the sulphonylurea [40], but not by high K^+ [41].

In conclusion, two steps of stimulus-secretion coupling display a distinct dependence on the energy state of B-cells. A step distal to the rise in $[Ca^{2+}]_i$ is rather resistant to relatively large decreases in the ATP/ADP ratio, whereas the control of the membrane potential is much more dependent on this ratio. This may be due to intrinsically different sensitivities of the two steps to the ATP/ADP ratio and/or to an inhomogeneity of this ratio within B-cells. Anyhow, generation of the triggering signal, high $[Ca^{2+}]_i$, through a metabolic control of membrane potential requires a higher global ATP/ADP ratio than does the activation of the secretory process itself.

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