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Direct effects of diazoxide on mitochondria in pancreatic B-cells and on isolated liver mitochondria

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1 The direct effects of diazoxide on mitochondrial membrane potential, Ca^{2+} transport, oxygen consumption and ATP generation were investigated in mouse pancreatic B-cells and rat liver mitochondria.

2 Diazoxide, at concentrations commonly used to open adenosine 5′-triphosphate (ATP)-dependent K^+-channels (K_{ATP} channels) in pancreatic B-cells (100 to 1000 μM), decreased mitochondrial membrane potential in mouse intact perifused B-cells, as evidenced by an increase of rhodamine 123 fluorescence. This reversible decrease of membrane potential occurred at non-stimulating (5 mM) and stimulating (20 mM) glucose concentrations.

3 A decrease of mitochondrial membrane potential in perifused B-cells was also caused by pinacidil, but no effect could be seen with levcromakalim (500 μM each).

4 Measurements by a tetraphenylphosphonium-sensitive electrode of the membrane potential of rat isolated liver mitochondria confirmed that diazoxide decreased mitochondrial membrane potential by a direct action. Pretreatment with glibenclamide (2 μM) did not antagonize the effects of diazoxide.

5 In Fura 2-loaded B-cells perifused with the Ca^{2+} channel blocker, D 600, a moderate, reversible increase of intracellular Ca^{2+} concentration could be seen in response to 500 μM diazoxide. This intracellular Ca^{2+} mobilization may be due to mitochondrial Ca^{2+} release, since the reduction of membrane potential of isolated liver mitochondria by diazoxide was accompanied by an accelerated release of Ca^{2+} stored in the mitochondria.

6 In the presence of 500 μM diazoxide, ATP content of pancreatic islets incubated in 20 mM glucose for 30 min was significantly decreased by 29%. However, insulin secretion from mouse perifused islets induced by 40 mM K^+ in the presence of 10 mM glucose was not inhibited by 500 μM diazoxide, suggesting that the energy-dependent processes of insulin secretion distal to Ca^{2+} influx were not affected by diazoxide at this concentration.

7 The effects of diazoxide on oxygen consumption and ATP production of liver mitochondria varied depending on the respiratory substrates (5 mM succinate, 10 mM α-ketoisocaproic acid, 2 mM tetramethyl phenylenediamine plus 5 mM ascorbic acid), indicating an inhibition of respiratory chain complex II. Pinacidil, but not levcromakalim, inhibited α-ketoisocaproic acid-fuelled ATP production.

8 In conclusion, diazoxide directly affects mitochondrial energy metabolism, which may be of relevance for stimulus-secretion coupling in pancreatic B-cells.

Keywords: Diazoxide; K_{ATP} channel openers; pancreatic B-cells; mitochondria; mitochondrial membrane potential; Ca^{2+}; ATP; insulin secretion

Introduction

The hyperglycaemic effect of the hypotensive benzothiadiazine, diazoxide, has been known since the introduction of this compound in clinical use and is due to an inhibition of insulin secretion which can be antagonized by sulphonylureas (Seltzer & Allen, 1965; Frerichs *et al*., 1966). This antagonism was shown to be due to opposite effects of these agents on B-cell K^+-permeability (Henquin & Meissner, 1982). Patch-clamp measurements then revealed that sulphonylureas act as inhibitors of adenosine 5′-triphosphate (ATP)-dependent K^+-channels (K_{ATP} channels) in pancreatic B-cells, while diazoxide is an opener of this type of channel (Sturgess *et al*., 1985; Trube *et al*., 1986; Zünkler *et al*., 1988). Although diazoxide is of distinctly lower potency than newer hypotensive vasodilators like cromakalim or pinacidil in opening smooth muscle K^+-channels (Edwards & Weston, 1993), it is the most potent and effective compound to open K_{ATP} channels in pancreatic B-cells (Garrino *et al*., 1989). For this reason diazoxide is widely used as an experimental tool in B-cell research. Areas of B-cell research in which the use of diazoxide is particularly important are the characterization of insulinotropic drugs acting on K_{ATP} channels (Chan & Morgan, 1990), the elucidation of metabolically regulated events in stimulus-secretion coupling which are independent of K_{ATP} channel closure (Gembal *et al*., 1992; Taguchi *et al*., 1995), and recently the functional characterization of cloned and mutated subunits of the K_{ATP} channel itself (Nichols *et al*., 1996).

Diazoxide is often used in concentrations of up to several hundred micromolar to achieve a maximal opening of K_{ATP} channels and possible additional effects are only rarely taken into account (Björklund & Grill, 1993; Taguchi *et al*., 1995). The close connection in pancreatic B-cells between energy metabolism and regulation of insulin secretion suggests that inhibitory effects of diazoxide on mitochondrial energetics should not be disregarded. This is currently the case, although effects of diazoxide on mitochondria have already been described: very early on, Schäfer *et al*. (1971) and Portenhäuser *et al*. (1971) published detailed studies on the effects of diazoxide on liver mitochondria. Later, inhibition by diazoxide

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of glycerol-3-phosphate dehydrogenase activity in islet homogenates was observed (MacDonald, 1981). This observation could not be confirmed by other authors, instead, inhibition of succinate dehydrogenase was found (Lenzen & Panten, 1983). Also, a reduced uptake of $^{45}Ca^{2+}$ by isolated B-cell mitochondria was observed in the presence of diazoxide, which was taken as indirect evidence for a reduced mitochondrial membrane potential (MacDonald, 1984). Since these findings were at least partially contradictory and of unclear relevance for intact B-cells, no further studies were conducted on this subject after a direct opening effect of diazoxide on B-cell K_{ATP} channels was described (Trube *et al*., 1986), which offered a logical explanation both for the inhibition of insulin secretion by diazoxide and for the antagonism between diazoxide and sulphonylureas. Recently, we noticed an increase of rhodamine 123 fluorescence in pancreatic B-cells in response to 250 μM diazoxide. This indicated a decrease of mitochondrial membrane potential, which was unexpected. A mitochondrial depolarization could be expected in response to depolarization-induced Ca^{2+} influx, but not in response to diazoxide-induced hyperpolarization of the B-cell plasma membrane. We thus tested whether diazoxide had direct effects on mitochondria and whether these effects might be relevant for stimulus-secretion coupling in pancreatic B-cells. The effects of diazoxide were compared with those of pinacidil and levcromakalim. Pinacidil was selected because it is the only K^+ channel opener in addition to diazoxide which has a significant inhibitory action on insulin secretion, whereas racemic cromakalim was shown to be without effect (Garrino *et al*., 1989). In addition to single B-cells from ob/ob mice, rat liver mitochondria were used for some experiments, because it is not feasible to obtain a sufficient amount of isolated mitochondria from collagenase-isolated islets.

Methods

Tissues

Islets were isolated from pancreata of ob/ob mice and NMRI mice by a conventional collagenase digestion technique. Single cells obtained by incubation of the islets for 10 min in a Ca^{2+}-free medium and subsequent vortexing for 2 min were cultivated in RPMI-1640 with 10% foetal calf serum. The glucose concentration was 5 mM to avoid continuous stimulation during the culture. Mitochondrial fractions were prepared from livers of Wistar rats by differential centrifugation as described previously (Rustenbeck *et al*., 1996).

Microfluorimetric measurements of $[Ca^{2+}]_i$ and of mitochondrial membrane potential in pancreatic B-cells

To increase the probability that B-cell responses are being registered, microfluorimetric measurements were performed in dispersed islet cells from ob/ob mice, the pancreatic islets of which contain around 95% B-cells (Hellman, 1965). The cells were cultured on glass cover slips in Petri dishes and were used from day 2 to 4 after isolation. Fura-2/AM (Grynkiewicz *et al*., 1985) was loaded at a concentration of 2 μM for 45 min at 37°C. The cover slip with the attached cells was inserted in a self-made perifusion chamber, which was placed on the stage of an upright epifluorescence microscope fitted with a Zeiss Fluar (40 ×) objective. A dual-wavelength illumination system was connected to the microscope by a quartz fiber light guide. The fluorescence (excitation at 340 or 380 nm, emission > 470 nm) was recorded by a slow-scan CCD camera. Illumination system, CCD camera and imaging software were supplied by TILL Photonics (Munich-Planegg, Germany). All perifusions were performed at 37°C with a HEPES-buffered Krebs-Ringer bicarbonate medium. Image pairs were taken at intervals as indicated in the figures, illumination time for each image was 600 ms, thus long perifusion times were possible without inducing photodamage to the B-cells. To measure mitochondrial membrane potential in intact B-cells, rhodamine 123 (Rh 123), a fluorescent lipophilic cation, was loaded at a concentration of 10 μg ml^{-1} for 10 min at 37°C (Chen, 1988). Rh 123 fluorescence was excited at 485 nm and emission > 530 nm was measured. All other conditions were the same as for Fura measurements. An increase of Rh 123 fluorescence corresponds to a decrease of mitochondrial membrane potential (Duchen *et al*., 1993). The use of Rh 123 does not permit corrections for variable loading of the indicator, therefore fluorescence of each cell was normalized with reference to the last value before addition of a test agent. In our system, a maximal depolarization corresponded to an increase of normalized Rh 123 fluorescence of around 300% and a maximal hyperpolarization to a decrease of around 25%.

Potentiometric measurements of Ca^{2+} concentration and membrane potential

The Ca^{2+} concentration in the incubation medium of rat isolated liver mitochondria was measured by a Ca^{2+}-sensitive minielectrode as described previously (Lenzen *et al*., 1992). The Ca^{2+} concentration of the medium was set at 10 μM, and Ca^{2+} uptake was started by adding mitochondria to yield a concentration of 1.25 mg protein ml^{-1} incubation medium. The mitochondrial membrane potential was determined by measuring the concentration of the lipophilic cation, tetraphenylphosphonium, which was added to the incubation medium of the mitochondria at a concentration of 8 μM (Kamo *et al*., 1979). The accumulation and release of this compound by mitochondria is a function of the mitochondrial membrane potential according to the equation:

$$\Delta\psi = 59 \log (v/V) - 59 \log[10^{(E-E_0)/59} - 1],$$

where v is the volume of the mitochondrial matrix in the incubation (0.1 μl), V the volume of the incubation chamber (40 μl), E the electrode potential, and E_0 the electrode potential before addition of the mitochondria (Muratsugu *et al*., 1977). Due to non-Nernstian behaviour of the indicator (Ritchie, 1984), low membrane potentials, such as occur during uncoupling, cannot be determined precisely. The potential of the Ca^{2+} or tetraphenylphosphonium electrode was measured by a pH-meter (0.1 mV resolution), which was connected to an A/D board in a microcomputer. Data acquisition was performed by Labtech Notebook software (Laboratory Technologies, Wilmington MA, U.S.A.).

Amperometric oxygen measurements

Respiration of rat liver mitochondria on incubation was measured in a thermostated glass vessel by use of a Clark-type electrode (Panten & Klein, 1982). Data acquisition and processing were the same as with the ion-sensitive electrode. All mitochondrial incubations were performed at 25°C.

Luminometric measurement of ATP content

The ATP content of incubated mouse islets and rat liver mitochondria was measured with a commercial luciferase assay

(Sigma). Islets were incubated for 30 min at 37°C in Krebs-Ringer medium with 20 mM glucose, isolated liver mitochondria were incubated for 4 min under the same conditions as used for respiration measurements.

Insulin secretion

The insulin content in the fractionated effluate of batches of 25 perifused NMRI mouse islets was determined by radioimmunoassay, by use of a locally produced antiserum, [^{125}I]-insulin (DuPont NEN, Dreieich, Germany) and crystalline mouse insulin (Novo) as standard (Panten *et al*., 1981).

Drugs

Diazoxide, rhodamine 123 (Rh 123) and N,N,N′,N′-tetramethyl-1,4-phenylenediamine, were from Sigma (Deisenhofen, Germany) and tetraphenylphosphonium and succinate from Fluka (Neu-Ulm, Germany). Pinacidil was obtained from RBI (Cologne, Germany) and levcromakalim was a kind gift of Smith Kline Beecham (Harlow, U.K.). Glibenclamide was donated by Hoechst (Frankfurt, Germany) and D 600 (methoxyverapamil) by Knoll (Ludwigshafen, Germany). Fura-2/AM was supplied by Molecular Probes (Leiden, The Netherlands). Collagenase P was obtained from Boehringer Mannheim (Mannheim, Germany). Cell culture medium RPMI 1640 (without glucose) was from Gibco BRL (Gaithersburg, MD, U.S.A.) and foetal calf serum from Biochrom (Berlin, Germany). Sodium azide (NaN_3), ascorbic acid and all other reagents of analytical grade were from E. Merck (Darmstadt, Germany). Diazoxide was dissolved in dry dimethylsulphoxide (DMSO) to prepare stock solutions of various concentrations. The maximal concentration of DMSO in the incubations was 1%. All control experiments were performed with the appropriate DMSO concentrations.

Data handling and statistics

Additional calculations and statistics (unpaired two-tailed *t* test, Wilcoxon signed rank test) were performed by Prism and Instat software (GraphPad, San Diego CA, U.S.A.).

Results

Our initial observation was that single ob/ob mouse B-cells, loaded with Rh 123, showed a prompt increase of fluorescence intensity when perifused with a Krebs-Ringer medium containing a non-stimulating glucose concentration and 250 μM diazoxide. When the perifusion medium was changed to a medium without diazoxide, a rapid reduction to prestimulant levels resulted. At 100 μM diazoxide, only a minimal increase of Rh 123 fluorescence could be seen, while 500 μM produced a marked increase and 1000 μM appeared to be a maximally effective concentration (Figure 1). There was only a very small increase of Rh 123 fluorescence when the concentration of diazoxide was raised from 1000 to 2000 μM (data not shown). The magnitude of the response varied considerably between the experiments, and occasionally only marginal increases of Rh 123 fluorescence were brought about by 250 μM diazoxide, but at 500 μM marked increases regularly occurred. Thus in all further experiments with B-cells, 500 μM diazoxide was used.

When diazoxide (500 μM) was added to a perifusion medium containing a stimulating concentration (20 mM) of glucose, a marked increase of Rh 123 fluorescence was noted. The relative fluorescence increase was similar to the one in the presence of 5 mM glucose (Figure 2). To see whether the decrease of mitochondrial membrane potential is a unique property of diazoxide or common for K^+ channel openers, pinacidil and levcromakalim were tested. Pinacidil at 500 μM led to a prompt increase of Rh 123 fluorescence of perifused B-cells, which was completely reversible after removal of the drug. Levcromakalim, the active enantiomer of cromakalim, was without effect at the same concentration (Figure 3).

To assess the degree of depolarization of B-cell mitochondria, the increase of Rh 123 fluorescence by 500 μM diazoxide was compared to the one brought about by a maximally effective concentration of sodium azide (NaN_3). The reduction

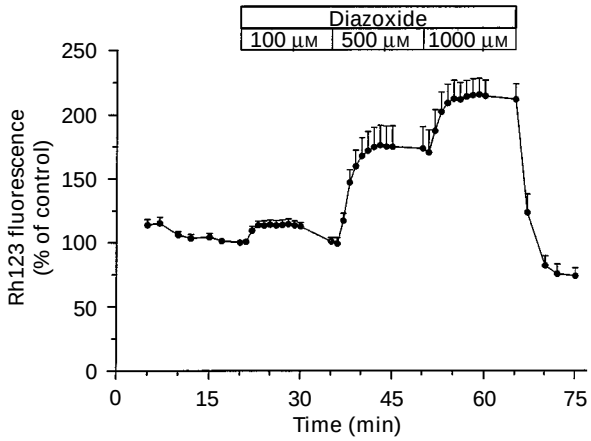

Figure 1 Concentration-dependent effect of diazoxide on mitochondrial membrane potential in intact pancreatic B-cells. After being loaded with Rh 123, ob/ob mouse B-cells were perifused with a HEPES-buffered Krebs-Ringer medium containing 5 mM glucose. Diazoxide was present from 20 to 65 min at concentrations of 100, 500 and 1000 μM. Each increase of diazoxide concentration yielded an increase of Rh 123 fluorescence. An increase of Rh 123 fluorescence indicates a decrease of mitochondrial membrane potential. Rh 123 fluorescence was normalized with respect to the last value before exposure to the drug. After withdrawal of diazoxide, membrane potential quickly returned to preinhibitory values. Data are means of 14 cells from 2 different preparations; vertical lines show s.e.mean.

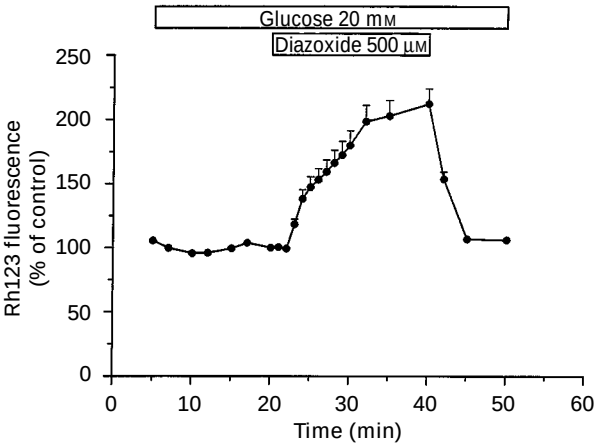

Figure 2 Effect of diazoxide on mitochondrial membrane potential in intact pancreatic B-cells in the presence of a stimulating glucose concentration. The Krebs-Ringer perifusion medium contained 20 mM glucose throughout the experiment and 500 μM diazoxide from 20 to 40 min. Data are means of 17 cells from 2 different preparations; vertical lines show s.e.mean.

of mitochondrial membrane potential by NaN_3, an inhibitor of cytochrome oxidase, is similar in extent to the virtually complete reduction elicited by the uncoupler of oxidative phosphorylation, carbonyl cyanide chlorophenylhydrazone (CCCP), but is much more easily reversible (Rustenbeck *et al*., 1997). When B-cells were perifused with a diazoxide-containing medium, 5 mM NaN_3 elicited a fast and markedly further increase of Rh 123 fluorescence in the B-cells. As expected, this decrease of membrane potential was quickly reversed when NaN_3 was removed and additional removal of diazoxide yielded a virtually unchanged mitochondrial membrane potential as compared to the initial values of Rh 123 fluorescence (Figure 4).

Since Rh 123 fluorescence is only a semi-quantitative measure of mitochondrial membrane potential and since a reduction of mitochondrial membrane potential in intact cells could also be an indirect effect of diazoxide, we tested whether diazoxide affected the membrane potential of isolated mitochondria. This was done by measuring the tetraphenylphosphonium concentration in the incubation medium of a mitochondrial fraction from rat liver by use of a tetraphenylphosphonium-sensitive minielectrode. In these experiments 100 μM diazoxide produced a small but significant ($P<0.05$, t test) reduction of mitochondrial membrane potential. As with intact B-cells, 5 mM NaN_3 had a much more pronounced effect than 500 μM diazoxide, leading to a nearly complete release of previously accumulated tetraphenylphosphonium (Figure 5). Transformation of the electrode potential into membrane potential values according to Muratsugu *et al*. (1977) yielded the following values: at the time point immediately before the addition of NaN_3, control mitochondria had a membrane potential of -156 ± 0.3 mV, while in the presence of 100 μM diazoxide it was reduced to -147 ± 2.0 mV and in the presence of 500 μM diazoxide to -138 ± 1.8 mV (all values are means$\pm$s.e.mean of 3 experiments). In contrast, 5 mM NaN_3 reduced the membrane potential down to at least -96 ± 2.9 mV. Neither tolbutamide (500 μM) not glibenclamide (2 μM) inhibited the diazoxide-induced decrease of membrane potential of isolated liver mitochondria (data not shown).

To test whether the diazoxide-induced reduction of mitochondrial membrane potential was accompanied by a mitochondrial Ca^{2+} release, $[Ca^{2+}]_i$ was measured in intact B-cells. When Fura-2 loaded ob/ob mouse B-cells were perifused with a medium containing 50 μM D 600, a moderate increase of the fluorescence ratio occurred during exposure to 500 μM diazoxide. This increase of $[Ca^{2+}]_i$ was completely reversible when exposure to diazoxide was discontinued (Figure 6). A subsequent depolarization with 40 mM K^+ did not result in an increase of cytoplasmic Ca^{2+} concentration, confirming the

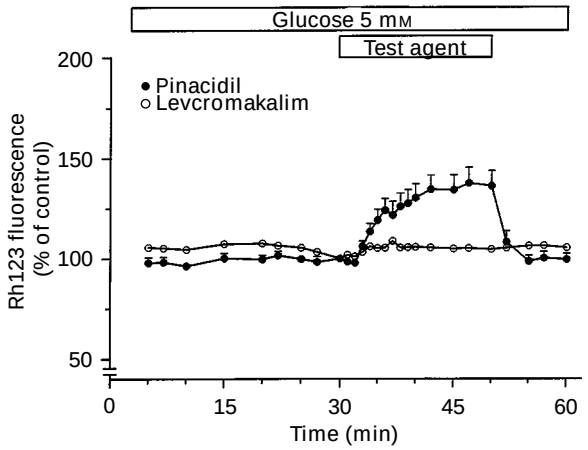

Figure 3 Effects of pinacidil and levcromakalim on mitochondrial membrane potential in intact pancreatic B-cells. Pinacidil (500 μM) or levcromakalim (500 μM) were present in the perifusion medium from 30 to 50 min. The data are means of 22 cells (for pinacidil) or 17 cells (for levcromakalim) from 2 different preparations; vertical lines show s.e.mean.

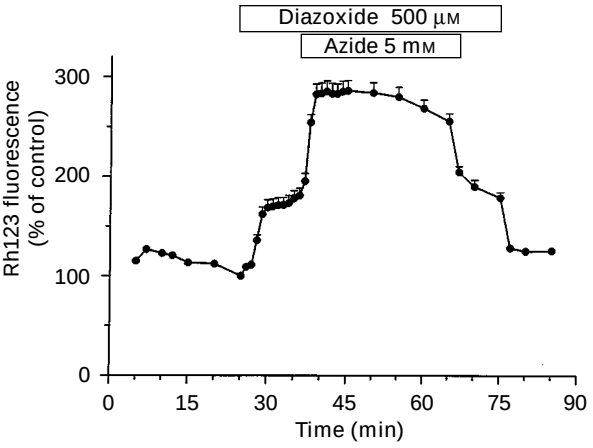

Figure 4 Effect of diazoxide on mitochondrial membrane potential in intact pancreatic B-cells in comparison with the effect of sodium azide (NaN_3). All media contained 5 mM glucose. After 10 min exposure to 500 μM diazoxide, the perifusion was switched to a medium which contained additionally 5 mM NaN_3. This led to an increase of Rh 123 fluorescence superimposed on elevated levels of Rh 123 fluorescence induced by diazoxide. The effects of diazoxide and of NaN_3 were quickly reversible. Data are means of 29 cells from 3 different preparations; vertical lines show s.e.mean.

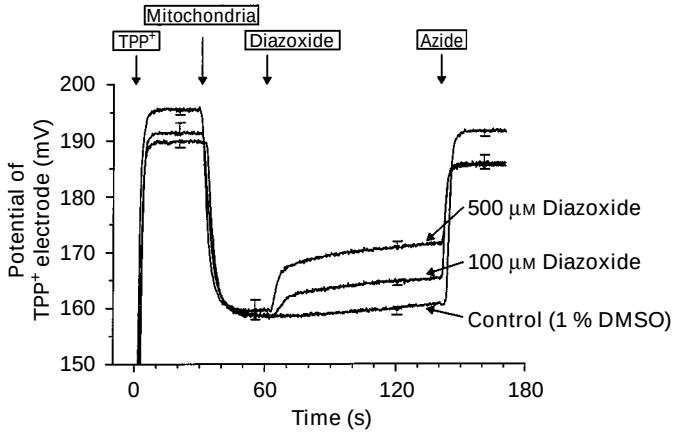

Figure 5 Effects of diazoxide and NaN_3 on membrane potential of rat isolated, incubated liver mitochondria. Mitochondrial membrane potential was measured as tetraphenylphosphonium (TPP^+) concentration in the incubation medium by means of a tetraphenylphosphonium-sensitive electrode. The incubation was started by adding 8 μM tetraphenylphosphonium to the incubation medium. Addition of mitochondria led to a fast decrease of tetraphenylphosphonium concentration in the medium due to membrane potential-dependent accumulation of the indicator. Addition of diazoxide (100 or 500 μM) led to a partial release within 10 s. The increase of tetraphenylphosphonium concentration between time points 60 s and 140 s was significant for both concentrations of diazoxide ($P<0.02$, t test), but not for the control experiments. Subsequent addition of 5 mM NaN_3 led to a nearly complete release of tetraphenylphosphonium. Each trace is a mean value of 3 experiments, the s.e.mean ranges are indicated at 4 time points.

effectiveness of D 600 as a blocker of L-type Ca^{2+} channels and, consequently, the intracellular origin of the $[Ca^{2+}]_i$ increase by diazoxide (Figure 6). A slight increase of $[Ca^{2+}]_i$ by diazoxide was also visible in the absence of D 600. The Ca^{2+} concentration in the incubation medium of rat isolated liver mitochondria was measured by a Ca^{2+}-sensitive minielectrode. In the presence of 100 or 500 μM diazoxide, the accumulation of Ca^{2+} by the mitochondria was not different from control, but subsequent release of Ca^{2+} from diazoxide-incubated mitochondria was significantly ($P<0.05$, t-test) faster at both concentrations (data not shown).

Since the mitochondrial membrane potential is the main component of the driving force for oxidative ATP synthesis (Erecinska *et al*., 1992), the effect of diazoxide on ATP production was also tested. When batches of 25 pancreatic islets from NMRI mice were incubated in the presence of 20 mM glucose for 30 min, 500 μM diazoxide decreased the ATP content to $71.3\pm4.9\%$ of the control value of 3.4 ± 0.4 pmol/islet ($n=12$). This effect, although moderate, was highly significant ($P<0.001$, Wilcoxon test). Since diazoxide is an essential experimental tool for the characterization of K_{ATP} channel-independent regulation of insulin secretion, its ability to affect the mechanisms of insulin secretion distal to Ca^{2+} influx, which are known to be energy-dependent (Detimary *et al*., 1994; Rustenbeck *et al*., 1997), was examined. Hence, insulin secretion of perifused mouse islets was stimulated by high K^+ (40 mM for 20 min) in the presence of a moderately stimulative glucose concentration (10 mM throughout the whole experiment). The secretory responses in the presence or absence of diazoxide (500 μM concomitantly with high K^+) were virtually identical (data not shown).

To characterize in more detail the mechanism(s) by which diazoxide decreased the mitochondrial membrane potential, the oxygen consumption and ATP production by rat isolated liver mitochondria were measured. The effect of diazoxide (500 μM) on mitochondrial oxygen consumption was dependent on the respiratory substrate. In the presence of 5 mM succinate, diazoxide decreased the rate of respiration down to $67\pm7\%$ of the control value of 4.8 nmol min^{-1} mg^{-1} protein ($P<0.05$, t test, $n=4$). The oxygen consumption in the presence of 2 mM tetramethyl phenylenediamine plus 5 mM ascorbate, which was 43.2 nmol min^{-1} mg^{-1} protein, was not influenced by diazoxide. The ability of ADP and of the uncoupler of oxidative phosphorylation, carbonyl cyanide

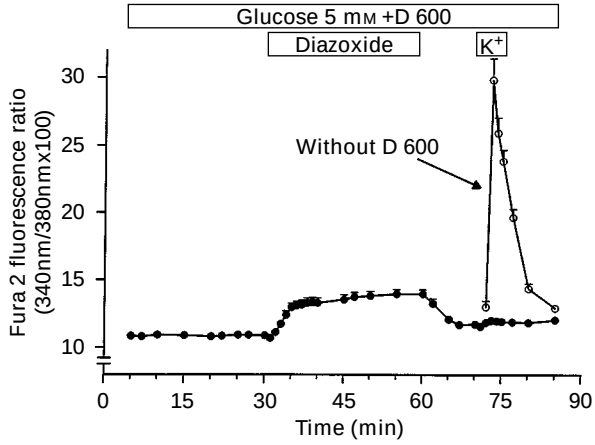

Figure 6 Effect of 500 μM diazoxide on cytoplasmic calcium concentration ($[Ca^{2+}]_i$) in intact pancreatic B-cells. Fura 2-loaded ob/ob mouse B-cells were perifused with Krebs-Ringer medium containing 50 μM D 600 throughout the experiment. From 30 to 60 min, 500 μM diazoxide was additionally present in the perifusion medium and from 70 to 75 min, 40 mM K^+. To assess the magnitude of the $[Ca^{2+}]_i$ increase by diazoxide, the effect of a 5 min-depolarization by 40 mM K^+ in the absence of D 600 is shown superimposed on the result in the presence of D 600. Data are means of 21 cells from 2 different preparations; vertical lines indicate s.e.mean.

Table 1 Effects of diazoxide on ATP production by liver mitochondria

Respiratory substrate	*Control*	*Diazoxide (500 μM)*	*Control +400 μM ADP*	*Diazoxide (500 μM) +400 μM ADP*
Succinate (5 mM)	435.51 ± 63.64	525.89 ± 33.32	73.98 ± 3.63	49.36 ± 3.37[a]
	($n=8$) 100%	($n=6$) 121%	($n=12$) 100%	($n=13$) 67%
Tetramethylphenylenediamine	152.80 ± 6.44	168.62 ± 12.18	55.99 ± 6.78	53.74 ± 7.68
(2 mM) + ascorbate (5 mM)	($n=4$) 100%	($n=3$) 111%	($n=7$) 100%	($n=7$) 96%
α-Ketoisocaproic acid (10 mM)	427.79 ± 22.69	35.28 ± 5.51[a]	22.36 ± 2.03	13.14 ± 2.56[b]
	($n=4$) 100%	($n=7$) 8%	($n=10$) 100%	($n=9$) 59%

The data are given as pmol ATP mg^{-1} protein or as nmol ATP mg^{-1} protein for ADP-stimulated ATP synthesis and are means ± s.e.mean of the number of experiments given in parentheses. Rat liver mitochondria (0.93 mg protein ml^{-1} incubation volume) were incubated for 4 min at 25°C, thereafter ATP was determined in an aliquot by a luciferase assay. The reduction of ATP synthesis by diazoxide has to be compared to the effect the uncoupler of oxidative phosphorylation, carbonyl cyanide chlorophenylhydrazone (10 μM), which reduced ADP-stimulated ATP synthesis to around 5% of control value irrespective of the respiratory substrate. Without ADP, ATP synthesis was reduced by carbonyl cyanide chlorophenylhydrazone to 6% of control in the presence of succinate and α-ketoisocaproic acid and to 17% in the presence of tetramethyl phenylenediamine plus ascorbate. [a]$P<0.001$, t test; [b]$P=0.011$, t test.

Table 2 Effects of pinacidil and levcromakalim on ATP production by liver mitochondria

Respiratory substrate	*Control +400 μM ADP*	*Levcromakalim 500 μM +400 μM ADP*	*Pinacidil 500 μM +400 μM ADP*
Succinate (5 mM)	73.98 ± 3.63 ($n=12$) 100%	73.42 ± 3.70 ($n=3$) 99%	73.07 ± 2.45 ($n=3$) 99%
α-Ketoisocaproic acid (10 mM)	22.36 ± 2.03 ($n=10$) 100%	27.16 ± 1.47 ($n=2$) 122%	15.77 ± 0.54[a] ($n=6$) 71%

The data are given as nmol ATP mg^{-1} protein and are mean values ± s.e.mean for the number of experiments given in parentheses. Rat liver mitochondria (0.93 mg protein ml^{-1} incubation volume) were incubated for 4 min at 25°C, thereafter ATP was determined in an aliquot by a luciferase assay. [a]$P=0.024$, t test.

chlorophenylhydrazone (10 μM), to stimulate respiration was abolished by diazoxide in the presence of succinate, but not in the presence of tetramethyl phenylenediamine plus ascorbate (data not shown). Correspondingly, the ADP-stimulated ATP synthesis was inhibited when succinate, but not tetramethyl phenylenediamine plus ascorbate was the respiratory substrate (Table 1). Virtually no effect of diazoxide on both succinate- and tetramethyl phenylenediamine plus ascorbate-sustained ATP synthesis was seen in the absence of ADP. Diazoxide also reduced ATP synthesis induced by α-ketoisocaproic acid. α-Ketoisocaproic acid is a model compound for an insulin secretagogue with an exclusively mitochondrial metabolism (Panten *et al*., 1981). Here, inhibition of ATP production by diazoxide was more efficient in the absence of ADP, a reduction down to 8% of control value (Table 1) came close to the effect of carbonyl cyanide chlorophenylhydrazone. Neither in the presence of succinate nor of α-ketoisocaproic acid did levcromakalim influence ADP-stimulated ATP production by liver mitochondria. Pinacidil significantly reduced ADP-stimulated ATP production in the presence of α-ketoisocaproic acid but had no effect in the presence of succinate (Table 2).

Discussion

In this study we have shown that diazoxide, which is generally regarded as a drug acting specifically on K_{ATP} channels, most probably by binding to the sulphonylurea receptor subunit (Ämmälä *et al*., 1996; Nichols *et al*., 1996), directly affects mitochondrial function, resulting in a decrease of mitochondrial membrane potential, an efflux of Ca^{2+} from mitochondria and a decreased ATP production. These effects are produced at concentrations of diazoxide which are often employed experimentally to open K_{ATP} channels and may be particularly relevant in pancreatic B-cells where energy metabolism is central for stimulus secretion coupling (Erecinska *et al*., 1992).

Three general mechanisms can be envisaged by which diazoxide might reduce mitochondrial membrane potential in B-cells: inhibition of respiratory chain complexes, an ionophoretic effect and opening of mitochondrial ion channels. The latter two effects would both result in an uncoupling of respiration from ATP synthesis. The data on oxygen consumption and ATP production by liver mitochondria suggest that diazoxide exerts mainly in inhibitory effect on the respiratory chain.

When tetramethyl phenylenediamine plus ascorbate are used as respiratory substrate, electrons are fed directly to cytochrome oxidase, the terminal multienzyme complex (complex IV) of the respiratory chain. Since diazoxide did not inhibit respiration and ATP synthesis in the presence of tetramethyl phenylenediamine plus ascorbate, it must inhibit the respiratory chain at an earlier site. The inhibition of succinate-supported respiration by diazoxide, which was accompanied by a reduction of ATP synthesis to a similar extent, indicates that the inhibition may be mainly exerted at respiratory chain complex II. Of note, both enzymes which have been described earlier to be inhibited by diazoxide in homogenates from pancreatic islets, glycerol-3-phosphate dehydrogenase (MacDonald, 1981) and succinate dehydrogenase (Lenzen & Panten, 1983) are closely linked to this complex. Inhibition of succinate dehydrogenase by diazoxide was also observed by Schäfer *et al*. (1971) in liver mitochondria. The observation, that α-ketoisocaproic acid-supported ATP generation was at least as efficiently inhibited as the one supported by succinate, may indicate that not only inhibition of electron transport at complex II may play a role, but also inhibition of the citric acid cycle, perhaps at succinate dehydrogenase, as described by Schäfer *et al*. (1971). In contrast to the findings of Portenhäuser *et al*. (1971) we could not verify an uncoupling action of diazoxide. The slight increase in respiration in the presence of tetramethyl phenylenediamine plus ascorbate was not significant. There are ATP-sensitive K^+ channels in the inner membrane of liver mitochondria (Inoue *et al*., 1991), which have been recently shown to be sensitive to glibenclamide and K^+ channel openers (Belyaeva *et al*., 1993; Garlid *et al*., 1996). Since we found neither an uncoupling effect of diazoxide nor an antagonism of the decrease of membrane potential by glibenclamide, mitochondrial ATP-sensitive K^+ channels are unlikely to be involved in the action of diazoxide.

It is obvious that data obtained with liver mitochondria should not be uncritically extrapolated to B-cell mitochondria, but there are several parallels which indicate that basically the same mechanisms are involved: with both isolated liver mitochondria and mitochondria in intact B-cells a decrease of membrane potential, an induction of a net Ca^{2+} efflux and a decrease of ATP production could be observed. In view of the finding that diazoxide inhibits succinate dehydrogenase (Lenzen & Panten, 1983) and/or glycerol 3-phosphate dehydrogenase (MacDonald, 1981) in homogenates from pancreatic B-cells, there seems to be sufficient evidence that the mechanism of action of diazoxide is essentially the same in liver and B-cell mitochondria. Earlier investigations on the effects of diazoxide on glucose metabolism in intact B-cells yielded conflicting results: while Ashcroft *et al*. (1970) did not find any significant effect on glucose oxidation, Hellman *et al*. (1974) observed a significant reduction (by 20%); both groups employed concentrations of diazoxide close to 500 μM and glucose concentrations around 10 mM. Taken together, the earlier data and the present results suggest that diazoxide moderately, but significantly decreases oxidative phosphorylation in B-cells by an inhibitory effect on the respiratory chain.

When considering possible acute consequences of the effects of diazoxide on B-cell energy metabolism, one has to distinguish between the energetic requirement of K_{ATP} channel regulation and energetic requirement of events distal to Ca^{2+} influx into the B-cell. It has long been known that diazoxide does not inhibit secretion elicited by high K^+ in the presence of a non-stimulative glucose concentration (Henquin *et al*., 1982). On the assumption that diazoxide affects the enhancement by nutrients of depolarization-induced insulin secretion, the effect of diazoxide on insulin secretion stimulated by 40 mM K^+ plus 10 mM glucose was measured. The lack of effect of diazoxide on the secretory response to this combination of stimuli confirms the validity of the studies in which diazoxide was used as a tool to dissect energetic requirements of distal events in stimulus-secretion coupling from the closure of K_{ATP} channels (Gembal *et al*., 1992; Taguchi *et al*., 1995).

The diazoxide-induced reversible increase of $[Ca^{2+}]_i$ in B-cells which was not inhibited by the Ca^{2+} channel blocker, D 600, most likely represents a net Ca^{2+} efflux from the mitochondria, because the mitochondrial membrane potential is the driving force for Ca^{2+} uptake via the Ca^{2+} uniporter (Gunter & Pfeiffer, 1990). When mitochondrial Ca^{2+} uptake is significantly impaired in the presence of diazoxide, this may in turn inhibit metabolism-induced increases of free Ca^{2+} concentration in the matrix space, which activate citric acid cycle dehydrogenases as a feed-forward effect (McCormack *et al*., 1990; Kennedy *et al*., 1996). Such a mechanism might not be relevant to acute inhibition of insulin secretion but might be

relevant for long-term effects, e.g. the phenomenon that diazoxide protects against glucose-induced desensitization of the B-cell, an effect which was found not to be directly associated with opening of the K_{ATP} channels (Björklund & Grill, 1993).

Could there be a relationship between the inhibition of mitochondrial functions by diazoxide and its opening effect on B-cell K_{ATP} channels? The ability of diazoxide to open K_{ATP} channels in excised (inside-out) membrane patches from B-cells and insulin-secreting cell lines argues against an indispensable role of the mitochondrial effects. Nevertheless, the observation is intriguing that levcromakalim, a compound which is virtually unable to open K_{ATP} channels in B-cells and insulin-secreting cells and to inhibit insulin secretion (Garrino *et al*., 1989; Kozlowski *et al*., 1989), was also the one compound which had no significant effect on mitochondrial membrane potential and ATP production, while pinacidil, which is somewhat less efficient than diazoxide in opening K_{ATP} channels and inhibiting insulin secretion (Garrino *et al*., 1989), also had intermediate position with regard to its potency at inducing mitochondrial effects. Effects of K_{ATP} channel openers on mitochondrial energy metabolism of pancreatic B-cells may deserve further attention.

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