Mitochondria Present in Excised Patches From Pancreatic B-cells May Form Microcompartments With ATP-Dependent Potassium Channels

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Experiments with inside-out patches excised from pancreatic B-cells have yielded evidence that mitochondria are often contained in the cytoplasmic plug protruding into the tip of patch pipette. When intact B-cells were loaded with the fluorescent mitochondrial stain, rhodamine 123, and membrane patches excised from these cells, a green fluorescence could be observed in the lumen at the tip of the patch pipette. The same result was obtained with the mitochondrial stain, MitoTracker Green FM, which is only fluorescent in a membrane-bound state. Furthermore, the open probability of ATP-dependent potassium (KATP) channels in inside-out patches was influenced by mitochondrial fuels and inhibitors. Respiratory substrates like tetramethyl phenylene diamine (2 mM) plus ascorbate (5 mM) or α-ketoisocaproic acid (10 mM) reduced the open probability of KATP channels in inside-out patches significantly (down to 57% or 65% of control, respectively). This effect was antagonized by the inhibitor of cytochrome oxidase, sodium azide (5 mM). Likewise, the inhibitor of succinate dehydrogenase, malonate (5 mM), increased the open probability of KATP channels in the presence of succinate (1 mM). However, oligomycin in combination with antimycin and rotenone did not increase open probability. Although it cannot be excluded that these effects result from a direct interaction with the KATP channels, the presence of mitochondria in the close vicinity permits the hypothesis that changes in mitochondrial metabolism are involved, mitochondria and KATP channels thus forming functional microcompartments.

KEY WORDS: KATP channels; pancreatic B-cells; mitochondria; patch-clamp technique.

ABBREVIATIONS: KATP channel, ATP-dependent potassium channel; KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; NaN3, sodium azide; Rh123, rhodamine 123; TMPD, tetramethyl phenylene diamine.

INTRODUCTION

ATP-dependent potassium channels (KATP channels) in pancreatic B-cells represent the main coupling site between the oxidative metabolism of fuel secretagogues and the ionic events that initiate insulin secretion [1, 2]. It is generally accepted that an enhanced oxidative phosphorylation results in an increase of the cytoplasmic ratio of ATP- to ADP-concentrations, which is regarded as determinant of the open probability of KATP channels. A decrease of potassium permeability and the ensuing

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depolarization trigger $\text{Ca}^{2+}$ influx and $\text{Ca}^{2+}$-induced exocytosis. While a relation between enhanced glucose metabolism in B-cells and closure of $K_{\text{ATP}}$ channels [3] could be shown shortly after the initial observation of this type of channel [4] it was only in 1995 that an increase of the ATP/ADP ratio in B-cell homogenates could be demonstrated in the course of glucose stimulation [5].

To explain the discrepancy between the millimolar ATP-concentration in the B-cell cytoplasm and the micromolar concentrations of this nucleotide that regulate the $K_{\text{ATP}}$ channels in excised patches from B-cell membranes (for an overview see [6]) it was suggested that adenine nucleotide concentrations in the vicinity of the $K_{\text{ATP}}$ channels were different from those in the bulk cytoplasmic phase [7]. Results obtained with a mutated sulfonylurea receptor subunit of the $K_{\text{ATP}}$ channel indicated that the role of ATP might be to exert a tonic inhibition, which is antagonized by the ADP concentration, a decrease of which would represent the physiological signal for channel closure [8].

Our recent observation that the potassium channel opener, diazoxide, directly affects energy metabolism of B-cell and liver mitochondria [9], prompted us to consider a possible role of these mitochondrial effects on $K_{\text{ATP}}$ channel opening by diazoxide. A major argument against such a role is the well-known ability of diazoxide to open $K_{\text{ATP}}$ channels in inside-out patches from B-cells [10,11]. These patches are generally regarded as excised pieces of plasma membrane, which permit the study of membrane channels without interference by intracellular structures. On the other hand, it is known that mitochondria adhere to several components of the cytoskeleton [12], and we imagined that they could be dragged into the patch pipette during seal formation and patch excision. Therefore we investigated whether mitochondria exist in the immediate vicinity of the plasma membrane by using fluorescent mitochondrial stains and measuring the effects of mitochondrial fuels and inhibitors of $K_{\text{ATP}}$ channel activity in inside-out patches from normal pancreatic B-cells.

**MATERIALS AND METHODS**

**Chemicals**

The following compounds were obtained from the named manufacturers. Antibiotics A, $\alpha$-ketoisocaproic acid (KIC), $\alpha$-ketoisovaleric acid (KIV), malonate, oligomycin, rhodamine 123 (Rh 123), rotenone, tetramethyl phenylene diamine (TMPD): Sigma (Deisenhofen, Germany); MitoTracker Green FM: Molecular Probes (Leiden, The Netherlands); succinic acid: Fluka (Neu-Ulm, Germany); ATP (disodium salt): Boehringer Mannheim (Mannheim, Germany); tolbutamide: Serva (Heidelberg, Germany); RPMI 1640 cell culture medium and fetal calf serum (FCS): Biochrom (Berlin, Germany); ascorbic acid, sodium azide and all other reagents of analytical grade: E. Merck (Darmstadt, Germany).

**Tissues**

Pancreatic islets were isolated from pancreata of NMRI mice by collagenase digestion and hand-picked under a stereomicroscope. Islets were dispersed into single cells by incubation for 11 min in a $\text{Ca}^{2+}$-free Krebs-Ringer bicarbonate buffer
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containing 0.5 mM EGTA and subsequent vortex-mixing for 1 min. Dispersed islet cells were sown on Petri dishes or glass coverslips and cultured for up to 3 days in RPMI 1640 medium (5 mM glucose) with 10% fetal calf serum. For fluorescence microphotography, coverslips with the adherent cells were sealed with vacuum grease over a hole in the bottom of a plastic Petri dish, which was placed on the stage of the inverted microscope of the patch-clamp setup.

**Fluorescent Staining of Mitochondria**

To stain mitochondria in intact B-cells, Rh 123, a fluorescent lipophilic cation, was loaded at a concentration of 10 µg/mL (26.3 µM) for 10 min at 37°C [13]. MitoTracker Green FM was loaded into intact B-cells at 37°C for 30 min using concentrations of 0.2; 0.5; 1 or 2 µM. Once inside the mitochondria, MitoTracker binds covalently to SH-groups and becomes irreversibly bound [14]. Fluorescence was excited at 490 nm by a monochromatic light source (TILL Photonics, München-Planegg, Germany) coupled by a quartz fiber to the inverted microscope (Zeiss Axiovert 135) of the patch-clamp setup. Dichroic beamsplitter (505 nm) and long-pass filter (530 nm) were from Omega Optical (Brattleboro, VT, USA). The objective was a Zeiss Fluar (magnification 100×, numerical aperture 1.3). Photomicrographs were taken by a Nikon SLR camera attached to the camera port of the microscope, using a Fujichrome sensia film (400 ASA) or a black and white Ilford HP 5 plus film (400 ASA).

**Electrophysiological Recordings**

The inside-out configuration of the patch-clamp technique [15] was used to measure K\textsubscript{ATP} channel activity. Currents were recorded using an EPC7 patch-clamp amplifier (List Electronic, Darmstadt, Germany), low pass-filtered by a 4-pole Bessel filter at 2 kHz, and stored on video tape. Data were analysed off-line using pClamp software (Axon Instruments, Foster City, CA, USA). The traces were printed by an Easygraf recorder (Gould Instruments, Dietzenbach, Germany). Pipettes were pulled from borosilicate glass capillaries (2 mm outer diameter, Hilgenberg, Germany) with a two-stage puller (List Electronic, Darmstadt, Germany) and had resistances between 3 and 8 MΩ when filled with pipette solution. The composition of the bath solution for inside-out experiments (intracellular solution) was: 140 mM KCl, 1.0 mM MgCl\textsubscript{2}, 10 mM EGTA, 2.0 mM CaCl\textsubscript{2} and 5 mM Hepes, pH 7.15. The pipette solution in these experiments consisted of 146 mM KCl, 2.6 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 10 mM Hepes and 3 mM glucose, pH 7.4. An ATP-containing solution (intracellular solution, supplemented with 1.0 mM MgATP and, additionally, 0.8 mM MgCl\textsubscript{2}) was used to close the K\textsubscript{ATP} channels completely and to inhibit channel run-down. The pipette potential was +50 mV. All experiments were performed at room temperature (21–23°C). Statistical calculations were performed using Instat software (GraphPad, San Diego, CA, USA).
RESULTS AND DISCUSSION

Initially, a morphological verification that mitochondria are present in inside-out patches from pancreatic B-cells was attempted by use of the fluorescent dye Rhodamine 123, which accumulates in mitochondria [13]. When B-cells (Fig. 1A) were loaded with Rh 123, the typical granular fluorescence in the cytoplasm could be seen (Fig. 1B). After formation of the gigaohm seal and excision of the patch by fast withdrawal of the pipette a small strip of Rh 123 fluorescence in the apical lumen of the patch pipette could be seen (Fig. 1B, Fig. 1C) in 15 out of 18 patches.

To exclude the possibility that Rh 123 fluorescence originated from the dye trapped in a cytoplasm-filled vesicle, we used the newly available fluorescent dye MitoTracker Green FM. Using this dye, a fluorescence could be seen with all excised patches (n = 13). In Fig. 2A two B-cells and a patch pipette are shown after excision of a membrane patch in transmitted light (magnification: 250×). In Fig. 2B the same view is shown under epifluorescence illumination at λ = 490 nm. MitoTracker fluorescence is visible in intact cells and inside the patch pipette. MitoTracker fluorescence in the patch pipette could be registered after staining of the intact B-cells at all loading concentrations from 0.2 to 2 μM.

To test whether the presence of mitochondria in inside-out patches has functional consequences, inhibitors of oxidative phosphorylation were used. These compounds, which are known to open K<sub>ATP</sub> channels when applied to intact B-cells, were applied to the bath solution of inside-out patches from pancreatic B-cells. But neither 5 mM sodium azide (NaN<sub>3</sub>, an inhibitor of cytochrome oxidase) nor 10 μM CCCP (a protonophoric uncoupler) significantly induced higher open probabilities than intracellular solution alone (n = 8 each). However, the combination of 2 mM TMPD and 5 mM ascorbate, which feeds electrons to cytochrome oxidase of isolated mitochondria and closes K<sub>ATP</sub> channels in intact B-cells [16], reduced open probability significantly down to 57.3 ± 4.7% of control (P < 0.01, one-sample t-test, n = 4). Thus, an experimental protocol was devised to supply a metabolic substrate first and then to test whether an appropriate metabolic inhibitor was able to antagonize the effect of the metabolic substrate (Fig. 3A–C).

In B-cell research, α-ketoisocaproic acid (KIC) is a model compound for a fuel secretagogue with an exclusively mitochondrial metabolism [17,18]. In fact, KIC (10 mM) reduced open probability of K<sub>ATP</sub> channels in inside-out patches down to 58.5 ± 5.3% (P < 0.001, one sample t-test, n = 12). When 5 mM NaN<sub>3</sub> was additionally present, there was a significant increase in open probability (83.7 ± 12.4% of control; P = 0.038, unpaired one-tailed t-test, n = 12; Fig. 3A), KIC was no longer able to close K<sub>ATP</sub> channels. α-Ketoisovaleric acid (KIV), a close structural analogue of KIC, which only weakly stimulates insulin secretion, reduced K<sub>ATP</sub> channel open probability moderately, but significantly (79.8 ± 3.9%, P = 0.014, one-sample t-test, n = 4) at a concentration of 10 mM. This effect of KIV was significantly smaller than the one of KIC (P = 0.043, unpaired two-tailed t-test).

Similarly, the reduced open probability induced by TMPD plus ascorbate (36.7 ± 6.2%, n = 6) was antagonized by NaN<sub>3</sub> (84.3 ± 18.7%, n = 6; P = 0.050, Welch's unpaired two-tailed t-test; Fig. 3B). In the presence of NaN<sub>3</sub>, K<sub>ATP</sub> channel activity was strongly reduced by 1 mM MgATP or by 500 μM tolbutamide, indicating that no unspecific or destructive effects had occurred (data not shown). However,
Fig. 1. Rh 123-loaded pancreatic B-cells and patch pipette with excised patch. (A) Immediately after patch excision from a cluster of B-cells a photograph was made in transmitted light to demonstrate the position of the patch pipette and the cells. (B) After switching to epifluorescence illumination (excitation at 490 nm, emission > 530 nm) fluorescent spots in the B-cell cluster and in the patch pipette (arrow) became visible. Note that B-cells and patch pipette are not in the same plane of focus, thus resolution of Rh 123 fluorescence is compromised. (C) Photographs A and B superimposed.
when a combination of 3 inhibitors was used (oligomycin, antimycin and rotenone, each at 5 μg/mL), which renders any mitochondrial ATP generation impossible, a significant antagonism of KIC-induced reduction of open probability could not be verified.

Finally, we tested whether an antagonism of nutrient-induced K$_{\text{ATP}}$ channel closure could be achieved by a compound structurally similar to the nutrient. To this end, succinate was used as respiratory substrate and malonate, a competitive inhibitor of succinate dehydrogenase, as antagonist (Fig. 3C). Succinate at 1 mM
produced only a weak, non-significant reduction of $K_{ATP}$ channel open probability ($78.6 \pm 10.6\%$, $P = 0.12$, one sample $t$-test, $n = 5$), but in the concomitant presence of malonate (5 mM) and succinate (1 mM), the open probability ($177.5 \pm 41.7\%$) was significantly higher than with succinate alone ($P = 0.016$, two-tailed Mann-Whitney U-test, $n = 5$).

The observation that mitochondria are present in inside-out patches from pancreatic B-cells is relevant for the interpretation of results obtained with inside-out patches in general and for the stimulus-secretion coupling of pancreatic B-cells in particular. To our knowledge the adherence of mitochondria to patch-clamped membranes has not yet been demonstrated. There are investigations on the structure of patch-clamped membranes from xenopus myocytes, using light microscopy and electron microscopy, which show that the excised patch is not a bilayer membrane, but a membrane-covered bleb of cytoplasm that may contain organelles and cytoskeleton [19, 20]. However, in these investigations it was not attempted to further specify the nature of the organelles.

Our morphological evidence relies on the selectivity of two mitochondrial stains, Rh 123 and a new derivative, MitoTracker Green FM. Rh 123 is a lipophilic cation with a delocalized charge, such compounds are accumulated by mitochondria as a function of their large, internally negative membrane potential [13, 21]. When intact cells are incubated with Rh 123, the mitochondria appear brightly fluorescent. However, Rh 123 fluorescence in the tip of the patch pipette (Fig. 1A–C) cannot be regarded as a direct proof that mitochondria exist in inside-out patches, because Rh 123 has also some fluorescence in an aqueous environment [22]. Theoretically, Rh 123 fluorescence might also originate from dye trapped in a cytoplasmic vesicle. For this reason we used MitoTracker Green FM, which has virtually no fluorescence in an aqueous environment [14]. Any MitoTracker fluorescence in the patch pipette must therefore originate from membranous structures (Fig. 2A–B). When used at a loading concentration of 0.2 $\mu$M, this compound can be regarded as a mitochondrion-selective stain [14]. The fluorescence in the pipette after loading of the B-cells with 0.2 $\mu$M, even though less bright than that after loading with 2 $\mu$M, confirmed the hypothesis that mitochondria are contained in inside-out patches from pancreatic B-cells. However, the demonstration that mitochondria are contained in inside-out patches does not yet prove a role for them in regulating $K_{ATP}$ channels.

While it is generally assumed that excised patches do possess some form of metabolism, this has been mainly regarded as a possible experimental pitfall (e.g., ref. 23), and we know of no systematic investigation. With respect to pancreatic B-cells, it is obvious that the existence of mitochondria in the vicinity of $K_{ATP}$ channels may be relevant, when the physiological role of the pancreatic islet as a “fuel sensor organ” is kept in mind. Earlier, it has been reported that $K_{ATP}$ channels in cardiac myocytes were mainly influenced by glycolytic ATP [24]. However, in pancreatic B-cells, mitochondrially metabolized compounds like KIC were shown to induce insulin secretion [17] involving the closure of $K_{ATP}$ channels [18]. Our observation that two different metabolic substrates for mitochondria decreased $K_{ATP}$ channel open probability which was antagonized by two different inhibitors of mitochondrial metabolism strongly suggests that mitochondria influence $K_{ATP}$ channels in excised
Fig. 3. Modulation of $\mathrm{K}_{\text{ATP}}$ channel activity in inside-out patches excised from pancreatic B-cells by perfusion with mitochondrial substrates and inhibitors. After patch excision, excised patches were alternatively perfused with intracellular solution (IC, perfusion time indicated by the bar) and with IC plus 1 mM MgATP to close channels completely and to inhibit run-down. (A) Antagonistic effects of KIC and sodium azide (Na$_3$N). After the first arrow, IC was used without further additions, after the second arrow IC contained 10 mM KIC and after the third arrow IC contained both 10 mM KIC and 5 mM azide. Thereafter (fourth arrow), IC contained again only 10 mM KIC. (B) Antagonistic effects of TMPD plus ascorbate and sodium azide (Na$_3$N). After the first arrow, IC contained no test agents, after the second arrow IC contained 2 mM TMPD plus 5 mM ascorbate (TMPD/Asc) and after the third arrow, IC contained both TMPD plus ascorbate (TMPD/Asc) and 5 mM Na$_3$N. Thereafter (fourth arrow) IC contained only TMPD plus ascorbate. (C) Antagonistic effects of succinate and malonate. After the first arrow IC was used without further additions, after the second arrow IC contained 1 mM succinate and after the third arrow IC contained both 1 mM succinate and 5 mM malonate.
patches. It is conceivable that all effective test agents directly influenced $K_{\text{ATP}}$ channel activity, however, this would invalidate a large amount of data on stimulus-secretion coupling in the B-cell, generated by use of these metabolic substrates or inhibitors. In fact, KIC was recently reported to have a direct inhibitory action on $K_{\text{ATP}}$ channels in addition to its stimulation of mitochondrial metabolism [25], but much of the evidence was based on the use of inside-out patches from B-cells. While we cannot exclude that $K_{\text{ATP}}$ channels were not influenced by the adherent mitochondria, but rather directly affected by the test agents, the simpler hypothesis is that the known mitochondrial effects of these compounds are responsible for the changes in $K_{\text{ATP}}$ channel activity.

If one accepts this hypothesis, there are two questions arising: (i) why does only NaN3 but not oligomycin (the latter in conjunction with rotenone and antimycin A) increase the $K_{\text{ATP}}$ channel open probability? (ii) why is the reduction of channel open probability by mitochondrial metabolic substrates incomplete? The first question cannot be satisfactorily answered at this stage, but it is noteworthy that the inhibitors which increased open probability were also those which lower mitochondrial membrane potential, while oligomycin inhibits ATP production in pancreatic B-cells without decreasing mitochondrial membrane potential [26]. The second question probably relates to the distance between the mitochondria and the $K_{\text{ATP}}$ channels. A reasonable explanation would be that the signal from the mitochondria (whatever its nature) is diluted by the bath medium, but not as completely as one would expect from this mode of patch-clamping.

It thus appears that mitochondria and $K_{\text{ATP}}$ channels form functional microcompartments. The existence of microcompartments between mitochondria and the B-cell plasma membrane would explain the recent observation of a direct influence of mitochondrial metabolism on insulin exocytosis [27]. In fact, interactions between ion channels in the plasma membrane and mitochondria in the close vicinity may be a more common phenomenon in cellular regulation than hitherto realized [28]. However, the presence of mitochondria in inside-out patches will most probably be affected by the techniques of seal formation and patch excision, whereby topological relations can be destroyed or distorted. Thus, it remains to be elucidated whether mitochondria form microcompartments with $K_{\text{ATP}}$ channels also in intact B-cells.

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