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Regulation of mitochondrial K_{ATP} channel by redox agents

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Abstract

The ATP-dependent K^+ channel (K_{ATP}) was purified from the inner mitochondrial membrane and reconstituted into lipid bilayer membranes. K_{ATP} activity was inhibited by high concentrations of ATP and ADP, but activated by low concentrations (up to 200 μM) of ADP. *p*-Diethylaminoethylbenzoate (DEB) acted as a K_{ATP} opener: at micromolar concentrations, it reversed inhibition by ATP and ADP and it also prevented K_{ATP} rundown. Pelargonidine, extracted from flowers of *Pelargonium*, reduced spontaneous activity of K_{ATP} channels and diminished their potentiation by DEB. Their opposite action on K_{ATP} corresponded with their opposite redox properties in reactions with free radicals: DEB behaved as an electron donor, whereas pelargonidine acted as an electron acceptor. We hypothesize that thiol groups on mitoK_{ATP} are targets for redox-active ligans.

Keywords: Mitochondria; K_{ATP} channel; Electron donor; Acceptor

1. Introduction

K^+ channels, whose activity is largely depressed by physiological concentrations of ATP, are found in cytoplasm membranes of many cell types [1]. They are thought to link intracellular metabolism with the membrane potential. Some regulators of cytoplasmic ATP-dependent K^+ channels (K_{ATP}) became useful as drugs. Blockers of these channels in pancreatic cells, derivatives of sulphonylurea such as glibenclamide and tolbutamide, are widely used as effective antidiabetics [2]. K_{ATP} channel openers, which have diverse structures, are expected to be used as potent regulators of blood vessel tone [3] and as antiischaemic agents [4].

Recently an ATP-dependent K channel was found in the mitochondrial inner membrane, characterized by patch-clamp technique [5], and reconstituted into liposomes [6]. The mitochondrial K_{ATP} (mitoK_{ATP}) channel was thought to play a significant role in mitochondrial volume regulation [7], thermoproduction [8] and cardioprotection [9]. In our laboratory, a single, 55 kDa protein was isolated from the mitochondrial inner membrane that formed K^+-selective ion channels when incorporated into the lipid bilayer [10]. Activity was inhibited by ATP in the millimolar range, similar to inhibition observed by Inoue et al. [5], but with much lower affinity than ATP inhibition observed with the complete mitoK_{ATP} by Paucek et al. [11]. The most likely explanation for this difference is that different ATP regulatory sites are being probed by the two techniques. The complete mitoK_{ATP} includes a sulphonylurea receptor [7], which binds ATP with high affinity, whereas the purified channel protein [10] binds ATP with low affinity.

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In the present work some electrical properties and regulation of the reconstituted mitoK_{ATP} channel by phosphonucleotides and new pharmacological agents were studied. We have used a benzocaine homologue, *p*-diethylaminoethylbenzoate (DEB) [13], and pelargonidine, a diglucose cyanide extracted from red flowers [14]. Fig. 1 indicates the structures of DEB and pelargonidine. Our choice of these drugs is based on their opposite redox properties: DEB behaved as an electron donor while pelargonidine acted as an electron acceptor towards free radicals. We aimed to test the hypothesis that redox properties might correspond with the regulation of the mitoK_{ATP} channel by these drugs, as the action of regulators of Na^+ and Ca^{2+} channels were found to correlate with their redox properties [15,16].

We showed that the electroneutral DEB largely potentiated the mitoK_{ATP} channel at low micromolar concentrations. As a rule, channel activity gradually declines after incorporation into BLM. This phenomenon, called 'channel rundown' [17], is routinely observed with cell membrane K_{ATP}. DEB prevented rundown and also abolished ATP or ADP inhibition of the channel. In contrast, pelargonidine depressed the mitoK_{ATP} channel and antagonized the potentiating effects of DEB. We also showed that ADP exerts a dual effect on the mitoK_{ATP} channel which was activated at concentrations below 100–200 μM and inhibited at concentrations above 1 mM. Moreover, the regulatory effects of ADP and ATP are discussed in view of their redox properties.

2. Materials and methods

Rat liver was perfused with 0.15 M NaCl and mitochondria were prepared by a conventional procedure [18]. The channel-forming protein was isolated and purified from inner mitochondria membrane by DEAE-cellulose chromatography [12]. The ion transport activity of the protein was measured by BLM technique [19]. Membrane was made from beef brain lipids (20 mg/ml), and 10% cardiolipin [20], dissolved in decane. The solution on both sides of the bilayer contained 100 mM KCl in 20 mM Tris-HCl, pH 7.4. Different potentials (10–100 mV) were applied across the membrane and the currents registered by operational amplifier (MAX 412) were presented as the value of membrane conductance in pS.

Methods for determination of redox properties of a biologically active compound by its reaction with free radicals were described elsewhere [21]. Briefly, they are as follows. Illumination of a deaerated solution of the dye eosin results in formation of free radical pairs – eosin anion and cation radicals which rapidly disintegrate. A compound with electron donor properties donates electrons to eosin cation radicals thus stabilizing and enhancing the concentration of eosin anion radicals. The enhanced concentration of dye anion radicals accelerates the photoreduction of haemin, an auxiliary electron acceptor, traced by an increase in its absorption at 420 nm. Haemin photoreduction in solution with the tested compound was compared to that in the control and taken as a measure of the compound's electron donor properties.

For examination of the electron acceptor properties of a compound, a high concentration of eosin anion radicals has been provided by addition of NADH to the deaerated dye solution. In the control this results in fast eosin photobleaching due to disproportionation of dye anion radicals and formation of colourless eosin. A compound with electron acceptor properties intercepts electrons from dye anion radicals and it slows the rate of dye photobleaching. Comparison of dye photobleaching in the control to

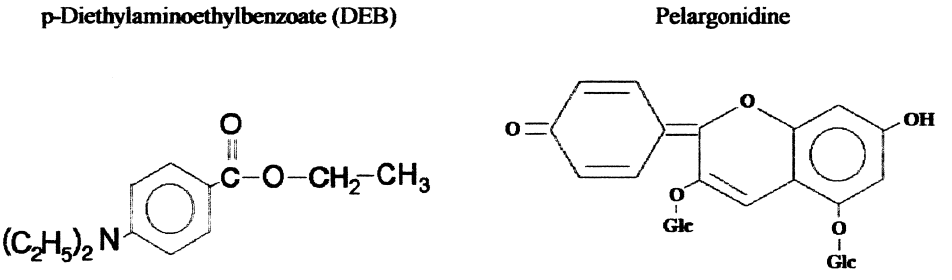

Fig. 1. Structures of *p*-diethylaminoethylbenzoate (DEB) and pelargonidine.

that in the solution with the compound tested manifests its electron acceptor properties. The photochemical reactions were performed in a hermetically closed quartz optical cuvette. Samples were deaerated by pumping during 30 min at 10^{-3} Torr, illuminated by filtered light from a tungsten lamp ($\lambda > 500$ nm, $P < 100$ W), and the changes in optical spectra were monitored by spectrophotometer Specord UV VIS (Germany, Jena). Eosin (Chimreactiv, Russia), NADH and haemin (Serva, Westbury) were used. All other reagents were obtained from Sigma. DEB was a generous gift from Prof. Ging Kuo Wang (Boston University Medical School). Pelargonidine was a gift from Dr. R. Rusieva (Institute of Photosynthesis, Pushchino, Russia).

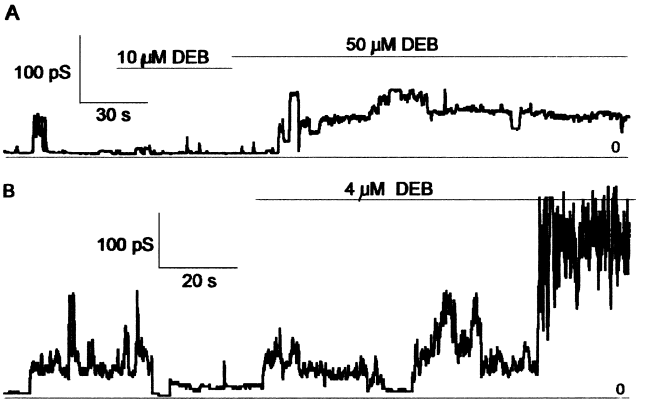

Fig. 3. Potentiation of the mitoK$_{ATP}$ channel by DEB. (A) Channels with low initial activity are activated by DEB. (B) Channels with high initial activity are still more activated at low concentrations of DEB.

3. Results and discussion

Conductance of the preformed thin 'black' BLM did not exceed 3–5 pS in symmetrical 100 mM KCl. When channel protein was added to the *trans* side of the membrane, conductance of BLM increased up to 100–200 pS after some delay. Electrical properties of the reconstituted channel have been described elsewhere [12].

The mitoK$_{ATP}$ channel often became silent (rundown) when the interior of the native was deprived. It seemed that activity of the reconstituted channel was gradually decreased (Fig. 2). This low spontaneous activity is enhanced by the uncharged local anesthetic DEB added to both sides of BLM to a final concentration of 50 μM (Fig. 3A). The activity remained high during the entire recording time. Fig. 3B shows initially more active channels with low rundown. These channels were highly activated at low concentration of DEB (4 μM) and the integral conductance rose to a high level. As a control experiment, DEB (50 μM) was added to the preformed black BLM without protein. This did not affect its low conductance (data not shown).

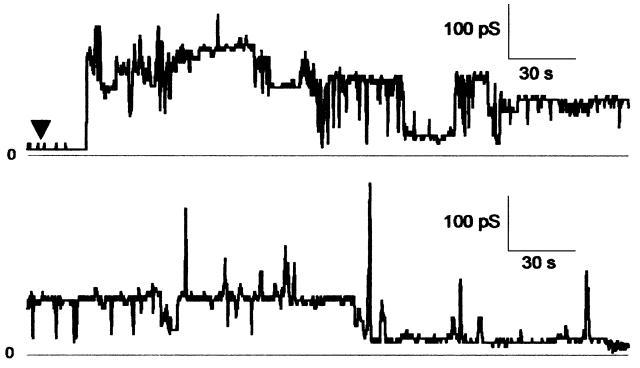

Fig. 2. Spontaneous decline with time (rundown) of multi-channel activity of BLM after incorporation of 55 kDa protein purified from the inner mitochondrial membrane. Channel-forming protein (3 μg) was added to the *cis* side of the membrane at the moment indicated by the arrow. The solution at both sides contained 100 mM KCl in 20 mM Tris-HCl buffer (pH 7.4).

The reconstituted mitoK$_{ATP}$ channels are specifically regulated by nucleotides [6,10]. Added to the *cis* side of BLM, MgATP decreased membrane conductance from 150 to 20 pS (Fig. 4, column 2). DEB antagonized the ATP blockade (Fig. 4, columns 3–5). At the concentration of 8 μM DEB, channel activity even exceeded the initial level.

DEB also affected the activity of the channels

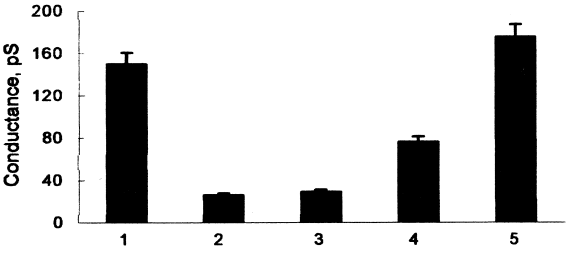

Fig. 4. Activation of mitoK$_{ATP}$ channels by DEB. 1, initial channel activity; 2, channel activity in the presence of 3 mM MgATP. Channel activity with MgATP (3 mM) and DEB 2 μM (3), 5 μM (4) and 8 μM (5), respectively. All data indicate the mean ± S.D.

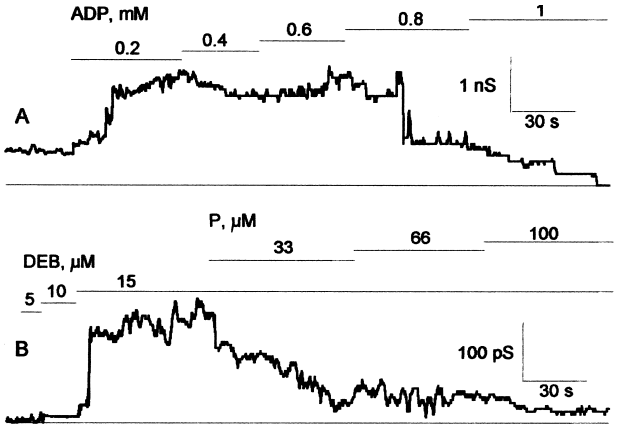

Fig. 5. Regulation of mitoK$_{ATP}$ channels by ADP and redox agents. (A) ADP was added to the *cis* side of the membrane. (B) DEB and pelargonidine (P) were added to both sides of the membrane. Horizontal bars represent onset and duration of application of channel regulators; concentrations of DEB are indicated above bars. Other conditions as in Fig. 1.

modified by the ADP. Low concentrations of ADP (200 μM) activated the channel (Fig. 5A). High [ADP] inhibited channel activity and at 1 mM channels became fully blocked. The blocked channels are reactivated by 10–15 μM DEB (Fig. 5B) in a manner similar to that seen in ATP inhibited channels. Thus, DEB overcomes the inhibitory action of both ATP and ADP and withstands channel rundown.

Pelargonidine, as yet not known as a channel regulator, abolished the activating effect of DEB. High channel activity promoted by DEB (15 μM) (Fig. 5B) is gradually diminished by pelargonidine and the channel became fully inhibited at 100 μM of pelargonidine.

To understand the molecular mechanisms of the opposite effects of DEB and pelargonidine it seems promising to find out whether these regulators differ in the opposite way in some physico-chemical properties. Interaction with dye radicals appears to be a useful approach to find differences in the redox properties of channel regulators [15,16]. DEB facilitated haemin photoreduction by the dye eosin under steady illumination of the deaerated solution. In the control sample containing haemin and eosin the haemin photoreduction is slow and less than 10% of haemin content was reduced at the end of 60 min illumination (Fig. 6A, curve 1). With 1 mM DEB more than 50% of haemin was photoreduced during a twofold shorter time (Fig. 6A, curve 2). Obviously, the reducing equivalents, electrons, were delivered by DEB, while the excited dye eosin served as a photocatalyst [21]. In this experiment DEB demonstrated its electron donor properties towards free radical intermediates of excited dye.

Under conditions providing high concentrations of eosin anion radicals, the electron acceptor properties of a compound may be revealed. In the control sample containing eosin and NADH, dye photobleaching is rapid and nearly complete – more than 60% of the initial eosin was bleached during 10 s of continuous illumination (Fig. 6B, curve 1). Pelargonidine (10 μM) slowed the initial rate of dye photobleaching more than twice and much eosin remained coloured even after 60 s of illumination (Fig. 6B, curve 2). In a higher pelargonidine concentration (100 μM) the dye bleached slowly during the whole illumination period (Fig. 6B, curve 3). In this experiment NADH favours

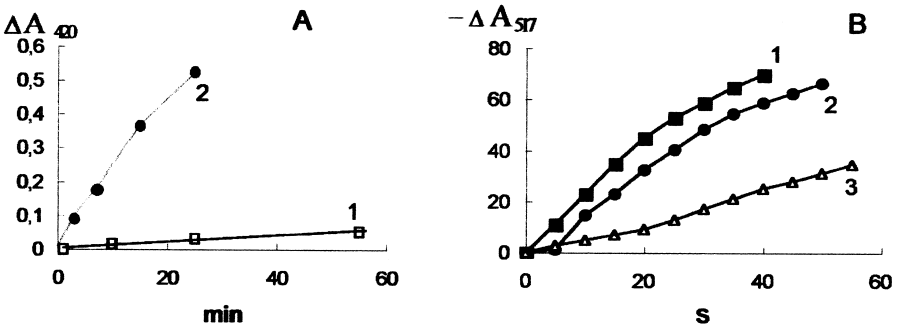

Fig. 6. Redox properties of DEB and pelargonidine. (A) DEB accelerates photoreduction of haemin acting as an electron donor. 1, control: 10 μM eosin and 10 μM haemin in the deaerated solution subjected to continuous visible light (a tungsten lamp $P = 10$ W, $\lambda > 500$ nM). 2, control+DEB (1 mM). (B) Pelargonidine slows down dye photobleaching facilitated by NADH, thus acting as an electron acceptor. 1, control: solution of 10 μM eosin and 100 μM NADH in Tris-HCl buffer (pH 7.5) is deaerated and subjected to continuous visible light (as in A). 2 and 3, control+pelargonidine 10 μM and 100 μM, respectively.

conversion of the excited dye to the high concentration of dye anion radicals [21] which accelerates formation of the colourless dye leuco form. Pelargonidine intercepts electrons from dye anion radicals, thus slowing down eosin photobleaching and demonstrating electron acceptor properties.

The inhibitory effect of ATP is possibly also determined by its electron acceptor properties. Under conditions providing high concentrations of eosin anion radicals photohydrolysis of ATP has been observed as judged by the appearance of inorganic phosphate, P_i [22]. The end phosphate and its ester bond in ATP have been thought to act as an electron acceptor site attacked by dye anion radicals thus resulting in ATP photohydrolysis. Taken together with the strong electron acceptor properties of pelargonidine, one can consider the acceptor activity of a substance as a determinant for inhibition of mitoK_{ATP} channels. Thus, this study demonstrates opposite effects of DEB and pelargonidine on the mitoK_{ATP} channel which may be related to their opposite redox properties. Though some K^+ channels are assumed to be activated by reactive oxygen species (ROS) [23,24], in our experiments the radical scavengers superoxide dismutase and catalase did not abolish potentiation by DEB of the mitoK_{ATP} channel (data not shown). It points to direct interaction of the regulators with the channel, in which the specific ligand-protein interaction is considered the primary step which may be followed by redox interactions.

The putative target for redox active ligands may be thiol groups in the channel-forming protein. Switching the neighbouring thiols from the oxidized to the reduced state (or vice versa) is supposed to change channel conformation and channel gating [25]. Recently, a thiol group(s) was found in the cytoplasmic domain of the cytoK_{ATP} and its oxidation by thimerosal caused channel closure while reduction by dithiothreitol (DTT) restored channel activity [26,27]. We observed a similar regulation of the mitoK_{ATP} channel by thiol agents (unpublished results). The oxidizing effect of thimerosal is equivalent to the action of an electron acceptor, while the reductive effect of DTT is equivalent to the action of an electron donor. We assume that these essential thiols may be the target for the opposite action of the electron donor (DEB) and the electron acceptor (pelargonidine).

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