Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 126-132

POTASSIUM CHANNEL OPENER, RP 66471, INDUCES MEMBRANE DEPOLARIZATION OF RAT LIVER MITOCHONDRIA

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Received December 5, 1994

Summary: Effect of potassium channel openers on membrane potential of rat liver mitochondria was studied. It has been found that potassium channel opener RP 66471 induces depolarization of the mitochondrial membrane. Since neither the inhibition of mitochondrial respiration nor the uncoupling of mitochondria was observed concomitantly, the specific effect on the mitochondrial potential is postulated. Most likely the effect is caused by the increase of permeability of the inner mitochondrial membrane to potassium ions. Interestingly, however, it was found that no other potassium channel openers tested but RP 66471 was able to induce depolarization of mitochondrial membrane.

Recently, the potassium channel sensitive to ATP (K_{ATP}) has been described in the inner membrane of rat liver mitochondria (1, 2). It was postulated that mitochondrial K_{ATP} channel may belong to the well known family of ATP dependent potassium channels described in plasma membranes of cardiac, smooth and skeletal muscle cells (3-5). The same channels play also a key role in insulin secretion from pancreatic ß-cells (6).

Despite of uncertainties about the functional role of mitochondrial K_{ATP} channel it has been shown that the channel may be involved in controlling mitochondrial matrix volume changes (7-9). It has also been suggested that the activation of mitochondrial K_{ATP} channel should lead to depolarization of the inner mitochondrial membrane potential (1). In order to

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Abbrevations: BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF/AM, acetoxymethyl ester of BCECF; EGTA, ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetate; DMSO, dimethylsulphoxide; TPP, tetraphenylphosphonium; CCCP, carbonyl cyanide p-chlorophenylhydrazone; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

0006-291X/95 $5.00

clarify this point, we used herewith several potassium channel openers as a tool to induce mitochondrial K_{ATP} channel activation.

Activators of K_{ATP} channels (potassium channel openers) constitute a chemically diverse group of compounds (10). It has been shown that these reagents are able to increase permeability to potassium ions of different cells containing K_{ATP} channels (11-13). The family of these compounds was enriched by new substances like Ro 31-6930 or RP 66471 (14,15).

In this report the effect of various potassium channel openers on membrane potential of respiring rat liver mitochondria is described.

MATERIALS AND METHODS

Materials. Drugs used in the present study were the generous gifts from: Rhone-Poulenc Rorer, France (aprykalim, nicorandil); Rhone-Poulenc Rorer, UK (RP 66471); Roche, UK (Ro 31-6930); Kirin Brewery Co., Japan (KRN 2391). Rotenone was from Sigma (U.S.A). BCECF/AM was purchased from Molecular Probes (Eugene, Oregon, U.S.A). All other chemicals were of the highest purity commercially available.

Isolation of rat liver mitochondria. Rat liver mitochondria were prepared according to Johnson & Lardy (16) using 75 mM sucrose, 225 mM mannitol, 3 mM Hepes-KOH pH 7.4 and 1 mM EGTA as the isolation medium.

Mitochondrial transmembrane potential measurements. Mitochondria (final concentration 1.2 mg of protein/ml) were suspended in the medium containing 100 mM KCl, 10 mM Mops-Tris pH 7.4, 5 mM succinate-Tris, 5 mM phosphate-Tris, 0.1 mM EGTA-Tris, 2 μM rotenone, 2 mM $MgCl_2$. Mitochondrial membrane potential was measured with a TPP-sensitive electrode (17) (Detektor, Poland) connected to the Ion Analyzer 13040 (Jenway, UK). The Ag/AgCl electrode served as a reference electrode. Electrical signals were digitalized and stored on a hard disc of an IBM PC for further analysis (18). Experiments were carried out at 25°C.

Respiration of mitochondria. Mitochondria (final concentration 2 mg of protein/ml) were added to the air-saturated medium containing 100 mM KCl, 1.25 μg/ml rotenone, 10 mM succinate, 5 mM phosphate, 10 Tris-HCl at pH 7.4. Respiration was measured with a Clarke-type oxygen electrode. Experiments were carried out at 25°C.

Measurements of pH by BCECF fluorescence. Measurements of pH in the mitochondrial matrix were performed with the fluorescent probe BCECF. Loading of rat liver mitochondria with BCECF/AM was performed as described previously (19). Fluorescence was recorded using Shimadzu fluorometer (RF-5000) with emission at 530 nm and excitation at 500 nm (as a pH-sensitive wavelength) and at 450 nm (as a pH-insensitive wavelength). The fluorescence data were used to compute the ratio of 500/450 nm and to convert it into the pH values (20).

Measurements of light scattering changes of mitochondrial suspension. The kinetics of light scattering changes, reflecting mitochondrial volume changes linked to solute transport, were measured at 540 nm with Shimadzu UV-visible recording spectrophotometer (UV-160A) at 25°C in the following medium: 150 mM KSCN, 20 mM Tris-Hepes pH 7.4, and 2 μM rotenone; final mitochondrial protein concentration was 1.0 mg/ml.

Planar bilayer technique. The planar lipid bilayer was formed by the folding method. A Teflon chamber with two compartments (each about 6 ml internal volume) was separated by a Teflon septum with an aperture of 85 μm in diameter. 40 to 60 μl solution of asolectin in n-hexane (2 mg/ml) was placed on the surface of 4.5 ml of the medium (1 M KCl). The solution level in each compartment was raised by the addition of the 0.5 ml of 1 M KCl through a glass pipette over the aperture where the lipid bilayer had formed. Ag/AgCl

electrodes were immersed in the medium. Formation of the bilayer was monitored by measuring of the membrane capacitance (the final capacitance was 50 - 60 pF). A solution of RP 66471 in DMSO was added to cis compartment. Membrane potential was defined as potential of the trans versus cis compartment. All measurements were carried out at room temperature. The current was measured using Bi-layer Membrane Admittance Meter (model ID 562, IDB, Gwynadd, U. K.).

Protein concentration. Protein concentration was measured with a protein-assay-kit (Bio-Rad) according to the method of Bradford (21).

Statistical analysis. All values are expressed as the means $\pm$ S.D. The significance of measured responses was assessed using the Student's t test or the analysis of variance.

RESULTS AND DISCUSSION

Functional role of K_{ATP} channel in mitochondria is not yet established. However, it has been suggested that K_{ATP} channel is involved in controlling mitochondrial membrane potential (1). Thus, opening of the channel should cause dissipation of membrane potential of energized mitochondria due to an influx of potassium cations into the mitochondrial matrix. Here, in order to induce potassium ions flux via mitochondrial K_{ATP} channel, so called "potassium channels openers" were used, i.e. substances shown to activate K_{ATP} channels in different tissues (10). Mitochondrial membrane potential changes were monitored with TPP-sensitive electrode, measuring the partitioning of a lipophilic TPP cation. Figure 1A presents the effect of RP 66471 (100 μM) on membrane potential of mitochondria respiring in the presence of succinate. It was found that 100 μM RP 66471 was able to induce depolarization of the mitochondrial membrane, similarly as it was observed in the presence of potassium ionophore valinomycin (Figure 1A). IC_{50} of the effect induced by the opener was found at 150 μM (Figure 1B). Interestingly the effect of RP 66471 was found to be specific. All the other potassium channel openers applied, as Ro 31-6930, KRN 2391, aprykalim and nicorandil (Figure 1A) were unable to depolarize membrane potential of energized mitochondria. To exclude an involvement of the inhibition of succinate dehydrogenase by RP 66471 in its effect on membrane potential, experiments in the presence of TMPD/ascorbate as substrate, were performed. As found RP 66471 was also able to depolarize mitochondria under these conditions (not shown). Figure 2 shows a comparison of RP 66471 induced depolarization in the presence of different monovalent cations: Li^+, Na^+, K^+ and Rb^+. As presented, the amplitude of depolarization induced in the presence of potassium is significantly larger than depolarization in the presence of lithium and sodium. However, rubidium ions were found to be able to replace potassium and to induce a significant depolarization of the membrane, similar to that observed in the presence of potassium. This suggests that RP 66471 induced ion permeability is specific to larger monovalent cations (K^+ and Rb^+), but not to the smaller ones (Li^+ and Na^+). In the monovalent cations free medium (sucrose medium), the observed depolarization of

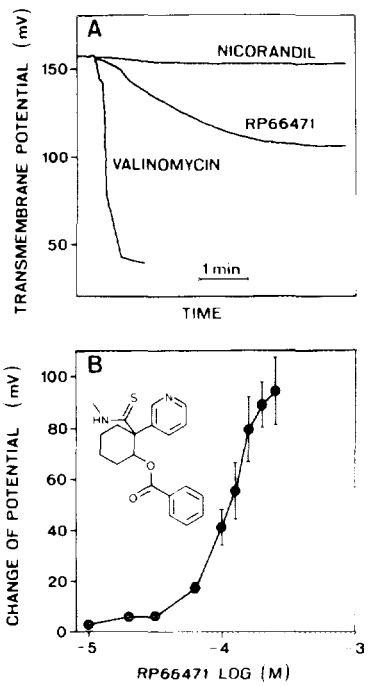

Figure 1. A. Depolarization of rat liver mitochondria membranes induced by RP 66471. Measurements of membrane potential were performed with a TPP-ion selective electrode as described under Material and Methods. Where indicated, 100 μM RP 66471 or valinomycin (150 pmol/mg protein) was added. B. Concentration dependence of RP 66471 induced membrane depolarization. Measurements were performed as described under Material and Methods. Mean values $\pm$ S.D. for three mitochondrial preparations are shown. Insert: Chemical structure of the potassium channel opener RP 66471.

mitochondrial membrane could be explained by the presence of potassium in the stock suspension of mitochondria which had leaked out from the particles during the isolation and storage (Figure 2).

It is known that the plasma membrane K_{ATP} channels are blocked by the nanomolar concentrations of antidiabetic sulfonylurea, glibenclamide, while mitochondrial K_{ATP} channel is blocked by micromolar concentration of this compound (1, 2). In addition, studies on binding of radioactive glibenclamide to mitochondrial membranes revealed only the existence of low affinity binding sites (Szewczyk, unpublished observation). Furthermore, application of micromolar concentration of glibenclamide on respiring mitochondria (generating membrane potential because of pH gradient formation on inner mitochondrial membrane) resulted in depolarization of the membrane (not shown), most likely due to protonophoric character of glibenclamide (22, 23). Taking all this into account, it should be expected that no diminution of the RP 66471 effect on respiring mitochondria can be observed in the presence of glibenclamide, what indeed was the case.

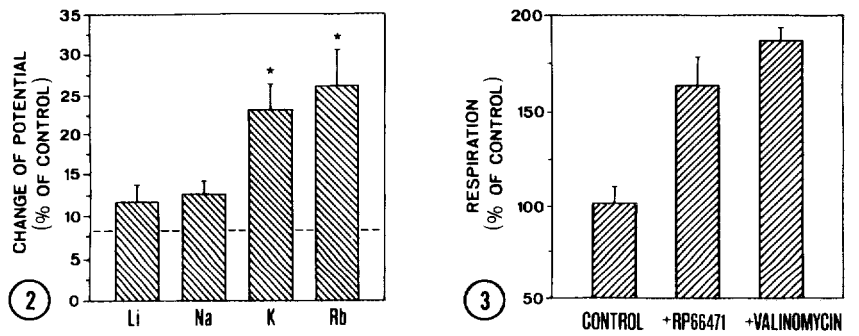

Figure 2. Ion specificity of RP 66471 induced mitochondrial membrane depolarization. Membrane potential was measured as described under Material and Methods. Instead of KCl LiCl, NaCl, RbCl and sucrose were also used. Depolarization of membrane induced by 200 μM RP 66471 is expressed as percentage of membrane potential before addition of the opener. Starting membrane potential in the presence of different salts was the following: for LiCl 175 $\pm$ 6 mV, for NaCl 185 $\pm$ 7 mV, for KCl 180 $\pm$ 3 mV and for RbCl 178 $\pm$ 3 mV. Starting membrane potential in the medium containing 200 mM sucrose was 189 $\pm$ 1 mV. Starting membrane potentials were not significantly different ($P < 0.05$, analysis of variance). Dotted line indicates RP 66471 induced depolarization, equal 8±1%, in the presence of 200 mM sucrose instead of 100 mM KCl. Mean values $\pm$ S.D. for three mitochondrial preparations are shown. * A significant ($P<0.05$, Student's t test) change from the depolarization in the presence of LiCl and NaCl.

Figure 3. Effect of RP 66471 on respiration of rat liver mitochondria. Results are expressed as percentage of control (respiration with succinate and without 100 μM RP 664671 added). Respiration measurements were performed as described under Material and Methods. Measurements were performed in triplicates ($\pm$ S.D.). Mean value of respiration in the presence of 100 μM RP 66471 was significantly different from control value ($P < 0.05$ Student's t test). As control value respiration 40 ng-atom of oxygen/min per mg of protein was taken.

In order to check whether depolarization of the membrane upon addition of RP 66471 results from an increased permeability of the inner mitochondrial membrane to ions, the mitochondrial respiration was measured concomitantly. The rate of respiration of mitochondria (in the presence of succinate) was recordered prior and after application of 100 μM RP 66471 or valinomycin. A clear increase of mitochondrial respiration was observed upon additions (Figure 3), what points to the depolarization of membrane potential as coming from the enhancement of mitochondrial membrane permeability to cations, both in the presence of RP 66471 and of valinomycin.

Moreover, measurements of the intramitochondrial pH excluded protonophoric effect of RP 66471 (Figure 4), since the raise of pH observed upon addition of succinate was not influenced by 100 μM RP 66471 in contrast to the mitochondrial uncoupler CCCP (Figure 4).

To show the direct influx of potassium ions into mitochondrial matrix, the rate of passive swelling of mitochondria in KSCN medium was measured. Under such conditions,

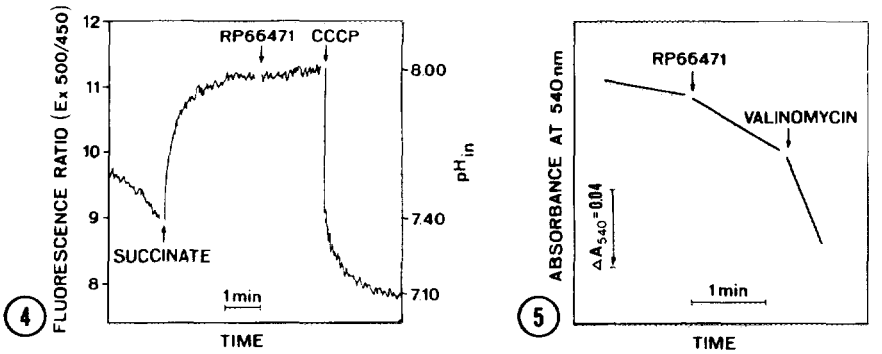

Figure 4. Changes of intramitochondrial pH in the presence of RP 66471. Measurements and calibration were performed as described under Material and Methods. Measurements were performed in medium containing 100 mM KCl, 10 mM Tris-HCl,pH 7.4, 0.5 mM EGTA-Tris, 2 μM rotenone and oligomycin 5 μg/ mg of protein. 10 mM succinate, 100 μM RP 66471 and 2 μM CCCP were added as indicated by arrows.

Figure 5. Effect of RP 66471 on mitochondrial swelling under non-respiring condition. Mitochondria (1 mg of protein/ml) were suspended in medium (see Materials and Methods) and their swelling was followed by measuring the rate of absorbance decrease at 450 nm. One representative measurement is shown; RP 66471 was 100 μM; valinomycin was 1 μM. Rate of swelling in untreated mitochondria varied within 0.007-0.036 absorbance unit/min, depending on mitochondrial preparation.

in the presence of a permeable anion (SCN^-), swelling of mitochondria directly reflects the influx of K^+ into the mitochondrial matrix. It was found that RP 66471 was indeed able to stimulate passive mitochondrial swelling, further increased by valinomycin (Figure 5). As reported earlier swelling of mitochondria induced by RP 66471 may be inhibited by the addition of glibenclamide (100 μM) (9).

To exclude the possibility that RP 66471 *per se* possesses ionophoric properties, its influence on the permeability of the model membrane was checked. No increase in potassium current measured with a planar bilayer technique (see Material and Methods) was found after application of RP 66471 (not shown).

Summarizing, our results suggest that potassium channel opener RP 66471 induces membrane depolarization of rat liver mitochondria through increasing of the K^+ flux catalyzed by the K_{ATP} channel of mitochondrial inner membrane. Therefore, it can be postulated that this channel is involved in regulation of membrane potential of liver mitochondria. Since changes of mitochondrial potential are sufficient to modulate some transport processes such as ATP/ADP exchange or calcium influx into mitochondria, it is of particular importance to establish whether the mitochondrial K_{ATP} channel could be, at least partially, responsible for the regulation of mitochondrial transport functions.

ACKNOWLEDGMENTS

This study was supported by grant No. 6 P203 003 04 from Polish Committee for Scientific Research to A.S. Ms. Beata Mikołajek is kindly acknowledged for her involvement in some of the preliminary experiments. The expert technical assistance of Mr. Tobias Roeser and Mr. Adam Jagielski is gratefully acknowledged.

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