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INHIBITION OF MITOCHONDRIAL METABOLISM BY THE DIABETOGENIC THIADIAZINE DIAZOXIDE—II

INTERACTION WITH ENERGY CONSERVATION AND ION TRANSPORT

R. PORTENHAUSER, G. SCHÄFER and R. TROLP
Department of Biochemistry, Medizinische Hochschule Hannover, Hannover, Germany

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Abstract—The interaction of diazoxide, a diabetogenic compound inhibiting tricarboxylic acid cycle oxidations, with the energy conservating system of mitochondria was studied. Changes of respiratory activity of livermitochondria with various substrates and corresponding changes of the redox states of respiratory pigments after addition of diazoxide were measured. The flux of reducing equivalents into the respiratory chain and the potential of the "energized state" is drastically lowered by the inhibitor. The drug developed only a weak uncoupling activity but a pronounced inhibition on substrate uptake. The interaction of diazoxide with accumulation of ^{14}C-labelled substrate anions was directly measured, and is of the competitive type. Diazoxide itself enters the mitochondrion in the anionic form, as revealed by anion exchange studies. ATP-ase is activated by diazoxide. This effect is reversed by sulfonylureas and is discussed as a mode for the antagonism of both compounds at the cellular membrane of pancreatic islets.

IN PRECEDING reports diazoxide was shown to inhibit integrated mitochondrial oxidations, when a complete operation of the citric acid cycle was involved.[1–3] This limitation of aerobic energy metabolism may be related to the diabetogenic action of the drug in general and to inhibition of insulin release in particular.[4–8] An hitherto undescribed observation is an interference of the drug with oxidative phosphorylation and with aniontransfer at the mitochondrial membrane and will be reported here. The compound behaves as a weak lipidsoluble acid, carrying the acidity in a dissociable NH-group. On this structural basis it shares the characteristic properties with classical uncouplers.[9–12] Although diazoxide is only a weak uncoupler its interaction with anion transfer may bear some significance for its capability to act on membrane-linked biological processes. Whereas it is phenomenologically established that sulfonylureas can release the diazoxide block of insulin liberation from pancreatic islets, the molecular mechanism of this antagonism is still unknown. The present investigation is the first to demonstrate an antagonistic effect of diazoxide and sulfonylurea in a subcellular model. That is the inhibition of diazoxide induced ATP-ase activity in isolated mitochondria.

METHODS

Preparation of mitochondria from tissues of male Wistar rats, polarographic registration of oxygen consumption, protein determination and measurement of the redox state of respiratory pigments were carried out as previously described.[13,14] For

ion exchange experiments rapid separation of mitochondria from the incubation medium was achieved by filtration-centrifugation[15] in the modification described by Pfaff,[16] Klingenberg,[17] and Harris.[18] All data on intramitochondrial substrate content were corrected for the amount present in the sucrose permeable space, which is freely permeable for anions. In parallel experiments with unlabelled substrate ^{14}C labelled sucrose and tritiated water were added, in order to determine the sucrose permeable space and the total mitochondrial water content.[19,20] In samples taken under constant conditions the standard error of mean for ^{14}C and tritium determinations was in the range of 3–5 per cent. Radioactive chemicals were obtained from the Radiochemical Centre Amersham, Great Britain. Diazoxide was a gift from Byck-Essex. Anion penetration studies were performed in a medium containing 0·25 M sucrose, 20 mM tris-buffer pH 7·3, 1 mM EDTA; all samples contained 8 μg rotenone and 16 μg antimycin A/ml to abolish oxidative metabolism. ATP-ase was measured by registration of proton liberation during ATP-hydrolysis with a sensitive "Ingold"-pH-electrode, connected to a radiometer pH-meter and a recorder, and in addition by determination of inorganic phosphate liberated.[21] Respiratory experiments were carried out in a medium containing 0·25 M sucrose, 10 mM triethanolamine pH 7·3; 10 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4 (RC-buffer). Liquid scintillation counting was used for radioactivity measurements.

RESULTS

Diazoxide action on redoxstates of respiratory pigments

Addition of diazoxide to liver mitochondria supplemented with succinate causes an oxidation of flavoproteins, as described earlier (Ref. 3, Fig. 2). Whereas this oxidation is explained by inhibition of succinate dehydrogenase, the same phenomenon could be observed with NAD-dependent substrates like HOB,* glutamate, malate. Moreover, an analogous response of cytochrome b and c could be registered,[22] but oxygenuptake was not inhibited, in contrast to the experiments with succinate. These latter findings suggested that indeed a slight uncoupling effect of diazoxide exists, being obscured when succinate was the substrate, due to specific inhibition of SDH. In respiratory experiments uncoupling could only be observed under special conditions (c.f. next section). Therefore, as another set-up for demonstration of the interference of diazoxide with energy conservation in the respiratory chain, anaerobic systems with ATP-driven reversed electron transfer were used. These react extremely sensitively to compounds dissipating the energy by either uncoupling or inhibition of energy transfer in the mitochondrial membrane.[23,24] In Fig. 1 cytochrome c of liver-mitochondria was reduced by anaerobiosis. ATP in presence of oxalacetate as hydrogen acceptor causes an energy driven oxidation of cytochrome c. The redox state of cytochrome c can be titrated to the fully reduced state by subsequent additions of diazoxide. This makes it evident that diazoxide indeed uncouples the energy linked reactions of the respiratory chain. For comparison similar experiments are shown with oligomycin, or the uncoupler m-Cl-CCP.† Compared to the latter the activity of diazoxide as an uncoupler is however, very low.

* β-hydroxybutyrate.
† Carbonylcyanid-(m,chloro-phenylhydrazone).

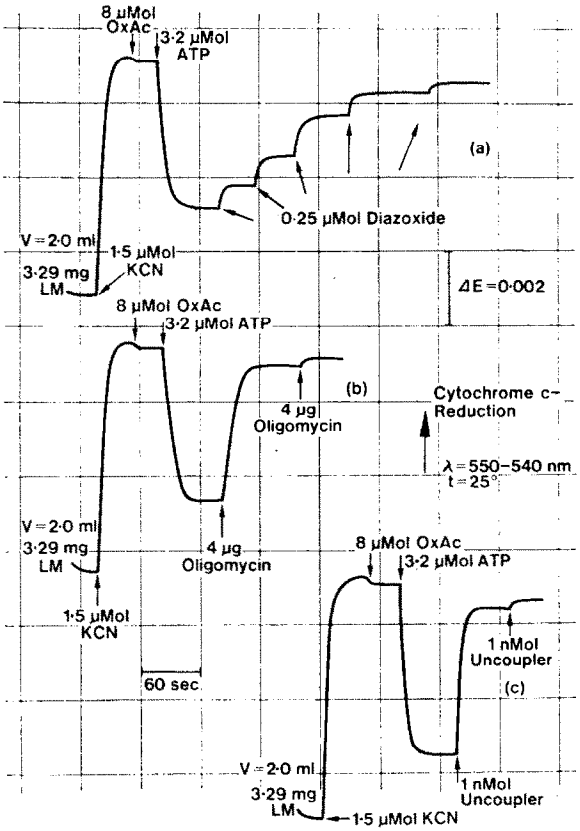

FIG. 1. Direct recording of the redox state of cytochrome c in rat liver mitochondria under the conditions of reversed electron transfer. The incubation medium contained 0·25 M sucrose, 10 mM triethanolamine buffer pH 7·3, 1 mM EDTA. Other conditions as indicated. Additions are given in absolute amounts. The uncoupler used was m-Cl-CCP.

Action of diazoxide on respiration

From the two experiments shown in Fig. 2, the right one illustrates that uncoupling, i.e. stimulation of oxygen uptake, by diazoxide could not be detected with succinate as substrate In contrast, when reducing equivalents are generated by the NAD-dependent dehydrogenation of β-HOB, addition of diazoxide stimulates respiration; left experiment. In both experiments respiratory control of the mitochondria was at first tested by a cycle of ADP phosphorylation. As an important observation it should be noted that (1) diazoxide does not activate oxygen uptake to its maximum degree in contrast to classical uncouplers; (2) that in the presence of diazoxide also ADP no longer achieves maximum respiration. Together with the finding (not shown here) that after addition of diazoxide to the system the mitochondrial pyridine nucleotides were largely oxidized—independent of the respiratory state—while respiration is only partially activated, one has to assume that in presence of this inhibitor the uptake of substrate or of inorganic phosphate, which enters the mitochondria in exchange for OH- or endogenous substrate anions,[25] becomes rate limiting. This interpretation is not only consistent with the experimental observations, but also with the properties

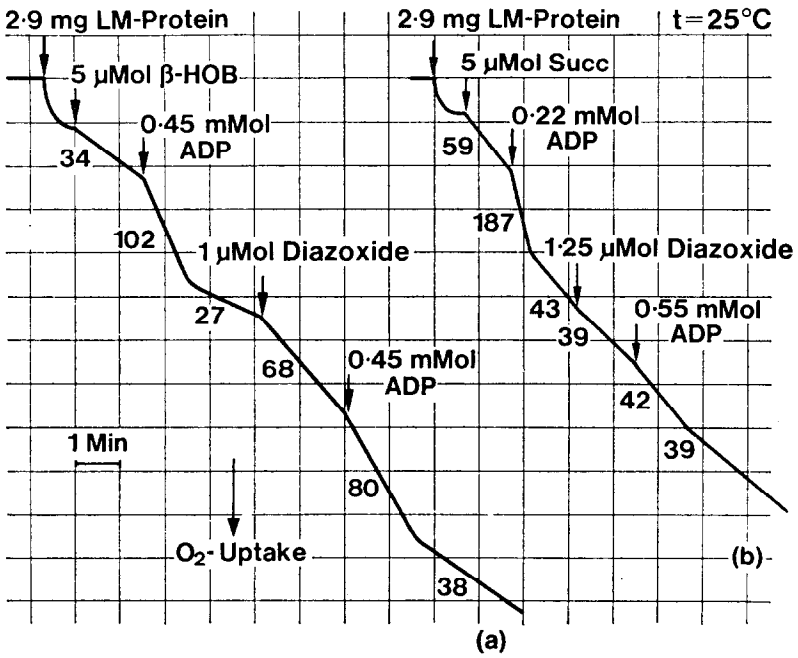

FIG. 2. Polarographic record of mitochondrial respiration. Total volume is 2·4 ml RC-buffer. Additions given in absolute amounts. The figures along the traces indicate the rate of oxygenuptake given as nA (O)/min/mg protein.

of uncouplers in general,[26,27] which may in the anionic form compete with substrate anions for penetration into the mitochondria.

On this basis the data summarized in Table 1 appear conclusive. With all substrates used an addition of diazoxide during state 4 of respiration (ADP absent) produced a slight respiratory increase (succinate excepted), due to uncoupling. When diazoxide was added in state 3 (ADP and P_i present) respiratory rate decreased in all cases, because the saturation of dehydrogenases and thus of the NAD-pool with reducing equivalents becomes rate limiting.

TABLE 1. INFLUENCE OF DIAZOXIDE ON THE RESPIRATORY RATE OF MITOCHONDRIA WITH VARIOUS SUBSTRATES IN STATE 3 AND STATE 4

Substrate	State 4	State 4 +Diazoxide	State 3	State 3 +Diazoxide
Glutamate	26·1 ± 1·8	36·4 ± 2·2	41·0 ± 2·2	32·2 ± 1·3
			(82·0 ± 6·2	64·2 ± 4·9)*
α-Ketoglutarate	25·1 ± 1·5	38·4 ± 5·1	(214·4 ± 2·3	82·3 ± 0·5)†
β-Hydroxybutyrate	32·1 ± 1·5	43·9 ± 1·5	91·3 ± 2·7	74·7 ± 1·8
Succinate	62·1 ± 4·3	48·1 ± 2·5	187·2 ± 5·8	91·4 ± 2·9
Pyruvate	—	—	(159·7 ± 3·7	91·3 ± 4·2)†

All figures give mean values; n = 5–8. The single substrates were tested in different preparations. Diazoxide concentration 0·4 mM.

* Measured in presence of malate.

† Measured with rabbit heart mitochondria, as described in a preceding paper.[8]

Substrate concentrations were 5 mM; figures are nA(O)/min/mg.

The results therefore characterise diazoxide as a weak uncoupler, which, however, inhibits mitochondrial anionexchange already at low concentrations, Potent uncouplers usually develop this inhibiting effect only at concentrations at least 10-times above their maximum uncoupling concentration. These differences of uncoupling-activity are to a large extent related to the mobility and partition of the uncoupling compounds in the mitochondrial membrane.[12,28]

In order to substantiate for the above assumptions, direct measurements on the action of diazoxide on mitochondrial uptake and release of substrate anions have been carried out.

Effect of diazoxide on mitochondrial substrate exchange

The interaction of diazoxide with substrate translocation across the mitochondrial membrane was studied via the distribution of ^{14}C-labelled substrates under varying conditions. Rapid separation of mitochondria from the incubation medium by filtration-centrifugation[15,17] allows the measurement of the rates and the amount of substrates taken up or exchanged. In the reported experiments succinate ^{14}C was used; it should be noticed however, that corresponding results were obtained with malate, for example. In all experiments metabolic activity was abolished by rotenone and antimycin A. Since the accumulation of succinate is a rapid process, to be resolved kinetically only at low temperature,[29] the present data from experiments carried out at room temperature represent the final equilibrium states of substrate distribution.

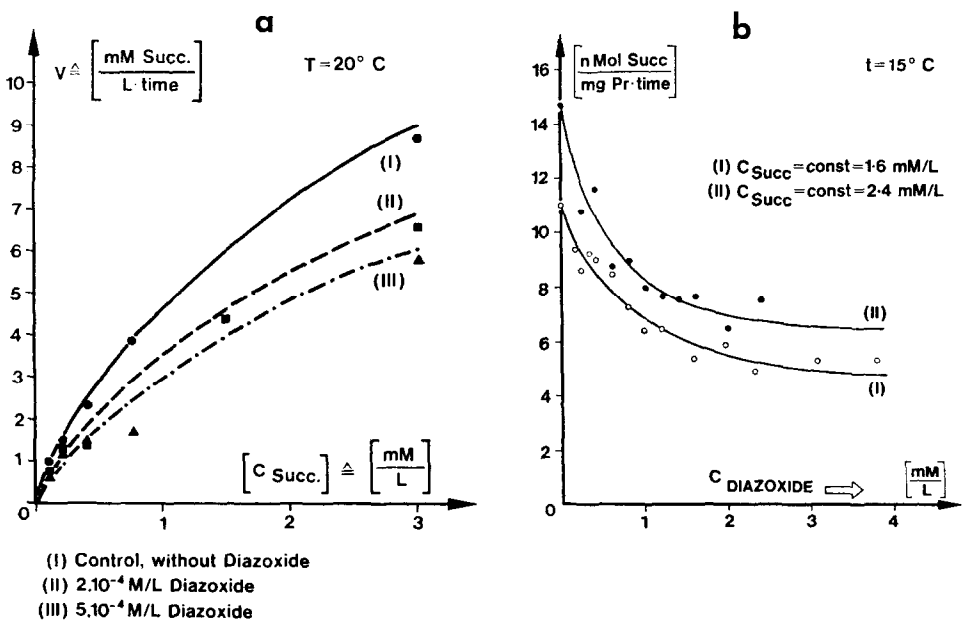

FIG. 3. Succinate uptake and extrusion by rat liver mitochondria in presence of diazoxide. Condition. as given in "methods". Total volume of each incubation 150 μl. Mitochondrial protein 1 mg/sample (a) succinate uptake with and without diazoxide present, dependent on succinate concentration. Incubation time 90 sec. (b) –○–○–○– succinate uptake in 1 min; diazoxide concentration increasing. –●–●–●– succinate extrusion from succinate-loaded mitochondria by increasing concentrations of diazoxide.

Figure 3 (a) demonstrates the accumulation of succinate in liver mitochondria as a function of external succinate concentration. The intramitochondrial concentration of succinate or of other divalent anions respectively, is successively lowered with increasing concentrations of diazoxide. From Lineweaver–Burk plots of these and corresponding data it appears that a competitive type of inhibition exists between accumulation of succinate and of diazoxide. In this regard diazoxide exactly resembles the properties of other uncoupling agents.[27] In Fig. 3 (b) the correlation of succinate uptake with diazoxide concentration is shown (trace I). The uptake reaction in these experiments was initiated by simultaneous addition of succinate and diazoxide and terminated after 90 sec. It is obvious from the experiments that by increasing the diazoxide concentration less succinate can be intramitochondrially accumulated.

Trace II in Fig. 3(b) shows experiments carried out in order to prove the assumption that the inhibitor itself enters the mitochondrion as an anion in exchange for endogenous anions. Livermitochondria were "loaded" with ^{14}C-succinate by preincubation for 1 min. Then the inhibitor was added to yield different concentrations and the particles were separated after 1 additional min of incubation. The results show, that by increasing the concentration of diazoxide, intramitochondrial succinate is gradually displaced, which is consistent with the above assumption. In line with these findings is the observation (not shown here),[22,30] that the level of pyridine nucleotide reduction in mitochondria supplemented with β-HOB was lowered by diazoxide, but thereafter could be increased again by raising the concentration of substrate significantly. This is expected on the basis of reversible anion equilibration at the mitochondrial membrane when a competition exists between single anionic species for carrier- or penetration-sites.

TABLE 2. ACTIVATION OF ATP-ASE IN HEART- AND LIVER MITOCHONDRIA OF THE RAT BY DIAZOXIDE

Diazoxide concentration (mM/l.)	Heart mitochondria ATP-ase (nVal H^+/min/mg)	Liver mitochondria ATP-ase (nVal H^+/min/mg)
Control	17·75	8·95
0·04	—	12·38
0·10	22·2	17·17
0·2	31·1	26·9
0·3	35·5	51·7
0·4	39·9	57·7
0·5	48·8	—
0·6	53·4	66·0
0·8	75·5	75·2
1·0	86·9	—
1·2	93·3	—

Incubation conditions: 2·5 ml medium containing 50 nM NaCl, 205 mM sucrose (heart), 125 mM sucrose (liver), 1 mM triethanolamine pH 7·4; 1·9 mg protein (heart), 3·05 mg protein (liver). Reaction started by addition of ATP to a final concentration of 1·6 mM. Proton liberation was monitored by a sensitive pH electrode and recorded. t = 25°. The figures are means of several determinations. The initial rate of the reaction during the first 90 sec was taken for calculation of ATP-ase activity.

Activation of ATP-ase by diazoxide and its reversal

Like usual uncouplers, diazoxide activates mitochondrial ATP-ase, although to a smaller extent. In Table 2 the rate of proton-release from mitochondria of rat heart and rat liver during ATP-hydrolysis is compared. Corresponding results were obtained by determination of P_i-liberation.[22] While we were unable to reverse any of the prior described[1-3] metabolic effects of diazoxide in subcellular systems by sulfonylureas, it is of particular interest that by the sulfonylurea glibenclamid* (HB 419) the activity of diazoxide induced ATP-ase was reversed. This is shown in Fig. 4, where also the action on heart- and livermitochondria is compared. With livermitochondria the effect is more pronounced, and ATP-ase can be almost titrated back to the initial endogenous activity by sulfonylurea. It should be mentioned that other sulfonylureas were much less effective as inhibitors of ATP-ase. Interestingly, glibenclamid is also the most effective compound to induce insulin release from pancreatic islets.[31] In addition also the DNP induced mitochondrial ATP-ase was decreased by the sulfonylurea glibenclamid, however to a smaller extent.

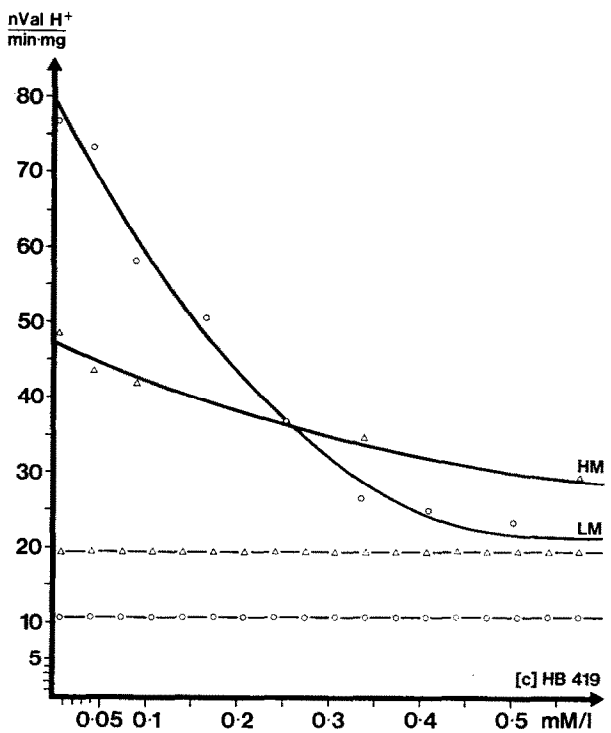

FIG. 4. Inhibition of diazoxide induced mitochondrial ATP-ase by increasing concentrations of sulfonylurea "glibenclamid". ATP-ase monitored via the proton liberation during ATP-hydrolysis. Activation of ATP-ase by 0·6 mM diazoxide. The incubation mixture contained in a total volume of 2·5 ml 125 mM sucrose, 50 mM NaCl, 1 mM triethanolamine pH 7·4; 3·5 mg liver mitochondria protein; start with 4 μmoles ATP added. With heart mitochondria sucrose was raised to 205 mM, other conditions identical, protein content was 1·1 mg protein per incubation. The dashed lines parallel to the abscissa are the initial values of ATP-ase without diazoxide added. t = 25°.

* *N*-4- [2-(5-Chlor-2-methoxybenzamido)-ethyl] phenylsulfonyl-*N*'-cyclohexylurea.

It may be speculated on the basis of these results, that by sulfonylureas, which like thiadiazines represent lipidsoluble compounds with an acidic NH-group, the inhibitor diazoxide may be displaced from a binding site at the mitochondrial membrane; a mechanism which may also hold for other membranes where both compounds affect transport phenomena.

DISCUSSION

Experimental facts of this and preceding reports have clearly revealed diazoxide as an inhibitor of oxidative metabolism.[1,3] Three points of action can be distinguished: (1) an inhibition of substrate oxidation on the enzymatic level; (2) an inhibition of energy conservation by weak uncoupling; (3) an inhibition of substrate anion exchange at the mitochondrial membrane. Since the different modes of action do not occur independently from each other, it is difficult to assign particular importance to only one of these effects. It seems more likely that those occur gradually superimposed at varying intensities in specific tissues.

In accordance with the mechanism of anionexchange, as proposed by Slater[32] and by Harris,[33] interactions of diazoxide with both, substrate uptake and oxidative phosphorylation may be understood on a common basis. It is supposed that diazoxide enters the mitochondrion as an anion using various anion carriers unspecifically. The competition with substrate anions for transport results in an inhibition of substrate uptake and respiratory activity. In addition the weak lipid soluble acid may exert its uncoupling effect acting as a proton carrier across the mitochondrial lipid-membrane, thus dissipating the energy dependent protongradient.[24,34,35] According to Finkelstein[36] evidence exists that uncoupling molecules enter the mitochondria as an anion of the dimer form $(A_2H)^-$. This negatively charged species accounts for both, competition with regular anion uptake, and the transport of protons in the appropriate direction for discharging the proton gradient, that means the inward direction. In the particular case of diazoxide the inhibiting action on anion transfer is high compared to the uncoupling activity, which is presumably based on a limited mobility of either the anionic or the undissociated form of the molecule in the membrane.

Although from studies with isolated mitochondria diazoxide appears as an unspecific inhibitor, it exerts specific effects in the intact organism or isolated pancreas. Its specificity to block insulin release for example[4–8] may result from a specific susceptibility of the islet cellular membrane for binding of this and chemically related compounds. A membrane effect of diazoxide has already been based on morphological studies by Creutzfeldt.[37] In this respect it should be mentioned that both, stimulation and inhibition of insulin release are brought about by compounds sharing similar physico-chemical properties, which points to a common receptor side accordingly. Under these aspects the antagonistic action of diazoxide and sulfonylureas on mitochondrial ATP-ase may represent a model for similar effects on the cell membrane. Although a convincing body of evidence exists, that the normal function of oxidative metabolism is a basic requirement in the mechanism of insulin secretion,[38–42] further investigations should be directed to the problem, to which extent generation and consumption of energy are dynamically linked to the process of insulin release and to structural and metabolic transitions in the cell membrane.

In mitochondria conditions which change the normal function of the energy conservating system frequently produce conformational changes of the membrane,

as monitored by the fluorochrome 1,8-ANS.[43,44] However, in order to elucidate the antagonistic action of diazoxide and sulfonylureas on the cellular membrane by this means other model systems have to be developed.

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