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Uncoupler-inhibitor titrations of ATP-driven reverse electron transfer in bovine submitochondrial particles provide evidence for direct interaction between ATPase and NADH:Q oxidoreductase

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(1) From the chemiosmotic hypothesis it follows that no change is expected in potency of an uncoupler to inhibit an energy-driven reaction in an energy-transducing membrane if the energy-requiring part of the reaction, the so-called secondary proton pump, is partially inhibited by a specific, tightly bound inhibitor. An increase in potency upon inhibition of the primary pump may be expected, due to a lower rate of the total proton flow that can be used by the secondary pump and dissipated by the uncoupler. (2) Contrary to this prediction several uncouplers (S13, SF6847, 2,4-dinitrophenol, valinomycin + nigericin) show an increase in uncoupling efficiency in ATP-driven reverse electron transfer (reversal) upon inhibition of the secondary pump in this reaction, the NADH:Q oxidoreductase, by rotenone. The increase in uncoupling efficiency is proportional to the decrease in the rate of reversal, that is to the decrease in concentration of active secondary pump. (3) Similarly, upon inhibition of the primary pump, the ATPase, with oligomycin, an increase in uncoupling efficiency was found, also proportional to the decrease in the rate of reversal. (4) When the pore-forming uncoupler gramicidin was used, no change in uncoupling potency was found upon inhibition of NADH:Q oxidoreductase. Inhibition of the ATPase, however, resulted in a proportionally lower uncoupling titre for gramicidin, just as was found for S13 in the presence of oligomycin. (5) A difference was also found in the relative concentrations of S13 and gramicidin required to stimulate ATP hydrolysis or to inhibit reversal. The amount of S13 needed to stimulate ATP hydrolysis was clearly higher than the amount needed to inhibit reversal. On the contrary, the titre of gramicidin for both actions was about the same. (6) To explain these results we propose that gramicidin uncouples via dissipation of the bulk $\Delta\tilde{\mu}_{H^+}$, whereas the carrier-type uncouplers preferentially interfere with the direct energy transduction between the ATPase and redox enzymes. This is in accordance with the recently developed collision hypothesis.

Introduction

According to the chemiosmotic theory an electrochemical proton gradient, the $\Delta\tilde{\mu}_{H^+}$, is the obligatory intermediate for energy transduction in biological membranes [1]. Although this postulate has been supported by many experimental data, the recent literature also reports experiments that seem to indicate that energy transduction cannot be satisfactorily explained by this theory (for reviews, see Refs. 2 and 3).

One type of the experiment that falls into this category is represented by the uncoupler-inhibitor

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzilidenemalononitrile; S13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide.

titrations in chromatophores reported by Hitchens and Kell [4,5]. In these experiments all uncouplers tested were more effective in inactivation of oxidative phosphorylation when the secondary proton pump, the ATP synthase, was inhibited. This seems not in agreement with the chemiosmotic theory, as $\Delta\tilde{\mu}_{H^+}$ would not decrease upon inhibition of the secondary proton pump, and hence an increase of the relative efficiency of an uncoupler upon inhibition of the secondary pump is not expected (see Discussion). However, when Cotton and Jackson [6] repeated some of these experiments under slightly modified conditions, in order to minimise time-dependent changes in the rate of ATP synthesis, they found results that were fully consistent with the chemiosmotic hypothesis. In submitochondrial particles one analogous experiment was published by Westerhoff et al. [7], showing that in particles that were partially inhibited by rotenone, an inhibitor of the NADH:Q oxidoreductase, the uncoupler FCCP was more effective in uncoupling the ATP-driven reduction of NAD^+ (reverse electron transfer or reversal) than in uninhibited particles.

These considerations prompted us to perform uncoupler-inhibitor titrations of reverse electron transfer in submitochondrial particles with four kinds of uncoupler: lipophilic anionic protonophores (SF6847, S13, FCCP), a less lipophilic anionic protonophore (2,4-dinitrophenol), cationic ionophores (valinomycin + nigericin) and the pore-forming uncoupler gramicidin. Titrations of reverse electron transfer were performed in the presence of an inhibitor of the secondary pump, but also some experiments were done in the presence of an inhibitor of the primary pump.

Furthermore, we extended the investigation of uncoupler effects to a second parameter: the stimulation of ATP hydrolysis by uncouplers. According to the chemiosmotic theory the turnover of the primary pump (ATPase in reverse electron transfer) is limited by $\Delta\tilde{\mu}_{H^+}$. So, if $\Delta\tilde{\mu}_{H^+}$ is the competent intermediate in reverse electron transfer in submitochondrial particles, a lowering of reversal activity by uncouplers should be paralleled by a stimulation of ATP hydrolysis.

The results of both sets of experiments are discussed in relation to the chemiosmotic theory, to the localised coupling unit concept of Kell [5] and the recently developed collsion hypothesis [8]. Some of these results have already been published in a preliminary form [9].

Methods and Materials

Methods

All experimental procedures were essentially as described in Ref. 10, except for some changes in the assay of ATP-driven reversal. Reversal was measured in a medium containing, at pH 7.5 and 30°C, 0.18 M sucrose/4 mM $MgCl_2$/10 mM Hepes-KOH/10 mM succinate/1.6 mM KCN/1.0 mM NAD^+/3 mM ATP. 0.1–0.3 mg particle protein was added to 2 ml assay medium and the reaction was followed spectrophotometrically at 340 nm. Usually preincubation was not necessary, since the succinate:Q oxidoreductase activity was fully activated during the preparation of the particles [11]. In some assays, however, ATP was left out of the medium and the particles were preincubated for several minutes with the uncoupler before starting the reaction with ATP. After a short lag phase reversal rates were always linear for several minutes.

To reach partial inhibition of the primary or secondary pump, the particles were photolabelled with 8-azido-ATP as described in Ref. 10, or incubated with rotenone or oligomycin at a protein concentration of about 10 mg/ml. Preincubation with these inhibitors for 30 min on ice resulted in a stable inhibition of the rate of reversal.

In uncoupler titrations a separate assay was performed for each concentration of uncoupler. When gramicidin was used, the particles were preincubated with the uncoupler for at least 20 min, at a protein concentration of about 10 mg/ml.

Materials

S13 was a gift from Dr. P. Hamm, Monsanto company, St. Louis, MO, U.S.A. FCCP was obtained from Fluka AG, Buchs, Switzerland. SF6847 was kindly supplied by Dr. Y. Nishizawa, Sumitomo Chemical Industry, Osaka, Japan. Oligomycin, nigericin, valinomycin and gramicidin were obtained from Sigma. All other 'bio'-chemicals were obtained from Boehringer Mannheim and all other chemicals were of analytical quality.

8-azido-ATP was synthesized in our laboratory

by Mr. A.F. Hartog, according to the method of Schäfer et al. [12].

Results

Uncoupler titrations of reversal in the presence or absence of inhibitors of the secondary or primary proton pump

Fig. 1 shows that the lipophilic uncoupler S13 inhibited ATP-driven reversal more potently when either rotenone, an inhibitor of the NADH:Q oxidoreductase, or oligomycin, an inhibitor of the ATP synthase, was present. Without rotenone or oligomycin, 50% inhibition of reversal was reached at an uncoupler concentration of 124 pmol per mg protein. If the particles were incubated with an amount of rotenone or oligomycin that caused 38–39% inhibition of the reversal rate, this concentration of uncoupler caused a larger decrease in the remaining activity. This can be seen more clearly in the inset of Fig. 1, where the activities in the presence of rotenone or oligomycin and in the absence of uncoupler are set at 100%.

S13 and also FCCP, that was used in the uncoupler-inhibitor titration of reversal reported by Westerhoff et al. [7], are very lipophilic uncouplers. They uncouple at concentrations that are about stoichiometric with the concentrations of respiratory chain complexes [13,14]. For instance, in the experiment given in Fig. 1, 50% uncoupling was reached at an S13 concentration equal to 0.4 mol per mol ATP synthase. To test the effect of lipophilicity of the uncoupler in this kind of experiment, we also performed uncoupler titrations with the less lipophilic uncoupler dinitrophenol.

Fig. 2 shows that the relative efficiency of the uncoupler 2,4-dinitrophenol was also increased by addition of rotenone. The smaller lipophilicity of this uncoupler is only reflected in the fact that the concentration of dinitrophenol needed to inhibit reversal by 50% was about 2000-times as high as the amount of S13 needed.

Whereas the increase of the relative efficiency can be deduced from the increasing slope of the normalised curves in the inset of Fig. 2, the non-normalised curves in Fig. 2 show that the absolute efficiency of the uncoupler remained the same upon inhibition of the secondary pump. At low concentrations of uncoupler the decrease in rate of reversal per mol of uncoupler added was constant. This indicates that the uncoupler has a certain capacity to de-energise the system and that it is not the ratio $\partial(\Delta\tilde{\mu}_{H^+})/\partial$(uncoupler) that is constant, but the ratio ∂(rate of energy transfer)/∂(uncoupler).

To avoid possible direct effects of the anionic uncouplers on ATP synthase and/or redox enzymes, as reported in the case of ATP synthase for DNP [15] and FCCP [16], ionophores were also used as uncouplers. Valinomycin in combination with nigericin transports protons over the membrane in the direction of the gradient. Both antibiotics work carrier-wise. They transport pro-

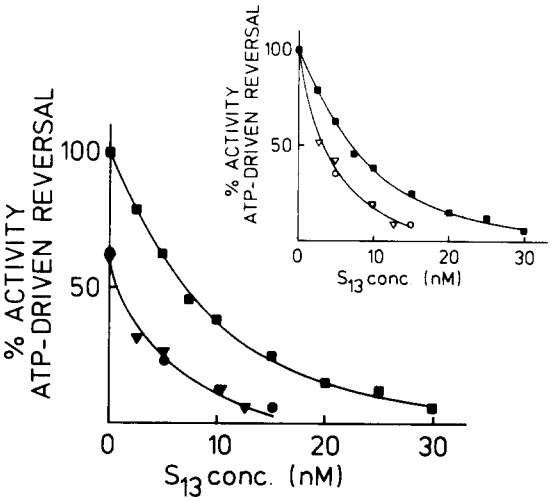

Fig. 1. Titration with S13 of ATP-driven reversal in submitochondrial particles in the presence or absence of rotenone or oligomycin. Particles were preincubated for at least 20 min at a concentration of 16.1 mg/ml with 152 pmol/mg rotenone or 150 pmol/mg oligomycin. Reversal was measured in 2 ml of a medium containing, at pH 7.5 and 30°C, 0.18 M sucrose, 4 mM $MgCl_2$, 10 mM Hepes/KOH, 10 mM succinate, 1.6 mM KCN, 1.0 mM NAD^+, 113 μg particles and the indicated amounts of S13. The reaction was started with 3 mM ATP. 100% activity of reversal was 364 nmol·min^{-1}·mg^{-1} and the inhibition by rotenone and oligomycin was 38 and 39%, respectively. The concentration at which 50% activity was reached was 124 pmol/mg in the control particles and 58 pmol/mg in the rotenone- or oligomycin-inhibited particles. ■———■, control particles; ●———●, particles incubated with rotenone; ▼———▼, particles incubated with oligomycin. In the inset the rotenone- or oligomycin-inhibited activity without added uncoupler was set at 100%. ○———○, normalised activity in the presence of rotenone; ▽———▽, normalised activity in the presence of oligomycin.

tons and/or potassium ions over the membrane one by one [17]. Gramicidin, on the other hand, is a pore-forming ionophore. Two molecules of gramicidin form a channel in the membrane through which protons can diffuse passively over the membrane [18].

Titration of control and rotenone-inhibited particles with valinomycin in the presence of excess nigericin (Fig. 3) also resulted in a higher relative efficiency of uncoupling in partially inhibited particles compared with the control particles.

However, when gramicidin was used to uncouple reversal (Fig. 4) the amount of uncoupler needed was not related to the percentage inhibition of the particles with rotenone. In the inset of Fig. 4 it can be seen that the normalised titration curves of control and of inhibited particles are the same, contrary to the results found with S13, dinitrophenol and valinomycin + nigericin. If determined from the separate titration curves, the titre for 50% uncoupling in the control particles is 85 pmol per mg protein; in the 41% inhibited particles it is 81 pmol per mg, and in the 60% inhibited particles it is 78 pmol per mg particle protein. So, almost no change in titre was obtained upon inhibition with rotenone.

So, in contrast to the experiments of Hitchens and Kell [5] in which they found identical effects of gramicidin and the protonophore SF6847 in chromatophores, our experiments in submitochondrial particles show that the uncoupling mechanism of gramicidin differs from that of the other uncouplers tested. The main difference between gramicidin and the other uncouplers used in these experiments is that gramicidin is a pore-forming uncoupler, whereas the other uncouplers, including valinomycin + nigericin, carry protons over the membrane one by one. To stress this

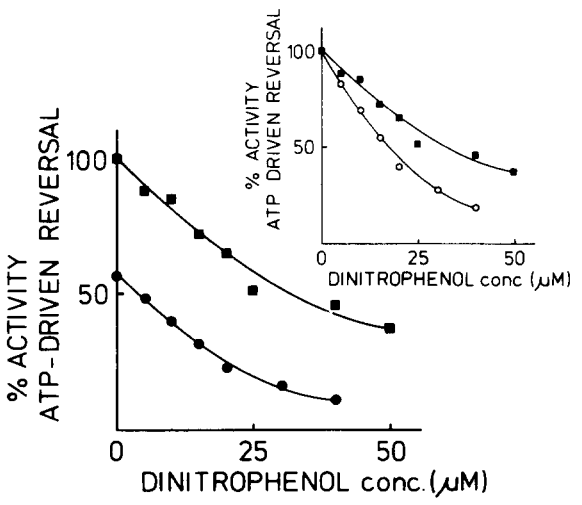

Fig. 2. Titration with 2,4-dinitrophenol of ATP-driven reversal in submitochondrial particles in the presence or absence of rotenone. Particles were preincubated with 66 pmol/mg rotenone and reversal was measured as described in the legend to Fig. 1, with the difference that 282 μg of protein was used in 2 ml of assay volume. 100% activity of reversal was 255 nmol·min^{-1}·mg^{-1}. The concentration at which 50% activity was reached was 32 μM or 255 nmol/mg in the control (■———■) particles and 17 μM or 121 nmol/mg in the particles that were 43% inhibited by rotenone (●———●). In the inset the rotenone-inhibited activity, without added uncoupler, was set at 100%. ○———○, normalised activity of the rotenone-inhibited particles.

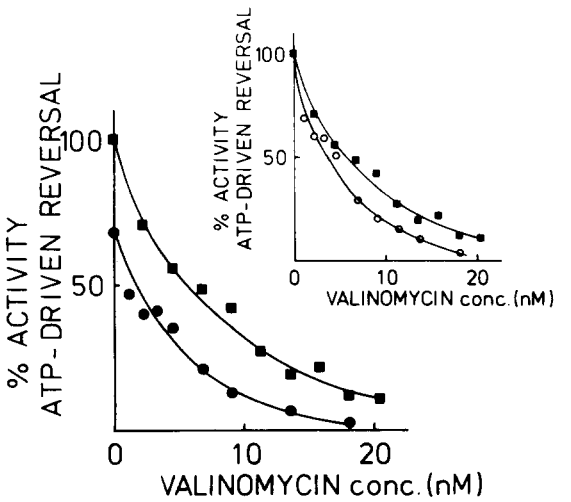

Fig. 3. Titration with valinomycin in the presence of nigericin of ATP-driven reversal in the presence or absence of rotenone. Particles were preincubated with 84 pmol/mg rotenone and reversal was measured as indicated in the legend to Fig. 1, with the difference that 225 μg of protein was incubated with 1.9 nmol/mg nigericin + the indicated concentrations of valinomycin in 2 ml of assay volume. After 10 min the reaction was started by addition of 3 mM ATP. Nigericin without valinomycin did not have any effect on reversal activity. 100% activity of reversal was 161 nmol·min^{-1}·mg^{-1}. The concentration at which 50% activity was reached was 45 pmol/mg in the control particles (■———■) and 30 pmol/mg in the particles that were 32% inhibited by rotenone (●———●). In the inset the rotenone-inhibited activity, without added uncoupler, was set at 100%. ○———○, normalised activity of the rotenone-inhibited particles.

difference we call the latter carrier-type uncouplers.

The data summarised in Table I show clearly that in the case of carrier-type uncouplers the decrease in titre for 50% uncoupling is proportional to the percentage inhibition by rotenone. This shows again that the efficiency of these uncouplers is proportional to the rate of energy transfer, and not, as expected for a delocalised chemiosmotic model, to the $\Delta\tilde{\mu}_{H^+}$ or the turnover of the primary pump. In the experiment with FCCP, the titre in the presence of rotenone was decreased even more than proportional. In the case of the pore-forming uncoupler gramicidin, however, the uncoupling efficiency was independent of the rate of energy transfer, as follows from the unchanged titre for 50% uncoupling upon inhibition with rotenone.

Fig. 5 shows that gramicidin uncoupled reversal more effectively when the ATPase was inhibited by photolabelling with 8-azido-ATP than in the absence of the inhibitor. The inset shows clearly that in this case the uncoupler was more potent in the partially inhibited particles. In the control particles 50% inhibition was reached at 48 pmol gramicidin per mg particle protein, and in the particles that were 37% inhibited by covalently bound 8-nitreno-ATP, the 50% uncoupling titre was 28 pmol per mg. Thus the uncoupling titre was decreased by 42%, which is close to the percentage inhibition of the primary pump. In Fig. 1 it was shown that the 50% uncoupling titre of S13 was also decreased proportionally with the percentage activity of the primary pump. Thus, in this respect, S13 and gramicidin behave similarly.

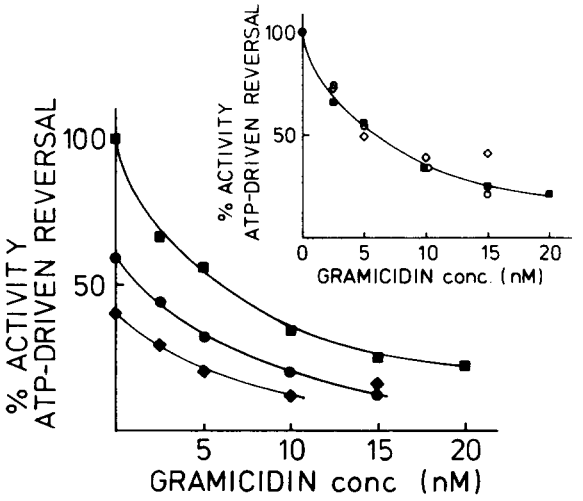

Fig. 4. Titration with gramicidin of ATP-driven reversal in submitochondrial particles in the presence or absence of rotenone. Particles were preincubated with 46 and 89 pmol/mg rotenone and reversal was measured as indicated in the legend to Fig. 1, with the difference that 142 μg of protein was used in 2 ml of assay volume. Before starting the reaction with ATP, the particles were incubated for 5–10 min in the cuvette with the indicated amounts of gramicidin. 100% activity of reversal was 257 nmol·min^{-1}·mg^{-1}. The concentration at which 50% activity was reached was 85 pmol/mg in the control particles (■———■), 81 pmol/mg in the particles that were 41% inhibited by rotenone (●———●) and 78 pmol/mg in the particles that were 60% inhibited by rotenone (◆———◆). In the inset the rotenone-inhibited activities without added uncoupler were set at 100%. ○———○, normalised activity of the 41% inhibited particles ◇———◇, normalised activity of the 60% inhibited particles.

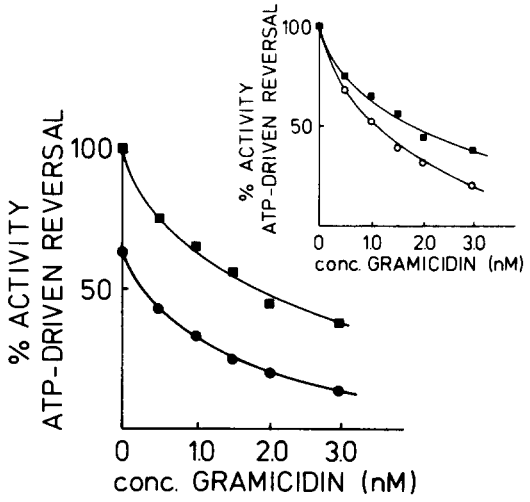

Fig. 5. Titration with gramicidin of ATP-driven reversal in submitochondrial particles that are partially inactivated by covalently bound 8-nitreno-ATP. Particles were incubated on icewater at a concentration of 5.2 mg/ml for 20 min with 0.5 mM 8-azido-ATP and 5 mM EDTA under ultraviolet light (λ_{max} = 360 nm). Afterwards reversal activity was measured as indicated in the legend to Fig. 1, with the difference that 95 μg particles were preincubated in 2 ml assay medium without ATP, but in the presence of the amounts of gramicidin indicated. After 10 min the reaction was started with ATP. 100% activity of reversal was 431 nmol·min^{-1}·mg^{-1}. The concentration of gramicidin at which the activity was 50% inhibited was 48 pmol/mg in the control particles (■———■) and 28 pmol/mg in the particles that were 37% inactivated by 8-nitreno-ATP (●———●). In the inset the rotenone-inhibited activity, without added uncoupler, was set at 100%. ○———○, normalised activity of ●———●.

Uncoupler titrations of stimulation of ATP hydrolysis

As can be seen in Fig. 6A, both gramicidin and S13 stimulated ATP hydrolysis to about the same extent. Stimulation varied somewhat with different batches of particles, but the maximal uncoupled rate was always between 1.6- and 2.7-times the rate without uncoupler. However, the difference between S13 and gramicidin can be seen when we compare Fig. 6A with Fig. 6B. Whereas the concentrations of S13 and gramicidin required to inhibit reversal (Fig. 6B) differed by a factor of about two, about 70-times as much S13 was needed to stimulate ATP hydrolysis as compared with the concentration of gramicidin needed (Fig. 6A).

Fig. 7 shows that the titres for 50% uncoupling of reversal and 50% stimulation of ATP hydrolysis were in the same order of magnitude when gramicidin was used as uncoupler (Fig. 7B), but differed greatly with S13. Stimulation of ATP hydrolysis by S13 was less than 20% at a concentration that caused more than 80% inhibition of reversal (Fig. 7A). In these experiments ATP hydrolysis and reversal were measured simultaneously. During the reversal assay samples were taken in which phosphate production was measured. When hydrolysis and reversal were measured in separate assays, the same difference between gramicidin and S13 was found. For instance, in the experiment of Fig. 6 half-maximal stimulation of ATP hydrolysis by gramicidin was reached at a concentration of 23 pmol per mg particle protein and for inhibition of reversal the 50% titre was 48 pmol per mg protein. For S13 these values are 1.49 nmol per mg for half-maximal stimulation of ATP hydrolysis and 77 pmol per mg for 50% inhibition of reversal.

Another assay to study the possible differences between the uncoupling mechanism of S13 and gramicidin resulted from the following considerations. According to the chemiosmotic theory, ATP hydrolysis is inhibited by the back-pressure of $\Delta\tilde{\mu}_{H^+}$ preventing proton influx. Thus, the rate of hydrolysis is limited by the rate of proton efflux through 'leaks' in submitochondrial particles and is increased by uncouplers which release the back-pressure. From this it follows that, if the leak remains constant, the turnover rate per active ATPase molecule will increase upon inhibition with an ATP synthase inhibitor, and since the maximal hydrolysis capacity of each uninhibited ATP synthase molecule does not change, the stimulation factor by addition of uncoupler would be expected to decrease. Again the effects of S13

TABLE I

SUMMARY OF UNCOUPLER-INHIBITOR TITRATION EXPERIMENTS

Reverse electron transfer was titrated with uncouplers in the presence or absence of rotenone. Experiments were performed as described in Methods and Materials and in the legends to Figs. 1–4. Unc_{50} is the amount of uncoupler per mg protein that causes 50% inhibition of reversal activity in control particles. Unc_{50}(rot) is the Unc_{50} in rotenone inhibited particles. In the experiment of the second mentioning of S13 piericidin was used to inhibit the NADH:Q oxidoreductase. The uncoupler valinomycin was in the presence of 0.3 μM nigericin.

Uncoupler (nmol/min per mg)	Unc_{50} (pmol/mg)	Unc_{50}(rot) (pmol/mg)	Reversal activity in rotenone inhibited particles (% of the activity of control particles)	$\frac{Unc_{50}(rot)}{Unc_{50}}$
S13	124	58	62	0.47
S13	47	24	46	0.51
FCCP	400	133	67	0.33
SF6847	120	78	75	0.65
Dinitrophenol	$2.25 \cdot 10^5$	$1.21 \cdot 10^5$	57	0.54
Valinomycin	45	30	68	0.67
Gramicidin	85	81	59	0.95
		78	40	0.92
Gramicidin	42	41	56	0.98

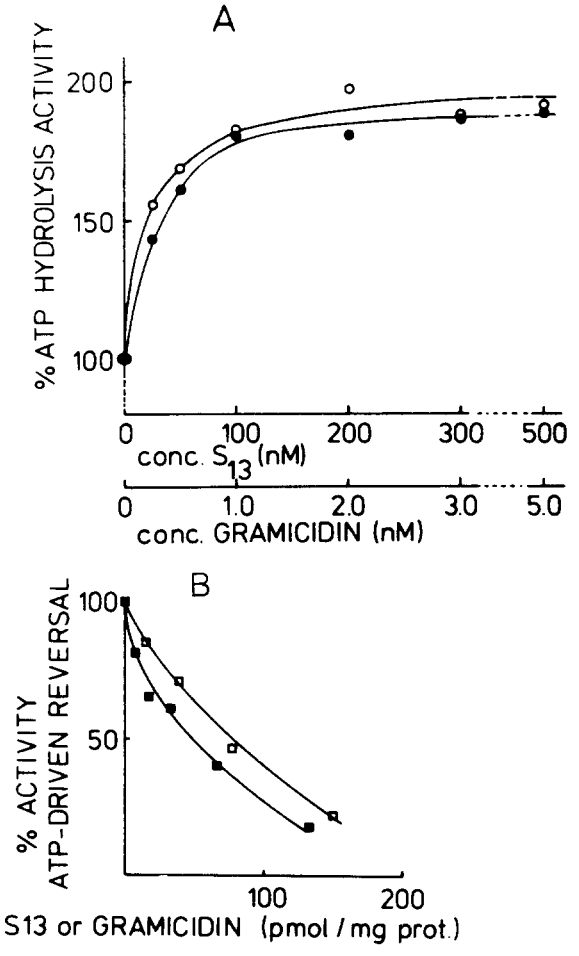

Fig. 6. Stimulation of ATP hydrolysis and inhibition of reversal by gramicidin and S13. Hydrolysis and reversal measured in separate assays. (A) ATP hydrolysis was measured as described previously [10] in 2 ml of a medium containing, at 30°C and pH 7.5, 83 mm sucrose, 6 mM $MgCl_2$, 33 mM Tris-HCl buffer, 5 mM ATP, 10 mM $NaHCO_3$, 0.5 mM phospho*enol*pyruvate, 0.25 mM NADH, 4 units of pyruvate kinase, 3 units of lactate dehydrogenase, 24 μg particles and S13 (●———●) or gramicidin (○———○) at the concentrations indicated. 100% activity of ATP hydrolysis was 3.24 μmol·min^{-1}·mg^{-1} in the S13 experiment and 3.58 μmol·min^{-1}·mg^{-1} in the gramicidin experiment. The values at which 50% stimulation was reached are 1.49 nmol S13 per mg and 23 pmol gramicidin per mg particle protein. (B) ATP-driven reversal was measured as described in the legend to Fig. 1 in 2 ml medium with 65 μg particles and S13 (■———■) at the concentrations indicated, or 150 μg particles and gramicidin (□———□) at the concentrations indicated. 100% activity of reversal was 336 nmol·min^{-1}·mg^{-1} in the S13 experiment and 300 nmol·min^{-1}·mg^{-1} in the experiment with gramicidin. The values at which 50% inhibition was reached were 77 pmol S13 per mg and 48 pmol gramicidin per mg particle protein.

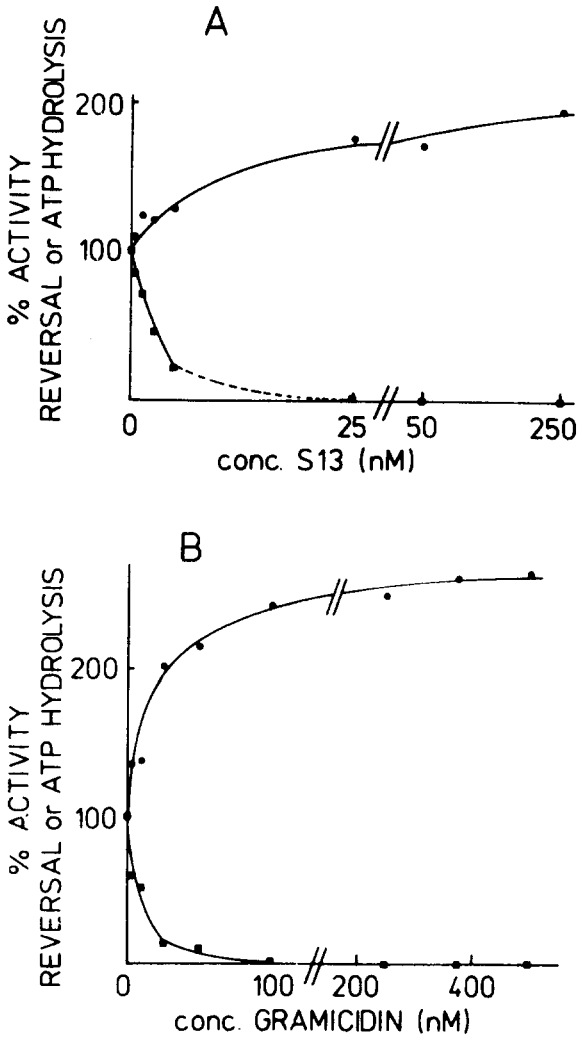

Fig. 7. Inhibition of ATP-driven reversal and stimulation of ATP hydrolysis by S13 and gramicidin. Reversal (■———■) and ATP hydrolysis (●———●) measured simultaneously in one assay. Reversal was measured as described in the legend to Fig. 1 in 4 ml medium with 130 μg particles and S13 at the concentrations indicated (Fig. 7A) or 240 μg particles and gramicidin at the concentrations indicated (Fig. 7B). In order to measure ATP hydrolysis during reversal, samples were taken and quenched in trichloroacetic acid. Afterwards the phosphate concentration in the sample was determined as described previously [10]. 100% activity of reversal was 336 nmol·min^{-1}·mg^{-1} in Fig. 7A and 497 nmol·min^{-1} in Fig. 7B. For ATP hydrolysis the values are 1.44 μmol·min^{-1}·mg^{-1} in Fig. 7A and 3.84 μmol·min^{-1}·mg^{-1} in Fig. 7B.

and gramicidin were compared.

Fig. 8 shows that the stimulation factor by either S13 or gramicidin was unchanged upon

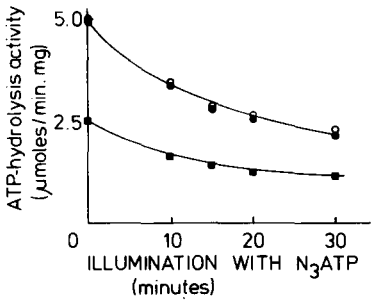

Fig. 8. Maximal stimulation of ATP hydrolysis by S13 and gramicidin after partial inactivation by 8-azido-ATP. Submitochondrial particles were incubated on icewater at a protein concentration of 9.5 mg/ml with 0.5 mM 8-azido-ATP and 5 mM EDTA for 0–30 min under ultraviolet light ($\lambda_{max} = 360$ nm). Samples were removed at intervals and stored in the dark on ice. Afterwards ATP hydrolysis was measured as indicated in the legend to Fig. 6, with 95 μg particles in 2 ml assay volume. Hydrolysis with and without uncoupler was measured in separate assays. No uncoupler added: ■———■; 0.5 μM S13 added: ○———○; 0.25 μM gramicidin added: ●———●.

inhibition of the primary pump by covalently bound 8-nitreno-ATP up to 54%. The same result was obtained earlier by Baum et al., using oligomycin to inhibit the ATPase [19]. These data can only be explained within the chemiosmotic theory if we assume that the leak through the membrane is of minor importance compared with that through the ATP synthase molecules. In that case the assumption that the leak does not change upon inhibition of the ATP synthase is no longer valid, since the ATPase itself causes the leak. However, respiration in the absence of ADP was not reduced by oligomycin in these particles (unpublished results). This suggests that back leakage of protons occurs mainly via the membrane.

Discussion

Uncoupler titrations are useful in the study of the mechanism of coupling between energy-producing and energy-requiring processes in biological energy-transducing membranes, as was pointed out already by Hitchens and Kell [5], since they make it possible to draw conclusions about the role of $\Delta\tilde{\mu}_{H^+}$ without actually measuring this parameter. One may argue that direct measurements of $\Delta\tilde{\mu}_{H^+}$ are preferable, but considering the continuing discussions about the validity of these measurements [20,21] experiments that can avoid these measurements are to be preferred.

The rationale behind the uncoupler-inhibitor titrations has been discussed heavily by several authors [22–25]. The discussion was mainly about the thesis, first formulated in Ref. 5, that in a chemiosmotic model, upon inhibition of the secondary pump, a decrease of the amount of uncoupler needed to inhibit the energy-transducing reaction cannot be expected. There are two main points of criticism.

The first point is the possible occurrence of direct kinetic regulation of the pumps by $\Delta\tilde{\mu}_{H^+}$ [23]. In chloroplasts this is well documented [23], but a comparable type of regulation in mitochondria is not established. In chloroplasts the dependence of the phosphorylation rate on $\Delta\tilde{\mu}_{H^+}$ is different, depending on the activation state of the ATPase (e.g., Fig. 4 in Ref. 26). In mitochondria no such differences are reported, as far as we know.

In the second place, as argued by Pietrobon and Caplan [25], the increase of $\Delta\tilde{\mu}_{H^+}$ upon inhibition of the secondary pump, although small, causes a decrease in the uncoupler titre. They showed this by simulating uncoupler-inhibitor titrations on the basis of proportional force-flow relationships in a chemiosmotic model. In the stimulations it is assumed that the only effect of uncouplers is to make the membrane more permeable to protons (for reviews about the mechanism of uncoupling, see Refs. 27– 29). However, when we apply this simulation method to our system, and use as parameters data from our own experiments, the decrease in titre upon inhibition of the secondary pump is just 1–2%. From this we conclude that the gramicidin results (Fig. 4) fit with a chemiosmotic scheme. The small increase in $\Delta\tilde{\mu}_{H^+}$ found in our simulations cannot account for the large decreases in uncoupling-titre found with the carrier-type uncouplers. However, there is one possible explanation we cannot exclude: the large decrease in uncoupling titre in the presence of rotenone may be compatible with a chemiosmotic scheme in which the force-flow relationships are strongly non-linear, as suggested in Refs. 23 and 25. This is not easy to simulate, so for the

moment we stay with the proportional force-flow simulations.

Using this method, with experimental data as parameters, we also simulated the different effects of primary-pump inhibitors on the uncoupling efficiency. Whereas nearly no effect of an inhibitor of the secondary pump was found, a primary pump inhibitor, however, decreased the uncoupling titre in the simulations considerably. Just as was found experimentally [10,25] there was in the simulation only a slight decrease in $\Delta\tilde{\mu}_{H^+}$ upon inhibition of the primary pump. The decrease in titre as calculated was more than 50% when the inhibition of the pump was set at 50%. With gramicidin we found, in fact, such a different effect of primary and secondary pump inhibitors (Figs. 4 and 5). With the carrier-type uncoupler S13, however, rotenone and oligomycin caused the same decrease in uncoupling titre (Fig. 1). The results with the primary pump inhibitors are in conformity with observations of Margolis et al. [14], who found an almost exact stoichiometry of FCCP with the number of active coupling sites upon inhibition of the primary pump of oxidative phosphorylation with rotenone, malonate or cyanide.

So according to the considerations given in the previous paragraphs the pore-former gramicidin behaves as a chemiosmotic uncoupler, whereas the carrier-type uncouplers deviate. Possibly these uncouplers interact with some form of direct energy transfer. Recently some experiments were published that support this point of view: in reconstituted vesicles containing bacteriorhodopsin and (yeast) ATP synthase, a system in which direct energy transfer is considered very unlikely, uncoupler titrations of light-driven ATP synthesis in control vesicles and vesicles partially inactivated by covalently bound 8-nitreno-ATP showed neither with gramicidin nor with FCCP an increase in uncoupling efficiency [30]. Yet in these vesicles both types of uncoupler do show an increase in efficiency upon decreasing the turnover rate of the primary pump, bacteriorhodopsin, by lowering the light intensity.

The apparently non-chemiosmotic results with the carrier-type uncouplers in the titrations in the presence of rotenone are not due to experimental problems, as was suggested by Cotton and Jackson [6] with respect to this kind of experiment in chromatophores. Reversal rates were linear, after a small lag phase, and for each point a separate assay was performed. In addition, with a different inhibitor of the NADH:Q oxidoreductase, piericidin, titrations with S13 and FCCP gave comparable results (not shown). Except for gramicidin, the results of our uncoupler titrations of reversal in the presence of rotenone largely agree with the data presented by Hitchens and Kell on the effect of uncouplers on oxidative phosphorylation in chromatophores in the presence of ATP synthase inhibitors. In rotenone-inhibited submitochondrial particles the efficiency of gramicidin, however, to uncouple reversal is unchanged, whereas in chromatophores gramicidin, just like the other uncouplers tested, also uncouples with a proportionally higher efficiency in partially inhibited chromatophores [5]. A possible explanation for this difference is that gramicidin inhibits phosphorylation in chromatophores by a mechanism that differs from that in submitochondrial particles. Saphon et al. [32] found that gramicidin inhibits oxidative phosphorylation in chromatophores, but, in contrast to the effects of other uncouplers, it did not stimulate the decay of the 515nm shift. They suggest that, due to the small diameter of the chromatophores (less than 60 nm), and the resulting curvature of the membrane, gramicidin dimers cannot be formed, and that gramidicin in its monomeric form possibly acts as carrier-type ionophore.

Trying to fit our gramicidin results with a fully localised coupling model along the lines of argumentation of Hitchens and Kell [5], we have to assume that due to the high rate of proton conductance of the gramicidin channel ($3 \cdot 10^8$ protons per s per channel [33]), the uncoupling process itself is less rate-limiting than the movement of the uncoupler between the coupling units. In that case the involvement of localised coupling units would remain undetected. However, this possibility is ruled out by the data in Fig. 5, which show that in the case of inhibition of the primary pump gramicidin uncouples with an efficiency that is increased proportionally with the inhibition of the ATPase. In a purely localised model this is not expected because it would make no difference whether the primary pump is inhibited or the

secondary one, because in both cases the coupling unit is fully inactivated and does not produce any usable membrane energisation [5]. So, inhibition of the primary or the secondary pump would have an identical effect upon uncoupling efficiency.

A good explanation of Figs. 4 and 5 can only be given if we propose different mechanisms of uncoupling for two types of uncouplers: carrier-type uncouplers and pore-forming uncouplers. The results with the pore-forming uncoupler gramicidin are in conformity with a chemiosmotic mechanism, so we propose that gramicidin indeed facilitates bulk-to-bulk proton leak over the membrane. The carrier-type uncouplers, however, act according to a different mechanism. The proportionality of the uncoupling titre with the degree of inhibition of the secondary pump suggests that these uncouplers interfere with a direct interaction between active primary and active secondary pumps, and that the uncoupling process itself and not the movement of the uncoupler from one site to another, is the rate-limiting step. So the concentration of uncoupler needed is only related to the concentration of colliding complexes that effectively transduce energy. Hitchens and Kell needed this same assumption to explain their results in a purely localised model.

If explained this way, our results fit nicely with the mechanism for energy transduction that we recently developed, the so-called collision hypothesis, which is described in detail in a separate paper [8]. Basically the hypothesis proposes that in oxidative phosphorylation energy is transduced as the result of collisions between an energised redox enzyme molecule and an ATP synthase molecule. Just as in the chemiosmotic hypothesis, redox enzymes and ATP synthase are considered to be proton pumps that can deliver protons to the bulk phases. The main route of energy transfer, however, is the transfer of the energy via a protonation–deprotonation reaction (or some form of conformational energy) from the energised primary pump to the non-energised secondary pump. The collision hypothesis can be considered as a molecular description of a parallel coupling hypothesis as formulated by Rottenberg [34]. The relatively slow rate of equilibration, under steady state conditions, between the protons on the enzyme and the bulk protons explains the finding that the factor by which the ATP hydrolysis is stimulated by gramicidin or S13 is not dependent on the concentration of active ATPases (Fig. 8).

In terms of the collision hypothesis the mechanism of reverse electron transfer can be written as follows:

$$E_{ATP} + ATP \rightleftharpoons E_{ATP} \sim h^+ + ADP + P_i \quad (1)$$

$$E_{ATP} \sim h^+ \rightleftharpoons E_{ATP} + \Delta\tilde{\mu}_{H^+} \quad (2)$$

$$E_{ATP} \sim h^+ + E_R \rightleftharpoons E_{ATP} \sim H^+ \sim E_R \rightleftharpoons E_R \sim h^+ + E_{ATP} \quad (3)$$

$$E_R \sim h^+ \rightleftharpoons E_R + \Delta\tilde{\mu}_{H^+} \quad (4)$$

$$E_R \sim h^+ + NAD^+ \rightleftharpoons E_R + NADH \quad (5)$$

E_{ATP} stands for the ATPase, E_R is a redox enzyme, in this case the NADH dehydrogenase, kept reduced by succinate. $\sim h^+$ stands for energisation that can result in a pumped proton, but that is not a priori accessible to a protonophore. $\sim H^+ \sim$ stands for proton transfer during the collision of the primary and secondary pump.

The different behaviour of carrier-type uncouplers and gramicidin is clearly pointed to by the relative concentrations required to stimulate the ATPase and to inhibit reverse electron flow. With gramicidin, lowering of the bulk $\Delta\tilde{\mu}_{H^+}$ is paralleled by a similar degree of stimulation of ATP hydrolysis and of inhibition of reversal (Fig. 7B). In contrast, the concentration at which ATP hydrolysis is maximally stimulated by S13 is much higher than the concentration required for inhibition of reversal (Fig. 7A). This strongly suggests that stimulation of ATP hydrolysis is brought about by a different mechanism from inhibition of reversal. Specifically, we suggest that interference with the direct interaction between NADH dehydrogenase and ATPase occurs at concentrations of S13 that have little effect on the rate of the transfer of protons over the membrane.

This is schematically depicted in Fig. 9. Our proposal differs from the models of Kell [5,35] and Westerhoff et al. [3] in that the direct interaction is not permanently localised. The transduction of energy is localised, at a certain moment at a certain place in the membrane, but the separate energised and non-energised complexes diffuse randomly in the membrane. The interference of carrier-type uncouplers with the direct interaction

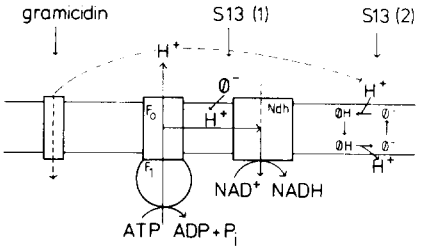

Fig. 9. A schematical depiction of the collision hypothesis, in case of reverse electron transfer. Proton, or energy, transfer in the membrane takes place when an ATP synthase molecule collides with a molecule of NADH:Q oxidoreductase. When no effective collisions are possible the ATPase delivers its protons to the bulk phase. Ndh, NAD:Q oxidoreductase; ØH, a carrier-type uncoupler.

between two colliding complexes is indicated by S13(1) in the figure. S13(2) visualizes the protonophoric mechanism of carrier-type uncouplers. The pore-forming uncoupler gramicidin is also indicated in the scheme. When direct energy transfer is fully uncoupled by S13, ATP hydrolysis is not yet maximally stimulated (Fig. 7A). Much more uncoupler is needed to increase the proton permeability of the membrane sufficiently to reach the maximal ATP hydrolysis rate. Such a difference in optimal efficiencies for the uncoupler in these two reactions is not surprising, since the lipophilic uncoupler will reside mainly in the membrane and will not be available to react with protons in the bulk phase. Gramicidin, on the other hand, is not able to react with protons in the membrane and can only function as a pore for protons from one bulk phase to another. Thus, uncoupling by gramicidin occurs via dissipation of $\Delta\tilde{\mu}_{H^+}$, and, according to the equations above, when $\Delta\tilde{\mu}_{H^+}$ is lowered by gramicidin, the rate of delivery of the protons to the bulk will increase, thereby decreasing the rate of direct energy transfer.

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