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Uncoupling of Oxidative Phosphorylation. 1. Protonophoric Effects Account Only Partially for Uncoupling†

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ABSTRACT: The mechanism of uncoupling of oxidative phosphorylation by carbonyl cyanide *p*-trifluoromethoxy)phenylhydrazone (FCCP), a typical weak acid protonophore, oleic acid, a fatty acid, and chloroform, a general anesthetic, has been investigated by measuring in mitochondria their effect on (i) the transmembrane proton electrochemical potential gradient ($\Delta\tilde{\mu}_H$) and the rates of electron transfer and adenosine 5′-triphosphate (ATP) hydrolysis in static head, (ii) $\Delta\tilde{\mu}_H$ and the rates of electron transfer and ATP synthesis in state 3, and (iii) the membrane proton conductance. Both FCCP and oleic acid increase the membrane proton conductance, and accordingly, they cause a depression of $\Delta\tilde{\mu}_H$ [generated by either the redox proton pumps or the adenosinetriphosphatase (ATPase) proton pumps]. Although their effects on ATP synthesis/hydrolysis, respiration, and $\Delta\tilde{\mu}_H$ are qualitatively consistent with a pure protonophoric uncoupling mechanism and an additional inhibitory action of oleic acid on both the ATPases and the electron-transfer enzymes, a quantitative comparison between the dissipative proton influx and the rate of either electron transfer or ATP hydrolysis (multiplied by either the H^+/e^- or the H^+/ATP stoichiometry, respectively) at the same $\Delta\tilde{\mu}_H$ shows that the increase in membrane conductance induced by FCCP and oleic acid accounts for the stimulation of the rate of ATP hydrolysis but not for that of the rate of electron transfer. Chloroform (at concentrations that fully inhibit ATP synthesis) only very slightly increases the proton conductance of the mitochondrial membrane and causes only a little depression of $\Delta\tilde{\mu}_H$. The negligible increase in the dissipative proton influx in the presence of chloroform does not account for the stimulation either of the rate of electron transfer or of ATP hydrolysis. The classical "chemiosmotic" explanation of the uncoupling of oxidative phosphorylation does not apply to the uncoupling action of chloroform.

The mechanism by which a number of substances uncouple oxidative phosphorylation, i.e., inhibit ATP[1] synthesis and stimulate resting respiration, has been the subject of intense research. In fact, any hypothesis on the mechanism of free energy coupling (between electron transfer and ATP synthesis) has to provide an explanation for the uncoupling consistent with the known properties of these substances. Thus, the observations that typical lipophilic weak acid uncouplers, such as 2,4-dinitrophenol (DNP) and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), increase the proton conductance of the inner mitochondrial membrane (Mitchell & Moyle, 1967) and of black lipid membranes (Hopfer et al., 1968) have been considered strong evidence in favor of the chemiosmotic hypothesis as formulated by Mitchell (1966).

Much work has been devoted to establishing if the protonophoric action could quantitatively account for the uncoupling of oxidative phosphorylation [for reviews see Hanstein (1976) and McLaughlin and Dilger (1980)]. From these studies it can be generally concluded that certainly there exists a correlation between protonophoric and uncoupling action. However, no clear-cut evidence that the uncoupling is exclusively and quantitatively due to the increase in membrane conductance has ever been reported. In fact, the quantitative comparison between the rate of passive proton influx in the presence of FCCP and the rate of electron transfer multiplied by the H^+/e^- stoichiometry made originally by Mitchell and Moyle (1967) was based on estimated values of $\Delta\tilde{\mu}_H$ and on measured values of H^+/e^- stoichiometries that were much too high for the former and too low for the latter with respect to the actual measurements (Wikstrom & Krab, 1980; Azzone et al., 1984). Instead, preliminary reports from our laboratory

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[1] Abbreviations: J_o^{sh}, rate of respiration in static head; J_o^{st3}, rate of respiration in state 3; J_o^{max}, maximal rate of respiration; J_e, rate of electron transfer; J_p, rate of ATP synthesis; J_{ATP}, rate of ATP hydrolysis; J_K^{eff}, rate of K^+ efflux; J_H^l, proton flux through leaks; $\Delta\psi$, transmembrane electrical potential gradient; ΔpH, transmembrane pH gradient; $\Delta\tilde{\mu}_H$, transmembrane proton electrochemical potential gradient (in absolute value); L_H^l, membrane proton-leak conductance; n_e, H^+/e^- stoichiometry; n_p, H^+/ATP stoichiometry; f_e, fraction of active redox pumps; f_p, fraction of active ATPase pumps; P_i, inorganic phosphate; DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium ion; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase; ADP, adenosine 5′-diphosphate.

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indicate that in mitochondria in the presence of a certain concentration of protonophore the rate of electron transfer multiplied by the H^+/e^- stoichiometry is higher than the dissipative proton influx at the same $\Delta\tilde{\mu}_H$ (Zoratti et al., 1984a). Such a result suggests that in addition to the protonophoric action at the level of the membrane the lipophilic weak acids may exert also a more specific effect at the level of the proton pumps. It has been proposed that this additional specific effect results in intrinsic uncoupling (slip) of the redox proton pumps whereby electrons can be transferred without proton pumping (Pietrobon et al., 1981; Walz, 1983). A similar type of mechanism has been invoked to explain the differential inhibition of electron transfer and proton pumping by fluorescamine and its derivatives (Ramirez et al., 1980; Tu et al., 1981).

Also, the results of the so-called uncoupler–inhibitor titrations (Hitchens & Kell, 1983; Herweijer et al., 1986), where the effectiveness of a given concentration of a lipophilic weak acid uncoupler or valinomycin in inhibiting the ATP synthesis appears to be proportional to the fraction of inhibited ATPases, point to a mechanism of uncoupling different from that proposed originally by Mitchell (Mitchell & Moyle, 1967). The uncoupler–inhibitor titration results have been considered evidence in favor of a local protonic mechanism of energy coupling and have been explained by postulating an interaction of the uncoupling agents with the localized coupling units (Hitchens & Kell, 1983; Westerhoff et al., 1984; Herweijer et al., 1986).

The existence of specific interactions of the typical protonophores with some proteins of the inner mitochondrial membrane (and also of bacterial membranes) has been amply documented (Hanstein & Hatefi, 1974; Hanstein, 1976; Katre & Wilson, 1978; 1980). That the specific binding of protonophores to the energy-transducing enzymes is not an epiphenomenon unrelated to their mechanism of action as uncouplers is strongly supported by the existence of uncoupler-resistant bacteria (Decker & Lang, 1977; Guffanti et al., 1981; Ito & Ohnishi, 1981) and mitochondria (Freeman et al., 1983).

Fatty acids and general anesthetics are well-known uncouplers of oxidative phosphorylation. However, on the basis of their structure and chemical properties they are not expected to act as protonophores with a mechanism similar to that established for the lipophilic weak acids (Benz & McLaughlin, 1983). Recently Rottenberg has addressed the problem of the mechanism of uncoupling of these substances by characterizing their effect on ATP synthesis, succinate oxidation, ATPase activity, and $\Delta\tilde{\mu}_H$ of rat liver mitochondria (Rottenberg, 1983; Rottenberg & Hashimoto, 1986). Rottenberg's results indicate that the collapse of $\Delta\tilde{\mu}_H$ is not the cause of uncoupling by these agents.

In this paper we further investigate the mechanism of uncoupling of a typical weak acid protonophore (FCCP), a fatty acid (oleic acid), and a general anesthetic (chloroform) by measuring their effect on the inner mitochondrial membrane conductance, in addition to their effects on the rates of electron transfer and ATP synthesis and on $\Delta\tilde{\mu}_H$. We find that both FCCP and oleic acid increase the membrane conductance, while chloroform has only a negligible effect on it. However, the comparison between the rate of electron transfer and the dissipative ionic current at the same $\Delta\tilde{\mu}_H$ in the presence of uncoupler shows that even for FCCP and oleic acid the increase in membrane conductance does not quantitatively account for the stimulation of the electron transfer. The comparison between the rate of ATP hydrolysis and the dissipative ionic current at the same $\Delta\tilde{\mu}_H$ shows that the increase in membrane conductance in the presence of FCCP and oleic acid accounts for most of the stimulation of the ATP hydrolysis, while the very small increase in membrane conductance in the presence of chloroform does not.

Materials and Methods

Materials. Rat liver mitochondria were prepared according to standard procedures (Massari et al., 1972), and all experiments were performed within 4 h of preparation. The mitochondrial protein was assayed with the biuret method using serum albumin as a standard. The composition of the reaction medium is given in the legends of the figures. All reagents were of maximal purity commercial grade. Enzymes, nucleotides, inhibitors, and valinomycin were obtained from Sigma and labeled probes from Amersham. The uncoupler FCCP was supplied by Dr. G. Heitler of Du Pont, chloroform by Riedel De Haen (99%), DCCD by EGA CHEMIE, and oleic acid by Kock & Lights.

Determination of Proton Fluxes. The passive proton flow through the mitochondrial inner membrane was determined essentially as described by Zoratti et al. (1986). The method consists of measuring the initial rate of potassium efflux, J_K^{eff}, upon addition of valinomycin to antimycin-inhibited mitochondria. The measurement relies on the principle that the rate of K^+ efflux down the K^+ electrochemical gradient must, for electroneutrality reasons, be equal to the sum of the rates of anion efflux and of influx of cations other than K^+. If, as under the experimental conditions used, essentially the only permeable ion besides K^+ is H^+ (OH^-), J_K^{eff} is equal to the rate of passive influx of protons, J_H^l. J_K^{eff} is higher than J_H^l if other ions move.

As discussed in Zoratti et al., (1986) the determination of J_H^l on the basis of J_K^{eff} eliminates artifactual underestimations of the proton fluxes while providing possible overestimation of the passive H^+ permeability. This procedure is therefore convenient in an investigation where the results may be misinterpreted by an underestimation but not by an overestimation of the rate of passive proton fluxes in the presence of uncoupling agents.

For the measurement of J_K^{eff}, mitochondria were suspended in 5 mL of buffered medium in a thermostated vessel, open to air. The suspension bathed a Schott K^+ electrode (response time 1 s) and a glass combination electrode (Beckman) serving as reference. The electrodes were connected to a Radiometer 26 pH meter, and the output was fed into a Perkin-Elmer Model R100 A chart recorder. The mitochondria were allowed to reach the stationary state (with succinate or ATP), and after 1 min of incubation with the uncoupling agent, antimycin (0.05 μg/mg of protein) and valinomycin (0.15 μg/mg of protein) were added to block the redox pumps and create a K^+ diffusion potential. The amount of valinomycin was selected so as to render the rate of K^+ diffusion nonlimiting with respect to that of the other ion species and thus to let the K^+ diffusion potential approach as close as possible the Nernst potential. Higher concentrations of valinomycin were not used because of the uncoupling effect of this ionophore at higher concentrations (Azzone et al., 1984). For each set of determinations, suitable calibrations with KCl standard solution were carried out for the calculations of the rates of K^+ efflux.

Determination of the Rates of Respiration, ATP Hydrolysis, and ATP Synthesis. The respiratory rates, J_o, were estimated from the rates of oxygen consumption, whose concentration in the medium was measured polarographically with a Clark electrode (Yellow Spring) equipped with a Teflon membrane in a closed thermostated and stirred vessel. The zero oxygen point was determined with an excess of dithionite.

Determination of medium oxygen content and calibration of the electrode response were carried out by allowing submitochondrial particles to oxidize known amounts of NADH. The operative conditions and times of incubations with the reagents are described in the legend of Figure 1.

The rate of ATP hydrolysis, J_{ATP}, was measured spectrophotometrically on an AMINCO DW 2a dual-wavelength spectrophotometer equipped with magnetic stirring and thermostatic control, following continuously the decrease in absorbance at 340 minus 374 nm due to NADH oxidation in the presence of excess phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. The rates of ATP hydrolysis were corrected for the contribution of extramitochondrial ATPases, which was determined in control samples in the presence of oligomycin (1 μg/mg of protein) and atractyloside (200 μM). The operative conditions and times of incubation with the reagents are described in Figure 2.

To determine the rate of ATP synthesis, J_p, three 1-mL samples were withdrawn from the suspension within 1 min after addition of ADP and were quenched in $HClO_4$ (5 M). After centrifugation of the denatured protein and neutralization of an aliquot of the supernatant with triethanolamine/KOH, the ATP content of the samples was determined by standard enzimatic methods, i.e., following fluorometrically with an Eppendorf spectrofluorometer the reduction of NADP in the presence of hexokinase, glucose, and glucose-6-phosphate dehydrogenase. The rate of ATP synthesis was calculated from the linear regression analysis of the ATP concentration vs time plot.

Determination of $\Delta\tilde{\mu}_H$. The transmembrane electrical potential, $\Delta\psi$, was evaluated from the distribution of the lipophilic ion triphenylmethylphosphonium ($TPMP^+$) [cf. Azzone et al. (1984)]. The concentration of TPMP in the incubation medium was followed continuously by using a TPMP-sensitive membrane electrode (response time about 10 s) as described in Zoratti and Petronilli (1985). The initial concentration of TPMP in the medium was 5 μM. The concentration of TPMP in the mitochondrial matrix was calculated from the amount of probe taken up by the mitochondria and the matrix volume, which was measured as described by Zoratti et al. (1984a,b). The matrix volume was 1 μL/mg of protein under the prevailing experimental conditions and did not change during TPMP uptake (Zoratti et al., 1984b). In the calculation of the membrane potential, no correction was used either for the TPMP binding or for the activity coefficient of TPMP in the matrix.

The $\Delta\psi$ values measured with the TPMP electrode were checked under steady-state conditions with the measurements of the distribution of the labeled [^{14}C]$TPMP^+$. In these assays [^{3}H]glycerol (approximately 0.5 μCi/mL) and 5 mM glycerol were included in the medium in order to determine the total pellet volume. Mitochondria (1 mg/mL) were incubated in 2-mL liquid-scintillation vials and centrifuged directly in an adapted SE-12 rotor in a Sorvall RC-5B centrifuge at 15 000 rpm for 6 min. After the supernatant was removed, the pellet was dissolved in 1% deoxycholate, and scintillation fluid was added to the vials. The amounts of protein in the pellet and in the supernatant were determined for each experiment in parallel samples, and the calculation was based on the pellet protein. The same procedure was used to assay ΔpH with the weak acid [^{14}C]DMO and the matrix volume with ^{45}Ca (Zoratti et al., 1984b). In the latter case, the incubation medium was supplemented with 2 mM EGTA–EDTA, and 1 nmol/mg Ruthenium Red was also added to inhibit the Ca^{2+} uniport. ^{45}Ca was added immediately before centrifugation.

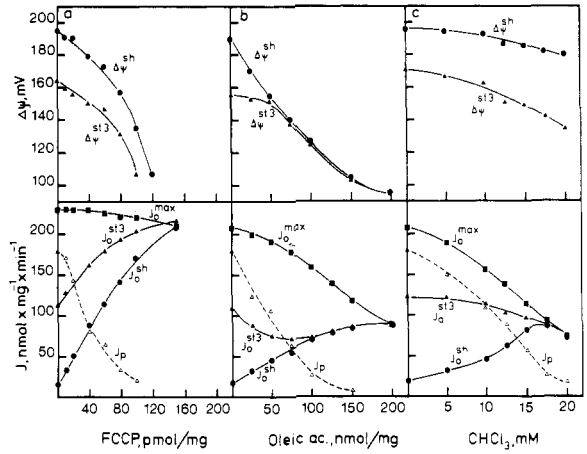

FIGURE 1: Lower panels: Mitochondrial rate of respiration in static head, J_o^{sh} (●), and in state 3, J_o^{st3} (▲), and maximal rate of respiration, J_o^{max} (■), and rate of ATP synthesis, J_p (△), as a function of the concentrations of FCCP (a), oleic acid (b), and chloroform (c). Upper panels: Difference of electrical potential across the inner mitochondrial membrane in static head, $\Delta\psi^{sh}$ (●), and in state 3, $\Delta\psi^{st3}$ (▲), as a function of the same uncoupling agent concentrations. Medium composition: 0.2 M sucrose, 30 mM Tris/Mops, 5 mM P_i/Tris, 1 mM EDTA, 5 μM rotenone; pH 7.4, T = 25 °C. After 3 min of incubation of rat liver mitochondria (1 mg of protein/mL), succinate (10 mM) was added, followed after 2 min by the addition of the uncoupling agent. After 2 min of incubation, either ADP (1 mM) or excess FCCP (0.2 μM) was added and either J_o in state 3, $\Delta\psi$ in state 3, and J_p or J_o^{max} were measured.

RESULTS

The continuous lines in the lower panels in Figure 1 show the effect of three different uncouplers (a typical protonophore, FCCP; a fatty acid, oleic acid; and an anesthetic, chloroform) on the mitochondrial rate of respiration in state 4 (static head) and in state 3 (stationary state of phosphorylation) and on the uncoupled (maximal) rate of respiration in the presence of excess FCCP. The dashed lines show the effect of the three uncouplers on the rate of ATP synthesis. All three agents decrease the rate of ATP synthesis and increase the rate of respiration in state 4, thus justifying their classification as uncouplers of oxidative phosphorylation. As shown by the inhibition of the uncoupled rate of respiration, chloroform and oleic acid act also as inhibitors of the redox enzymes. In static head the uncoupling effect leading to stimulation of the electron transfer prevails over the inhibitory effect (at least in a certain range of oleic acid and chloroform concentrations), while in state 3 the two effects appear to balance each other.

The inhibition of the uncoupled rate of respiration by chloroform and oleic acid shown in Figure 1 is at variance from the results of analogous experiments performed by Rottenberg (1983) [see also Rottenberg and Hashimoto (1986)] where no inhibition of the uncoupled rate was observed. In the experiment with chloroform we have used a procedure different from that used by Rottenberg (1983) since the excess FCCP is not added after the incubation of mitochondria for 5 min in the presence of chloroform and successive additions of succinate and ADP, but rather it is added 2 min after the addition of chloroform to mitochondria previously incubated and already respiring. We have performed a control experiment in which the rate of respiration in state 4 and in the presence of excess FCCP has been measured at increasing concentrations of chloroform in the same medium and conditions of Rottenberg (1983) following the two different procedures. We have found a result similar to that shown in Figure 1c following our procedure and a result similar to that found by Rottenberg (1983) following his procedure (not shown). A possible explanation for the discrepancy is the

partial evaporation of the volatile anesthetic during the longer incubation adopted by Rottenberg (1983). This explanation does not apply to the discrepancy found with oleic acid. Control experiments have shown that the discrepancy still remains when dilute ethanol solutions (3 mM) of fatty acid are used, prepared and sealed under nitrogen as suggested in Rottenberg and Hashimoto (1986). However, when dilute solutions of fatty acid were used, the maximal stimulation of the rate of respiration in state 4 is shifted at lower concentration of oleic acid (30 nmol/mg; not shown) in accord with the results of Rottenberg and Hashimoto (1986).

The upper panels in Figure 1 show the effect of increasing concentrations of the three uncouplers on $\Delta\psi$ in state 4 and 3. Control experiments have shown that ΔpH, as measured from the distribution of DMO, is very small (10–20 mV) and goes to zero at high concentrations of uncoupler. Therefore, in our conditions, the variations of $\Delta\psi$ can be considered to reflect to a good approximation those of $\Delta\tilde{\mu}_H$.

A given stimulation of the rate of respiration in state 4 is accompanied by different depressions of $\Delta\tilde{\mu}_H$ for the different uncouplers. Oleic acid decreases $\Delta\tilde{\mu}_H$ more than FCCP and FCCP more than chloroform for a certain stimulation of the rate of respiration in state 4. If these agents were pure classical uncouplers, i.e., their only effect was that of increasing the passive conductance of the membrane without interfering with the redox pumps, a given rate of respiration should correspond to a unique value of $\Delta\tilde{\mu}_H$ independent of the type of uncoupler. We have already noted that oleic acid and chloroform also act as inhibitors of the redox pumps. The difference between the depression of $\Delta\tilde{\mu}_H$ induced by oleic acid and FCCP is in the direction predicted by the inhibitory effect on respiration of oleic acid. In contrast, that between chloroform and FCCP is in the opposite direction. Since part, if not all, of the small chloroform-induced depression of $\Delta\tilde{\mu}_H$ in static head is presumably due to its inhibitory effect on respiration, the pattern of the relationship between J_o^{sh} and $\Delta\tilde{\mu}_H^{sh}$ with chloroform can be considered as the first evidence that the stimulation of the rate of respiration in state 4 induced by this agent cannot be explained with the classical mechanism of uncoupling (increase of the conductance of the membrane and consequent decrease of $\Delta\tilde{\mu}_H$).

The rapid decrease of $\Delta\tilde{\mu}_H$ in state 4 at increasing concentrations of oleic acid is in sharp contrast with the constancy of $\Delta\tilde{\mu}_H$ reported by Rottenberg and Hashimoto (1986) in an analogous experiment. We have no explanation for this discrepancy. In control experiments the same rapid decrease of $\Delta\tilde{\mu}_H$ has been measured when dilute solutions of oleic acid prepared and sealed under nitrogen were used (not shown). As to the effect of oleic acid on $\Delta\tilde{\mu}_H$ in state 3 (see upper panel of Figure 1b), there is a range of oleic acid concentrations that have practically no effect on $\Delta\tilde{\mu}_H^{st3}$. Thus, in this range $\Delta\tilde{\mu}_H^{st3}$ remains practically constant, almost reaching the value of $\Delta\tilde{\mu}_H^{st4}$, and then decreases in parallel with the latter. This behavior, together with the decrease of the rate of respiration in state 3 in the same range of oleic acid concentrations, suggests a specific inhibitory effect of oleic acid on the ATPases and/or on the adenine nucleotide translocator. From here on we will refer only to an effect on the ATPases by implicitly meaning the lack of discrimination between the ATPase proton pump and the adenine nucleotide translocator.

The existence of an inhibitory effect of oleic acid on the ATPases is more directly supported by the behavior of the rate of ATP hydrolysis as a function of increasing concentrations of oleic acid shown in Figure 2b (lower panel). The bell-shaped dependence of the rate of ATP hydrolysis in static head on the concentration of oleic acid can be interpreted as the result

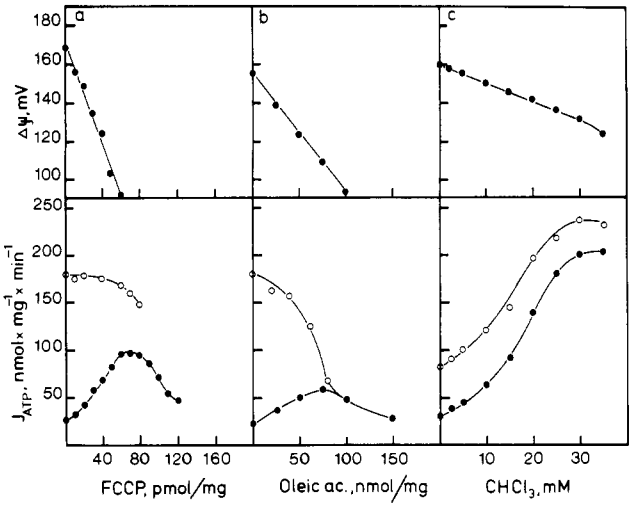

FIGURE 2: Rate of ATP hydrolysis, J_{ATP}, and $\Delta\psi$ in static head (●) as a function of the concentrations of FCCP (a), oleic acid (b), and chloroform (c). The open symbols (○) represent the rate of ATP hydrolysis in the presence of 25 mM chloroform in panels a and b and in the presence of 60 pmol/mg of FCCP in panel c. Medium composition: 0.2 M sucrose, 30 mM Tris/Mops, 0.2 mM EGTA, 2 mM $MgCl_2$, 5 mM P_i/Tris, 1 mM phosphoenolpyruvate, 0.1 mM NADH, excess pyruvate kinase, and lactate dehydrogenase; pH 7.4, T = 25 °C. Same procedure as in Figure 1 with ATP (3 mM) instead of succinate and chloroform (25 mM) in (a) and (b) instead of the excess FCCP and 0.5 mg of protein/mL instead of 1. The shift to higher values of the range of uncoupling concentrations of chloroform with respect to that shown in Figure 1c is in large part due to the lower concentration of mitochondria used in here. The uncoupling efficiency of chloroform increases with the mitochondrial concentration in the range 0.25–1 mg/mL and decreases above 1 mg/mL (not shown).

of two contrasting effects: the stimulation of the ATP hydrolysis due to the general ionophoric properties of oleic acid (cf. the depression of $\Delta\tilde{\mu}_H$ in the upper panel) and the inhibition of the rate of ATP hydrolysis due to specific inhibitory properties. A specific inhibition of the ATPases by oleic acid is also suggested by the observation that the rate of ATP hydrolysis maximally stimulated by chloroform undergoes a progressive inhibition in the presence of increasing concentrations of oleic acid. This inhibition explains also the less marked degree of stimulation of the rate of ATP hydrolysis obtained with oleic acid with respect to that obtained with FCCP and especially with chloroform (cf. lower panels in parts a and c of Figure 2).

The maximal rate of ATP hydrolysis obtained with FCCP is about 2 times that obtained with oleic acid but only about half that obtained with chloroform. The bell-shaped dependence of the rate of ATP hydrolysis in static head on the concentration of FCCP in Figure 2a is compatible with the presence of a natural inhibitor protein whose binding to the ATPase is $\Delta\tilde{\mu}_H$ dependent and also with the regulation of the activity of the ATPases by tightly bound ADP, whose binding is also $\Delta\tilde{\mu}_H$ dependent (Bertina & Slater, 1975; Chernyak & Kozlov, 1986). Both regulatory mechanisms give rise to inactivation of the ATPases as $\Delta\tilde{\mu}_H$ decreases.

The upper panels in Figure 2 show the effect of increasing concentrations of FCCP, oleic acid, and chloroform on $\Delta\psi$ generated by the hydrolysis of ATP in static head. Also in these experiments the variations of $\Delta\psi$ can be considered to reflect those of $\Delta\tilde{\mu}_H$ to a good approximation. A certain depression of $\Delta\tilde{\mu}_H$ is accompanied by a much higher stimulation of the rate of ATP hydrolysis with chloroform than with FCCP or oleic acid. As in the case of the stimulation of the rate of respiration in state 4, this result can be considered as evidence that chloroform does not act simply as a classical

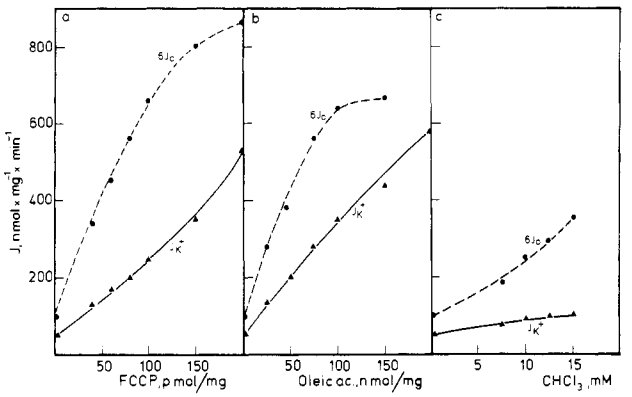

FIGURE 3: Rate of respiration in static head multiplied by the H^+/O stoichiometry and initial rate of K^+ efflux after addition of valinomycin to respiration-inhibited mitochondria as a function of the concentrations of FCCP (a), oleic (b), and chloroform (c). Medium composition as in Figure 1 except for the presence of 1 μg/mg of oligomycin. After incubation of mitochondria for 2 min in the presence of succinate (10 mM), the uncoupling agent was added, and after 1 min, the rate of respiration and, in parallel sample the initial rate of K^+ efflux immediately after addition of excess antimycin (0.05 μg/mg and valinomycin (0.15 μg/mg), were measured.

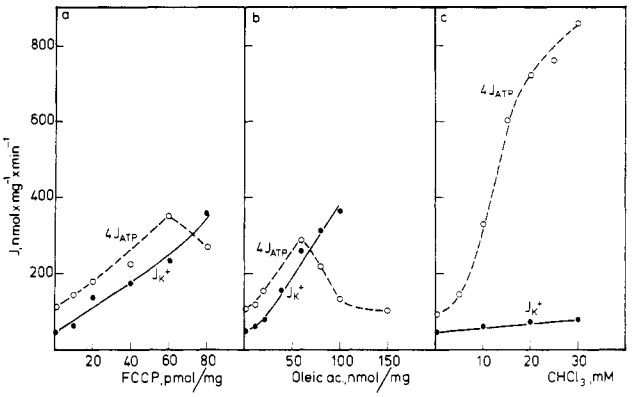

FIGURE 4: Rate of ATP hydrolysis in static head multiplied by the H^+/ATP stoichiometry and initial rate of K^+ efflux as a function of the concentrations of FCCP (a), oleic acid (b), and chloroform (c). Medium composition as in Figure 2 and procedure as in Figure 3 with ATP (3 mM) instead of succinate and excess oligomycin (1.6 μg/mg) instead of antimycin.

uncoupler which increases the conductance of the membrane (and as a consequence decreases $\Delta\tilde{\mu}_H$, thus releasing the thermodynamic control on the ATPases) and suggests a direct interaction of chloroform with the ATPases. Another interesting result in Figure 2c [cf. also Rottenberg (1983)] pointing to the same conclusion is that, at FCCP concentrations which maximally stimulate the rate of ATP hydrolysis (cf. Figure 2a), chloroform causes a further stimulation, which almost parallels that of the ATP hydrolysis in static head. Moreover, the process of ATP hydrolysis stimulated with chloroform is only slightly inhibited at concentrations of oligomycin 10 times higher than those that completely inhibit the rate of ATP hydrolysis stimulated with FCCP or oleic acid, while it is completely inhibited at similar concentrations of DCCD (not shown).

To obtain more direct evidence supporting these conclusions, we have measured the dissipative ionic current across the membrane in the presence of increasing concentrations of chloroform. The method adopted [amply discussed in Zoratti et al. (1986)] consists in measuring the initial rate of K^+ efflux upon addition of valinomycin to inhibited mitochondria. The condition of electroneutrality requires that the rate of potassium efflux be equal to the sum of the rates of influx of cations other than K^+ and of efflux of anions. The initial rate of potassium efflux then measures the dissipative ionic current, which is equal to the rate of passive influx, of protons if essentially the only permeable ion besides K^+ is H^+ (OH^-) (as is probably the case in our conditions) or higher than the rate of H^+ influx if other ions move.

Figure 3c shows that chloroform increases only slightly the dissipative ionic current. What is more relevant, the small increase in dissipative ionic current does not account for the increase in the proton efflux by the redox pumps as calculated from the rate of electron transfer and a stoichiometry $H^+/e^- = 3$ (from succinate to oxygen) assuming fully coupled redox pumps. A control experiment, where the distribution of $TPMP^+$ has been measured both in static head and after addition of valinomycin to inhibited mitochondria, shows that at each concentration of chloroform the diffusion potential following the addition of valinomycin is higher than $\Delta\psi$ in static head (not shown). ΔpH is similar in the two conditions (Zoratti et al., 1986). Therefore, the difference between dissipative ionic current and rate of electron transfer (multiplied by the stoichiometry) at any given $\Delta\tilde{\mu}_H$ is even higher than that shown in Figure 3c.

Parts a and b of Figure 3 show the results of the same type of experiment as a function of increasing concentrations of FCCP and oleic acid. In agreement with the decrease of $\Delta\tilde{\mu}_H$[st4] shown in Figure 1 and 2, both agents increase considerably the passive conductance of the membrane, as indicated by the increase of the ionic dissipative current. However, in both cases this increase does not completely account for the stimulation of the rate of electron transfer in static head. In control experiments, analogous to those performed with chloroform, it has been ascertained that at each concentration of uncoupler the diffusion potential is higher than the corresponding $\Delta\psi$ in static head. Therefore, the discrepancy between the rate of ionic current and the rate of electron transfer can be even higher.

Figure 4 shows the ionic dissipative current and the rate of ATP hydrolysis in static head measured in parallel at increasing concentrations of FCCP, oleic acid, and chloroform. The small increase in the passive conductance of the membrane as a function of chloroform does not account for the marked stimulation of the rate of ATP hydrolysis (Figure 4c). In contrast, in the range where the ATP hydrolysis is stimulated by either FCCP or oleic acid, the difference between the rate of K^+ efflux and the rate of ATP hydrolysis multiplied by the stoichiometry H^+/ATP remains more or less constant at the value in static head without uncouplers. This difference may be considered to reflect the intrinsic uncoupling of the ATPases (Pietrobon et al., 1983). If, at each concentration of uncoupler, $\Delta\psi$ in static head and during K^+ efflux was equal, Figure 4 would show that the stimulation of the rate of ATP hydrolysis by FCCP and oleic acid is completely accounted for by the increase in passive conductance of the membrane. However, also in this experiment, the diffusion potential is higher than the corresponding $\Delta\psi$ in static head at each concentration of uncoupler. Therefore, this experiment does not completely eliminate the possibility of a direct interaction of FCCP and oleic acid with the ATPases but suggests that, if present, the effect is small.

Discussion

The evidence that FCCP and other lipophilic weak acids act as protonophores increasing the proton conductance of both natural and artificial phospholipid membranes is overwhelming [for review see McLaughlin and Dilger (1980)]. Accordingly, our results show that FCCP causes an increase of the dissipative ionic flux across the inner mitochondrial membrane

(Figure 3a) and a depression of the proton electrochemical gradient, $\Delta\tilde{\mu}_H$, generated by either the redox proton pumps (Figure 1a) or the ATPase proton pumps (Figure 2a).

By the same criteria we can conclude that oleic acid also increases the passive conductance of the membrane. In fact, oleic acid increases the dissipative ionic flux (Figure 3b) and decreases $\Delta\tilde{\mu}_H$ (Figures 1b and 2b). This conclusion is at variance from that reached by Rottenberg and Hashimoto (1986), who negate the protonophoric action of oleic acid from the evidence of a constant $\Delta\tilde{\mu}_H$ (when generated by the redox pumps) in the presence of increasing concentrations of this agent. The same authors, however, report that fatty acids cause a depression of the ATPase-generated $\Delta\tilde{\mu}_H$, which is confirmed by the present study (Rottenberg & Hashimoto, 1986). The agreement between the results of independent experiments carried out under different experimental conditions, i.e., the agreement between the increase of the dissipative ionic flux in the presence of oleic acid shown in Figures 3b and 4b and the depression of $\Delta\tilde{\mu}_H$ shown in the upper panels of Figures 1b and 2b [cf. also the results of Pietrobon et al. (1987), following paper in this issue], supports the validity of the present $\Delta\tilde{\mu}_H$ measurements. We also note that the discrepancy cannot be ascribed to the fact that we have used more concentrated solutions of oleic acid not sealed under nitrogen, since control experiments have shown that the only effect of this type of solution is to induce the same $\Delta\tilde{\mu}_H$ depression at lower oleic acid concentrations.

The chemiosmotic hypothesis predicts that a decrease of $\Delta\tilde{\mu}_H$ is accompanied by a stimulation of the rate of electron transfer and a depression of the rate of ATP synthesis. Indeed, these are the well-known effects of FCCP on oxidative phosphorylation shown in Figure 1a.

Oleic acid, in addition to its protonophoric action at the level of the membrane, has an inhibitory effect on the redox enzymes (cf. the inhibition of the maximal rate of respiration in Figure 1b). The inhibition of the maximal rate of ATP hydrolysis in Figure 2b also suggests an inhibitory effect of oleic acid on the ATPase and/or the adenine nucleotide translocator. Simulations performed on the basis of the kinetic model of chemiosmotic free energy coupling recently described by Pietrobon and Caplan (1986) [cf. also Pietrobon (1986)] show (cf. following paper) that these additional inhibitory effects of oleic acid can account for the lack of stimulation of the rate of respiration in state 3 and for the near constancy of $\Delta\tilde{\mu}_H$ in state 3 shown in Figure 1b (and also for the lower, if compared with FCCP, stimulation of the rate of respiration in state 4 at any given $\Delta\tilde{\mu}_H$).

In contrast with FCCP and oleic acid, chloroform only slightly increases the dissipative ionic flux across the membrane (Figures 3c and 4c) and only slightly decreases $\Delta\tilde{\mu}_H$ at concentrations that completely inhibit the rate of ATP synthesis [Figures 1c and 2c; cf. also Rottenberg (1983)]. That the uncoupling by chloroform is not accounted for by the increase in membrane conductance is shown directly by the finding that the increase in protonic dissipative flux across the membrane induced by chloroform is lower than the corresponding increase in the rate of electron transfer multiplied by the H^+/e^- stoichiometry (Figure 3c) and is also lower than the corresponding increase in the rate of ATP hydrolysis multiplied by the H^+/ATP stoichiometry (Figure 4c).

The condition of zero net proton flux in static head requires the rate of proton efflux through the $\Delta\tilde{\mu}_H$-generating pump (either redox or ATPase) to be equal to the rate of passive proton influx. If the proton pumps are assumed to be fully coupled, the rate of proton efflux through the pumps can be calculated from the rate of either electron transfer or ATP hydrolysis multiplied by the H^+/e^- (n_e) or the H^+/ATP (n_p) stoichiometry, respectively. However, even in static head, without external agents the rate of electron transfer multiplied by the H^+/e^- stoichiometry is higher than the rate of passive proton influx at the same $\Delta\tilde{\mu}_H$ (Zoratti et al., 1986; see also in Figure 3 the rates in the absence of uncoupling agent). The difference between n_eJ_e and the rate of passive proton influx has been considered to reflect the presence of a certain extent of intrinsic uncoupling (slip) in the redox pumps, whereby electrons can be transferred without proton pumping (Zoratti et al., 1986). Analogously, the difference between the rates of passive proton influx and of ATP hydrolysis shown in Figure 4 in static head without uncoupling agent can be considered a consequence of intrinsic uncoupling of the ATPases. The hypothesis of slipping proton pumps had been previously formulated to explain the nonlinear behavior of the rates of electron transfer and of ATP hydrolysis as a function of $\Delta\tilde{\mu}_H$ in titrations with inhibitors of the pumps (Pietrobon et al., 1981, 1983). Modeling studies have shown that this behavior can indeed be simulated by assuming a certain degree of intrinsic uncoupling in the pumps (Pietrobon et al., 1986).

If the effect of adding chloroform were only that of increasing the proton conductance, the initial difference between the rate of passive proton influx and the rate of electron transfer multiplied by the stoichiometry in Figure 3c should remain constant or even slightly decrease (if it is due to a $\Delta\tilde{\mu}_H$-dependent slip; Pietrobon et al., 1986). The fact that the difference between the rate of proton influx and the rate of electron transfer (multiplied by the H^+/e^- stoichiometry) in Figure 3c increases at increasing concentrations of chloroform indicates that chloroform increases the fraction of electrons which are transferred without stoichiometric proton translocation in the external medium. Analogously, the comparison between the rate of proton influx and the rate of ATP hydrolysis (Figure 4c) indicates that chloroform increases also the fraction of ATP molecules hydrolyzed without stoichiometric proton translocation in the external medium.

Although the effects of FCCP and oleic acid on ATP synthesis, respiration, and $\Delta\tilde{\mu}_H$ are consistent with the view that these agents act as pure classical uncouplers increasing the membrane proton conductance, the quantitative comparison between the rate of passive proton influx and the rate of respiration (multiplied by the H^+/O stoichiometry) in Figure 3a,b shows that FCCP and oleic acid have actually a mixed behavior. They increase the proton conductance, but in addition they also increase the fraction of electrons transferred without proton pumping. On the other hand, the experiment in Figure 4 suggests that FCCP and oleic acid do not significantly increase the fraction of ATP molecules hydrolyzed without proton pumping.

In conclusion, in this paper we have shown that the uncoupling of oxidative phosphorylation by three different types of uncouplers (a lipophilic weak acid, a fatty acid, and a general anesthetic) is not quantitatively accounted for by the increase in membrane conductance. All three agents appear to cause the transfer of a certain number of electrons without translocation of a stoichiometric number of protons across the membrane from the mitochondrial matrix to the external medium. In addition, chloroform gives rise also to hydrolysis of ATP not accompanied by the translocation across the membrane of a stoichiometric number of protons. At least two different mechanisms of uncoupling (not necessarily alternative ones with respect to each other) can give rise to electron transfer and ATP hydrolysis without apparent

translocation of protons across the membrane: (1) intrinsic uncoupling of the proton pumps (Pietrobon et al., 1981, 1986; Walz, 1983); and (2) "decoupling" of a localized protonic coupling mechanism (Williams, 1961; Rottenberg, 1978, 1983; Hitchens & Kell, 1983; Westerhoff et al., 1984; Slater et al., 1985). In the following paper we further investigate, with new experiments, the mechanism of uncoupling by FCCP, oleic acid, and chloroform, and we discuss the consistency of the results with the above-mentioned different mechanisms of uncoupling.

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