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Uncoupling of oxidative phosphorylation in rat liver mitochondria by general anesthetics

(membrane potential/ΔpH/membrane fluidity/ATPase/chemiosmosis)

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ABSTRACT **The general anesthetics chloroform and halothane inhibit ATP synthesis in rat liver mitochondria, in the millimolar concentration range (1–12 mM), in parallel with a reduction of respiratory control and the ratio of ATP produced to oxygen consumed. In these effects, halothane and chloroform are similar to classical, protonophoric, uncouplers. The rate of ADP-stimulated respiration or the rate of uncoupler-stimulated respiration is not affected. Like classical uncouplers, halothane and chloroform also stimulate mitochondrial ATPase activity. However, the extent of stimulation by these agents is larger than by protonophoric uncouplers and, more significantly, ATPase activity stimulated by carbonylcyanide *m*-chlorophenylhydrazone is further stimulated by these agents. In the presence of the Ca^{2+} chelator EGTA, halothane and chloroform have no measurable effect on the magnitude of the proton electrochemical potential, $\Delta\tilde{\mu}_H$. In the absence of EGTA these anesthetics have a small effect on $\Delta\tilde{\mu}_H$, apparently due to stimulation of Ca^{2+} cycling. Under these conditions the membrane potential is decreased while ΔpH is increased, but the total value of $\Delta\tilde{\mu}_H$ is only slightly decreased. The uncoupling activity of the anesthetics is the same in the presence or absence of EGTA. Thus, in contrast to protonophoric uncouplers, the uncoupling effect of general anesthetics does not depend on the collapse of $\Delta\tilde{\mu}_H$. In the same concentration range in which anesthetics uncouple oxidative phosphorylation both halothane and chloroform increase membrane fluidity, as measured by the partitioning of the hydrophobic spin probe 5-doxyldecane. These findings suggest a role for intramembrane processes in energy conversion that is not dependent on the bulk $\Delta\tilde{\mu}_H$.**

Oxidative phosphorylation in mitochondria is believed to be mediated by the proton electrochemical potential gradient ($\Delta\tilde{\mu}_H$) as postulated by Mitchell in the chemiosmotic hypothesis (1–3). According to the chemiosmotic scheme, uncouplers are protonophores that catalyze the transport of protons across the membrane, thereby collapsing $\Delta\tilde{\mu}_H$, which is the direct driving force for ATP synthesis. The evidence for the protonophoric activity of uncouplers in both natural membranes and phospholipid bilayers is overwhelming (for review see ref. 4). In mitochondria, where ΔpH is relatively small, the collapse of membrane potential by electrophoretic transport of cations (e.g., Ca^{2+} or K^+ in the presence of valinomycin) is sufficient to decrease $\Delta\tilde{\mu}_H$ and lead to uncoupling as well. Thus, to date, all known uncouplers of oxidative phosphorylation and photophosphorylation appear to collapse $\Delta\tilde{\mu}_H$ in correlation with the inhibition of ATP synthesis (but see *Discussion*). However, several agents whose mechanism of action is still unknown are known to inhibit oxidative phosphorylation. Of particular interest are the general anesthetics, which are similar to protonophoric uncouplers in their effect on succinate respiration and on ATP synthesis and hydrolysis in mitochondria. Earlier studies, mostly with halothane, indicated that general anesthetics inhibit NADH respiration and uncouple succinate respiration, but they did not resolve the mechanism by which anesthetics affect mitochondria (5–9). General anesthetics are not expected to be protonophores, *per se*, but might affect proton transport or other ion transport indirectly, leading to the collapse of $\Delta\tilde{\mu}_H$ (8–10). On the other hand, general anesthetics are known to affect membrane fluidity, the membrane dielectric constant, and lipid–protein and protein–protein interactions (11). These latter effects may cause uncoupling. In this study we characterize the effects of the general anesthetics halothane and chloroform on ATP synthesis, succinate oxidation, and the ATPase activity of rat liver mitochondria. We compare these effects on mitochondrial coupling to their effects on $\Delta\tilde{\mu}_H$ and on membrane fluidity. Our results indicate that the collapse of $\Delta\tilde{\mu}_H$ does not appear to be the cause of uncoupling by these agents. Although general anesthetics significantly increase mitochondrial membrane fluidity, it is unlikely that uncoupling is the direct result of this effect. We postulate that the uncoupling is due to interference with delicate intramembrane processes that mediate direct energy transfer between the electron transport complexes and the ATPase. General anesthetics may be useful probes in a search for such interactions.

EXPERIMENTAL

Rat liver mitochondria were prepared from male Sprague–Dawley rats (Charles River Laboratories) by differential centrifugation as described (12). Mitochondrial protein was determined by the cyanide biuret procedure (13). Rates of respiration were measured by following oxygen consumption with a Clark type oxygen electrode in a thermostated cell (14). Phosphorylation and ATPase rates were measured routinely by following the rate of proton consumption or production with the aid of a glass pH electrode (15). In several experiments ATP synthesis in the presence of glucose and hexokinase was measured by enzymatic determination of the glucose 6-phosphate content in extracts from the mitochondrial suspension (16). Membrane potential and ΔpH were determined from the distribution of $^{86}Rb^+$ (in the presence of valinomycin) and from the distribution of ^{14}C-labeled 5,5-dimethyl-2,4-oxazolidinedione (DMO) as described (17). The concentration ratio was calculated by using ^3H_2O as a measure of the pellet water volume and [^{14}C]sucrose as a marker for the volume of externally trapped water (17). In all experiments the mitochondrial suspension was incubated with anesthetics for 5 min prior to the initiation of the experiments by the addition of the proper substrate. To

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; diS-C_3-(5), 3,3-dipropylthiocarbocyanine; 5N10, 2,2-dimethyl-4-butyl-4-pentyl-*N*-oxylazolidine; DMO, 5,5-dimethyl-2,4-oxazolidinedione.

minimize evaporation of the volatile anesthetics the reactions were carried out in sealed vessels and extra care was taken to minimize exposure to the atmosphere on addition of reagents or withdrawal of samples. All the experiments presented in this report were performed at least three times with different mitochondrial preparations. The partition of the doxyldecane 2,2-dimethyl-4-butyl-4-pentyl-*N*-oxylazolidine (5N10) was determined from the EPR spectra of the probe–membrane system by using a Varian E-109 spectrometer (18). The fluorescence of 3,3-dipropylthiocarbocyanine [diS-C_3-(5)] was followed with an Eppendorf fluorimeter (17).

RESULTS

Fig. 1 shows the effect of halothane and chloroform on the rate of succinate oxidation and ATP synthesis by rat liver mitochondria. This experiment, like all experiments shown in this report, was conducted at 37°C. Essentially identical results were obtained at 25°C, but higher concentrations of anesthetics were required at this temperature. Halothane and chloroform both inhibit ATP synthesis. The inhibition depends linearly on the anesthetic concentration, with half-maximal inhibition at 6 mM and maximal inhibition at 12 mM. When ATP synthesis was measured in the presence of excess hexokinase and glucose, which lowered the phosphate potential, 50% inhibition was observed at somewhat higher concentrations (7–8 mM), and the inhibition was complete at 20 mM anesthetic. Succinate respiration in the absence of ADP (state 4) is stimulated by these anesthetics in a linear fashion, with half-maximal stimulation obtained at 4 mM for halothane and 6 mM for chloroform. Hence, respiratory control is gradually abolished and the P/O ratio (the number of phosphates incorporated per atom of oxygen consumed) approaches zero. The maximal stimulation of the rate

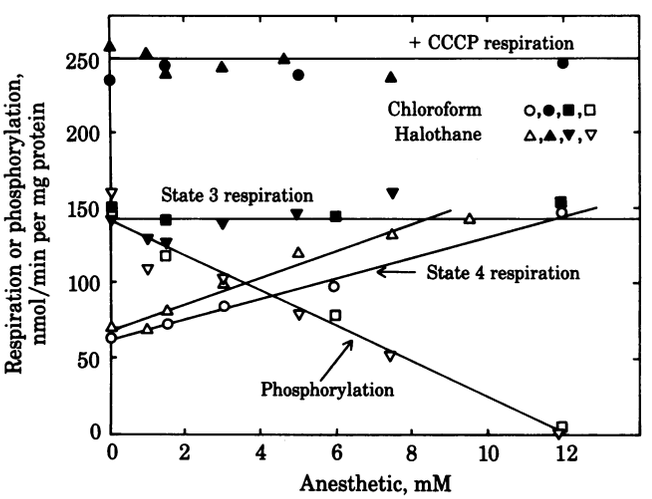

FIG. 1. Effects of halothane and chloroform on the rate of electron transport and ATP synthesis. The mitochondrial suspension was incubated with the indicated amount of anesthetic for 5 min prior to the addition of sodium succinate (10 mM), which initiated the reactions. The basic medium contained 0.2 M sucrose, 5 mM Na_2HPO_4 (pH 8.0), 50 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, and 2 μM rotenone. In the respiration experiments 10 mM Tris·HCl (pH 8.0) was added. State 3 respiration was induced by the addition of 100 μM ADP, and CCCP stimulation was measured at 0.2 μM. The mitochondrial content was 1.5 mg of protein per ml. State 4 respiration: △, halothane; ○, chloroform. State 3 respiration: ▼, halothane; ■, chloroform. CCCP-stimulated respiration: ▲, halothane; ●, chloroform. In the phosphorylation experiments the basic medium was supplemented with only 5 mM Tris and the phosphorylation was initiated by the addition of 1 mM ADP. The mitochondrial content was 2 mg of protein per ml. Temperature, 37°C.

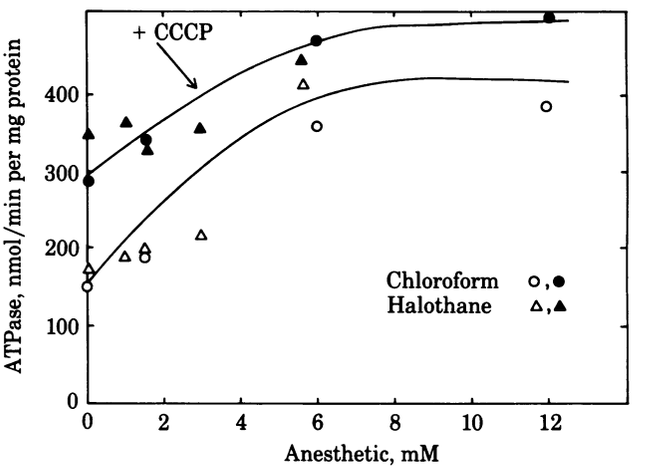

FIG. 2. Effect of halothane and chloroform on the rate of ATP hydrolysis. ATP hydrolysis was measured by the rate of acidification of the medium. The mitochondrial suspension was incubated for 5 min with the indicated amount of anesthetic prior to initiation of the reaction by the addition of 2 mM ATP. The basic medium was the same as for Fig. 1 except that Na_2HPO_4 was omitted and Tris·HCl (pH 8.0) was only 2 mM. CCCP-stimulated ATPase was measured after the addition of 0.2 μM CCCP. The mitochondrial content was 1.5 mg of protein per ml. Temperature, 37°C.

of respiration is identical to the maximal stimulation obtained by ADP, under these conditions, but far less than the maximal stimulation obtained with the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Neither the rate of ADP-stimulated respiration (state 3) nor the rate of CCCP-stimulated respiration is affected by the anesthetic in this concentration range. The data of Fig. 1 suggest that the stimulation of state 4 respiration is due to specific release of the acceptor (ADP) control of respiration. It is important to notice that under these experimental conditions protonophoric uncouplers stimulate state 3 respiration, presumably by releasing the control of $\Delta\tilde{\mu}_H$ on respiration.

Fig. 2 shows the effect of halothane and chloroform on the rates of coupled and CCCP-stimulated ATP hydrolysis. Under these experimental conditions CCCP stimulated the ATPase only by about 100% (19). The anesthetics stimulate ATPase to a larger extent than CCCP does. More importantly, even CCCP-stimulated ATPase activity is further stimulated by the anesthetics. Half-maximal stimulation is obtained at about 4 mM. Table 1 shows the effect of chloroform on the inhibition by oligomycin of the mitochondrial ATPase activity. At an oligomycin

Table 1. Effect of chloroform on the inhibition by oligomycin of uncoupler-stimulated ATPase

Chloroform, mM	CCCP, μM	Oligomycin, μg/mg protein	ATPase nmol/min per mg protein	ATPase % of control
—	0.2	—	214	100
—	—	—	63	29
—	—	1.3	31	15
—	0.2	1.3	36	17
5	0.2	—	366	100
5	—	—	268	73
5	—	1.3	67	18
5	0.2	1.3	67	18

Medium and conditions were as in Fig. 2 except that 2 mM Na_2HPO_4 was added and the medium pH was 7.5.

concentration (1.3 μg/mg of protein) that inhibits CCCP-stimulated ATPase activity by 83%, the chloroform + CCCP-stimulated activity is inhibited by 82%. Oligomycin inhibits chloroform-stimulated ATPase to a greater extent than coupled ATPase. Oligomycin had no effect on the stimulation of state 4 respiration by the anesthetics (not shown). Thus, the effects of the anesthetics do not appear to result from a direct interaction with the ATPase system. The relative effectiveness of CCCP and anesthetics on ATPase activity contrasts with their effects on respiration (Fig. 1), in which CCCP is a more effective stimulator of respiration and in which the anesthetics do not stimulate either ADP- or CCCP-stimulated respiration.

The results of Figs. 1 and 2 suggest that the effects of anesthetics are more closely associated with the ATPase system and do not directly affect the electron transport system. To find whether the uncoupling of oxidative phosphorylation is a result of the collapse of $\Delta\tilde{\mu}_H$ we have determined the effect of halothane and chloroform on membrane potential Ψ and ΔpH, from which we calculated the effect on $\Delta\tilde{\mu}_H$ ($\Delta\tilde{\mu}_H = -\Delta\Psi + 0.06$ ΔpH). In these experiments (Fig. 3) $\Delta\tilde{\mu}_H$ was generated by succinate oxidation. In the presence of up to 20 mM anesthetics there is no measurable effect on $\Delta\tilde{\mu}_H$. In these experiments (as in the experiments of Figs. 1 and 2), EGTA and Mg^{2+} were included in the incubation medium. In the absence of EGTA there is a slight reduction of $\Delta\tilde{\mu}_H$ (Fig. 4). This is apparently due to Ca^{2+} cycling (8), but it is not related to the uncoupling, which is unaffected by EGTA. Similar results were observed

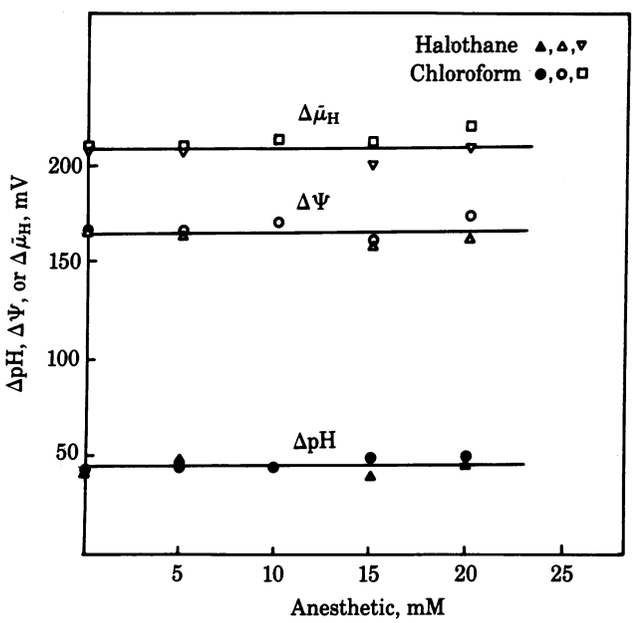

FIG. 3. Effect of halothane and chloroform on $\Delta\tilde{\mu}_H$. The mitochondrial suspension was incubated with the indicated concentration of anesthetic and the appropriate tracer for 5 min prior to addition of 10 mM succinate. After addition of succinate the suspension was incubated for another 2 min, after which the mitochondria were sedimented in a micro centrifuge and dissolved in $HClO_4$, and radioactivities were measured as described (17). The basic medium was the same as for Fig. 1 except for the omission of Na_2HPO_4 and the addition of 10 mM Hepes (pH 7.5) and 0.4 μM valinomycin. The mitochondrial content was 2 mg of protein per ml. Each reaction system was divided into three equal portions: one was incubated with $^{86}RbCl$ [0.1 μCi/ml (1 Ci = 3.7×10^{10} Bq)] and ^3H_2O (2 μCi/ml) for $\Delta\Psi$ determination, the second was incubated with [^{14}C]DMO (0.5 μCi/ml) and ^3H_2O (2 μCi/ml) for ΔpH determination, and the third was incubated with [^{14}C]sucrose (0.5 μCi/ml) and ^3H_2O (2 μCi/ml) for matrix space determination. Each determination was done in triplicate. The values shown are the average of three separate experiments. Temperature, 37°C.

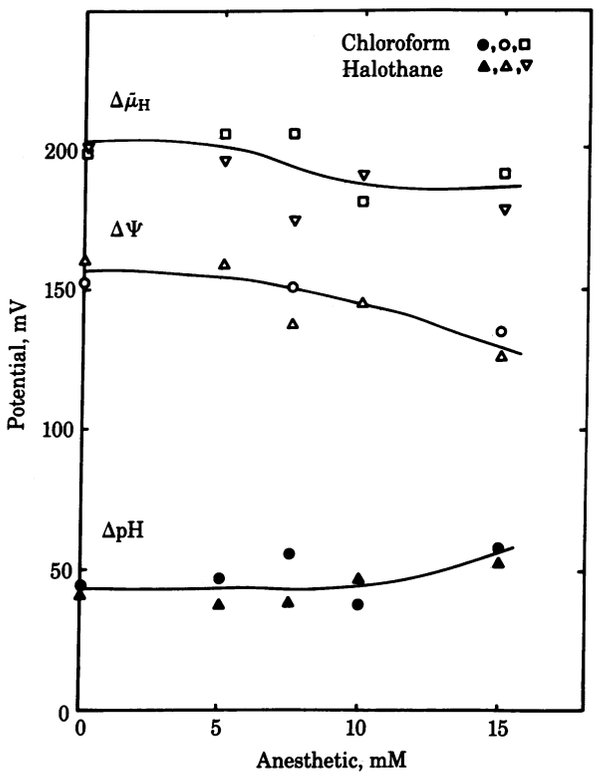

FIG. 4. Effect of halothane and chloroform on $\Delta\tilde{\mu}_H$ in the absence of EGTA. Conditions are as for Fig. 3 except for the omission of EGTA. Temperature, 37°C.

at 25°C and also when ATP was used to generate $\Delta\tilde{\mu}_H$. The results of Fig. 3, when compared to those of Figs. 1 and 2, indicate that the uncoupling of oxidative phosphorylation and the stimulation of the ATPase by the anesthetics are not mediated by an effect on $\Delta\tilde{\mu}_H$. In the absence of EGTA (Fig. 4) the effect of the anesthetics on $\Delta\tilde{\mu}_H$ qualitatively resemble that of valinomycin + potassium (20), but the magnitude of the effect is negligible. To get an equivalent stimulation of respiration or inhibition of phosphorylation by valinomycin + potassium, $\Delta\tilde{\mu}_H$ must be decreased by more than 50% (20). Chloroform greatly accelerated the relaxation of valinomycin-induced potassium diffusion potential as measured by the fluorescence of diS-C_3-(5) (unpublished data). This observation suggests that chloroform increased the permeability to some ions, probably Ca^{2+}, which is the cause of the slight effect on $\Delta\tilde{\mu}_H$ (8–10). General anesthetics are known to increase membrane fluidity (11). We have previously observed that halothane greatly increases the fluidity of mitochondrial membranes as measured by the order parameter of 5-doxylstearate and the partition of the doxyldecane 5N10 (21). In Fig. 5 we show that halothane and chloroform are equally effective in increasing the membrane fluidity at the concentration range in which they uncouple oxidative phosphorylation. Similar effects were observed on the fluorescence polarization of diphenylhexatriene (results not shown).

DISCUSSION

Because it is now generally accepted that uncouplers increase proton permeability and collapse $\Delta\tilde{\mu}_H$ it is difficult to classify general anesthetics as uncouplers. Uncoupling, in general, refers to the release of "coupling" as expressed by the inhibition of ATP synthesis and release of respiratory control; in this sense general anesthetics clearly uncouple oxidative phosphorylation and yet $\Delta\tilde{\mu}_H$ is not collapsed. We could not test whether gen-

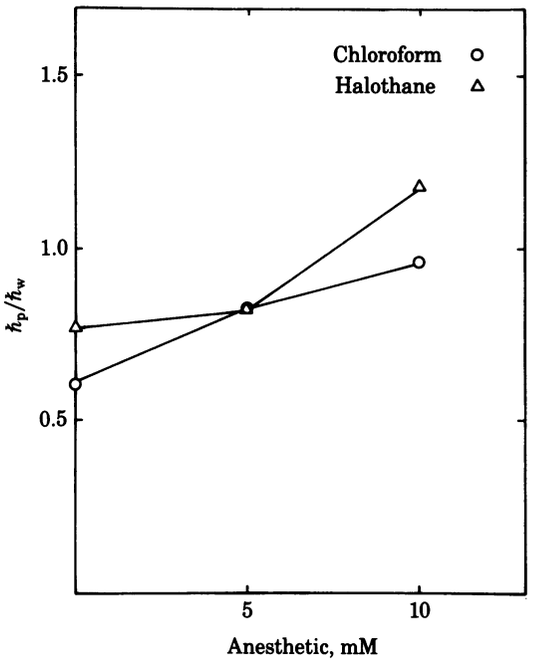

FIG. 5. Effect of halothane and chloroform on the partition of 5N10 into the mitochondrial membrane. 5N10 (25 μM) was incubated with mitochondria (10 mg of mitochondrial protein per ml) for 5 min prior to measurement of the EPR spectra. The partition was calculated as described (18). h_p and h_w are the amplitude of the high-field signal of the bound and free probe, respectively. Temperature, 37°C.

eral anesthetics also uncouple reverse electron transport, as expected of uncouplers, because of their apparent inhibition of NADH oxidase. It might be of great significance that under the conditions in which protonophoric uncouplers stimulate respiration to a greater extent than ADP, anesthetics do not stimulate state 3 respiration. This may indicate that the control of respiration by $\Delta\tilde{\mu}_H$ and by phosphorylation occur by separate pathways and that the anesthetic specifically uncouples the phosphorylation control pathway. A discrepancy between the effects of ADP and uncouplers on respiratory control and $\Delta\tilde{\mu}_H$, which is not compatible with the chemiosmotic theory, was observed previously by Padan and Rottenberg (20). The validity of the chemiosmotic hypothesis of energy conversion in oxidative phosphorylation and photophosphorylation depends on several well-established lines of evidence (3). (*i*) Several transport complexes function as proton pumps utilizing the free energy derived from substrate oxidation (or light) to pump protons across the membrane and build a substantial $\Delta\tilde{\mu}_H$. (*ii*) The mitochondrial, chloroplast, and bacterial ATPases are also reversible proton pumps, which may utilize the energy derived from ATP hydrolysis to build $\Delta\tilde{\mu}_H$ or, under the appropriate thermodynamic balance, utilize an existing $\Delta\tilde{\mu}_H$ to synthesize ATP against a large positive free energy change of the ATPase reaction. The feasibility of combining systems *i* and *ii* into a coupled process was proven by reconstitution experiments (22) and by the fact that during oxidative phosphorylation and photophosphorylation a substantial $\Delta\tilde{\mu}_H$ is generated in these systems (2). The evidence that bulk $\Delta\tilde{\mu}_H$ is the *only* mode of coupling between the redox and the ATPase system is considerably weaker and rests entirely on the effects of uncouplers (and ionophores) on oxidative phosphorylation or photophosphorylation. Qualitatively, all uncouplers were found to collapse $\Delta\tilde{\mu}_H$, apparently by shuttling of protons across the membrane (4). Ionophores and permeant ions uncouple energy conversion by collapsing $\Delta\Psi$ due to electrogenic transfer of various ions, ΔpH due to exchange of protons with cations, or both in proper combination (e.g., valinomycin + nigericin). However, the quantitative relationships between the reduction of $\Delta\tilde{\mu}_H$ and the effect on either respiration or phosphorylation do not conform to the prediction of the chemiosmotic theory. In the presence of valinomycin and potassium mitochondria continue to phosphorylate with high efficiency even when $\Delta\tilde{\mu}_H$ is greatly reduced (23, 24). The opposite is the case with protonophoric uncouplers and electron transport inhibitors (24–26). The phosphate potential that can be built up by the coupled ATPase is not reduced in proportion to the reduction of $\Delta\tilde{\mu}_H$ by uncouplers or ionophores (27, 28). The stimulation of respiration by ADP is accompanied by only a small reduction of $\Delta\tilde{\mu}_H$, far less than the reduction necessary with uncouplers or ionophores to obtain a comparable stimulation of respiration (20, 29). In several bacterial systems oxidative phosphorylation proceeds efficiently under conditions of greatly reduced $\Delta\tilde{\mu}_H$ (30, 31). All these observations suggest that in addition to $\Delta\tilde{\mu}_H$ as measured in the bulk phases across the membrane there is another more direct route of energy conversion between the redox and the ATPase system (20). The most feasible and minimal modification of the chemiosmotic model is to suggest that a fraction of the proton current moves directly between the systems (19). However, to date there is little direct evidence for such a scheme. The results of this study may provide a clue for the mechanism of the intramembrane coupling.

While the mechanism for anesthesia is still largely unknown, a great deal is known about the effect of anesthetics on biological membranes. There is very little doubt that the site of action of these agents (at least at low concentration) is in the membranes (32). Whether their effect is due to disordering of the lipid structure in the membrane, interactions with membrane proteins, or interference with lipid–protein interactions is still a matter of hot debate. The fact that anesthetics increase mitochondrial lipid fluidity, as shown here and elsewhere (21), does not necessarily mean that this is the cause of the uncoupling. It is possible that these changes in membrane fluidity simply reflect the partition of the agent into the membrane. In this concentration range, the anesthetic concentration in the membranes is so high that a very large fraction of the membrane is occupied by the anesthetic molecules, which move more freely than the lipid acyl chains. However, precisely because such a large fraction of the membrane is occupied by the anesthetics one may expect that protein–lipid interactions and possibly protein–protein interactions would be drastically modified. If the putative intramembrane coupling depends on such interaction it is quite likely that general anesthetics would disturb these interactions. Another known effect of general anesthetics on membranes is to increase their dielectric constant (33). This may facilitate ion movements, thus increasing the leak of protons (or other charged species) from the putative intramembrane coupling pathway. This may also cause the effects on ΔpH and $\Delta\Psi$ in the absence of EGTA, presumably due to an increase in Ca^{2+} permeability (8–10). It is interesting to note that the uncoupling action of the anesthetics appears to be more closely related to the ATPase than to the electron transport chain even though no direct effect on the ATPase is indicated. In this respect we notice that several lipophilic cations act as energy transfer inhibitors (34, 35).

Recent results (unpublished) indicate that these agents do not act by inhibiting the ATPase reaction directly but by accumulating on the matrix face of the inner membrane, where they interfere with proton access to the ATPase complex. Also, recent results obtained by Winkler and Kornstein on ATP synthesis driven by an external electric field in chloroplasts (36) may be interpreted to suggest that the ATPase complex in the

membrane has the capacity to store a large amount of energy, sufficient to provide for many turnovers of ATP synthesis. These observations may indicate the existence of a pool of protons (or other charged species) in the vicinity of the ATPase complex that may be directly coupled to the electron transport system. In this hypothetical scheme, anesthetics uncouple oxidative phosphorylation by dissipating this pool; lipophilic cations inhibit energy transfer by preventing access of protons to or from the pool, and the external electric field is capable of directly charging this pool. Regardless of the exact mechanism by which anesthetics uncouple oxidative phosphorylation, it is clear that the uncoupling occurs in the membrane without affecting bulk $\Delta\tilde{\mu}_H$. Thus, these findings strongly support the notion that, in addition to the chemiosmotic coupling through $\Delta\tilde{\mu}_H$, there is an additional intramembrane coupling in oxidative phosphorylation.

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